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(71) **Demandeur/Applicant:**
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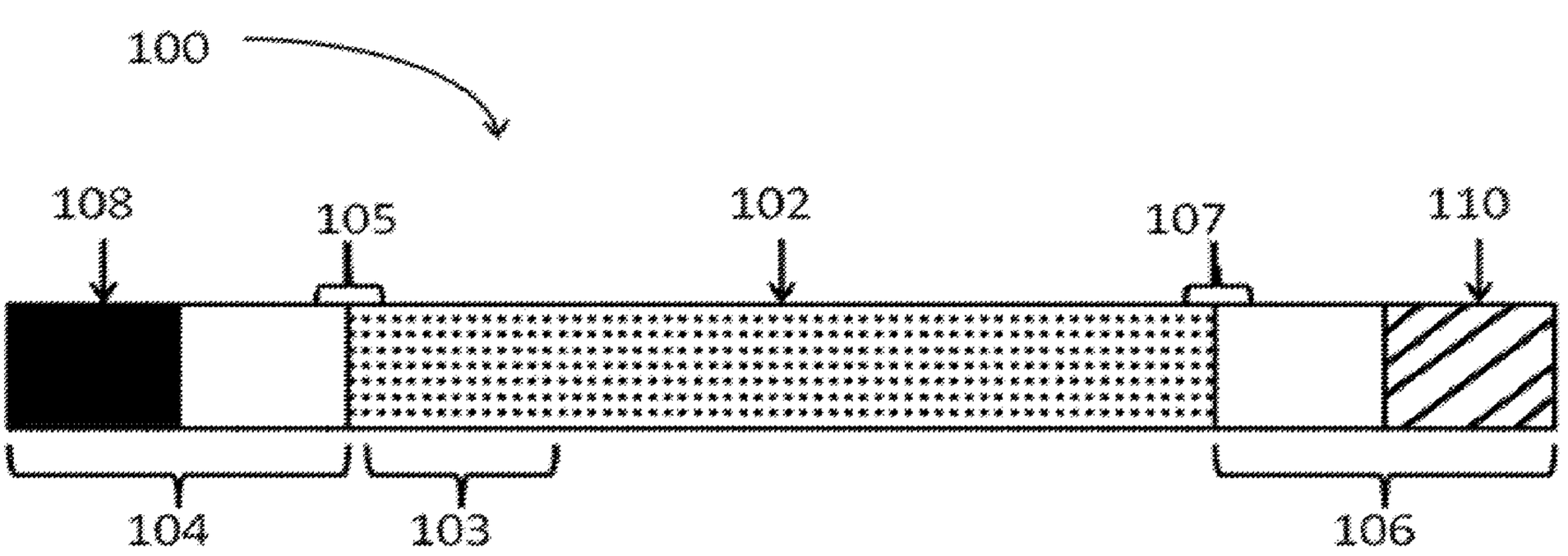
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(54) **Titre : POLYNUCLEOTIDES MODIFIES DESTINES A LA PRODUCTION DE PRODUITS BIOLOGIQUES ET DE PROTEINES ASSOCIEES A UNE MALADIE HUMAINE**

(54) **Title: MODIFIED POLYNUCLEOTIDES FOR THE PRODUCTION OF BIOLOGICS AND PROTEINS ASSOCIATED WITH HUMAN DISEASE**

FIGURE 1



(57) **Abrégé/Abstract:**
The invention relates to compositions and methods for the preparation, manufacture and therapeutic use of polynucleotides, primary transcripts and mmRNA molecules.

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(54) Title: MODIFIED POLYNUCLEOTIDES FOR THE PRODUCTION OF BIOLOGICS AND PROTEINS ASSOCIATED WITH HUMAN DISEASE

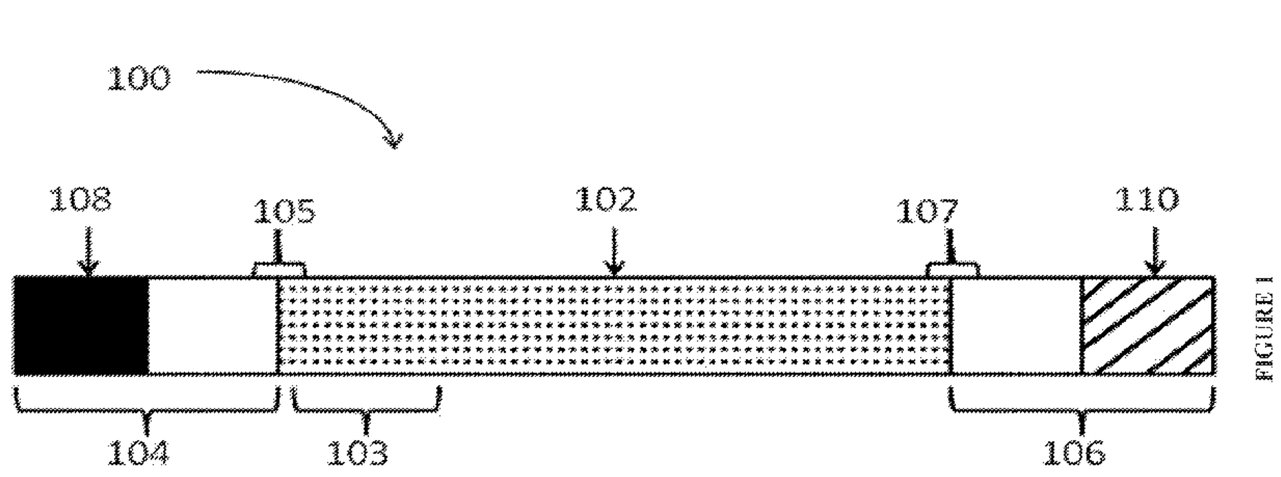


FIGURE 1

(57) Abstract: The invention relates to compositions and methods for the preparation, manufacture and therapeutic use of polynucleotides, primary transcripts and mmRNA molecules.

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JUMBO APPLICATIONS/PATENTS

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MODIFIED POLYNUCLEOTIDES FOR THE PRODUCTION OF BIOLOGICS AND PROTEINS ASSOCIATED WITH HUMAN DISEASE

REFERENCE TO SEQUENCE LISTING

[0001] The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing file entitled M300PCTSQLST.txt, was created on March 9, 2013 and is 49,417,315 bytes in size. The information in electronic format of the Sequence Listing is incorporated herein by reference in its entirety.

CROSS REFERENCE TO RELATED APPLICATIONS

[0002] This application claims priority to U.S. Provisional Patent Application No 61/681,742, filed, August 10, 2012, entitled Modified Polynucleotides for the Production of Oncology-Related Proteins and Peptides, U.S. Provisional Patent Application No 61/737,224, filed December 14, 2012, entitled Terminally Optimized Modified RNAs, International Application No PCT/US2012/069610, filed December 14, 2012, entitled Modified Nucleoside, Nucleotide, and Nucleic Acid Compositions, U.S. Provisional Patent Application No 61/618,862, filed April 2, 2012, entitled Modified Polynucleotides for the Production of Biologics, U.S. Provisional Patent Application No 61/681,645, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Biologics, U.S. Provisional Patent Application No 61/737,130, filed December 14, 2012, entitled Modified Polynucleotides for the Production of Biologics, U.S. Provisional Patent Application No 61/618,866, filed April 2, 2012, entitled Modified Polynucleotides for the Production of Antibodies, U.S. Provisional Patent Application No 61/681,647, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Antibodies, U.S. Provisional Patent Application No 61/737,134, filed December 14, 2012, entitled Modified Polynucleotides for the Production of Antibodies, U.S. Provisional Patent Application No 61/618,868, filed April 2, 2012, entitled Modified Polynucleotides for the Production of Vaccines, U.S. Provisional Patent Application No 61/681,648, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Vaccines, U.S. Provisional Patent Application No 61/737,135, filed December 14, 2012, entitled Modified Polynucleotides for the Production of Vaccines, U.S. Provisional Patent Application No 61/618,870, filed April 2, 2012, entitled Modified Polynucleotides for the Production of Therapeutic Proteins and Peptides, U.S. Provisional Patent Application No

61/681,649, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Therapeutic Proteins and Peptides, U.S. Provisional Patent Application No 61/737,139, filed December 14, 2012, Modified Polynucleotides for the Production of Therapeutic Proteins and Peptides, U.S. Provisional Patent Application No 61/618,873, filed April 2, 2012, entitled Modified Polynucleotides for the Production of Secreted Proteins, U.S. Provisional Patent Application No 61/681,650, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Secreted Proteins, U.S. Provisional Patent Application No 61/737,147, filed December 14, 2012, entitled Modified Polynucleotides for the Production of Secreted Proteins, U.S. Provisional Patent Application No 61/618,878, filed April 2, 2012, entitled Modified Polynucleotides for the Production of Plasma Membrane Proteins, U.S. Provisional Patent Application No 61/681,654, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Plasma Membrane Proteins, U.S. Provisional Patent Application No 61/737,152, filed December 14, 2012, entitled Modified Polynucleotides for the Production of Plasma Membrane Proteins, U.S. Provisional Patent Application No 61/618,885, filed April 2, 2012, entitled Modified Polynucleotides for the Production of Cytoplasmic and Cytoskeletal Proteins, U.S. Provisional Patent Application No 61/681,658, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Cytoplasmic and Cytoskeletal Proteins, U.S. Provisional Patent Application No 61/737,155, filed December 14, 2012, entitled Modified Polynucleotides for the Production of Cytoplasmic and Cytoskeletal Proteins, U.S. Provisional Patent Application No 61/618,896, filed April 2, 2012, entitled Modified Polynucleotides for the Production of Intracellular Membrane Bound Proteins, U.S. Provisional Patent Application No 61/668,157, filed July 5, 2012, entitled Modified Polynucleotides for the Production of Intracellular Membrane Bound Proteins, U.S. Provisional Patent Application No 61/681,661, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Intracellular Membrane Bound Proteins, U.S. Provisional Patent Application No 61/737,160, filed December 14, 2012, entitled Modified Polynucleotides for the Production of Intracellular Membrane Bound Proteins, U.S. Provisional Patent Application No 61/618,911, filed April 2, 2012, entitled Modified Polynucleotides for the Production of Nuclear Proteins, U.S. Provisional Patent Application No 61/681,667, filed August 10, 2012, entitled Modified Polynucleotides for

the Production of Nuclear Proteins, U.S. Provisional Patent Application No 61/737,168, filed December 14, 2012, entitled Modified Polynucleotides for the Production of Nuclear Proteins, U.S. Provisional Patent Application No 61/618,922, filed April 2, 2012, entitled Modified Polynucleotides for the Production of Proteins, U.S. Provisional Patent Application No 61/681,675, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Proteins, U.S. Provisional Patent Application No 61/737,174, filed December 14, 2012, entitled Modified Polynucleotides for the Production of Proteins, U.S. Provisional Patent Application No 61/618,935, filed April 2, 2012, entitled Modified Polynucleotides for the Production of Proteins Associated with Human Disease, U.S. Provisional Patent Application No 61/681,687, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Proteins Associated with Human Disease, U.S. Provisional Patent Application No 61/737,184, filed December 14, 2012, entitled Modified Polynucleotides for the Production of Proteins Associated with Human Disease, U.S. Provisional Patent Application No 61/618,945, filed April 2, 2012, entitled Modified Polynucleotides for the Production of Proteins Associated with Human Disease, U.S. Provisional Patent Application No 61/681,696, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Proteins Associated with Human Disease, U.S. Provisional Patent Application No 61/737,191, filed December 14, 2012, entitled Modified Polynucleotides for the Production of Proteins Associated with Human Disease, U.S. Provisional Patent Application No 61/618,953, filed April 2, 2012, entitled Modified Polynucleotides for the Production of Proteins Associated with Human Disease, U.S. Provisional Patent Application No 61/681,704, filed August 10, 2012, entitled Modified Polynucleotides for the Production of Proteins Associated with Human Disease, U.S. Provisional Patent Application No 61/737,203, filed December 14, 2012, entitled Modified Polynucleotides for the Production of Proteins Associated with Human Disease, U.S. Provisional Patent Application No 61/618,961, filed April 2, 2012, entitled Dosing Methods for Modified mRNA, U.S. Provisional Patent Application No 61/648,286, filed May 17, 2012, entitled Dosing Methods for Modified mRNA, the contents of each of which are herein incorporated by reference in its entirety.

[0003] This application is also related to International Publication No. PCT/US2012/58519, filed October 3, 2012, entitled Modified Nucleosides, Nucleotides,

and Nucleic Acids, and Uses Thereof and International Publication No. PCT/US2012/69610, filed December 14, 2012, entitled Modified Nucleoside, Nucleotide, and Nucleic Acid Compositions.

[0004] The instant application is also related to co-pending applications, each filed concurrently herewith on March 9, 2013 and having Attorney Docket Number M301.20, (PCT/US13/XXXXX) entitled Modified Polynucleotides; Attorney Docket Number M304.20 (PCT/US13/XXXXX), entitled Modified Polynucleotides for the Production of Secreted Proteins; Attorney Docket Number M305.20 (PCT/US13/XXXXX), entitled Modified Polynucleotides for the Production of Membrane Proteins; Attorney Docket Number M306.20 (PCT/US13/XXXXX), entitled Modified Polynucleotides for the Production of Cytoplasmic and Cytoskeletal Proteins; Attorney Docket Number M308.20 (PCT/US13/XXXXX), entitled Modified Polynucleotides for the Production of Nuclear Proteins; Attorney Docket Number M309.20 (PCT/US13/XXXXX), entitled Modified Polynucleotides for the Production of Proteins; Attorney Docket Number M310.20 (PCT/US13/XXXXX), entitled Modified Polynucleotides for the Production of Proteins Associated with Human Disease; Attorney Docket Number MNC1.20 (PCT/US13/XXXXX), entitled Modified Polynucleotides for the Production of Cosmetic Proteins and Peptides and Attorney Docket Number MNC2.20 (PCT/US13/XXXXX), entitled Modified Polynucleotides for the Production of Oncology-Related Proteins and Peptides, the contents of each of which are herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

[0005] The invention relates to compositions, methods, processes, kits and devices for the design, preparation, manufacture and/or formulation of polynucleotides, primary constructs and modified mRNA molecules (mmRNA).

BACKGROUND OF THE INVENTION

[0006] There are multiple problems with prior methodologies of effecting protein expression. For example, introduced DNA can integrate into host cell genomic DNA at some frequency, resulting in alterations and/or damage to the host cell genomic DNA. Alternatively, the heterologous deoxyribonucleic acid (DNA) introduced into a cell can be inherited by daughter cells (whether or not the heterologous DNA has integrated into

the chromosome) or by offspring. In addition, assuming proper delivery and no damage or integration into the host genome, there are multiple steps which must occur before the encoded protein is made. Once inside the cell, DNA must be transported into the nucleus where it is transcribed into RNA. The RNA transcribed from DNA must then enter the cytoplasm where it is translated into protein. Not only do the multiple processing steps from administered DNA to protein create lag times before the generation of the functional protein, each step represents an opportunity for error and damage to the cell. Further, it is known to be difficult to obtain DNA expression in cells as DNA frequently enters a cell but is not expressed or not expressed at reasonable rates or concentrations. This can be a particular problem when DNA is introduced into primary cells or modified cell lines.

[0007] In the early 1990's Bloom and colleagues successfully rescued vasopressin-deficient rats by injecting in vitro-transcribed vasopressin mRNA into the hypothalamus (Science 255: 996–998; 1992). However, the low levels of translation and the immunogenicity of the molecules hampered the development of mRNA as a therapeutic and efforts have since focused on alternative applications that could instead exploit these pitfalls, i.e. immunization with mRNAs coding for cancer antigens.

[0008] Others have investigated the use of mRNA to deliver a polypeptide of interest and shown that certain chemical modifications of mRNA molecules, particularly pseudouridine and 5-methyl-cytosine, have reduced immunostimulatory effect.

[0009] These studies are disclosed in, for example, Ribostem Limited in United Kingdom patent application serial number 0316089.2 filed on July 9, 2003 now abandoned, PCT application number PCT/GB2004/002981 filed on July 9, 2004 published as WO2005005622, United States patent application national phase entry serial number 10/563,897 filed on June 8, 2006 published as US20060247195 now abandoned, and European patent application national phase entry serial number EP2004743322 filed on July 9, 2004 published as EP1646714 now withdrawn; Novozymes, Inc. in PCT application number PCT/US2007/88060 filed on December 19, 2007 published as WO2008140615, United States patent application national phase entry serial number 12/520,072 filed on July 2, 2009 published as US20100028943 and European patent application national phase entry serial number EP2007874376 filed on July 7, 2009

published as EP2104739; University of Rochester in PCT application number PCT/US2006/46120 filed on December 4, 2006 published as WO2007064952 and United States patent application serial number 11/606,995 filed on December 1, 2006 published as US20070141030; BioNTech AG in European patent application serial number EP2007024312 filed December 14, 2007 now abandoned, PCT application number PCT/EP2008/01059 filed on December 12, 2008 published as WO2009077134, European patent application national phase entry serial number EP2008861423 filed on June 2, 2010 published as EP2240572, United States patent application national phase entry serial number 12/,735,060 filed November 24, 2010 published as US20110065103, German patent application serial number DE 10 2005 046 490 filed September 28, 2005, PCT application PCT/EP2006/0448 filed September 28, 2006 published as WO2007036366, national phase European patent EP1934345 published March, 21, 2012 and national phase US patent application serial number 11/992,638 filed August 14, 2009 published as 20100129877; Immune Disease Institute Inc. in United States patent application serial number 13/088,009 filed April 15, 2011 published as US20120046346 and PCT application PCT/US2011/32679 filed April 15, 2011 published as WO20110130624; Shire Human Genetic Therapeutics in United States patent application serial number 12/957,340 filed on November 20, 2010 published as US20110244026; Sequitur Inc. in PCT application PCT/US1998/019492 filed on September 18, 1998 published as WO1999014346; The Scripps Research Institute in PCT application number PCT/US2010/00567 filed on February 24, 2010 published as WO2010098861, and United States patent application national phase entry serial number 13/203,229 filed November 3, 2011 published as US20120053333; Ludwig-Maximillians University in PCT application number PCT/EP2010/004681 filed on July 30, 2010 published as WO2011012316; Cellscript Inc. in United States patent number 8,039,214 filed June 30, 2008 and granted October 18, 2011, United States patent application serial numbers 12/962,498 filed on December 7, 2010 published as US20110143436, 12/962,468 filed on December 7, 2010 published as US20110143397, 13/237,451 filed on September 20, 2011 published as US20120009649, and PCT applications PCT/US2010/59305 filed December 7, 2010 published as WO2011071931 and PCT/US2010/59317 filed on December 7, 2010 published as WO2011071936; The Trustees of the University of

Pennsylvania in PCT application number PCT/US2006/32372 filed on August 21, 2006 published as WO2007024708, and United States patent application national phase entry serial number 11/990,646 filed on March 27, 2009 published as US20090286852; Curevac GMBH in German patent application serial numbers DE10 2001 027 283.9 filed June 5, 2001, DE10 2001 062 480.8 filed December 19, 2001, and DE 20 2006 051 516 filed October 31, 2006 all abandoned, European patent numbers EP1392341 granted March 30, 2005 and EP1458410 granted January 2, 2008, PCT application numbers PCT/EP2002/06180 filed June 5, 2002 published as WO2002098443, PCT/EP2002/14577 filed on December 19, 2002 published as WO2003051401, PCT/EP2007/09469 filed on December 31, 2007 published as WO2008052770, PCT/EP2008/03033 filed on April 16, 2008 published as WO2009127230, PCT/EP2006/004784 filed on May 19, 2005 published as WO2006122828, PCT/EP2008/00081 filed on January 9, 2007 published as WO2008083949, and United States patent application serial numbers 10/729,830 filed on December 5, 2003 published as US20050032730, 10/870,110 filed on June 18, 2004 published as US20050059624, 11/914,945 filed on July 7, 2008 published as US20080267873, 12/446,912 filed on October 27, 2009 published as US2010047261 now abandoned, 12/522,214 filed on January 4, 2010 published as US20100189729, 12/787,566 filed on May 26, 2010 published as US20110077287, 12/787,755 filed on May 26, 2010 published as US20100239608, 13/185,119 filed on July 18, 2011 published as US20110269950, and 13/106,548 filed on May 12, 2011 published as US20110311472 all of which are herein incorporated by reference in their entirety.

[00010] Notwithstanding these reports which are limited to a selection of chemical modifications including pseudouridine and 5-methyl-cytosine, there remains a need in the art for therapeutic modalities to address the myriad of barriers surrounding the efficacious modulation of intracellular translation and processing of nucleic acids encoding polypeptides or fragments thereof.

[00011] To this end, the inventors have shown that certain modified mRNA sequences have the potential as therapeutics with benefits beyond just evading, avoiding or diminishing the immune response. Such studies are detailed in published co-pending applications International Application PCT/US2011/046861 filed August 5, 2011 and

PCT/US2011/054636 filed October 3, 2011, International Application number PCT/US2011/054617 filed October 3, 2011, the contents of which are incorporated herein by reference in their entirety.

[00012] The present invention addresses this need by providing nucleic acid based compounds or polynucleotides which encode a polypeptide of interest (e.g., modified mRNA or mmRNA) and which have structural and/or chemical features that avoid one or more of the problems in the art, for example, features which are useful for optimizing formulation and delivery of nucleic acid-based therapeutics while retaining structural and functional integrity, overcoming the threshold of expression, improving expression rates, half life and/or protein concentrations, optimizing protein localization, and avoiding deleterious bio-responses such as the immune response and/or degradation pathways.

SUMMARY OF THE INVENTION

[00013] Described herein are compositions, methods, processes, kits and devices for the design, preparation, manufacture and/or formulation of modified mRNA (mmRNA) molecules.

[00014] The details of various embodiments of the invention are set forth in the description below. Other features, objects, and advantages of the invention will be apparent from the description and the drawings, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[00015] The foregoing and other objects, features and advantages will be apparent from the following description of particular embodiments of the invention, as illustrated in the accompanying drawings in which like reference characters refer to the same parts throughout the different views. The drawings are not necessarily to scale, emphasis instead being placed upon illustrating the principles of various embodiments of the invention.

[00016] FIG. 1 is a schematic of a primary construct of the present invention.

[00017] FIG. 2 illustrates lipid structures in the prior art useful in the present invention. Shown are the structures for 98N12-5 (TETA5-LAP), DLin-DMA, DLin-K-DMA (2,2-Dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane), DLin-KC2-DMA, DLin-MC3-DMA and C12-200.

[00018] FIG. 3 is a representative plasmid useful in the IVT reactions taught herein. The plasmid contains Insert 64818, designed by the instant inventors.

[00019] FIG. 4 is a gel profile of modified mRNA encapsulated in PLGA microspheres.

[00020] FIG. 5 is a histogram of Factor IX protein production PLGA formulation Factor IX modified mRNA.

[00021] FIG. 6 is a histogram showing VEGF protein production in human keratinocyte cells after transfection of modified mRNA at a range of doses. Figure 6A shows protein production after transfection of modified mRNA comprising natural nucleoside triphosphate (NTP). Figure 6B shows protein production after transfection of modified mRNA fully modified with pseudouridine (Pseudo-U) and 5-methylcytosine (5mC). Figure 6C shows protein production after transfection of modified mRNA fully modified with N1-methyl-pseudouridine (N1-methyl-Pseudo-U) and 5-methylcytosine (5mC).

[00022] FIG. 7 is a histogram of VEGF protein production in HEK293 cells.

[00023] FIG. 8 is a histogram of VEGF expression and IFN-alpha induction after transfection of VEGF modified mRNA in peripheral blood mononuclear cells (PBMC). Figure 8A shows VEGF expression. Figure 8B shows IFN-alpha induction.

[00024] FIG. 9 is a histogram of VEGF protein production in HeLa cells from VEGF modified mRNA.

[00025] FIG. 10 is a histogram of VEGF protein production from lipoplexed VEGF modified mRNA in mice.

[00026] FIG. 11 is a histogram of G-CSF protein production in HeLa cells from G-CSF modified mRNA.

[00027] FIG. 12 is a histogram of G-CSF protein production in mice from lipoplexedG-CSF modified mRNA.

[00028] FIG. 13 is a histogram of Factor IX protein production in HeLa cell supernatant from Factor IX modified mRNA.

[00029] FIG. 14 is a histogram of APOA1 protein production in HeLa cells from APOA1 wild-type modified mRNA, APOA1 Milano modified mRNA or APOA1 Paris modified mRNA.

[00030] FIG. 15 is a gel profile of APOA1 protein from APOA1 wild-type modified mRNA.

[00031] FIG. 16 is a gel profile of APOA1 protein from APOA1 Paris modified mRNA.

[00032] FIG. 17 is a gel profile of APOA1 protein from APOA1 Milano modified mRNA.

[00033] FIG. 18 is a gel profile of Fibrinogen alpha (FGA) protein from FGA modified mRNA.

[00034] FIG. 19 is a histogram of Plasminogen protein production in HeLa cell supernatant from Plasminogen modified mRNA.

[00035] FIG. 20 is a gel profile of Plasminogen protein from Plasminogen modified mRNA.

[00036] FIG. 21 is a gel profile of galactose-1-phosphate uridylyltransferase (GALT) protein from GALT modified mRNA.

[00037] FIG. 22 is a gel profile of argininosuccinate lyase (ASL) protein from ASL modified mRNA.

[00038] FIG. 23 is a gel profile of tyrosine aminotransferase (TAT) protein from TAT modified mRNA.

[00039] FIG. 24 is a gel profile of glucan (1,4-alpha-), branching enzyme 1 (GBE1) protein from GBE1 modified mRNA.

[00040] FIG. 25 is a histogram of Prothrombin protein production in HeLa cell supernatant from Prothrombin modified mRNA.

[00041] FIG. 26 is a histogram of Prothrombin protein production in HeLa cell supernatant from Prothrombin modified mRNA.

[00042] FIG. 27 is a gel profile of ceruloplasmin (CP or CLP) protein from CP modified mRNA.

[00043] FIG. 28 is a histogram of transforming growth factor beta 1 (TGF-beta1) protein production in HeLa cell supernatant from TGF-beta1 modified mRNA.

[00044] FIG. 29 is a gel profile of ornithine carbamoyltransferase (OTC) protein from OTC modified mRNA.

[00045] FIG. 30 is a flow cytometry plot of low density lipoprotein receptor (LDLR) modified mRNA.

[00046] FIG. 31 is a gel profile of UDP glucuronosyltransferase 1 family, polypeptide A1 (UGT1A1) protein from UGT1A1 modified mRNA.

[00047] FIG. 32 is a histogram of Factor XI protein production in HEK293 cells.

[00048] FIG. 33 is a gel profile of Aquaporin-5 protein from Aquaporin-5 modified mRNA.

[00049] FIG. 34 is a histogram of Factor VII protein production in HeLa cells from Factor VII modified mRNA.

[00050] FIG. 35 is a histogram of Insulin Glargine protein production in HeLa cells from Insulin Glargine modified mRNA.

[00051] FIG. 36 is a histogram of Tissue Factor protein production in HeLa cells from Tissue Factor modified mRNA.

[00052] FIG. 37 is a histogram of Factor XI protein production in HeLa cells from Factor XI modified mRNA.

[00053] FIG. 38 is a histogram of Factor XI protein production in HeLa cell supernatant from Factor XI modified mRNA.

[00054] FIG. 39 is a histogram of Insulin Aspart protein production in HeLa cells from Insulin Aspart modified mRNA.

[00055] FIG. 40 is a histogram of Insulin Lispro protein production in HeLa cells from Insulin Lispro modified mRNA.

[00056] FIG. 41 is a histogram of Insulin Glulisine protein production in HeLa cells from Insulin Glulisine modified mRNA.

[00057] FIG. 42 is a histogram of human growth hormone protein production in HeLa cells from human growth hormone modified mRNA.

[00058] FIG. 43 is a gel profile of tumor protein 53 (p53) protein from p53 modified mRNA. Figure 43A shows the expected size of p53. Figure 43B shows the expected size of p53.

[00059] FIG. 44 is a gel profile of tuftelin (TUFT1) protein from TUFT1 modified mRNA.

[00060] FIG. 45 is a gel profile of galactokinase 1 (GALK1) protein from GALK1 modified mRNA. Figure 45A shows the expected size of GALK1. Figure 45B shows the expected size of GALK1.

[00061] FIG. 46 is a gel profile of defensin, beta 103A (DEFB103A) protein from DEFB103A modified mRNA.

[00062] FIG. 47 is a flow cytometry plot of LDLR modified mRNA.

[00063] FIG. 48 is a histogram of vascular endothelial growth factor expression in HeLa.

[00064] FIG. 49 is a histogram of the cell vitality of HeLa cells transfected with vascular endothelial growth factor mRNA.

[00065] FIG. 50 is a histogram of Insulin Aspart protein expression.

[00066] FIG. 51 is a histogram of Insulin Glargine protein expression.

[00067] FIG. 52 is a histogram of Insulin Glulisine protein expression.

[00068] FIG. 53 is a histogram of Interleukin 7 (IL-7) protein expression.

[00069] FIG. 54 is a histogram of Erythropoietin (EPO) protein expression.

[00070] FIG. 55 is a gel profile of Lysosomal Acid Lipase protein from Lysosomal Acid Lipase modified mRNA.

[00071] FIG. 56 is a gel profile of Glucocerebrosidase protein from Glucocerebrosidase modified mRNA.

[00072] FIG. 57 is a gel profile of Iduronate 2-Sulfatase protein from Iduronate 2-Sulfatase modified mRNA.

[00073] FIG. 58 is a gel profile of Luciferase protein from Luciferase modified mRNA.

[00074] FIG. 59 is a histogram of IgG concentration after administration with formulated Herceptin modified mRNA in mammals.

[00075] FIG. 60 is a histogram of IgG concentration (in ng/ml) after transfection with Herceptin modified mRNA.

[00076] FIG. 61 is a gel profile of Herceptin protein from Herceptin modified mRNA.

[00077] FIG. 62 is a histogram of Glucocerebrosidase enzyme activity.

[00078] FIG. 63 is a histogram of Lysosomal Acid Lipase enzyme activity.

[00079] FIG. 64 a is histogram of Factor VIII protein expression.

[00080] FIG. 65 is a histogram of Factor VIII Chromogenic Activity.

[00081] FIG. 66 is a graph of LDLR Expression. Figure 66A shows LDL Receptor Expression of cells compared to LDLR mRNA added. Figure 66B shows LDL Receptor Expression of cells post transfection. Figure 66C shows the saturation of BODIPY® labeled LRL. Figure 66D shows the binding affinity of BODIPY-LDL to cells.

[00082] FIG. 67 is a graph showing the percent positive cells for UGT1A1 Expression.

[00083] FIG. 68 is a graph showing UGT1A1 protein accumulation.

[00084] FIG. 69 is a gel profile of UGT1A1 protein and OTC from eith UGT1A1 or OTC modified mRNA.

[00085] FIG. 70 is a flow cytometry plot of HEK293 cells transfected with PAh or UGT1A1.

[00086] FIG. 71 is a gel profile of UGT1A1 protein from UGT1A1 modified mRNA.

[00087] FIG. 72 is a gel profile of microsomal extracts of mice treated with LNPs containing UGT1A1.

DETAILED DESCRIPTION

[00088] It is of great interest in the fields of therapeutics, diagnostics, reagents and for biological assays to be able to deliver a nucleic acid, e.g., a ribonucleic acid (RNA) inside a cell, whether *in vitro*, *in vivo, in situ* or *ex vivo*, such as to cause intracellular translation of the nucleic acid and production of an encoded polypeptide of interest. Of particular importance is the delivery and function of a non-integrative polynucleotide.

[00089] Described herein are compositions (including pharmaceutical compositions) and methods for the design, preparation, manufacture and/or formulation of polynucleotides encoding one or more polypeptides of interest. Also provided are systems, processes, devices and kits for the selection, design and/or utilization of the polynucleotides encoding the polypeptides of interest described herein.

[00090] According to the present invention, these polynucleotides are preferably modified as to avoid the deficiencies of other polypeptide-encoding molecules of the art. Hence these polynucleotides are referred to as modified mRNA or mmRNA.

[00091] The use of modified polynucleotides in the fields of antibodies, viruses, veterinary applications and a variety of *in vivo* settings has been explored by the

inventors and these studies are disclosed in for example, co-pending and co-owned United States provisional patent application serial numbers 61/470,451 filed March 31, 2011 teaching in vivo applications of mmRNA; 61/517,784 filed on April 26, 2011 teaching engineered nucleic acids for the production of antibody polypeptides; 61/519,158 filed May 17, 2011 teaching veterinary applications of mmRNA technology; 61/533, 537 filed on September 12, 2011 teaching antimicrobial applications of mmRNA technology; 61/533,554 filed on September 12, 2011 teaching viral applications of mmRNA technology, 61/542,533 filed on October 3, 2011 teaching various chemical modifications for use in mmRNA technology; 61/570,690 filed on December 14, 2011 teaching mobile devices for use in making or using mmRNA technology; 61/570,708 filed on December 14, 2011 teaching the use of mmRNA in acute care situations; 61/576,651 filed on December 16, 2011 teaching terminal modification architecture for mmRNA; 61/576,705 filed on December 16, 2011 teaching delivery methods using lipidoids for mmRNA; 61/578,271 filed on December 21, 2011 teaching methods to increase the viability of organs or tissues using mmRNA; 61/581,322 filed on December 29, 2011 teaching mmRNA encoding cell penetrating peptides; 61/581,352 filed on December 29, 2011 teaching the incorporation of cytotoxic nucleosides in mmRNA and 61/631,729 filed on January 10, 2012 teaching methods of using mmRNA for crossing the blood brain barrier; all of which are herein incorporated by reference in their entirety.

[00092] Provided herein, in part, are polynucleotides, primary constructs and/or mmRNA encoding polypeptides of interest which have been designed to improve one or more of the stability and/or clearance in tissues, receptor uptake and/or kinetics, cellular access by the compositions, engagement with translational machinery, mRNA half-life, translation efficiency, immune evasion, protein production capacity, secretion efficiency (when applicable), accessibility to circulation, protein half-life and/or modulation of a cell's status, function and/or activity.

I. Compositions of the Invention (mmRNA)

[00093] The present invention provides nucleic acid molecules, specifically polynucleotides, primary constructs and/or mmRNA which encode one or more polypeptides of interest. The term "nucleic acid," in its broadest sense, includes any compound and/or substance that comprise a polymer of nucleotides. These polymers are

often referred to as polynucleotides. Exemplary nucleic acids or polynucleotides of the invention include, but are not limited to, ribonucleic acids (RNAs), deoxyribonucleic acids (DNAs), threose nucleic acids (TNAs), glycol nucleic acids (GNAs), peptide nucleic acids (PNAs), locked nucleic acids (LNAs, including LNA having a β- D-ribo configuration, α-LNA having an α-L-ribo configuration (a diastereomer of LNA), 2′-amino-LNA having a 2′-amino functionalization, and 2′-amino- α-LNA having a 2′-amino functionalization) or hybrids thereof.

[00094] In preferred embodiments, the nucleic acid molecule is a messenger RNA (mRNA). As used herein, the term "messenger RNA" (mRNA) refers to any polynucleotide which encodes a polypeptide of interest and which is capable of being translated to produce the encoded polypeptide of interest *in vitro, in vivo, in situ* or *ex vivo*.

[00095] Traditionally, the basic components of an mRNA molecule include at least a coding region, a 5′UTR, a 3′UTR, a 5′ cap and a poly-A tail. Building on this wild type modular structure, the present invention expands the scope of functionality of traditional mRNA molecules by providing polynucleotides or primary RNA constructs which maintain a modular organization, but which comprise one or more structural and/or chemical modifications or alterations which impart useful properties to the polynucleotide including, in some embodiments, the lack of a substantial induction of the innate immune response of a cell into which the polynucleotide is introduced. As such, modified mRNA molecules of the present invention are termed "mmRNA." As used herein, a "structural" feature or modification is one in which two or more linked nucleotides are inserted, deleted, duplicated, inverted or randomized in a polynucleotide, primary construct or mmRNA without significant chemical modification to the nucleotides themselves. Because chemical bonds will necessarily be broken and reformed to effect a structural modification, structural modifications are of a chemical nature and hence are chemical modifications. However, structural modifications will result in a different sequence of nucleotides. For example, the polynucleotide "ATCG" may be chemically modified to "AT-5meC-G". The same polynucleotide may be structurally modified from "ATCG" to "ATCCCG". Here, the dinucleotide "CC" has been inserted, resulting in a structural modification to the polynucleotide.

mmRNA Architecture

[00096] The mmRNA of the present invention are distinguished from wild type mRNA in their functional and/or structural design features which serve to, as evidenced herein, overcome existing problems of effective polypeptide production using nucleic acid-based therapeutics.

[00097] Figure 1 shows a representative polynucleotide primary construct **100** of the present invention. As used herein, the term "primary construct" or "primary mRNA construct" refers to a polynucleotide transcript which encodes one or more polypeptides of interest and which retains sufficient structural and/or chemical features to allow the polypeptide of interest encoded therein to be translated. Primary constructs may be polynucleotides of the invention. When structurally or chemically modified, the primary construct may be referred to as an mmRNA.

[00098] Returning to FIG. **1**, the primary construct **100** here contains a first region of linked nucleotides **102** that is flanked by a first flanking region **104** and a second flaking region **106**. As used herein, the "first region" may be referred to as a "coding region" or "region encoding" or simply the "first region." This first region may include, but is not limited to, the encoded polypeptide of interest. The polypeptide of interest may comprise at its 5' terminus one or more signal sequences encoded by a signal sequence region **103**. The flanking region **104** may comprise a region of linked nucleotides comprising one or more complete or incomplete 5′ UTRs sequences. The flanking region **104** may also comprise a 5′ terminal cap **108**. The second flanking region **106** may comprise a region of linked nucleotides comprising one or more complete or incomplete 3′ UTRs. The flanking region **106** may also comprise a 3′ tailing sequence **110**.

[00099] Bridging the 5′ terminus of the first region **102** and the first flanking region **104** is a first operational region **105**. Traditionally this operational region comprises a Start codon. The operational region may alternatively comprise any translation initiation sequence or signal including a Start codon.

[000100] Bridging the 3′ terminus of the first region **102** and the second flanking region **106** is a second operational region **107**. Traditionally this operational region comprises a Stop codon. The operational region may alternatively comprise any translation initiation

sequence or signal including a Stop codon. According to the present invention, multiple serial stop codons may also be used.

[000101] Generally, the shortest length of the first region of the primary construct of the present invention can be the length of a nucleic acid sequence that is sufficient to encode for a dipeptide, a tripeptide, a tetrapeptide, a pentapeptide, a hexapeptide, a heptapeptide, an octapeptide, a nonapeptide, or a decapeptide. In another embodiment, the length may be sufficient to encode a peptide of 2-30 amino acids, e.g. 5-30, 10-30, 2-25, 5-25, 10-25, or 10-20 amino acids. The length may be sufficient to encode for a peptide of at least 11, 12, 13, 14, 15, 17, 20, 25 or 30 amino acids, or a peptide that is no longer than 40 amino acids, e.g. no longer than 35, 30, 25, 20, 17, 15, 14, 13, 12, 11 or 10 amino acids. Examples of dipeptides that the polynucleotide sequences can encode or include, but are not limited to, carnosine and anserine.

[000102] Generally, the length of the first region encoding the polypeptide of interest of the present invention is greater than about 30 nucleotides in length (e.g., at least or greater than about 35, 40, 45, 50, 55, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1,000, 1,100, 1,200, 1,300, 1,400, 1,500, 1,600, 1,700, 1,800, 1,900, 2,000, 2,500, and 3,000, 4,000, 5,000, 6,000, 7,000, 8,000, 9,000, 10,000, 20,000, 30,000, 40,000, 50,000, 60,000, 70,000, 80,000, 90,000 or up to and including 100,000 nucleotides). As used herein, the "first region" may be referred to as a "coding region" or "region encoding" or simply the "first region."

[000103] In some embodiments, the polynucleotide, primary construct, or mmRNA includes from about 30 to about 100,000 nucleotides (e.g., from 30 to 50, from 30 to 100, from 30 to 250, from 30 to 500, from 30 to 1,000, from 30 to 1,500, from 30 to 3,000, from 30 to 5,000, from 30 to 7,000, from 30 to 10,000, from 30 to 25,000, from 30 to 50,000, from 30 to 70,000, from 100 to 250, from 100 to 500, from 100 to 1,000, from 100 to 1,500, from 100 to 3,000, from 100 to 5,000, from 100 to 7,000, from 100 to 10,000, from 100 to 25,000, from 100 to 50,000, from 100 to 70,000, from 100 to 100,000, from 500 to 1,000, from 500 to 1,500, from 500 to 2,000, from 500 to 3,000, from 500 to 5,000, from 500 to 7,000, from 500 to 10,000, from 500 to 25,000, from 500 to 50,000, from 500 to 70,000, from 500 to 100,000, from 1,000 to 1,500, from 1,000 to 2,000, from 1,000 to 3,000, from 1,000 to 5,000, from 1,000 to 7,000, from 1,000 to

10,000, from 1,000 to 25,000, from 1,000 to 50,000, from 1,000 to 70,000, from 1,000 to 100,000, from 1,500 to 3,000, from 1,500 to 5,000, from 1,500 to 7,000, from 1,500 to 10,000, from 1,500 to 25,000, from 1,500 to 50,000, from 1,500 to 70,000, from 1,500 to 100,000, from 2,000 to 3,000, from 2,000 to 5,000, from 2,000 to 7,000, from 2,000 to 10,000, from 2,000 to 25,000, from 2,000 to 50,000, from 2,000 to 70,000, and from 2,000 to 100,000).

[000104] According to the present invention, the first and second flanking regions may range independently from 15-1,000 nucleotides in length (e.g., greater than 30, 40, 45, 50, 55, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, and 900 nucleotides or at least 30, 40, 45, 50, 55, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, and 1,000 nucleotides).

[000105] According to the present invention, the tailing sequence may range from absent to 500 nucleotides in length (e.g., at least 60, 70, 80, 90, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, or 500 nucleotides). Where the tailing region is a polyA tail, the length may be determined in units of or as a function of polyA Binding Protein binding. In this embodiment, the polyA tail is long enough to bind at least 4 monomers of PolyA Binding Protein. PolyA Binding Protein monomers bind to stretches of approximately 38 nucleotides. As such, it has been observed that polyA tails of about 80 nucleotides and 160 nucleotides are functional.

[000106] According to the present invention, the capping region may comprise a single cap or a series of nucleotides forming the cap. In this embodiment the capping region may be from 1 to 10, e.g. 2-9, 3-8, 4-7, 1-5, 5-10, or at least 2, or 10 or fewer nucleotides in length. In some embodiments, the cap is absent.

[000107] According to the present invention, the first and second operational regions may range from 3 to 40, e.g., 5-30, 10-20, 15, or at least 4, or 30 or fewer nucleotides in length and may comprise, in addition to a Start and/or Stop codon, one or more signal and/or restriction sequences.

Cyclic mmRNA

[000108] According to the present invention, a primary construct or mmRNA may be cyclized, or concatemerized, to generate a translation competent molecule to assist interactions between poly-A binding proteins and 5′-end binding proteins. The

mechanism of cyclization or concatemerization may occur through at least 3 different routes: 1) chemical, 2) enzymatic, and 3) ribozyme catalyzed. The newly formed 5′-/3′-linkage may be intramolecular or intermolecular.

[000109] In the first route, the 5′-end and the 3′-end of the nucleic acid contain chemically reactive groups that, when close together, form a new covalent linkage between the 5′-end and the 3′-end of the molecule. The 5′-end may contain an NHS-ester reactive group and the 3′-end may contain a 3′-amino-terminated nucleotide such that in an organic solvent the 3′-amino-terminated nucleotide on the 3′-end of a synthetic mRNA molecule will undergo a nucleophilic attack on the 5′-NHS-ester moiety forming a new 5′-/3′-amide bond.

[000110] In the second route, T4 RNA ligase may be used to enzymatically link a 5′-phosphorylated nucleic acid molecule to the 3′-hydroxyl group of a nucleic acid forming a new phosphorodiester linkage. In an example reaction, 1μg of a nucleic acid molecule is incubated at 37°C for 1 hour with 1-10 units of T4 RNA ligase (New England Biolabs, Ipswich, MA) according to the manufacturer's protocol. The ligation reaction may occur in the presence of a split oligonucleotide capable of base-pairing with both the 5′- and 3′-region in juxtaposition to assist the enzymatic ligation reaction.

[000111] In the third route, either the 5′-or 3′-end of the cDNA template encodes a ligase ribozyme sequence such that during *in vitro* transcription, the resultant nucleic acid molecule can contain an active ribozyme sequence capable of ligating the 5′-end of a nucleic acid molecule to the 3′-end of a nucleic acid molecule. The ligase ribozyme may be derived from the Group I Intron, Group I Intron, Hepatitis Delta Virus, Hairpin ribozyme or may be selected by SELEX (systematic evolution of ligands by exponential enrichment). The ribozyme ligase reaction may take 1 to 24 hours at temperatures between 0 and 37°C.

mmRNA Multimers

[000112] According to the present invention, multiple distinct polynucleotides, primary constructs or mmRNA may be linked together through the 3′-end using nucleotides which are modified at the 3′-terminus. Chemical conjugation may be used to control the stoichiometry of delivery into cells. For example, the glyoxylate cycle enzymes, isocitrate lyase and malate synthase, may be supplied into HepG2 cells at a 1:1 ratio to

alter cellular fatty acid metabolism. This ratio may be controlled by chemically linking polynucleotides, primary constructs or mmRNA using a 3′-azido terminated nucleotide on one polynucleotide, primary construct or mmRNA species and a C5-ethynyl or alkynyl-containing nucleotide on the opposite polynucleotide, primary construct or mmRNA species. The modified nucleotide is added post-transcriptionally using terminal transferase (New England Biolabs, Ipswich, MA) according to the manufacturer's protocol. After the addition of the 3′-modified nucleotide, the two polynucleotide, primary construct or mmRNA species may be combined in an aqueous solution, in the presence or absence of copper, to form a new covalent linkage via a click chemistry mechanism as described in the literature.

[000113] In another example, more than two polynucleotides may be linked together using a functionalized linker molecule. For example, a functionalized saccharide molecule may be chemically modified to contain multiple chemical reactive groups (SH-, NH_2-, N_3, etc…) to react with the cognate moiety on a 3′-functionalized mRNA molecule (i.e., a 3′-maleimide ester, 3′-NHS-ester, alkynyl). The number of reactive groups on the modified saccharide can be controlled in a stoichiometric fashion to directly control the stoichiometric ratio of conjugated polynucleotide, primary construct or mmRNA.

mmRNA Conjugates and Combinations

[000114] In order to further enhance protein production, primary constructs or mmRNA of the present invention can be designed to be conjugated to other polynucleotides, dyes, intercalating agents (*e.g.* acridines), cross-linkers (*e.g.* psoralene, mitomycin C), porphyrins (TPPC4, texaphyrin, Sapphyrin), polycyclic aromatic hydrocarbons (*e.g.*, phenazine, dihydrophenazine), artificial endonucleases (*e.g.* EDTA), alkylating agents, phosphate, amino, mercapto, PEG (*e.g.*, PEG-40K), MPEG, $[MPEG]_2$, polyamino, alkyl, substituted alkyl, radiolabeled markers, enzymes, haptens (*e.g.* biotin), transport/absorption facilitators (*e.g.*, aspirin, vitamin E, folic acid), synthetic ribonucleases, proteins, e.g., glycoproteins, or peptides, e.g., molecules having a specific affinity for a co-ligand, or antibodies *e.g.*, an antibody, that binds to a specified cell type such as a cancer cell, endothelial cell, or bone cell, hormones and hormone receptors, non-peptidic species, such as lipids, lectins, carbohydrates, vitamins, cofactors, or a drug.

[000115] Conjugation may result in increased stability and/or half life and may be particularly useful in targeting the polynucleotides, primary constructs or mmRNA to specific sites in the cell, tissue or organism.

[000116] According to the present invention, the mmRNA or primary constructs may be administered with, or further encode one or more of RNAi agents, siRNAs, shRNAs, miRNAs, miRNA binding sites, antisense RNAs, ribozymes, catalytic DNA, tRNA, RNAs that induce triple helix formation, aptamers or vectors, and the like.

Bifunctional mmRNA

[000117] In one embodiment of the invention are bifunctional polynucleotides (e.g., bifunctional primary constructs or bifunctional mmRNA). As the name implies, bifunctional polynucleotides are those having or capable of at least two functions. These molecules may also by convention be referred to as multi-functional.

[000118] The multiple functionalities of bifunctional polynucleotides may be encoded by the RNA (the function may not manifest until the encoded product is translated) or may be a property of the polynucleotide itself. It may be structural or chemical. Bifunctional modified polynucleotides may comprise a function that is covalently or electrostatically associated with the polynucleotides. Further, the two functions may be provided in the context of a complex of a mmRNA and another molecule.

[000119] Bifunctional polynucleotides may encode peptides which are anti-proliferative. These peptides may be linear, cyclic, constrained or random coil. They may function as aptamers, signaling molecules, ligands or mimics or mimetics thereof. Anti-proliferative peptides may, as translated, be from 3 to 50 amino acids in length. They may be 5-40, 10-30, or approximately 15 amino acids long. They may be single chain, multichain or branched and may form complexes, aggregates or any multi-unit structure once translated.

Noncoding polynucleotides and primary constructs

[000120] As described herein, provided are polynucleotides and primary constructs having sequences that are partially or substantially not translatable, e.g., having a noncoding region. Such noncoding region may be the "first region" of the primary construct. Alternatively, the noncoding region may be a region other than the first region. Such molecules are generally not translated, but can exert an effect on protein production

by one or more of binding to and sequestering one or more translational machinery components such as a ribosomal protein or a transfer RNA (tRNA), thereby effectively reducing protein expression in the cell or modulating one or more pathways or cascades in a cell which in turn alters protein levels. The polynucleotide or primary construct may contain or encode one or more long noncoding RNA (lncRNA, or lincRNA) or portion thereof, a small nucleolar RNA (sno-RNA), micro RNA (miRNA), small interfering RNA (siRNA) or Piwi-interacting RNA (piRNA).

Polypeptides of interest

[000121] According to the present invention, the primary construct is designed to encode one or more polypeptides of interest or fragments thereof. A polypeptide of interest may include, but is not limited to, whole polypeptides, a plurality of polypeptides or fragments of polypeptides, which independently may be encoded by one or more nucleic acids, a plurality of nucleic acids, fragments of nucleic acids or variants of any of the aforementioned. As used herein, the term "polypeptides of interest" refer to any polypeptide which is selected to be encoded in the primary construct of the present invention. As used herein, "polypeptide" means a polymer of amino acid residues (natural or unnatural) linked together most often by peptide bonds. The term, as used herein, refers to proteins, polypeptides, and peptides of any size, structure, or function. In some instances the polypeptide encoded is smaller than about 50 amino acids and the polypeptide is then termed a peptide. If the polypeptide is a peptide, it will be at least about 2, 3, 4, or at least 5 amino acid residues long. Thus, polypeptides include gene products, naturally occurring polypeptides, synthetic polypeptides, homologs, orthologs, paralogs, fragments and other equivalents, variants, and analogs of the foregoing. A polypeptide may be a single molecule or may be a multi-molecular complex such as a dimer, trimer or tetramer. They may also comprise single chain or multichain polypeptides such as antibodies or insulin and may be associated or linked. Most commonly disulfide linkages are found in multichain polypeptides. The term polypeptide may also apply to amino acid polymers in which one or more amino acid residues are an artificial chemical analogue of a corresponding naturally occurring amino acid.

[000122] The term "polypeptide variant" refers to molecules which differ in their amino acid sequence from a native or reference sequence. The amino acid sequence variants

may possess substitutions, deletions, and/or insertions at certain positions within the amino acid sequence, as compared to a native or reference sequence. Ordinarily, variants will possess at least about 50% identity (homology) to a native or reference sequence, and preferably, they will be at least about 80%, more preferably at least about 90% identical (homologous) to a native or reference sequence.

[000123] In some embodiments "variant mimics" are provided. As used herein, the term "variant mimic" is one which contains one or more amino acids which would mimic an activated sequence. For example, glutamate may serve as a mimic for phosphoro-threonine and/or phosphoro-serine. Alternatively, variant mimics may result in deactivation or in an inactivated product containing the mimic, e.g., phenylalanine may act as an inactivating substitution for tyrosine; or alanine may act as an inactivating substitution for serine.

[000124] "Homology" as it applies to amino acid sequences is defined as the percentage of residues in the candidate amino acid sequence that are identical with the residues in the amino acid sequence of a second sequence after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology. Methods and computer programs for the alignment are well known in the art. It is understood that homology depends on a calculation of percent identity but may differ in value due to gaps and penalties introduced in the calculation.

[000125] By "homologs" as it applies to polypeptide sequences means the corresponding sequence of other species having substantial identity to a second sequence of a second species.

[000126] "Analogs" is meant to include polypeptide variants which differ by one or more amino acid alterations, e.g., substitutions, additions or deletions of amino acid residues that still maintain one or more of the properties of the parent or starting polypeptide.

[000127] The present invention contemplates several types of compositions which are polypeptide based including variants and derivatives. These include substitutional, insertional, deletion and covalent variants and derivatives. The term "derivative" is used synonymously with the term "variant" but generally refers to a molecule that has been modified and/or changed in any way relative to a reference molecule or starting molecule.

[000128] As such, mmRNA encoding polypeptides containing substitutions, insertions and/or additions, deletions and covalent modifications with respect to reference sequences, in particular the polypeptide sequences disclosed herein, are included within the scope of this invention. For example, sequence tags or amino acids, such as one or more lysines, can be added to the peptide sequences of the invention (e.g., at the N-terminal or C-terminal ends). Sequence tags can be used for peptide purification or localization. Lysines can be used to increase peptide solubility or to allow for biotinylation. Alternatively, amino acid residues located at the carboxy and amino terminal regions of the amino acid sequence of a peptide or protein may optionally be deleted providing for truncated sequences. Certain amino acids (e.g., C-terminal or N-terminal residues) may alternatively be deleted depending on the use of the sequence, as for example, expression of the sequence as part of a larger sequence which is soluble, or linked to a solid support.

[000129] "Substitutional variants" when referring to polypeptides are those that have at least one amino acid residue in a native or starting sequence removed and a different amino acid inserted in its place at the same position. The substitutions may be single, where only one amino acid in the molecule has been substituted, or they may be multiple, where two or more amino acids have been substituted in the same molecule.

[000130] As used herein the term "conservative amino acid substitution" refers to the substitution of an amino acid that is normally present in the sequence with a different amino acid of similar size, charge, or polarity. Examples of conservative substitutions include the substitution of a non-polar (hydrophobic) residue such as isoleucine, valine and leucine for another non-polar residue. Likewise, examples of conservative substitutions include the substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, and between glycine and serine. Additionally, the substitution of a basic residue such as lysine, arginine or histidine for another, or the substitution of one acidic residue such as aspartic acid or glutamic acid for another acidic residue are additional examples of conservative substitutions. Examples of non-conservative substitutions include the substitution of a non-polar (hydrophobic) amino acid residue such as isoleucine, valine, leucine, alanine,

methionine for a polar (hydrophilic) residue such as cysteine, glutamine, glutamic acid or lysine and/or a polar residue for a non-polar residue.

[000131] "Insertional variants" when referring to polypeptides are those with one or more amino acids inserted immediately adjacent to an amino acid at a particular position in a native or starting sequence. "Immediately adjacent" to an amino acid means connected to either the alpha-carboxy or alpha-amino functional group of the amino acid.

[000132] "Deletional variants" when referring to polypeptides are those with one or more amino acids in the native or starting amino acid sequence removed. Ordinarily, deletional variants will have one or more amino acids deleted in a particular region of the molecule.

[000133] "Covalent derivatives" when referring to polypeptides include modifications of a native or starting protein with an organic proteinaceous or non-proteinaceous derivatizing agent, and/or post-translational modifications. Covalent modifications are traditionally introduced by reacting targeted amino acid residues of the protein with an organic derivatizing agent that is capable of reacting with selected side-chains or terminal residues, or by harnessing mechanisms of post-translational modifications that function in selected recombinant host cells. The resultant covalent derivatives are useful in programs directed at identifying residues important for biological activity, for immunoassays, or for the preparation of anti-protein antibodies for immunoaffinity purification of the recombinant glycoprotein. Such modifications are within the ordinary skill in the art and are performed without undue experimentation.

[000134] Certain post-translational modifications are the result of the action of recombinant host cells on the expressed polypeptide. Glutaminyl and asparaginyl residues are frequently post-translationally deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues may be present in the polypeptides produced in accordance with the present invention.

[000135] Other post-translational modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the alpha-amino groups of lysine, arginine, and histidine side chains (T. E. Creighton,

Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)).

[000136] "Features" when referring to polypeptides are defined as distinct amino acid sequence-based components of a molecule. Features of the polypeptides encoded by the mmRNA of the present invention include surface manifestations, local conformational shape, folds, loops, half-loops, domains, half-domains, sites, termini or any combination thereof.

[000137] As used herein when referring to polypeptides the term "surface manifestation" refers to a polypeptide based component of a protein appearing on an outermost surface.

[000138] As used herein when referring to polypeptides the term "local conformational shape" means a polypeptide based structural manifestation of a protein which is located within a definable space of the protein.

[000139] As used herein when referring to polypeptides the term "fold" refers to the resultant conformation of an amino acid sequence upon energy minimization. A fold may occur at the secondary or tertiary level of the folding process. Examples of secondary level folds include beta sheets and alpha helices. Examples of tertiary folds include domains and regions formed due to aggregation or separation of energetic forces. Regions formed in this way include hydrophobic and hydrophilic pockets, and the like.

[000140] As used herein the term "turn" as it relates to protein conformation means a bend which alters the direction of the backbone of a peptide or polypeptide and may involve one, two, three or more amino acid residues.

[000141] As used herein when referring to polypeptides the term "loop" refers to a structural feature of a polypeptide which may serve to reverse the direction of the backbone of a peptide or polypeptide. Where the loop is found in a polypeptide and only alters the direction of the backbone, it may comprise four or more amino acid residues. Oliva et al. have identified at least 5 classes of protein loops (J. Mol Biol 266 (4): 814-830; 1997). Loops may be open or closed. Closed loops or "cyclic" loops may comprise 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acids between the bridging moieties. Such bridging moieties may comprise a cysteine-cysteine bridge (Cys-Cys) typical in polypeptides

having disulfide bridges or alternatively bridging moieties may be non-protein based such as the dibromozylyl agents used herein.

[000142] As used herein when referring to polypeptides the term "half-loop" refers to a portion of an identified loop having at least half the number of amino acid resides as the loop from which it is derived. It is understood that loops may not always contain an even number of amino acid residues. Therefore, in those cases where a loop contains or is identified to comprise an odd number of amino acids, a half-loop of the odd-numbered loop will comprise the whole number portion or next whole number portion of the loop (number of amino acids of the loop/2+/-0.5 amino acids). For example, a loop identified as a 7 amino acid loop could produce half-loops of 3 amino acids or 4 amino acids (7/2=3.5+/-0.5 being 3 or 4).

[000143] As used herein when referring to polypeptides the term "domain" refers to a motif of a polypeptide having one or more identifiable structural or functional characteristics or properties (e.g., binding capacity, serving as a site for protein-protein interactions).

[000144] As used herein when referring to polypeptides the term "half-domain" means a portion of an identified domain having at least half the number of amino acid resides as the domain from which it is derived. It is understood that domains may not always contain an even number of amino acid residues. Therefore, in those cases where a domain contains or is identified to comprise an odd number of amino acids, a half-domain of the odd-numbered domain will comprise the whole number portion or next whole number portion of the domain (number of amino acids of the domain/2+/-0.5 amino acids). For example, a domain identified as a 7 amino acid domain could produce half-domains of 3 amino acids or 4 amino acids (7/2=3.5+/-0.5 being 3 or 4). It is also understood that sub-domains may be identified within domains or half-domains, these subdomains possessing less than all of the structural or functional properties identified in the domains or half domains from which they were derived. It is also understood that the amino acids that comprise any of the domain types herein need not be contiguous along the backbone of the polypeptide (i.e., nonadjacent amino acids may fold structurally to produce a domain, half-domain or subdomain).

[000145] As used herein when referring to polypeptides the terms "site" as it pertains to amino acid based embodiments is used synonymously with "amino acid residue" and "amino acid side chain." A site represents a position within a peptide or polypeptide that may be modified, manipulated, altered, derivatized or varied within the polypeptide based molecules of the present invention.

[000146] As used herein the terms "termini" or "terminus" when referring to polypeptides refers to an extremity of a peptide or polypeptide. Such extremity is not limited only to the first or final site of the peptide or polypeptide but may include additional amino acids in the terminal regions. The polypeptide based molecules of the present invention may be characterized as having both an N-terminus (terminated by an amino acid with a free amino group (NH2)) and a C-terminus (terminated by an amino acid with a free carboxyl group (COOH)). Proteins of the invention are in some cases made up of multiple polypeptide chains brought together by disulfide bonds or by non-covalent forces (multimers, oligomers). These sorts of proteins will have multiple N- and C-termini. Alternatively, the termini of the polypeptides may be modified such that they begin or end, as the case may be, with a non-polypeptide based moiety such as an organic conjugate.

[000147] Once any of the features have been identified or defined as a desired component of a polypeptide to be encoded by the primary construct or mmRNA of the invention, any of several manipulations and/or modifications of these features may be performed by moving, swapping, inverting, deleting, randomizing or duplicating. Furthermore, it is understood that manipulation of features may result in the same outcome as a modification to the molecules of the invention. For example, a manipulation which involved deleting a domain would result in the alteration of the length of a molecule just as modification of a nucleic acid to encode less than a full length molecule would.

[000148] Modifications and manipulations can be accomplished by methods known in the art such as, but not limited to, site directed mutagenesis. The resulting modified molecules may then be tested for activity using *in vitro* or *in vivo* assays such as those described herein or any other suitable screening assay known in the art.

[000149] According to the present invention, the polypeptides may comprise a consensus sequence which is discovered through rounds of experimentation. As used herein a "consensus" sequence is a single sequence which represents a collective population of sequences allowing for variability at one or more sites.

[000150] As recognized by those skilled in the art, protein fragments, functional protein domains, and homologous proteins are also considered to be within the scope of polypeptides of interest of this invention. For example, provided herein is any protein fragment (meaning a polypeptide sequence at least one amino acid residue shorter than a reference polypeptide sequence but otherwise identical) of a reference protein 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 or greater than 100 amino acids in length. In another example, any protein that includes a stretch of about 20, about 30, about 40, about 50, or about 100 amino acids which are about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, or about 100% identical to any of the sequences described herein can be utilized in accordance with the invention. In certain embodiments, a polypeptide to be utilized in accordance with the invention includes 2, 3, 4, 5, 6, 7, 8, 9, 10, or more mutations as shown in any of the sequences provided or referenced herein.

Encoded Polypeptides

[000151] The primary constructs or mmRNA of the present invention may be designed to encode polypeptides of interest selected from any of several target categories including, but not limited to, biologics, antibodies, vaccines, therapeutic proteins or peptides, cell penetrating peptides, secreted proteins, plasma membrane proteins, cytoplasmic or cytoskeletal proteins, intracellular membrane bound proteins, nuclear proteins, proteins associated with human disease, targeting moieties or those proteins encoded by the human genome for which no therapeutic indication has been identified but which nonetheless have utility in areas of research and discovery.

[000152] In one embodiment primary constructs or mmRNA may encode variant polypeptides which have a certain identity with a reference polypeptide sequence. As used herein, a "reference polypeptide sequence" refers to a starting polypeptide sequence. Reference sequences may be wild type sequences or any sequence to which reference is made in the design of another sequence. A "reference polypeptide sequence" may, e.g., be any one of SEQ ID NOs: 769-1392as disclosed herein, e.g., any of SEQ ID NOs 769,

770, 771, 772, 773, 774, 775, 776, 777, 778, 779, 780, 781, 782, 783, 784, 785, 786, 787, 788, 789, 790, 791, 792, 793, 794, 795, 796, 797, 798, 799, 800, 801, 802, 803, 804, 805, 806, 807, 808, 809, 810, 811, 812, 813, 814, 815, 816, 817, 818, 819, 820, 821, 822, 823, 824, 825, 826, 827, 828, 829, 830, 831, 832, 833, 834, 835, 836, 837, 838, 839, 840, 841, 842, 843, 844, 845, 846, 847, 848, 849, 850, 851, 852, 853, 854, 855, 856, 857, 858, 859, 860, 861, 862, 863, 864, 865, 866, 867, 868, 869, 870, 871, 872, 873, 874, 875, 876, 877, 878, 879, 880, 881, 882, 883, 884, 885, 886, 887, 888, 889, 890, 891, 892, 893, 894, 895, 896, 897, 898, 899, 900, 901, 902, 903, 904, 905, 906, 907, 908, 909, 910, 911, 912, 913, 914, 915, 916, 917, 918, 919, 920, 921, 922, 923, 924, 925, 926, 927, 928, 929, 930, 931, 932, 933, 934, 935, 936, 937, 938, 939, 940, 941, 942, 943, 944, 945, 946, 947, 948, 949, 950, 951, 952, 953, 954, 955, 956, 957, 958, 959, 960, 961, 962, 963, 964, 965, 966, 967, 968, 969, 970, 971, 972, 973, 974, 975, 976, 977, 978, 979, 980, 981, 982, 983, 984, 985, 986, 987, 988, 989, 990, 991, 992, 993, 994, 995, 996, 997, 998, 999, 1000, 1001, 1002, 1003, 1004, 1005, 1006, 1007, 1008, 1009, 1010, 1011, 1012, 1013, 1014, 1015, 1016, 1017, 1018, 1019, 1020, 1021, 1022, 1023, 1024, 1025, 1026, 1027, 1028, 1029, 1030, 1031, 1032, 1033, 1034, 1035, 1036, 1037, 1038, 1039, 1040, 1041, 1042, 1043, 1044, 1045, 1046, 1047, 1048, 1049, 1050, 1051, 1052, 1053, 1054, 1055, 1056, 1057, 1058, 1059, 1060, 1061, 1062, 1063, 1064, 1065, 1066, 1067, 1068, 1069, 1070, 1071, 1072, 1073, 1074, 1075, 1076, 1077, 1078, 1079, 1080, 1081, 1082, 1083, 1084, 1085, 1086, 1087, 1088, 1089, 1090, 1091, 1092, 1093, 1094, 1095, 1096, 1097, 1098, 1099, 1100, 1101, 1102, 1103, 1104, 1105, 1106, 1107, 1108, 1109, 1110, 1111, 1112, 1113, 1114, 1115, 1116, 1117, 1118, 1119, 1120, 1121, 1122, 1123, 1124, 1125, 1126, 1127, 1128, 1129, 1130, 1131, 1132, 1133, 1134, 1135, 1136, 1137, 1138, 1139, 1140, 1141, 1142, 1143, 1144, 1145, 1146, 1147, 1148, 1149, 1150, 1151, 1152, 1153, 1154, 1155, 1156, 1157, 1158, 1159, 1160, 1161, 1162, 1163, 1164, 1165, 1166, 1167, 1168, 1169, 1170, 1171, 1172, 1173, 1174, 1175, 1176, 1177, 1178, 1179, 1180, 1181, 1182, 1183, 1184, 1185, 1186, 1187, 1188, 1189, 1190, 1191, 1192, 1193, 1194, 1195, 1196, 1197, 1198, 1199, 1200, 1201, 1202, 1203, 1204, 1205, 1206, 1207, 1208, 1209, 1210, 1211, 1212, 1213, 1214, 1215, 1216, 1217, 1218, 1219, 1220, 1221, 1222, 1223, 1224, 1225, 1226, 1227, 1228, 1229, 1230, 1231, 1232, 1233, 1234, 1235, 1236, 1237, 1238, 1239, 1240, 1241, 1242, 1243, 1244, 1245, 1246, 1247, 1248, 1249, 1250, 1251, 1252, 1253, 1254,

1255, 1256, 1257, 1258, 1259, 1260, 1261, 1262, 1263, 1264, 1265, 1266, 1267, 1268, 1269, 1270, 1271, 1272, 1273, 1274, 1275, 1276, 1277, 1278, 1279, 1280, 1281, 1282, 1283, 1284, 1285, 1286, 1287, 1288, 1289, 1290, 1291, 1292, 1293, 1294, 1295, 1296, 1297, 1298, 1299, 1300, 1301, 1302, 1303, 1304, 1305, 1306, 1307, 1308, 1309, 1310, 1311, 1312, 1313, 1314, 1315, 1316, 1317, 1318, 1319, 1320, 1321, 1322, 1323, 1324, 1325, 1326, 1327, 1328, 1329, 1330, 1331, 1332, 1333, 1334, 1335, 1336, 1337, 1338, 1339, 1340, 1341, 1342, 1343, 1344, 1345, 1346, 1347, 1348, 1349, 1350, 1351, 1352, 1353, 1354, 1355, 1356, 1357, 1358, 1359, 1360, 1361, 1362, 1363, 1364, 1365, 1366, 1367, 1368, 1369, 1370, 1371, 1372, 1373, 1374, 1375, 1376, 1377, 1378, 1379, 1380, 1381, 1382, 1383, 1384, 1385, 1386, 1387, 1388, 1389, 1390, 1391, 1392.

[000153] The term "identity" as known in the art, refers to a relationship between the sequences of two or more peptides, as determined by comparing the sequences. In the art, identity also means the degree of sequence relatedness between peptides, as determined by the number of matches between strings of two or more amino acid residues. Identity measures the percent of identical matches between the smaller of two or more sequences with gap alignments (if any) addressed by a particular mathematical model or computer program (i.e., "algorithms"). Identity of related peptides can be readily calculated by known methods. Such methods include, but are not limited to, those described in Computational Molecular Biology, Lesk, A. M., ed., Oxford University Press, New York, 1988; Biocomputing: Informatics and Genome Projects, Smith, D. W., ed., Academic Press, New York, 1993; Computer Analysis of Sequence Data, Part 1, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987; Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M. Stockton Press, New York, 1991; and Carillo et al., SIAM J. Applied Math. 48, 1073 (1988).

[000154] In some embodiments, the polypeptide variant may have the same or a similar activity as the reference polypeptide. Alternatively, the variant may have an altered activity (e.g., increased or decreased) relative to a reference polypeptide. Generally, variants of a particular polynucleotide or polypeptide of the invention will have at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% but less than 100% sequence identity to that particular

reference polynucleotide or polypeptide as determined by sequence alignment programs and parameters described herein and known to those skilled in the art. Such tools for alignment include those of the BLAST suite (Stephen F. Altschul, Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.) Other tools are described herein, specifically in the definition of "Identity."

[000155] Default parameters in the BLAST algorithm include, for example, an expect threshold of 10, Word size of 28, Match/Mismatch Scores 1, -2, Gap costs Linear. Any filter can be applied as well as a selection for species specific repeats, e.g., Homo sapiens.

Biologics

[000156] The polynucleotides, primary constructs or mmRNA disclosed herein, may encode one or more biologics. As used herein, a "biologic" is a polypeptide-based molecule produced by the methods provided herein and which may be used to treat, cure, mitigate, prevent, or diagnose a serious or life-threatening disease or medical condition. Biologics, according to the present invention include, but are not limited to, allergenic extracts (e.g. for allergy shots and tests), blood components, gene therapy products, human tissue or cellular products used in transplantation, vaccines, monoclonal antibodies, cytokines, growth factors, enzymes, thrombolytics, and immunomodulators, among others.

[000157] According to the present invention, one or more biologics currently being marketed or in development may be encoded by the polynucleotides, primary constructs or mmRNA of the present invention. While not wishing to be bound by theory, it is believed that incorporation of the encoding polynucleotides of a known biologic into the primary constructs or mmRNA of the invention will result in improved therapeutic efficacy due at least in part to the specificity, purity and/or selectivity of the construct designs.

Antibodies

[000158] The primary constructs or mmRNA disclosed herein, may encode one or more antibodies or fragments thereof. The term "antibody" includes monoclonal antibodies (including full length antibodies which have an immunoglobulin Fc region), antibody

compositions with polyepitopic specificity, multispecific antibodies (e.g., bispecific antibodies, diabodies, and single-chain molecules), as well as antibody fragments. The term "immunoglobulin" (Ig) is used interchangeably with "antibody" herein. As used herein, the term "monoclonal antibody" refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations and/or post-translation modifications (e.g., isomerizations, amidations) that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site.

[000159] The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is(are) identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity. Chimeric antibodies of interest herein include, but are not limited to, "primatized" antibodies comprising variable domain antigen-binding sequences derived from a non-human primate (e.g., Old World Monkey, Ape etc.) and human constant region sequences.

[000160] An "antibody fragment" comprises a portion of an intact antibody, preferably the antigen binding and/or the variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', $F(ab')_2$ and Fv fragments; diabodies; linear antibodies; nanobodies; single-chain antibody molecules and multispecific antibodies formed from antibody fragments.

[000161] Any of the five classes of immunoglobulins, IgA, IgD, IgE, IgG and IgM, may be encoded by the mmRNA of the invention, including the heavy chains designated alpha, delta, epsilon, gamma and mu, respectively. Also included are polynucleotide sequences encoding the subclasses, gamma and mu. Hence any of the subclasses of antibodies may be encoded in part or in whole and include the following subclasses: IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2.

[000162] According to the present invention, one or more antibodies or fragments currently being marketed or in development may be encoded by the polynucleotides, primary constructs or mmRNA of the present invention. While not wishing to be bound by theory, it is believed that incorporation into the primary constructs of the invention will result in improved therapeutic efficacy due at least in part to the specificity, purity and selectivity of the mmRNA designs.

[000163] Antibodies encoded in the polynucleotides, primary constructs or mmRNA of the invention may be utilized to treat conditions or diseases in many therapeutic areas such as, but not limited to, blood, cardiovascular, CNS, poisoning (including antivenoms), dermatology, endocrinology, gastrointestinal, medical imaging, musculoskeletal, oncology, immunology, respiratory, sensory and anti-infective.

[000164] In one embodiment, primary constructs or mmRNA disclosed herein may encode monoclonal antibodies and/or variants thereof. Variants of antibodies may also include, but are not limited to, substitutional variants, conservative amino acid substitution, insertional variants, deletional variants and/or covalent derivatives. In one embodiment, the primary construct and/or mmRNA disclosed herein may encode an immunoglobulin Fc region. In another embodiment, the primary constructs and/or mmRNA may encode a variant immunoglobulin Fc region. As a non-limiting example, the primary constructs and/or mmRNA may encode an antibody having a variant immunoglobulin Fc region as described in U.S. Pat. No. 8,217,147 herein incorporated by reference in its entirety.

Vaccines

[000165] The primary constructs or mmRNA disclosed herein, may encode one or more vaccines. As used herein, a "vaccine" is a biological preparation that improves immunity to a particular disease or infectious agent. According to the present invention, one or more vaccines currently being marketed or in development may be encoded by the polynucleotides, primary constructs or mmRNA of the present invention. While not wishing to be bound by theory, it is believed that incorporation into the primary constructs or mmRNA of the invention will result in improved therapeutic efficacy due at least in part to the specificity, purity and selectivity of the construct designs.

[000166] Vaccines encoded in the polynucleotides, primary constructs or mmRNA of the invention may be utilized to treat conditions or diseases in many therapeutic areas such as, but not limited to, cardiovascular, CNS, dermatology, endocrinology, oncology, immunology, respiratory, and anti-infective.

Therapeutic proteins or peptides

[000167] The primary constructs or mmRNA disclosed herein, may encode one or more validated or "in testing" therapeutic proteins or peptides.

[000168] According to the present invention, one or more therapeutic proteins or peptides currently being marketed or in development may be encoded by the polynucleotides, primary constructs or mmRNA of the present invention. While not wishing to be bound by theory, it is believed that incorporation into the primary constructs or mmRNA of the invention will result in improved therapeutic efficacy due at least in part to the specificity, purity and selectivity of the construct designs.

[000169] Therapeutic proteins and peptides encoded in the polynucleotides, primary constructs or mmRNA of the invention may be utilized to treat conditions or diseases in many therapeutic areas such as, but not limited to, blood, cardiovascular, CNS, poisoning (including antivenoms), dermatology, endocrinology, genetic, genitourinary, gastrointestinal, musculoskeletal, oncology, and immunology, respiratory, sensory and anti-infective.

Cell-Penetrating Polypeptides

[000170] The primary constructs or mmRNA disclosed herein, may encode one or more cell-penetrating polypeptides. As used herein, "cell-penetrating polypeptide" or CPP refers to a polypeptide which may facilitate the cellular uptake of molecules. A cell-penetrating polypeptide of the present invention may contain one or more detectable labels. The polypeptides may be partially labeled or completely labeled throughout. The polynucleotide, primary construct or mmRNA may encode the detectable label completely, partially or not at all. The cell-penetrating peptide may also include a signal sequence. As used herein, a "signal sequence" refers to a sequence of amino acid residues bound at the amino terminus of a nascent protein during protein translation. The signal sequence may be used to signal the secretion of the cell-penetrating polypeptide.

[000171] In one embodiment, the polynucleotides, primary constructs or mmRNA may also encode a fusion protein. The fusion protein may be created by operably linking a charged protein to a therapeutic protein. As used herein, "operably linked" refers to the therapeutic protein and the charged protein being connected in such a way to permit the expression of the complex when introduced into the cell. As used herein, "charged protein" refers to a protein that carries a positive, negative or overall neutral electrical charge. Preferably, the therapeutic protein may be covalently linked to the charged protein in the formation of the fusion protein. The ratio of surface charge to total or surface amino acids may be approximately 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 or 0.9.

[000172] The cell-penetrating polypeptide encoded by the polynucleotides, primary constructs or mmRNA may form a complex after being translated. The complex may comprise a charged protein linked, e.g. covalently linked, to the cell-penetrating polypeptide. "Therapeutic protein" refers to a protein that, when administered to a cell has a therapeutic, diagnostic, and/or prophylactic effect and/or elicits a desired biological and/or pharmacological effect.

[000173] In one embodiment, the cell-penetrating polypeptide may comprise a first domain and a second domain. The first domain may comprise a supercharged polypeptide. The second domain may comprise a protein-binding partner. As used herein, "protein-binding partner" includes, but is not limited to, antibodies and functional fragments thereof, scaffold proteins, or peptides. The cell-penetrating polypeptide may further comprise an intracellular binding partner for the protein-binding partner. The cell-penetrating polypeptide may be capable of being secreted from a cell where the polynucleotide, primary construct or mmRNA may be introduced. The cell-penetrating polypeptide may also be capable of penetrating the first cell.

[000174] In a further embodiment, the cell-penetrating polypeptide is capable of penetrating a second cell. The second cell may be from the same area as the first cell, or it may be from a different area. The area may include, but is not limited to, tissues and organs. The second cell may also be proximal or distal to the first cell.

[000175] In one embodiment, the polynucleotides, primary constructs or mmRNA may encode a cell-penetrating polypeptide which may comprise a protein-binding partner. The protein binding partner may include, but is not limited to, an antibody, a

supercharged antibody or a functional fragment. The polynucleotides, primary constructs or mmRNA may be introduced into the cell where a cell-penetrating polypeptide comprising the protein-binding partner is introduced.

S*ecreted proteins*

[000176] Human and other eukaryotic cells are subdivided by membranes into many functionally distinct compartments. Each membrane-bounded compartment, or organelle, contains different proteins essential for the function of the organelle. The cell uses "sorting signals," which are amino acid motifs located within the protein, to target proteins to particular cellular organelles.

[000177] One type of sorting signal, called a signal sequence, a signal peptide, or a leader sequence, directs a class of proteins to an organelle called the endoplasmic reticulum (ER).

[000178] Proteins targeted to the ER by a signal sequence can be released into the extracellular space as a secreted protein. Similarly, proteins residing on the cell membrane can also be secreted into the extracellular space by proteolytic cleavage of a "linker" holding the protein to the membrane. While not wishing to be bound by theory, the molecules of the present invention may be used to exploit the cellular trafficking described above. As such, in some embodiments of the invention, polynucleotides, primary constructs or mmRNA are provided to express a secreted protein. The secreted proteins may be selected from those described herein or those in US Patent Publication, 20100255574, the contents of which are incorporated herein by reference in their entirety.

[000179] In one embodiment, these may be used in the manufacture of large quantities of valuable human gene products.

Plasma membrane proteins

[000180] In some embodiments of the invention, polynucleotides, primary constructs or mmRNA are provided to express a protein of the plasma membrane.

Cytoplasmic or cytoskeletal proteins

[000181] In some embodiments of the invention, polynucleotides, primary constructs or mmRNA are provided to express a cytoplasmic or cytoskeletal protein.

Intracellular membrane bound proteins

[000182] In some embodiments of the invention, polynucleotides, primary constructs or mmRNA are provided to express an intracellular membrane bound protein.

Nuclear proteins

[000183] In some embodiments of the invention, polynucleotides, primary constructs or mmRNA are provided to express a nuclear protein.

Proteins associated with human disease

[000184] In some embodiments of the invention, polynucleotides, primary constructs or mmRNA are provided to express a protein associated with human disease.

Miscellaneous proteins

[000185] In some embodiments of the invention, polynucleotides, primary constructs or mmRNA are provided to express a protein with a presently unknown therapeutic function.

Targeting Moieties

[000186] In some embodiments of the invention, polynucleotides, primary constructs or mmRNA are provided to express a targeting moiety. These include a protein-binding partner or a receptor on the surface of the cell, which functions to target the cell to a specific tissue space or to interact with a specific moiety, either *in vivo* or *in vitro*. Suitable protein-binding partners include, but are not limited to, antibodies and functional fragments thereof, scaffold proteins, or peptides. Additionally, polynucleotide, primary construct or mmRNA can be employed to direct the synthesis and extracellular localization of lipids, carbohydrates, or other biological moieties or biomolecules.

Polypeptide Libraries

[000187] In one embodiment, the polynucleotides, primary constructs or mmRNA may be used to produce polypeptide libraries. These libraries may arise from the production of a population of polynucleotides, primary constructs or mmRNA, each containing various structural or chemical modification designs. In this embodiment, a population of polynucleotides, primary constructs or mmRNA may comprise a plurality of encoded polypeptides, including but not limited to, an antibody or antibody fragment, protein binding partner, scaffold protein, and other polypeptides taught herein or known in the art. In a preferred embodiment, the polynucleotides are primary constructs of the present

invention, including mmRNA which may be suitable for direct introduction into a target cell or culture which in turn may synthesize the encoded polypeptides.

[000188] In certain embodiments, multiple variants of a protein, each with different amino acid modification(s), may be produced and tested to determine the best variant in terms of pharmacokinetics, stability, biocompatibility, and/or biological activity, or a biophysical property such as expression level. Such a library may contain 10, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, or over 10^9 possible variants (including, but not limited to, substitutions, deletions of one or more residues, and insertion of one or more residues).

Anti-Microbial and Anti-viral Polypeptides

[000189] The polynucleotides, primary constructs and mmRNA of the present invention may be designed to encode on or more antimicrobial peptides (AMP) or antiviral peptides (AVP). AMPs and AVPs have been isolated and described from a wide range of animals such as, but not limited to, microorganisms, invertebrates, plants, amphibians, birds, fish, and mammals (Wang *et al.*, *Nucleic Acids Res.* 2009; 37 (Database issue):D933-7). For example, anti-microbial polypeptides are described in Antimicrobial Peptide Database (http://aps.unmc.edu/AP/main.php; Wang *et al., Nucleic Acids Res.* 2009; 37 (Database issue):D933-7), CAMP: Collection of Anti-Microbial Peptides (http://www.bicnirrh.res.in/antimicrobial/); Thomas *et al., Nucleic Acids Res.* 2010; 38 (Database issue):D774-80), US 5221732, US 5447914, US 5519115, US 5607914, US 5714577, US 5734015, US 5798336, US 5821224, US 5849490, US 5856127, US 5905187, US 5994308, US 5998374, US 6107460, US 6191254, US 6211148, US 6300489, US 6329504, US 6399370, US 6476189, US 6478825, US 6492328, US 6514701, US 6573361, US 6573361, US 6576755, US 6605698, US 6624140, US 6638531, US 6642203, US 6653280, US 6696238, US 6727066, US 6730659, US 6743598, US 6743769, US 6747007, US 6790833, US 6794490, US 6818407, US 6835536, US 6835713, US 6838435, US 6872705, US 6875907, US 6884776, US 6887847, US 6906035, US 6911524, US 6936432, US 7001924, US 7071293, US 7078380, US 7091185, US 7094759, US 7166769, US 7244710, US 7314858, and US 7582301, the contents of which are incorporated by reference in their entirety.

[000190] The anti-microbial polypeptides described herein may block cell fusion and/or viral entry by one or more enveloped viruses (*e.g.*, HIV, HCV). For example, the anti-

microbial polypeptide can comprise or consist of a synthetic peptide corresponding to a region, *e.g.*, a consecutive sequence of at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, or 60 amino acids of the transmembrane subunit of a viral envelope protein, *e.g.*, HIV-1 gp120 or gp41. The amino acid and nucleotide sequences of HIV-1 gp120 or gp41 are described in, *e.g.*, Kuiken *et al.*, (2008). *"HIV Sequence Compendium,"* Los Alamos National Laboratory.

[000191] In some embodiments, the anti-microbial polypeptide may have at least about 75%, 80%, 85%, 90%, 95%, 100% sequence homology to the corresponding viral protein sequence. In some embodiments, the anti-microbial polypeptide may have at least about 75%, 80%, 85%, 90%, 95%, or 100% sequence homology to the corresponding viral protein sequence.

[000192] In other embodiments, the anti-microbial polypeptide may comprise or consist of a synthetic peptide corresponding to a region, *e.g.*, a consecutive sequence of at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, or 60 amino acids of the binding domain of a capsid binding protein. In some embodiments, the anti-microbial polypeptide may have at least about 75%, 80%, 85%, 90%, 95%, or 100% sequence homology to the corresponding sequence of the capsid binding protein.

[000193] The anti-microbial polypeptides described herein may block protease dimerization and inhibit cleavage of viral proproteins (*e.g.*, HIV Gag-pol processing) into functional proteins thereby preventing release of one or more enveloped viruses (*e.g.*, HIV, HCV). In some embodiments, the anti-microbial polypeptide may have at least about 75%, 80%, 85%, 90%, 95%, 100% sequence homology to the corresponding viral protein sequence.

[000194] In other embodiments, the anti-microbial polypeptide can comprise or consist of a synthetic peptide corresponding to a region, *e.g.*, a consecutive sequence of at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, or 60 amino acids of the binding domain of a protease binding protein. In some embodiments, the anti-microbial polypeptide may have at least about 75%, 80%, 85%, 90%, 95%, 100% sequence homology to the corresponding sequence of the protease binding protein.

[000195] The anti-microbial polypeptides described herein can include an *in vitro*-evolved polypeptide directed against a viral pathogen.

Anti-Microbial Polypeptides

[000196] Anti-microbial polypeptides (AMPs) are small peptides of variable length, sequence and structure with broad spectrum activity against a wide range of microorganisms including, but not limited to, bacteria, viruses, fungi, protozoa, parasites, prions, and tumor/cancer cells. (See, *e.g.*, Zaiou, J Mol Med, 2007; 85:317; herein incorporated by reference in its entirety). It has been shown that AMPs have broad-spectrum of rapid onset of killing activities, with potentially low levels of induced resistance and concomitant broad anti-inflammatory effects.

[000197] In some embodiments, the anti-microbial polypeptide (*e.g.*, an anti-bacterial polypeptide) may be under 10kDa, *e.g.*, under 8kDa, 6kDa, 4kDa, 2kDa, or 1kDa. In some embodiments, the anti-microbial polypeptide (*e.g.*, an anti-bacterial polypeptide) consists of from about 6 to about 100 amino acids, *e.g.*, from about 6 to about 75 amino acids, about 6 to about 50 amino acids, about 6 to about 25 amino acids, about 25 to about 100 amino acids, about 50 to about 100 amino acids, or about 75 to about 100 amino acids. In certain embodiments, the anti-microbial polypeptide (*e.g.*, an anti-bacterial polypeptide) may consist of from about 15 to about 45 amino acids. In some embodiments, the anti-microbial polypeptide (*e.g.*, an anti-bacterial polypeptide) is substantially cationic.

[000198] In some embodiments, the anti-microbial polypeptide (*e.g.*, an anti-bacterial polypeptide) may be substantially amphipathic. In certain embodiments, the anti-microbial polypeptide (*e.g.*, an anti-bacterial polypeptide) may be substantially cationic and amphipathic. In some embodiments, the anti-microbial polypeptide (*e.g.*, an anti-bacterial polypeptide) may be cytostatic to a Gram-positive bacterium. In some embodiments, the anti-microbial polypeptide (*e.g.*, an anti-bacterial polypeptide) may be cytotoxic to a Gram-positive bacterium. In some embodiments, the anti-microbial polypeptide (*e.g.*, an anti-bacterial polypeptide) may be cytostatic and cytotoxic to a Gram-positive bacterium. In some embodiments, the anti-microbial polypeptide (*e.g.*, an anti-bacterial polypeptide) may be cytostatic to a Gram-negative bacterium. In some embodiments, the anti-microbial polypeptide (*e.g.*, an anti-bacterial polypeptide) may be cytotoxic to a Gram-negative bacterium. In some embodiments, the anti-microbial polypeptide (*e.g.*, an anti-bacterial polypeptide) may be cytostatic and cytotoxic to a

Gram-positive bacterium. In some embodiments, the anti-microbial polypeptide may be cytostatic to a virus, fungus, protozoan, parasite, prion, or a combination thereof. In some embodiments, the anti-microbial polypeptide may be cytotoxic to a virus, fungus, protozoan, parasite, prion, or a combination thereof. In certain embodiments, the anti-microbial polypeptide may be cytostatic and cytotoxic to a virus, fungus, protozoan, parasite, prion, or a combination thereof. In some embodiments, the anti-microbial polypeptide may be cytotoxic to a tumor or cancer cell (*e.g.*, a human tumor and/or cancer cell). In some embodiments, the anti-microbial polypeptide may be cytostatic to a tumor or cancer cell (*e.g.*, a human tumor and/or cancer cell). In certain embodiments, the anti-microbial polypeptide may be cytotoxic and cytostatic to a tumor or cancer cell (*e.g.*, a human tumor or cancer cell). In some embodiments, the anti-microbial polypeptide (*e.g.*, an anti-bacterial polypeptide) may be a secreted polypeptide.

[000199] In some embodiments, the anti-microbial polypeptide comprises or consists of a defensin. Exemplary defensins include, but are not limited to, α-defensins (*e.g.*, neutrophil defensin 1, defensin alpha 1, neutrophil defensin 3, neutrophil defensin 4, defensin 5, defensin 6), β-defensins (*e.g.*, beta-defensin 1, beta-defensin 2, beta-defensin 103, beta-defensin 107, beta-defensin 110, beta-defensin 136), and θ-defensins. In other embodiments, the anti-microbial polypeptide comprises or consists of a cathelicidin (*e.g.*, hCAP18).

Anti-Viral Polypeptides

[000200] Anti-viral polypeptides (AVPs) are small peptides of variable length, sequence and structure with broad spectrum activity against a wide range of viruses. See, *e.g.*, Zaiou, J Mol Med, 2007; 85:317. It has been shown that AVPs have a broad-spectrum of rapid onset of killing activities, with potentially low levels of induced resistance and concomitant broad anti-inflammatory effects. In some embodiments, the anti-viral polypeptide is under 10kDa, *e.g.*, under 8kDa, 6kDa, 4kDa, 2kDa, or 1kDa. In some embodiments, the anti-viral polypeptide comprises or consists of from about 6 to about 100 amino acids, *e.g.*, from about 6 to about 75 amino acids, about 6 to about 50 amino acids, about 6 to about 25 amino acids, about 25 to about 100 amino acids, about 50 to about 100 amino acids, or about 75 to about 100 amino acids. In certain embodiments, the anti-viral polypeptide comprises or consists of from about 15 to about

45 amino acids. In some embodiments, the anti-viral polypeptide is substantially cationic. In some embodiments, the anti-viral polypeptide is substantially amphipathic. In certain embodiments, the anti-viral polypeptide is substantially cationic and amphipathic. In some embodiments, the anti-viral polypeptide is cytostatic to a virus. In some embodiments, the anti-viral polypeptide is cytotoxic to a virus. In some embodiments, the anti-viral polypeptide is cytostatic and cytotoxic to a virus. In some embodiments, the anti-viral polypeptide is cytostatic to a bacterium, fungus, protozoan, parasite, prion, or a combination thereof. In some embodiments, the anti-viral polypeptide is cytotoxic to a bacterium, fungus, protozoan, parasite, prion or a combination thereof. In certain embodiments, the anti-viral polypeptide is cytostatic and cytotoxic to a bacterium, fungus, protozoan, parasite, prion, or a combination thereof. In some embodiments, the anti-viral polypeptide is cytotoxic to a tumor or cancer cell (*e.g.*, a human cancer cell). In some embodiments, the anti-viral polypeptide is cytostatic to a tumor or cancer cell (*e.g.*, a human cancer cell). In certain embodiments, the anti-viral polypeptide is cytotoxic and cytostatic to a tumor or cancer cell (*e.g.*, a human cancer cell). In some embodiments, the anti-viral polypeptide is a secreted polypeptide.

Cytotoxic Nucleosides

[000201] In one embodiment, the polynucleotides, primary constructs or mmRNA of the present invention may incorporate one or more cytotoxic nucleosides. For example, cytotoxic nucleosides may be incorporated into polynucleotides, primary constructs or mmRNA such as bifunctional modified RNAs or mRNAs. Cytotoxic nucleoside anti-cancer agents include, but are not limited to, adenosine arabinoside, cytarabine, cytosine arabinoside, 5-fluorouracil, fludarabine, floxuridine, FTORAFUR® (a combination of tegafur and uracil), tegafur ((RS)-5-fluoro-1-(tetrahydrofuran-2-yl)pyrimidine-2,4(1H,3H)-dione), and 6-mercaptopurine.

[000202] A number of cytotoxic nucleoside analogues are in clinical use, or have been the subject of clinical trials, as anticancer agents. Examples of such analogues include, but are not limited to, cytarabine, gemcitabine, troxacitabine, decitabine, tezacitabine, 2′-deoxy-2′-methylidenecytidine (DMDC), cladribine, clofarabine, 5-azacytidine, 4'-thio-aracytidine, cyclopentenylcytosine and 1-(2-C-cyano-2-deoxy-beta-D-arabino-pentofuranosyl)-cytosine. Another example of such a compound is fludarabine

phosphate. These compounds may be administered systemically and may have side effects which are typical of cytotoxic agents such as, but not limited to, little or no specificity for tumor cells over proliferating normal cells.

[000203] A number of prodrugs of cytotoxic nucleoside analogues are also reported in the art. Examples include, but are not limited to, N4-behenoyl-1-beta-D-arabinofuranosylcytosine, N4-octadecyl-1-beta-D-arabinofuranosylcytosine, N4-palmitoyl-1-(2-C-cyano-2-deoxy-beta-D-arabino-pentofuranosyl) cytosine, and P-4055 (cytarabine 5'-elaidic acid ester). In general, these prodrugs may be converted into the active drugs mainly in the liver and systemic circulation and display little or no selective release of active drug in the tumor tissue. For example, capecitabine, a prodrug of 5'-deoxy-5-fluorocytidine (and eventually of 5-fluorouracil), is metabolized both in the liver and in the tumor tissue. A series of capecitabine analogues containing "an easily hydrolysable radical under physiological conditions" has been claimed by Fujiu et al. (U.S. Pat. No. 4,966,891) and is herein incorporated by reference. The series described by Fujiu includes N4 alkyl and aralkyl carbamates of 5'-deoxy-5-fluorocytidine and the implication that these compounds will be activated by hydrolysis under normal physiological conditions to provide 5'-deoxy-5-fluorocytidine.

[000204] A series of cytarabine N4-carbamates has been by reported by Fadl et al (Pharmazie. 1995, 50, 382-7, herein incorporated by reference) in which compounds were designed to convert into cytarabine in the liver and plasma. WO 2004/041203, herein incorporated by reference, discloses prodrugs of gemcitabine, where some of the prodrugs are N4-carbamates. These compounds were designed to overcome the gastrointestinal toxicity of gemcitabine and were intended to provide gemcitabine by hydrolytic release in the liver and plasma after absorption of the intact prodrug from the gastrointestinal tract. Nomura et al (Bioorg Med. Chem. 2003, 11, 2453-61, herein incorporated by reference) have described acetal derivatives of 1-(3-C-ethynyl-β-D-ribo-pentofaranosyl) cytosine which, on bioreduction, produced an intermediate that required further hydrolysis under acidic conditions to produce a cytotoxic nucleoside compound.

[000205] Cytotoxic nucleotides which may be chemotherapeutic also include, but are not limited to, pyrazolo [3,4-D]-pyrimidines, allopurinol, azathioprine, capecitabine, cytosine arabinoside, fluorouracil, mercaptopurine, 6-thioguanine, acyclovir, ara-

adenosine, ribavirin, 7-deaza-adenosine, 7-deaza-guanosine, 6-aza-uracil, 6-aza-cytidine, thymidine ribonucleotide, 5-bromodeoxyuridine, 2-chloro-purine, and inosine, or combinations thereof.

Flanking Regions: Untranslated Regions (UTRs)

[000206] Untranslated regions (UTRs) of a gene are transcribed but not translated. The 5′UTR starts at the transcription start site and continues to the start codon but does not include the start codon; whereas, the 3′UTR starts immediately following the stop codon and continues until the transcriptional termination signal. There is growing body of evidence about the regulatory roles played by the UTRs in terms of stability of the nucleic acid molecule and translation. The regulatory features of a UTR can be incorporated into the polynucleotides, primary constructs and/or mmRNA of the present invention to enhance the stability of the molecule. The specific features can also be incorporated to ensure controlled down-regulation of the transcript in case they are misdirected to undesired organs sites.

5′ UTR and Translation Initiation

[000207] Natural 5′UTRs bear features which play roles in for translation initiation. They harbor signatures like Kozak sequences which are commonly known to be involved in the process by which the ribosome initiates translation of many genes. Kozak sequences have the consensus CCR(A/G)CCAUGG, where R is a purine (adenine or guanine) three bases upstream of the start codon (AUG), which is followed by another 'G'. 5′UTR also have been known to form secondary structures which are involved in elongation factor binding.

[000208] By engineering the features typically found in abundantly expressed genes of specific target organs, one can enhance the stability and protein production of the polynucleotides, primary constructs or mmRNA of the invention. For example, introduction of 5′ UTR of liver-expressed mRNA, such as albumin, serum amyloid A, Apolipoprotein A/B/E, transferrin, alpha fetoprotein, erythropoietin, or Factor VIII, could be used to enhance expression of a nucleic acid molecule, such as a mmRNA, in hepatic cell lines or liver. Likewise, use of 5′ UTR from other tissue-specific mRNA to improve expression in that tissue is possible for muscle (MyoD, Myosin, Myoglobin, Myogenin, Herculin), for endothelial cells (Tie-1, CD36), for myeloid cells (C/EBP, AML1, G-CSF,

GM-CSF, CD11b, MSR, Fr-1, i-NOS), for leukocytes (CD45, CD18), for adipose tissue (CD36, GLUT4, ACRP30, adiponectin) and for lung epithelial cells (SP-A/B/C/D).

[000209] Other non-UTR sequences may be incorporated into the 5′ (or 3′ UTR) UTRs. For example, introns or portions of introns sequences may be incorporated into the flanking regions of the polynucleotides, primary constructs or mmRNA of the invention. Incorporation of intronic sequences may increase protein production as well as mRNA levels.

3′ UTR and the AU Rich Elements

[000210] 3′ UTRs are known to have stretches of Adenosines and Uridines embedded in them. These AU rich signatures are particularly prevalent in genes with high rates of turnover. Based on their sequence features and functional properties, the AU rich elements (AREs) can be separated into three classes (Chen et al, 1995): Class I AREs contain several dispersed copies of an AUUUA motif within U-rich regions. C-Myc and MyoD contain class I AREs. Class II AREs possess two or more overlapping UUAUUUA(U/A)(U/A) nonamers. Molecules containing this type of AREs include GM-CSF and TNF-a. Class III ARES are less well defined. These U rich regions do not contain an AUUUA motif. c-Jun and Myogenin are two well-studied examples of this class. Most proteins binding to the AREs are known to destabilize the messenger, whereas members of the ELAV family, most notably HuR, have been documented to increase the stability of mRNA. HuR binds to AREs of all the three classes. Engineering the HuR specific binding sites into the 3′ UTR of nucleic acid molecules will lead to HuR binding and thus, stabilization of the message *in vivo*.

[000211] Introduction, removal or modification of 3′ UTR AU rich elements (AREs) can be used to modulate the stability of polynucleotides, primary constructs or mmRNA of the invention. When engineering specific polynucleotides, primary constructs or mmRNA, one or more copies of an ARE can be introduced to make polynucleotides, primary constructs or mmRNA of the invention less stable and thereby curtail translation and decrease production of the resultant protein. Likewise, AREs can be identified and removed or mutated to increase the intracellular stability and thus increase translation and production of the resultant protein. Transfection experiments can be conducted in relevant cell lines, using polynucleotides, primary constructs or mmRNA of the invention

and protein production can be assayed at various time points post-transfection. For example, cells can be transfected with different ARE-engineering molecules and by using an ELISA kit to the relevant protein and assaying protein produced at 6 hour, 12 hour, 24 hour, 48 hour, and 7 days post-transfection.

Incorporating microRNA Binding Sites

[000212] microRNAs (or miRNA) are 19-25 nucleotide long noncoding RNAs that bind to the 3′UTR of nucleic acid molecules and down-regulate gene expression either by reducing nucleic acid molecule stability or by inhibiting translation. The polynucleotides, primary constructs or mmRNA of the invention may comprise one or more microRNA target sequences, microRNA seqences, or microRNA seeds. Such sequences may correspond to any known microRNA such as those taught in US Publication US2005/0261218 and US Publication US2005/0059005, the contents of which are incorporated herein by reference in their entirety.

[000213] A microRNA sequence comprises a "seed" region, i.e., a sequence in the region of positions 2-8 of the mature microRNA, which sequence has perfect Watson-Crick complementarity to the miRNA target sequence. A microRNA seed may comprise positions 2-8 or 2-7 of the mature microRNA. In some embodiments, a microRNA seed may comprise 7 nucleotides (e.g., nucleotides 2-8 of the mature microRNA), wherein the seed-complementary site in the corresponding miRNA target is flanked by an adenine (A) opposed to microRNA position 1. In some embodiments, a microRNA seed may comprise 6 nucleotides (e.g., nucleotides 2-7 of the mature microRNA), wherein the seed-complementary site in the corresponding miRNA target is flanked byan adenine (A) opposed to microRNA position 1. See for example, Grimson A, Farh KK, Johnston WK, Garrett-Engele P, Lim LP, Bartel DP; Mol Cell. 2007 Jul 6;27(1):91-105; each of which is herein incorporated by reference in their entirety. The bases of the microRNA seed have complete complementarity with the target sequence. By engineering microRNA target sequences into the 3′UTR of polynucleotides, primary constructs or mmRNA of the invention one can target the molecule for degradation or reduced translation, provided the microRNA in question is available. This process will reduce the hazard of off target effects upon nucleic acid molecule delivery. Identification of microRNA, microRNA target regions, and their expression patterns and role in biology have been reported

(Bonauer et al., Curr Drug Targets 2010 11:943-949; Anand and Cheresh Curr Opin Hematol 2011 18:171-176; Contreras and Rao Leukemia 2012 26:404-413 (2011 Dec 20. doi: 10.1038/leu.2011.356); Bartel Cell 2009 136:215-233; Landgraf et al, Cell, 2007 129:1401-1414; each of which is herein incorporated by reference in its entirety).

[000214] For example, if the nucleic acid molecule is an mRNA and is not intended to be delivered to the liver but ends up there, then miR-122, a microRNA abundant in liver, can inhibit the expression of the gene of interest if one or multiple target sites of miR-122 are engineered into the 3′ UTR of the polynucleotides, primary constructs or mmRNA. Introduction of one or multiple binding sites for different microRNA can be engineered to further decrease the longevity, stability, and protein translation of a polynucleotides, primary constructs or mmRNA.

[000215] As used herein, the term "microRNA site" refers to a microRNA target site or a microRNA recognition site, or any nucleotide sequence to which a microRNA binds or associates. It should be understood that "binding" may follow traditional Watson-Crick hybridization rules or may reflect any stable association of the microRNA with the target sequence at or adjacent to the microRNA site.

[000216] Conversely, for the purposes of the polynucleotides, primary constructs or mmRNA of the present invention, microRNA binding sites can be engineered out of (i.e. removed from) sequences in which they naturally occur in order to increase protein expression in specific tissues. For example, miR-122 binding sites may be removed to improve protein expression in the liver. Regulation of expression in multiple tissues can be accomplished through introduction or removal or one or several microRNA binding sites.

[000217] Examples of tissues where microRNA are known to regulate mRNA, and thereby protein expression, include, but are not limited to, liver (miR-122), muscle (miR-133, miR-206, miR-208), endothelial cells (miR-17-92, miR-126), myeloid cells (miR-142-3p, miR-142-5p, miR-16, miR-21, miR-223, miR-24, miR-27), adipose tissue (let-7, miR-30c), heart (miR-1d, miR-149), kidney (miR-192, miR-194, miR-204), and lung epithelial cells (let-7, miR-133, miR-126). MicroRNA can also regulate complex biological processes such as angiogenesis (miR-132) (Anand and Cheresh Curr Opin Hematol 2011 18:171-176; herein incorporated by reference in its entirety). In the

polynucleotides, primary constructs or mmRNA of the present invention, binding sites for microRNAs that are involved in such processes may be removed or introduced, in order to tailor the expression of the polynucleotides, primary constructs or mmRNA expression to biologically relevant cell types or to the context of relevant biological processes. A listing of MicroRNA, miR sequences and miR binding sites is listed in Table 9 of U.S. Provisional Application No. 61/753,661 filed January 17, 2013, in Table 9 of U.S. Provisional Application No. 61/754,159 filed January 18, 2013, and in Table 7 of U.S. Provisional Application No. 61/758,921 filed January 31, 2013, each of which are herein incorporated by reference in their entireties.

[000218] Lastly, through an understanding of the expression patterns of microRNA in different cell types, polynucleotides, primary constructs or mmRNA can be engineered for more targeted expression in specific cell types or only under specific biological conditions. Through introduction of tissue-specific microRNA binding sites, polynucleotides, primary constructs or mmRNA could be designed that would be optimal for protein expression in a tissue or in the context of a biological condition. Examples of use of microRNA to drive tissue or disease-specific gene expression are listed (Getner and Naldini, Tissue Antigens. 2012, 80:393-403; herein incoroporated by reference in its entirety). In addition, microRNA seed sites can be incorporated into mRNA to decrease expression in certain cells which results in a biological improvement. An example of this is incorporation of miR-142 sites into a UGT1A1-expressing lentiviral vector. The presence of miR-142 seed sites reduced expression in hematopoietic cells, and as a consequence reduced expression in antigen-presentating cells, leading to the absence of an immune response against the virally expressed UGT1A1 (Schmitt et al., Gastroenterology 2010; 139:999-1007; Gonzalez-Asequinolaza et al. Gastroenterology 2010, 139:726-729; both herein incorporated by reference in its entirety) . Incorporation of miR-142 sites into modified mRNA could not only reduce expression of the encoded protein in hematopoietic cells, but could also reduce or abolish immune responses to the mRNA-encoded protein. Incorporation of miR-142 seed sites (one or multiple) into mRNA would be important in the case of treatment of patients with complete protein deficiencies (UGT1A1 type I, LDLR-deficient patients, CRIM-negative Pompe patients, etc.) .

[000219] Transfection experiments can be conducted in relevant cell lines, using engineered polynucleotides, primary constructs or mmRNA and protein production can be assayed at various time points post-transfection. For example, cells can be transfected with different microRNA binding site-engineering polynucleotides, primary constructs or mmRNA and by using an ELISA kit to the relevant protein and assaying protein produced at 6 hour, 12 hour, 24 hour, 48 hour, 72 hour and 7 days post-transfection. *In vivo* experiments can also be conducted using microRNA-binding site-engineered molecules to examine changes in tissue-specific expression of formulated polynucleotides, primary constructs or mmRNA.

5′ Capping

[000220] The 5′ cap structure of an mRNA is involved in nuclear export, increasing mRNA stability and binds the mRNA Cap Binding Protein (CBP), which is responsibile for mRNA stability in the cell and translation competency through the association of CBP with poly(A) binding protein to form the mature cyclic mRNA species. The cap further assists the removal of 5′ proximal introns removal during mRNA splicing.

[000221] Endogenous mRNA molecules may be 5′-end capped generating a 5′-ppp-5′-triphosphate linkage between a terminal guanosine cap residue and the 5′-terminal transcribed sense nucleotide of the mRNA molecule. This 5′-guanylate cap may then be methylated to generate an N7-methyl-guanylate residue. The ribose sugars of the terminal and/or anteterminal transcribed nucleotides of the 5′ end of the mRNA may optionally also be 2′-O-methylated. 5′-decapping through hydrolysis and cleavage of the guanylate cap structure may target a nucleic acid molecule, such as an mRNA molecule, for degradation.

[000222] Modifications to the polynucleotides, primary constructs, and mmRNA of the present invention may generate a non-hydrolyzable cap structure preventing decapping and thus increasing mRNA half-life. Because cap structure hydrolysis requires cleavage of 5′-ppp-5′ phosphorodiester linkages, modified nucleotides may be used during the capping reaction. For example, a Vaccinia Capping Enzyme from New England Biolabs (Ipswich, MA) may be used with α-thio-guanosine nucleotides according to the manufacturer's instructions to create a phosphorothioate linkage in the 5′-ppp-5′ cap.

Additional modified guanosine nucleotides may be used such as α-methyl-phosphonate and seleno-phosphate nucleotides.

[000223] Additional modifications include, but are not limited to, 2′-O-methylation of the ribose sugars of 5′-terminal and/or 5′-anteterminal nucleotides of the mRNA (as mentioned above) on the 2′-hydroxyl group of the sugar ring. Multiple distinct 5′-cap structures can be used to generate the 5′-cap of a nucleic acid molecule, such as an mRNA molecule.

[000224] Cap analogs, which herein are also referred to as synthetic cap analogs, chemical caps, chemical cap analogs, or structural or functional cap analogs, differ from natural (i.e. endogenous, wild-type or physiological) 5′-caps in their chemical structure, while retaining cap function. Cap analogs may be chemically (i.e. non-enzymatically) or enzymatically synthesized and/or linked to a nucleic acid molecule.

[000225] For example, the Anti-Reverse Cap Analog (ARCA) cap contains two guanines linked by a 5′-5′-triphosphate group, wherein one guanine contains an N7 methyl group as well as a 3′-O-methyl group (i.e., N7,3′-O-dimethyl-guanosine-5′-triphosphate-5′-guanosine (m^7G-3′mppp-G; which may equivaliently be designated 3′ O-Me-m7G(5')ppp(5')G). The 3′-O atom of the other, unmodified, guanine becomes linked to the 5′-terminal nucleotide of the capped nucleic acid molecule (e.g. an mRNA or mmRNA). The N7- and 3′-O-methlyated guanine provides the terminal moiety of the capped nucleic acid molecule (e.g. mRNA or mmRNA).

[000226] Another exemplary cap is mCAP, which is similar to ARCA but has a 2′-O-methyl group on guanosine (i.e., N7,2′-O-dimethyl-guanosine-5′-triphosphate-5′-guanosine, m^7Gm-ppp-G).

[000227] While cap analogs allow for the concomitant capping of a nucleic acid molecule in an in vitro transcription reaction, up to 20% of transcripts can remain uncapped. This, as well as the structural differences of a cap analog from an endogenous 5′-cap structures of nucleic acids produced by the endogenous, cellular transcription machinery, may lead to reduced translational competency and reduced cellular stability.

[000228] Polynucleotides, primary constructs and mmRNA of the invention may also be capped post-transcriptionally, using enzymes, in order to generate more authentic 5′-cap structures. As used herein, the phrase "more authentic" refers to a feature that

closely mirrors or mimics, either structurally or functionally, an endogenous or wild type feature. That is, a "more authentic" feature is better representative of an endogenous, wild-type, natural or physiological cellular function and/or structure as compared to synthetic features or analogs, etc., of the prior art, or which outperforms the corresponding endogenous, wild-type, natural or physiological feature in one or more respects. Non-limiting examples of more authentic 5′cap structures of the present invention are those which, among other things, have enhanced binding of cap binding proteins, increased half life, reduced susceptibility to 5′ endonucleases and/or reduced 5′decapping, as compared to synthetic 5′cap structures known in the art (or to a wild-type, natural or physiological 5′cap structure). For example, recombinant Vaccinia Virus Capping Enzyme and recombinant 2′-O-methyltransferase enzyme can create a canonical 5′-5′-triphosphate linkage between the 5′-terminal nucleotide of an mRNA and a guanine cap nucleotide wherein the cap guanine contains an N7 methylation and the 5′-terminal nucleotide of the mRNA contains a 2′-O-methyl. Such a structure is termed the Cap1 structure. This cap results in a higher translational-competency and cellular stability and a reduced activation of cellular pro-inflammatory cytokines, as compared, e.g., to other 5′cap analog structures known in the art. Cap structures include, but are not limited to, 7mG(5')ppp(5')N,pN2p (cap 0), 7mG(5')ppp(5')NlmpNp (cap 1), and 7mG(5')-ppp(5')NlmpN2mp (cap 2).

[000229] Because the polynucleotides, primary constructs or mmRNA may be capped post-transcriptionally, and because this process is more efficient, nearly 100% of the polynucleotides, primary constructs or mmRNA may be capped. This is in contrast to ~80% when a cap analog is linked to an mRNA in the course of an in vitro transcription reaction.

[000230] According to the present invention, 5′ terminal caps may include endogenous caps or cap analogs. According to the present invention, a 5′ terminal cap may comprise a guanine analog. Useful guanine analogs include, but are not limited to, inosine, N1-methyl-guanosine, 2′fluoro-guanosine, 7-deaza-guanosine, 8-oxo-guanosine, 2-amino-guanosine, LNA-guanosine, and 2-azido-guanosine.

Viral Sequences

[000231] Additional viral sequences such as, but not limited to, the translation enhancer sequence of the barley yellow dwarf virus (BYDV-PAV), the Jaagsiekte sheep retrovirus (JSRV) and/or the Enzootic nasal tumor virus (See e.g., International Pub. No. WO2012129648; herein incorporated by reference in its entirety) can be engineered and inserted in the 3′ UTR of the polynucleotides, primary constructs or mmRNA of the invention and can stimulate the translation of the construct *in vitro* and *in vivo*. Transfection experiments can be conducted in relevant cell lines at and protein production can be assayed by ELISA at 12hr, 24hr, 48hr, 72 hr and day 7 post-transfection.

IRES Sequences

[000232] Further, provided are polynucleotides, primary constructs or mmRNA which may contain an internal ribosome entry site (IRES). First identified as a feature Picorna virus RNA, IRES plays an important role in initiating protein synthesis in absence of the 5′ cap structure. An IRES may act as the sole ribosome binding site, or may serve as one of multiple ribosome binding sites of an mRNA. Polynucleotides, primary constructs or mmRNA containing more than one functional ribosome binding site may encode several peptides or polypeptides that are translated independently by the ribosomes ("multicistronic nucleic acid molecules"). When polynucleotides, primary constructs or mmRNA are provided with an IRES, further optionally provided is a second translatable region. Examples of IRES sequences that can be used according to the invention include without limitation, those from picornaviruses (e.g. FMDV), pest viruses (CFFV), polio viruses (PV), encephalomyocarditis viruses (ECMV), foot-and-mouth disease viruses (FMDV), hepatitis C viruses (HCV), classical swine fever viruses (CSFV), murine leukemia virus (MLV), simian immune deficiency viruses (SIV) or cricket paralysis viruses (CrPV).

Poly-A tails

[000233] During RNA processing, a long chain of adenine nucleotides (poly-A tail) may be added to a polynucleotide such as an mRNA molecules in order to increase stability. Immediately after transcription, the 3' end of the transcript may be cleaved to free a 3' hydroxyl. Then poly-A polymerase adds a chain of adenine nucleotides to the

RNA. The process, called polyadenylation, adds a poly-A tail that can be between, for example, approximately 100 and 250 residues long.

[000234] It has been discovered that unique poly-A tail lengths provide certain advantages to the polynucleotides, primary constructs or mmRNA of the present invention.

[000235] Generally, the length of a poly-A tail of the present invention is greater than 30 nucleotides in length. In another embodiment, the poly-A tail is greater than 35 nucleotides in length (e.g., at least or greater than about 35, 40, 45, 50, 55, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1,000, 1,100, 1,200, 1,300, 1,400, 1,500, 1,600, 1,700, 1,800, 1,900, 2,000, 2,500, and 3,000 nucleotides). In some embodiments, the polynucleotide, primary construct, or mmRNA includes from about 30 to about 3,000 nucleotides (e.g., from 30 to 50, from 30 to 100, from 30 to 250, from 30 to 500, from 30 to 750, from 30 to 1,000, from 30 to 1,500, from 30 to 2,000, from 30 to 2,500, from 50 to 100, from 50 to 250, from 50 to 500, from 50 to 750, from 50 to 1,000, from 50 to 1,500, from 50 to 2,000, from 50 to 2,500, from 50 to 3,000, from 100 to 500, from 100 to 750, from 100 to 1,000, from 100 to 1,500, from 100 to 2,000, from 100 to 2,500, from 100 to 3,000, from 500 to 750, from 500 to 1,000, from 500 to 1,500, from 500 to 2,000, from 500 to 2,500, from 500 to 3,000, from 1,000 to 1,500, from 1,000 to 2,000, from 1,000 to 2,500, from 1,000 to 3,000, from 1,500 to 2,000, from 1,500 to 2,500, from 1,500 to 3,000, from 2,000 to 3,000, from 2,000 to 2,500, and from 2,500 to 3,000).

[000236] In one embodiment, the poly-A tail is designed relative to the length of the overall polynucleotides, primary constructs or mmRNA. This design may be based on the length of the coding region, the length of a particular feature or region (such as the first or flanking regions), or based on the length of the ultimate product expressed from the polynucleotides, primary constructs or mmRNA.

[000237] In this context the poly-A tail may be 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100% greater in length than the polynucleotides, primary constructs or mmRNA or feature thereof. The poly-A tail may also be designed as a fraction of polynucleotides, primary constructs or mmRNA to which it belongs. In this context, the poly-A tail may be 10, 20, 30, 40, 50, 60, 70, 80, or 90% or more of the total length of the construct or the

total length of the construct minus the poly-A tail. Further, engineered binding sites and conjugation of polynucleotides, primary constructs or mmRNA for Poly-A binding protein may enhance expression.

[000238] Additionally, multiple distinct polynucleotides, primary constructs or mmRNA may be linked together to the PABP (Poly-A binding protein) through the 3′-end using modified nucleotides at the 3′-terminus of the poly-A tail. Transfection experiments can be conducted in relevant cell lines at and protein production can be assayed by ELISA at 12hr, 24hr, 48hr, 72 hr and day 7 post-transfection.

[000239] In one embodiment, the polynucleotide primary constructs of the present invention are designed to include a polyA-G Quartet. The G-quartet is a cyclic hydrogen bonded array of four guanine nucleotides that can be formed by G-rich sequences in both DNA and RNA. In this embodiment, the G-quartet is incorporated at the end of the poly-A tail. The resultant mmRNA construct is assayed for stability, protein production and other parameters including half-life at various time points. It has been discovered that the polyA-G quartet results in protein production equivalent to at least 75% of that seen using a poly-A tail of 120 nucleotides alone.

Quantification

[000240] In one embodiment, the polynucleotides, primary constructs or mmRNA of the present invention may be quantified in exosomes derived from one or more bodily fluid. As used herein “bodily fluids” include peripheral blood, serum, plasma, ascites, urine, cerebrospinal fluid (CSF), sputum, saliva, bone marrow, synovial fluid, aqueous humor, amniotic fluid, cerumen, breast milk, broncheoalveolar lavage fluid, semen, prostatic fluid, cowper's fluid or pre-ejaculatory fluid, sweat, fecal matter, hair, tears, cyst fluid, pleural and peritoneal fluid, pericardial fluid, lymph, chyme, chyle, bile, interstitial fluid, menses, pus, sebum, vomit, vaginal secretions, mucosal secretion, stool water, pancreatic juice, lavage fluids from sinus cavities, bronchopulmonary aspirates, blastocyl cavity fluid, and umbilical cord blood. Alternatively, exosomes may be retrieved from an organ selected from the group consisting of lung, heart, pancreas, stomach, intestine, bladder, kidney, ovary, testis, skin, colon, breast, prostate, brain, esophagus, liver, and placenta.

[000241] In the quantification method, a sample of not more than 2mL is obtained from the subject and the exosomes isolated by size exclusion chromatography, density gradient centrifugation, differential centrifugation, nanomembrane ultrafiltration, immunoabsorbent capture, affinity purification, microfluidic separation, or combinations thereof. In the analysis, the level or concentration of a polynucleotide, primary construct or mmRNA may be an expression level, presence, absence, truncation or alteration of the administered construct. It is advantageous to correlate the level with one or more clinical phenotypes or with an assay for a human disease biomarker. The assay may be performed using construct specific probes, cytometry, qRT-PCR, real-time PCR, PCR, flow cytometry, electrophoresis, mass spectrometry, or combinations thereof while the exosomes may be isolated using immunohistochemical methods such as enzyme linked immunosorbent assay (ELISA) methods. Exosomes may also be isolated by size exclusion chromatography, density gradient centrifugation, differential centrifugation, nanomembrane ultrafiltration, immunoabsorbent capture, affinity purification, microfluidic separation, or combinations thereof.

[000242] These methods afford the investigator the ability to monitor, in real time, the level of polynucleotides, primary constructs or mmRNA remaining or delivered. This is possible because the polynucleotides, primary constructs or mmRNA of the present invention differ from the endogenous forms due to the structural or chemical modifications.

II. Design and synthesis of mmRNA

[000243] Polynucleotides, primary constructs or mmRNA for use in accordance with the invention may be prepared according to any available technique including, but not limited to chemical synthesis, enzymatic synthesis, which is generally termed *in vitro* transcription (IVT) or enzymatic or chemical cleavage of a longer precursor, etc. Methods of synthesizing RNAs are known in the art (see, *e.g.*, Gait, M.J. (ed.) *Oligonucleotide synthesis: a practical approach*, Oxford [Oxfordshire], Washington, DC: IRL Press, 1984; and Herdewijn, P. (ed.) *Oligonucleotide synthesis: methods and applications*, Methods in Molecular Biology, v. 288 (Clifton, N.J.) Totowa, N.J.: Humana Press, 2005; both of which are incorporated herein by reference).

[000244] The process of design and synthesis of the primary constructs of the invention generally includes the steps of gene construction, mRNA production (either with or without modifications) and purification. In the enzymatic synthesis method, a target polynucleotide sequence encoding the polypeptide of interest is first selected for incorporation into a vector which will be amplified to produce a cDNA template. Optionally, the target polynucleotide sequence and/or any flanking sequences may be codon optimized. The cDNA template is then used to produce mRNA through *in vitro* transcription (IVT). After production, the mRNA may undergo purification and clean-up processes. The steps of which are provided in more detail below.

Gene Construction

[000245] The step of gene construction may include, but is not limited to gene synthesis, vector amplification, plasmid purification, plasmid linearization and clean-up, and cDNA template synthesis and clean-up.

Gene Synthesis

[000246] Once a polypeptide of interest, or target, is selected for production, a primary construct is designed. Within the primary construct, a first region of linked nucleosides encoding the polypeptide of interest may be constructed using an open reading frame (ORF) of a selected nucleic acid (DNA or RNA) transcript. The ORF may comprise the wild type ORF, an isoform, variant or a fragment thereof. As used herein, an "open reading frame" or "ORF" is meant to refer to a nucleic acid sequence (DNA or RNA) which is capable of encoding a polypeptide of interest. ORFs often begin with the start codon, ATG and end with a nonsense or termination codon or signal.

[000247] Further, the nucleotide sequence of the first region may be codon optimized. Codon optimization methods are known in the art and may be useful in efforts to achieve one or more of several goals. These goals include to match codon frequencies in target and host organisms to ensure proper folding, bias GC content to increase mRNA stability or reduce secondary structures, minimize tandem repeat codons or base runs that may impair gene construction or expression, customize transcriptional and translational control regions, insert or remove protein trafficking sequences, remove/add post translation modification sites in encoded protein (e.g. glycosylation sites), add, remove or shuffle protein domains, insert or delete restriction sites, modify ribosome binding sites

and mRNA degradation sites, to adjust translational rates to allow the various domains of the protein to fold properly, or to reduce or eliminate problem secondary structures within the mRNA. Codon optimization tools, algorithms and services are known in the art, non-limiting examples include services from GeneArt (Life Technologies), DNA2.0 (Menlo Park CA) and/or proprietary methods. In one embodiment, the ORF sequence is optimized using optimization algorithms. Codon options for each amino acid are given in Table 1.

Table 1. Codon Options

Amino Acid	Single Letter Code	Codon Options
Isoleucine	I	ATT, ATC, ATA
Leucine	L	CTT, CTC, CTA, CTG, TTA, TTG
Valine	V	GTT, GTC, GTA, GTG
Phenylalanine	F	TTT, TTC
Methionine	M	ATG
Cysteine	C	TGT, TGC
Alanine	A	GCT, GCC, GCA, GCG
Glycine	G	GGT, GGC, GGA, GGG
Proline	P	CCT, CCC, CCA, CCG
Threonine	T	ACT, ACC, ACA, ACG
Serine	S	TCT, TCC, TCA, TCG, AGT, AGC
Tyrosine	Y	TAT, TAC
Tryptophan	W	TGG
Glutamine	Q	CAA, CAG
Asparagine	N	AAT, AAC
Histidine	H	CAT, CAC
Glutamic acid	E	GAA, GAG
Aspartic acid	D	GAT, GAC
Lysine	K	AAA, AAG
Arginine	R	CGT, CGC, CGA, CGG, AGA, AGG
Selenocysteine	Sec	UGA in mRNA in presence of Selenocystein insertion element (SECIS)
Stop codons	Stop	TAA, TAG, TGA

[000248] Features, which may be considered beneficial in some embodiments of the present invention, may be encoded by the primary construct and may flank the ORF as a first or second flanking region. The flanking regions may be incorporated into the primary construct before and/or after optimization of the ORF. It is not required that a primary construct contain both a 5′ and 3′ flanking region. Examples of such features include, but are not limited to, untranslated regions (UTRs), Kozak sequences, an

oligo(dT) sequence, and detectable tags and may include multiple cloning sites which may have XbaI recognition.

[000249] In some embodiments, a 5′ UTR and/or a 3′ UTR may be provided as flanking regions. Multiple 5′ or 3′ UTRs may be included in the flanking regions and may be the same or of different sequences. Any portion of the flanking regions, including none, may be codon optimized and any may independently contain one or more different structural or chemical modifications, before and/or after codon optimization. Combinations of features may be included in the first and second flanking regions and may be contained within other features. For example, the ORF may be flanked by a 5' UTR which may contain a strong Kozak translational initiation signal and/or a 3' UTR which may include an oligo(dT) sequence for templated addition of a poly-A tail. 5'UTR may comprise a first polynucleotide fragment and a second polynucleotide fragment from the same and/or different genes such as the 5'UTRs described in US Patent Application Publication No. 20100293625, herein incorporated by reference in its entirety.

[000250] Tables 2 and 3 provide a listing of exemplary UTRs which may be utilized in the primary construct of the present invention as flanking regions. Shown in Table 2 is a listing of a 5′-untranslated region of the invention. Variants of 5' UTRs may be utilized wherein one or more nucleotides are added or removed to the termini, including A, T, C or G.

Table 2. 5′-Untranslated Regions

5′ UTR Identifier	Name/Description	Sequence	SEQ ID NO.
5UTR-001	Upstream UTR	GGGAAATAAGAGAGAAAAGAAGAGTAAGAAGAAATATAAGAGCCACC	1
5UTR-002	Upstream UTR	GGGAGATCAGAGAGAAAAGAAGAGTAAGAAGAAATATAAGAGCCACC	2
5UTR-003	Upstream UTR	GGAATAAAAGTCTCAACACAACATATACAAAACAAACGAATCTCAAGCAATCAAGCATTCTACTTCTATTGCAGCAATTTAAATCATTTCTTTTAAAGCAAAAGCAATTTTCTGAAAATTTTCACCATTTACGAACGATAGCAAC	3
5UTR-004	Upstream UTR	GGGAGACAAGCUUGGCAUUCCGGUACUGUUGGUAAAGCCACC	4

[000251] Shown in Table 3 is a representative listing of 3′-untranslated regions of the invention. Variants of 3' UTRs may be utilized wherein one or more nucleotides are added or removed to the termini, including A, T, C or G.

Table 3. 3′-Untranslated Regions

3′ UTR Identifier	Name/Description	Sequence	SEQ ID NO.
3UTR-001	Creatine Kinase	GCGCCTGCCCACCTGCCACCGACTGCTGGA ACCCAGCCAGTGGGAGGGCCTGGCCCACC AGAGTCCTGCTCCCTCACTCCTCGCCCCGC CCCCTGTCCCAGAGTCCCACCTGGGGGCTC TCTCCACCCTTCTCAGAGTTCCAGTTTCAAC CAGAGTTCCAACCAATGGGCTCCATCCTCT GGATTCTGGCCAATGAAATATCTCCCTGGC AGGGTCCTCTTCTTTTCCCAGAGCTCCACCC CAACCAGGAGCTCTAGTTAATGGAGAGCTC CCAGCACACTCGGAGCTTGTGCTTTGTCTC CACGCAAAGCGATAAATAAAAGCATTGGT GGCCTTTGGTCTTTGAATAAAGCCTGAGTA GGAAGTCTAGA	5
3UTR-002	Myoglobin	GCCCCTGCCGCTCCCACCCCCACCCATCTG GGCCCCGGGTTCAAGAGAGAGCGGGGTCT GATCTCGTGTAGCCATATAGAGTTTGCTTC TGAGTGTCTGCTTTGTTTAGTAGAGGTGGG CAGGAGGAGCTGAGGGGCTGGGGCTGGGG TGTTGAAGTTGGCTTTGCATGCCCAGCGAT GCGCCTCCCTGTGGGATGTCATCACCCTGG GAACCGGGAGTGGCCCTTGGCTCACTGTGT TCTGCATGGTTTGGATCTGAATTAATTGTCC TTTCTTCTAAATCCCAACCGAACTTCTTCCA ACCTCCAAACTGGCTGTAACCCCAAATCCA AGCCATTAACTACACCTGACAGTAGCAATT GTCTGATTAATCACTGGCCCCTTGAAGACA GCAGAATGTCCCTTTGCAATGAGGAGGAG ATCTGGGCTGGGCGGGCCAGCTGGGGAAG CATTTGACTATCTGGAACTTGTGTGTGCCTC CTCAGGTATGGCAGTGACTCACCTGGTTTT AATAAAACAACCTGCAACATCTCATGGTCT TTGAATAAAGCCTGAGTAGGAAGTCTAGA	6
3UTR-003	α-actin	ACACACTCCACCTCCAGCACGCGACTTCTC AGGACGACGAATCTTCTCAATGGGGGGGC GGCTGAGCTCCAGCCACCCCGCAGTCACTT TCTTTGTAACAACTTCCGTTGCTGCCATCGT AAACTGACACAGTGTTTATAACGTGTACAT ACATTAACTTATTACCTCATTTTGTTATTTT TCGAAACAAAGCCCTGTGGAAGAAAATGG AAAACTTGAAGAAGCATTAAAGTCATTCTG TTAAGCTGCGTAAATGGTCTTTGAATAAAG	7

		CCTGAGTAGGAAGTCTAGA	
3UTR-004	Albumin	CATCACATTTAAAAGCATCTCAGCCTACCA TGAGAATAAGAGAAAGAAAATGAAGATCA AAAGCTTATTCATCTGTTTTTCTTTTTCGTT GGTGTAAAGCCAACACCCTGTCTAAAAAAC ATAAATTTCTTTAATCATTTTGCCTCTTTTC TCTGTGCTTCAATTAATAAAAAATGGAAAG AATCTAATAGAGTGGTACAGCACTGTTATT TTTCAAAGATGTGTTGCTATCCTGAAAATT CTGTAGGTTCTGTGGAAGTTCCAGTGTTCT CTCTTATTCCACTTCGGTAGAGGATTTCTAG TTTCTTGTGGGCTAATTAAATAAATCATTA ATACTCTTCTAATGGTCTTTGAATAAAGCC TGAGTAGGAAGTCTAGA	8
3UTR-005	α-globin	GCTGCCTTCTGCGGGGCTTGCCTTCTGGCC ATGCCCTTCTTCTCTCCCTTGCACCTGTACC TCTTGGTCTTTGAATAAAGCCTGAGTAGGA AGGCGGCCGCTCGAGCATGCATCTAGA	9
3UTR-006	G-CSF	GCCAAGCCCTCCCCATCCCATGTATTTATCT CTATTTAATATTTATGTCTATTTAAGCCTCA TATTTAAAGACAGGGAAGAGCAGAACGGA GCCCCAGGCCTCTGTGTCCTTCCCTGCATTT CTGAGTTTCATTCTCCTGCCTGTAGCAGTG AGAAAAAGCTCCTGTCCTCCCATCCCCTGG ACTGGGAGGTAGATAGGTAAATACCAAGT ATTTATTACTATGACTGCTCCCCAGCCCTG GCTCTGCAATGGGCACTGGGATGAGCCGCT GTGAGCCCCTGGTCCTGAGGGTCCCCACCT GGGACCCTTGAGAGTATCAGGTCTCCCACG TGGGAGACAAGAAATCCCTGTTTAATATTT AAACAGCAGTGTTCCCCATCTGGGTCCTTG CACCCCTCACTCTGGCCTCAGCCGACTGCA CAGCGGCCCCTGCATCCCCTTGGCTGTGAG GCCCCTGGACAAGCAGAGGTGGCCAGAGC TGGGAGGCATGGCCCTGGGGTCCCACGAAT TTGCTGGGGAATCTCGTTTTTCTTCTTAAGA CTTTTGGGACATGGTTTGACTCCCGAACAT CACCGACGCGTCTCCTGTTTTTCTGGGTGG CCTCGGGACACCTGCCCTGCCCCCACGAGG GTCAGGACTGTGACTCTTTTTAGGGCCAGG CAGGTGCCTGGACATTTGCCTTGCTGGACG GGGACTGGGGATGTGGGAGGGAGCAGACA GGAGGAATCATGTCAGGCCTGTGTGTGAAA GGAAGCTCCACTGTCACCCTCCACCTCTTC ACCCCCCACTCACCAGTGTCCCCTCCACTG TCACATTGTAACTGAACTTCAGGATAATAA AGTGTTTGCCTCCATGGTCTTTGAATAAAG CCTGAGTAGGAAGGCGGCCGCTCGAGCAT GCATCTAGA	10
3UTR-007	Col1a2;	ACTCAATCTAAATTAAAAAAGAAAGAAAT	11

	collagen, type I, alpha 2	TTGAAAAAACTTTCTCTTTGCCATTTCTTCT TCTTCTTTTTTAACTGAAAGCTGAATCCTTC CATTTCTTCTGCACATCTACTTGCTTAAATT GTGGGCAAAAGAGAAAAAGAAGGATTGAT CAGAGCATTGTGCAATACAGTTTCATTAAC TCCTTCCCCCGCTCCCCCAAAAATTTGAATT TTTTTTTCAACACTCTTACACCTGTTATGGA AAATGTCAACCTTTGTAAGAAAACCAAAAT AAAAATTGAAAAATAAAAACCATAAACAT TTGCACCACTTGTGGCTTTTGAATATCTTCC ACAGAGGGAAGTTTAAAACCCAAACTTCC AAAGGTTTAAACTACCTCAAAACACTTTCC CATGAGTGTGATCCACATTGTTAGGTGCTG ACCTAGACAGAGATGAACTGAGGTCCTTGT TTTGTTTTGTTCATAATACAAAGGTGCTAAT TAATAGTATTTCAGATACTTGAAGAATGTT GATGGTGCTAGAAGAATTTGAGAAGAAAT ACTCCTGTATTGAGTTGTATCGTGTGGTGT ATTTTTTAAAAAATTTGATTTAGCATTCATA TTTTCCATCTTATTCCCAATTAAAAGTATGC AGATTATTTGCCCAAATCTTCTTCAGATTCA GCATTTGTTCTTTGCCAGTCTCATTTTCATC TTCTTCCATGGTTCCACAGAAGCTTTGTTTC TTGGGCAAGCAGAAAAATTAAATTGTACCT ATTTTGTATATGTGAGATGTTTAAATAAAT TGTGAAAAAAATGAAATAAAGCATGTTTG GTTTTCCAAAAGAACATAT	
3UTR-008	Col6a2; collagen, type VI, alpha 2	CGCCGCCGCCCGGGCCCCGCAGTCGAGGGT CGTGAGCCCACCCCGTCCATGGTGCTAAGC GGGCCCGGGTCCCACACGGCCAGCACCGCT GCTCACTCGGACGACGCCCTGGGCCTGCAC CTCTCCAGCTCCTCCCACGGGGTCCCCGTA GCCCCGGCCCCCGCCCAGCCCCAGGTCTCC CCAGGCCCTCCGCAGGCTGCCCGGCCTCCC TCCCCCTGCAGCCATCCCAAGGCTCCTGAC CTACCTGGCCCCTGAGCTCTGGAGCAAGCC CTGACCCAATAAAGGCTTTGAACCCAT	12
3UTR-009	RPN1; ribophorin I	GGGGCTAGAGCCCTCTCCGCACAGCGTGGA GACGGGGCAAGGAGGGGGGTTATTAGGAT TGGTGGTTTTGTTTTGCTTTGTTTAAAGCCG TGGGAAAATGGCACAACTTTACCTCTGTGG GAGATGCAACACTGAGAGCCAAGGGGTGG GAGTTGGGATAATTTTTATATAAAAGAAGT TTTTCCACTTTGAATTGCTAAAAGTGGCATT TTTCCTATGTGCAGTCACTCCTCTCATTTCT AAAATAGGGACGTGGCCAGGCACGGTGGC TCATGCCTGTAATCCCAGCACTTTGGGAGG CCGAGGCAGGCGGCTCACGAGGTCAGGAG ATCGAGACTATCCTGGCTAACACGGTAAAA CCCTGTCTCTACTAAAAGTACAAAAAATTA GCTGGGCGTGGTGGTGGGCACCTGTAGTCC	13

		CAGCTACTCGGGAGGCTGAGGCAGGAGAA AGGCATGAATCCAAGAGGCAGAGCTTGCA GTGAGCTGAGATCACGCCATTGCACTCCAG CCTGGGCAACAGTGTTAAGACTCTGTCTCA AATATAAATAAATAAATAAATAAATAAAT AAATAAATAAAAATAAAGCGAGATGTTGC CCTCAAA	
3UTR-010	LRP1; low density lipoprotein receptor-related protein 1	GGCCCTGCCCCGTCGGACTGCCCCCAGAAA GCCTCCTGCCCCCTGCCAGTGAAGTCCTTC AGTGAGCCCCTCCCCAGCCAGCCCTTCCCT GGCCCCGCCGGATGTATAAATGTAAAAATG AAGGAATTACATTTTATATGTGAGCGAGCA AGCCGGCAAGCGAGCACAGTATTATTTCTC CATCCCCTCCCTGCCTGCTCCTTGGCACCCC CATGCTGCCTTCAGGGAGACAGGCAGGGA GGGCTTGGGGCTGCACCTCCTACCCTCCCA CCAGAACGCACCCCACTGGGAGAGCTGGT GGTGCAGCCTTCCCCTCCCTGTATAAGACA CTTTGCCAAGGCTCTCCCCTCTCGCCCCATC CCTGCTTGCCCGCTCCCACAGCTTCCTGAG GGCTAATTCTGGGAAGGGAGAGTTCTTTGC TGCCCCTGTCTGGAAGACGTGGCTCTGGGT GAGGTAGGCGGGAAAGGATGGAGTGTTTT AGTTCTTGGGGGAGGCCACCCCAAACCCCA GCCCCAACTCCAGGGGCACCTATGAGATGG CCATGCTCAACCCCCCTCCCAGACAGGCCC TCCCTGTCTCCAGGGCCCCCACCGAGGTTC CCAGGGCTGGAGACTTCCTCTGGTAAACAT TCCTCCAGCCTCCCCTCCCCTGGGGACGCC AAGGAGGTGGGCCACACCCAGGAAGGGAA AGCGGGCAGCCCCGTTTTGGGGACGTGAAC GTTTTAATAATTTTTGCTGAATTCCTTTACA ACTAAATAACACAGATATTGTTATAAATAA AATTGT	14
3UTR-011	Nnt1; cardiotrophin-like cytokine factor 1	ATATTAAGGATCAAGCTGTTAGCTAATAAT GCCACCTCTGCAGTTTTGGGAACAGGCAAA TAAAGTATCAGTATACATGGTGATGTACAT CTGTAGCAAAGCTCTTGGAGAAAATGAAG ACTGAAGAAAGCAAAGCAAAAACTGTATA GAGAGATTTTTCAAAAGCAGTAATCCCTCA ATTTTAAAAAAGGATTGAAAATTCTAAATG TCTTTCTGTGCATATTTTTTGTGTTAGGAAT CAAAAGTATTTTATAAAAGGAGAAAGAAC AGCCTCATTTTAGATGTAGTCCTGTTGGATT TTTTATGCCTCCTCAGTAACCAGAAATGTTT TAAAAAACTAAGTGTTTAGGATTTCAAGAC AACATTATACATGGCTCTGAAATATCTGAC ACAATGTAAACATTGCAGGCACCTGCATTT TATGTTTTTTTTTTCAACAAATGTGACTAAT TTGAAACTTTTATGAACTTCTGAGCTGTCCC CTTGCAATTCAACCGCAGTTTGAATTAATC	15

		ATATCAAATCAGTTTTAATTTTTTAAATTGT ACTTCAGAGTCTATATTTCAAGGGCACATT TTCTCACTACTATTTTAATACATTAAAGGA CTAAATAATCTTTCAGAGATGCTGGAAACA AATCATTTGCTTTATATGTTTCATTAGAATA CCAATGAAACATACAACTTGAAAATTAGTA ATAGTATTTTTGAAGATCCCATTTCTAATTG GAGATCTCTTTAATTTCGATCAACTTATAAT GTGTAGTACTATATTAAGTGCACTTGAGTG GAATTCAACATTTGACTAATAAAATGAGTT CATCATGTTGGCAAGTGATGTGGCAATTAT CTCTGGTGACAAAAGAGTAAAATCAAATAT TTCTGCCTGTTACAAATATCAAGGAAGACC TGCTACTATGAAATAGATGACATTAATCTG TCTTCACTGTTTATAATACGGATGGATTTTT TTTCAAATCAGTGTGTGTTTTGAGGTCTTAT GTAATTGATGACATTTGAGAGAAATGGTGG CTTTTTTTAGCTACCTCTTTGTTCATTTAAG CACCAGTAAAGATCATGTCTTTTTATAGAA GTGTAGATTTTCTTTGTGACTTTGCTATCGT GCCTAAAGCTCTAAATATAGGTGAATGTGT GATGAATACTCAGATTATTTGTCTCTCTATA TAATTAGTTTGGTACTAAGTTTCTCAAAAA ATTATTAACACATGAAAGACAATCTCTAAA CCAGAAAAAGAAGTAGTACAAATTTTGTTA CTGTAATGCTCGCGTTTAGTGAGTTTAAAA CACACAGTATCTTTTGGTTTTATAATCAGTT TCTATTTTGCTGTGCCTGAGATTAAGATCTG TGTATGTGTGTGTGTGTGTGTGTGCGTTTGT GTGTTAAAGCAGAAAAGACTTTTTTAAAAG TTTTAAGTGATAAATGCAATTTGTTAATTG ATCTTAGATCACTAGTAAACTCAGGGCTGA ATTATACCATGTATATTCTATTAGAAGAAA GTAAACACCATCTTTATTCCTGCCCTTTTTC TTCTCTCAAAGTAGTTGTAGTTATATCTAG AAAGAAGCAATTTTGATTTCTTGAAAAGGT AGTTCCTGCACTCAGTTTAAACTAAAAATA ATCATACTTGGATTTTATTTATTTTTGTCAT AGTAAAAATTTTAATTTATATATATTTTTAT TTAGTATTATCTTATTCTTTGCTATTTGCCA ATCCTTTGTCATCAATTGTGTTAAATGAATT GAAAATTCATGCCCTGTTCATTTTATTTTAC TTTATTGGTTAGGATATTTAAAGGATTTTTG TATATATAATTTCTTAAATTAATATTCCAAA AGGTTAGTGGACTTAGATTATAAATTATGG CAAAAATCTAAAAACAACAAAAATGATTTT TATACATTCTATTTCATTATTCCTCTTTTTCC AATAAGTCATACAATTGGTAGATATGACTT ATTTTATTTTTGTATTATTCACTATATCTTT ATGATATTTAAGTATAAATAATTAAAAAAA TTTATTGTACCTTATAGTCTGTCACCAAAA	

		AAAAAAAATTATCTGTAGGTAGTGAAATGC TAATGTTGATTTGTCTTTAAGGGCTTGTTAA CTATCCTTTATTTTCTCATTTGTCTTAAATT AGGAGTTTGTGTTTAAATTACTCATCTAAG CAAAAAATGTATATAAATCCCATTACTGGG TATATACCCAAAGGATTATAAATCATGCTG CTATAAAGACACATGCACACGTATGTTTAT TGCAGCACTATTCACAATAGCAAAGACTTG GAACCAACCCAAATGTCCATCAATGATAGA CTTGATTAAGAAAATGTGCACATATACACC ATGGAATACTATGCAGCCATAAAAAAGGA TGAGTTCATGTCCTTTGTAGGGACATGGAT AAAGCTGGAAACCATCATTCTGAGCAAACT ATTGCAAGGACAGAAAACCAAACACTGCA TGTTCTCACTCATAGGTGGGAATTGAACAA TGAGAACACTTGGACACAAGGTGGGGAAC ACCACACACCAGGGCCTGTCATGGGGTGG GGGGAGTGGGGAGGGATAGCATTAGGAGA TATACCTAATGTAAATGATGAGTTAATGGG TGCAGCACACCAACATGGCACATGTATACA TATGTAGCAAACCTGCACGTTGTGCACATG TACCCTAGAACTTAAAGTATAATTAAAAAA AAAAAGAAAACAGAAGCTATTTATAAAGA AGTTATTTGCTGAAATAAATGTGATCTTTC CCATTAAAAAAATAAAGAAATTTTGGGGTA AAAAAACACAATATATTGTATTCTTGAAAA ATTCTAAGAGAGTGGATGTGAAGTGTTCTC ACCACAAAAGTGATAACTAATTGAGGTAAT GCACATATTAATTAGAAAGATTTTGTCATT CCACAATGTATATATACTTAAAAATATGTT ATACACAATAAATACATACATTAAAAAATA AGTAAATGTA	
3UTR-012	Col6a1; collagen, type VI, alpha 1	CCCACCCTGCACGCCGGCACCAAACCCTGT CCTCCCACCCCTCCCCACTCATCACTAAAC AGAGTAAAATGTGATGCGAATTTTCCCGAC CAACCTGATTCGCTAGATTTTTTTTAAGGA AAAGCTTGGAAAGCCAGGACACAACGCTG CTGCCTGCTTTGTGCAGGGTCCTCCGGGGC TCAGCCCTGAGTTGGCATCACCTGCGCAGG GCCCTCTGGGGCTCAGCCCTGAGCTAGTGT CACCTGCACAGGGCCCTCTGAGGCTCAGCC CTGAGCTGGCGTCACCTGTGCAGGGCCCTC TGGGGCTCAGCCCTGAGCTGGCCTCACCTG GGTTCCCCACCCCGGGCTCTCCTGCCCTGC CCTCCTGCCCGCCCTCCCTCCTGCCTGCGCA GCTCCTTCCCTAGGCACCTCTGTGCTGCATC CCACCAGCCTGAGCAAGACGCCCTCTCGGG GCCTGTGCCGCACTAGCCTCCCTCTCCTCTG TCCCCATAGCTGGTTTTTCCCACCAATCCTC ACCTAACAGTTACTTTACAATTAAACTCAA AGCAAGCTCTTCTCCTCAGCTTGGGGCAGC	16

		CATTGGCCTCTGTCTCGTTTTGGGAAACCA AGGTCAGGAGGCCGTTGCAGACATAAATCT CGGCGACTCGGCCCCGTCTCCTGAGGGTCC TGCTGGTGACCGGCCTGGACCTTGGCCCTA CAGCCCTGGAGGCCGCTGCTGACCAGCACT GACCCCGACCTCAGAGAGTACTCGCAGGG GCGCTGGCTGCACTCAAGACCCTCGAGATT AACGGTGCTAACCCCGTCTGCTCCTCCCTC CCGCAGAGACTGGGGCCTGGACTGGACAT GAGAGCCCCTTGGTGCCACAGAGGGCTGTG TCTTACTAGAAACAACGCAAACCTCTCCTT CCTCAGAATAGTGATGTGTTCGACGTTTTA TCAAAGGCCCCCTTTCTATGTTCATGTTAGT TTTGCTCCTTCTGTGTTTTTTTCTGAACCAT ATCCATGTTGCTGACTTTTCCAAATAAAGG TTTTCACTCCTCTC	
3UTR-013	Calr; calreticulin	AGAGGCCTGCCTCCAGGGCTGGACTGAGG CCTGAGCGCTCCTGCCGCAGAGCTGGCCGC GCCAAATAATGTCTCTGTGAGACTCGAGAA CTTTCATTTTTTTCCAGGCTGGTTCGGATTT GGGGTGGATTTTGGTTTTGTTCCCCTCCTCC ACTCTCCCCCACCCCCTCCCCGCCCTTTTTT TTTTTTTTTTTTAAACTGGTATTTTATCTTTG ATTCTCCTTCAGCCCTCACCCCTGGTTCTCA TCTTTCTTGATCAACATCTTTTCTTGCCTCT GTCCCCTTCTCTCATCTCTTAGCTCCCCTCC AACCTGGGGGGCAGTGGTGTGGAGAAGCC ACAGGCCTGAGATTTCATCTGCTCTCCTTCC TGGAGCCCAGAGGAGGGCAGCAGAAGGGG GTGGTGTCTCCAACCCCCCAGCACTGAGGA AGAACGGGGCTCTTCTCATTTCACCCCTCC CTTTCTCCCCTGCCCCCAGGACTGGGCCAC TTCTGGGTGGGGCAGTGGGTCCCAGATTGG CTCACACTGAGAATGTAAGAACTACAAAC AAAATTTCTATTAAATTAAATTTTGTGTCTC C	17
3UTR-014	Col1a1; collagen, type I, alpha 1	CTCCCTCCATCCCAACCTGGCTCCCTCCCAC CCAACCAACTTTCCCCCCAACCCGGAAACA GACAAGCAACCCAAACTGAACCCCCTCAA AAGCCAAAAAATGGGAGACAATTTCACAT GGACTTTGGAAAATATTTTTTCCTTTGCAT TCATCTCTCAAACTTAGTTTTTATCTTTGAC CAACCGAACATGACCAAAAACCAAAAGTG CATTCAACCTTACCAAAAAAAAAAAAAAAA AAAAGAATAAATAAATAACTTTTTAAAAA AGGAAGCTTGGTCCACTTGCTTGAAGACCC ATGCGGGGGTAAGTCCCTTTCTGCCCGTTG GGCTTATGAAACCCCAATGCTGCCCTTTCT GCTCCTTTCTCCACACCCCCCTTGGGGCCTC CCCTCCACTCCTTCCCAAATCTGTCTCCCCA GAAGACACAGGAAACAATGTATTGTCTGCC	18

		CAGCAATCAAAGGCAATGCTCAAACACCC AAGTGGCCCCCACCCTCAGCCCGCTCCTGC CCGCCCAGCACCCCCAGGCCCTGGGGGACC TGGGGTTCTCAGACTGCCAAAGAAGCCTTG CCATCTGGCGCTCCCATGGCTCTTGCAACA TCTCCCCTTCGTTTTTGAGGGGGTCATGCCG GGGGAGCCACCAGCCCCTCACTGGGTTCGG AGGAGAGTCAGGAAGGGCCACGACAAAGC AGAAACATCGGATTTGGGGAACGCGTGTC AATCCCTTGTGCCGCAGGGCTGGGCGGGAG AGACTGTTCTGTTCCTTGTGTAACTGTGTTG CTGAAAGACTACCTCGTTCTTGTCTTGATGT GTCACCGGGGCAACTGCCTGGGGGCGGGG ATGGGGGCAGGGTGGAAGCGGCTCCCCAT TTTATACCAAAGGTGCTACATCTATGTGAT GGGTGGGGTGGGGAGGGAATCACTGGTGC TATAGAAATTGAGATGCCCCCCCAGGCCAG CAAATGTTCCTTTTTGTTCAAAGTCTATTTT TATTCCTTGATATTTTTCTTTTTTTTTTTTT TTTTTGTGGATGGGGACTTGTGAATTTTTCT AAAGGTGCTATTTAACATGGGAGGAGAGC GTGTGCGGCTCCAGCCCAGCCCGCTGCTCA CTTTCCACCCTCTCTCCACCTGCCTCTGGCT TCTCAGGCCTCTGCTCTCCGACCTCTCTCCT CTGAAACCCTCCTCCACAGCTGCAGCCCAT CCTCCCGGCTCCCTCCTAGTCTGTCCTGCGT CCTCTGTCCCCGGGTTTCAGAGACAACTTC CCAAAGCACAAAGCAGTTTTTCCCCCTAGG GGTGGGAGGAAGCAAAAGACTCTGTACCT ATTTTGTATGTGTATAATAATTTGAGATGTT TTTAATTATTTTGATTGCTGGAATAAAGCA TGTGGAAATGACCCAAACATAATCCGCAGT GGCCTCCTAATTTCCTTCTTTGGAGTTGGGG GAGGGGTAGACATGGGGAAGGGGCTTTGG GGTGATGGGCTTGCCTTCCATTCCTGCCCTT TCCCTCCCCACTATTCTCTTCTAGATCCCTC CATAACCCCACTCCCCTTTCTCTCACCCTTC TTATACCGCAAACCTTTCTACTTCCTCTTTC ATTTTCTATTCTTGCAATTTCCTTGCACCTT TTCCAAATCCTCTTCTCCCCTGCAATACCAT ACAGGCAATCCACGTGCACAACACACACA CACACTCTTCACATCTGGGGTTGTCCAAAC CTCATACCCACTCCCCTTCAAGCCCATCCA CTCTCCACCCCCTGGATGCCCTGCACTTGG TGGCGGTGGGATGCTCATGGATACTGGGAG GGTGAGGGGAGTGGAACCCGTGAGGAGGA CCTGGGGGCCTCTCCTTGAACTGACATGAA GGGTCATCTGGCCTCTGCTCCCTTCTCACCC ACGCTGACCTCCTGCCGAAGGAGCAACGC AACAGGAGAGGGGTCTGCTGAGCCTGGCG AGGGTCTGGGAGGGACCAGGAGGAAGGCG	

		TGCTCCCTGCTCGCTGTCCTGGCCCTGGGG GAGTGAGGGAGACAGACACCTGGGAGAGC TGTGGGGAAGGCACTCGCACCGTGCTCTTG GGAAGGAAGGAGACCTGGCCCTGCTCACC ACGGACTGGGTGCCTCGACCTCCTGAATCC CCAGAACACAACCCCCCTGGGCTGGGGTG GTCTGGGGAACCATCGTGCCCCCGCCTCCC GCCTACTCCTTTTTAAGCTT	
3UTR-015	Plod1; procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1	TTGGCCAGGCCTGACCCTCTTGGACCTTTCT TCTTTGCCGACAACCACTGCCCAGCAGCCT CTGGGACCTCGGGGTCCCAGGGAACCCAGT CCAGCCTCCTGGCTGTTGACTTCCCATTGCT CTTGGAGCCACCAATCAAAGAGATTCAAA GAGATTCCTGCAGGCCAGAGGCGGAACAC ACCTTTATGGCTGGGGCTCTCCGTGGTGTT CTGGACCCAGCCCCTGGAGACACCATTCAC TTTTACTGCTTTGTAGTGACTCGTGCTCTCC AACCTGTCTTCCTGAAAAACCAAGGCCCCC TTCCCCCACCTCTTCCATGGGGTGAGACTT GAGCAGAACAGGGGCTTCCCCAAGTTGCCC AGAAAGACTGTCTGGGTGAGAAGCCATGG CCAGAGCTTCTCCCAGGCACAGGTGTTGCA CCAGGGACTTCTGCTTCAAGTTTTGGGGTA AAGACACCTGGATCAGACTCCAAGGGCTG CCCTGAGTCTGGGACTTCTGCCTCCATGGC TGGTCATGAGAGCAAACCGTAGTCCCCTGG AGACAGCGACTCCAGAGAACCTCTTGGGA GACAGAAGAGGCATCTGTGCACAGCTCGA TCTTCTACTTGCCTGTGGGGAGGGGAGTGA CAGGTCCACACACCACACTGGGTCACCCTG TCCTGGATGCCTCTGAAGAGAGGGACAGA CCGTCAGAAACTGGAGAGTTTCTATTAAAG GTCATTTAAACCA	19
3UTR-016	Nucb1; nucleobindin 1	TCCTCCGGGACCCCAGCCCTCAGGATTCCT GATGCTCCAAGGCGACTGATGGGCGCTGG ATGAAGTGGCACAGTCAGCTTCCCTGGGGG CTGGTGTCATGTTGGGCTCCTGGGGCGGGG GCACGGCCTGGCATTTCACGCATTGCTGCC ACCCCAGGTCCACCTGTCTCCACTTTCACA GCCTCCAAGTCTGTGGCTCTTCCCTTCTGTC CTCCGAGGGGCTTGCCTTCTCTCGTGTCCA GTGAGGTGCTCAGTGATCGGCTTAACTTAG AGAAGCCCGCCCCCTCCCCTTCTCCGTCTG TCCCAAGAGGGTCTGCTCTGAGCCTGCGTT CCTAGGTGGCTCGGCCTCAGCTGCCTGGGT TGTGGCCGCCCTAGCATCCTGTATGCCCAC AGCTACTGGAATCCCCGCTGCTGCTCCGGG CCAAGCTTCTGGTTGATTAATGAGGGCATG GGGTGGTCCCTCAAGACCTTCCCCTACCTT TTGTGGAACCAGTGATGCCTCAAAGACAGT GTCCCCTCCACAGCTGGGTGCCAGGGGCAG	20

		GGGATCCTCAGTATAGCCGGTGAACCCTGA TACCAGGAGCCTGGGCCTCCCTGAACCCCT GGCTTCCAGCCATCTCATCGCCAGCCTCCT CCTGGACCTCTTGGCCCCCAGCCCCTTCCC CACACAGCCCCAGAAGGGTCCCAGAGCTG ACCCCACTCCAGGACCTAGGCCCAGCCCCT CAGCCTCATCTGGAGCCCCTGAAGACCAGT CCCACCCACCTTTCTGGCCTCATCTGACACT GCTCCGCATCCTGCTGTGTGTCCTGTTCCAT GTTCCGGTTCCATCCAAATACACTTTCTGG AACAAA	
3UTR-017	α-globin	GCTGGAGCCTCGGTGGCCATGCTTCTTGCC CCTTGGGCCTCCCCCCAGCCCCTCCTCCCCT TCCTGCACCCGTACCCCCGTGGTCTTTGAA TAAAGTCTGAGTGGGCGGC	21

[000252] It should be understood that those listed in the previous tables are examples and that any UTR from any gene may be incorporated into the respective first or second flanking region of the primary construct. Furthermore, multiple wild-type UTRs of any known gene may be utilized. It is also within the scope of the present invention to provide artificial UTRs which are not variants of wild type genes. These UTRs or portions thereof may be placed in the same orientation as in the transcript from which they were selected or may be altered in orientation or location. Hence a 5′ or 3′ UTR may be inverted, shortened, lengthened, made chimeric with one or more other 5′ UTRs or 3′ UTRs. As used herein, the term "altered" as it relates to a UTR sequence, means that the UTR has been changed in some way in relation to a reference sequence. For example, a 3′ or 5′ UTR may be altered relative to a wild type or native UTR by the change in orientation or location as taught above or may be altered by the inclusion of additional nucleotides, deletion of nucleotides, swapping or transposition of nucleotides. Any of these changes producing an "altered" UTR (whether 3′ or 5′) comprise a variant UTR.

[000253] In one embodiment, a double, triple or quadruple UTR such as a 5′ or 3′ UTR may be used. As used herein, a "double" UTR is one in which two copies of the same UTR are encoded either in series or substantially in series. For example, a double beta-globin 3′ UTR may be used as described in US Patent publication 20100129877, the contents of which are incorporated herein by reference in its entirety.

[000254] It is also within the scope of the present invention to have patterned UTRs. As used herein "patterned UTRs" are those UTRs which reflect a repeating or alternating pattern, such as ABABAB or AABBAABBAABB or ABCABCABC or variants thereof repeated once, twice, or more than 3 times. In these patterns, each letter, A, B, or C represent a different UTR at the nucleotide level.

[000255] In one embodiment, flanking regions are selected from a family of transcripts whose proteins share a common function, structure, feature of property. For example, polypeptides of interest may belong to a family of proteins which are expressed in a particular cell, tissue or at some time during development. The UTRs from any of these genes may be swapped for any other UTR of the same or different family of proteins to create a new chimeric primary transcript. As used herein, a "family of proteins" is used in the broadest sense to refer to a group of two or more polypeptides of interest which share at least one function, structure, feature, localization, origin, or expression pattern.

[000256] After optimization (if desired), the primary construct components are reconstituted and transformed into a vector such as, but not limited to, plasmids, viruses, cosmids, and artificial chromosomes. For example, the optimized construct may be reconstituted and transformed into chemically competent *E. coli,* yeast, neurospora, maize, drosophila, etc. where high copy plasmid-like or chromosome structures occur by methods described herein.

[000257] The untranslated region may also include translation enhancer elements (TEE). As a non-limiting example, the TEE may include those described in US Application No. 20090226470, herein incorporated by reference in its entirety, and those known in the art.

Stop Codons

[000258] In one embodiment, the primary constructs of the present invention may include at least two stop codons before the 3' untranslated region (UTR). The stop codon may be selected from TGA, TAA and TAG. In one embodiment, the primary constructs of the present invention include the stop codon TGA and one additional stop codon. In a further embodiment the addition stop codon may be TAA. In another embodiment, the primary constructs of the present invention include three stop codons.

Vector Amplification

[000259] The vector containing the primary construct is then amplified and the plasmid isolated and purified using methods known in the art such as, but not limited to, a maxi prep using the Invitrogen PURELINK™ HiPure Maxiprep Kit (Carlsbad, CA).

Plasmid Linearization

[000260] The plasmid may then be linearized using methods known in the art such as, but not limited to, the use of restriction enzymes and buffers. The linearization reaction may be purified using methods including, for example Invitrogen's PURELINK™ PCR Micro Kit (Carlsbad, CA), and HPLC based purification methods such as, but not limited to, strong anion exchange HPLC, weak anion exchange HPLC, reverse phase HPLC (RP-HPLC), and hydrophobic interaction HPLC (HIC-HPLC) and Invitrogen's standard PURELINK™ PCR Kit (Carlsbad, CA). The purification method may be modified depending on the size of the linearization reaction which was conducted. The linearized plasmid is then used to generate cDNA for *in vitro* transcription (IVT) reactions.

cDNA Template Synthesis

[000261] A cDNA template may be synthesized by having a linearized plasmid undergo polymerase chain reaction (PCR). Table 4 is a listing of primers and probes that may be usefully in the PCR reactions of the present invention. It should be understood that the listing is not exhaustive and that primer-probe design for any amplification is within the skill of those in the art. Probes may also contain chemically modified bases to increase base-pairing fidelity to the target molecule and base-pairing strength. Such modifications may include 5-methyl-Cytidine, 2, 6-di-amino-purine, 2′-fluoro, phosphoro-thioate, or locked nucleic acids.

Table 4. Primers and Probes

Primer/ Probe Identifier	**Sequence (5′-3′)**	**Hybridization target**	**SEQ ID NO.**
UFP	TTGGACCCTCGTACAGAAGCTAATACG	cDNA Template	22
URP	T_{x160}CTTCCTACTCAGGCTTTATTCAAAGACCA	cDNA Template	23
GBA1	CCTTGACCTTCTGGAACTTC	Acid glucocerebrosidase	24
GBA2	CCAAGCACTGAAACGGATAT	Acid glucocerebrosidase	25
LUC1	GATGAAAAGTGCTCCAAGGA	Luciferase	26

LUC2	AACCGTGATGAAAAGGTACC	Luciferase	27
LUC3	TCATGCAGATTGGAAAGGTC	Luciferase	28
GCSF1	CTTCTTGGACTGTCCAGAGG	G-CSF	29
GCSF2	GCAGTCCCTGATACAAGAAC	G-CSF	30
GCSF3	GATTGAAGGTGGCTCGCTAC	G-CSF	31

*UFP is universal forward primer; URP is universal reverse primer.

[000262] In one embodiment, the cDNA may be submitted for sequencing analysis before undergoing transcription.

mRNA Production

[000263] The process of mRNA or mmRNA production may include, but is not limited to, *in vitro* transcription, cDNA template removal and RNA clean-up, and mRNA capping and/or tailing reactions.

In Vitro Transcription

[000264] The cDNA produced in the previous step may be transcribed using an *in vitro* transcription (IVT) system. The system typically comprises a transcription buffer, nucleotide triphosphates (NTPs), an RNase inhibitor and a polymerase. The NTPs may be manufactured in house, may be selected from a supplier, or may be synthesized as described herein. The NTPs may be selected from, but are not limited to, those described herein including natural and unnatural (modified) NTPs. The polymerase may be selected from, but is not limited to, T7 RNA polymerase, T3 RNA polymerase and mutant polymerases such as, but not limited to, polymerases able to incorporate modified nucleic acids.

RNA Polymerases

[000265] Any number of RNA polymerases or variants may be used in the design of the primary constructs of the present invention.

[000266] RNA polymerases may be modified by inserting or deleting amino acids of the RNA polymerase sequence. As a non-limiting example, the RNA polymerase may be modified to exhibit an increased ability to incorporate a 2'-modified nucleotide triphosphate compared to an unmodified RNA polymerase (see International Publication

WO2008078180 and U.S. Patent 8,101,385; herein incorporated by reference in their entireties).

[000267] Variants may be obtained by evolving an RNA polymerase, optimizing the RNA polymerase amino acid and/or nucleic acid sequence and/or by using other methods known in the art. As a non-limiting example, T7 RNA polymerase variants may be evolved using the continuous directed evolution system set out by Esvelt *et al.* (Nature (2011) 472(7344):499-503; herein incorporated by reference in its entirety) where clones of T7 RNA polymerase may encode at least one mutation such as, but not limited to, lysine at position 93 substituted for threonine (K93T), I4M, A7T, E63V, V64D, A65E, D66Y, T76N, C125R, S128R, A136T, N165S, G175R, H176L, Y178H, F182L, L196F, G198V, D208Y, E222K, S228A, Q239R, T243N, G259D, M267I, G280C, H300R, D351A, A354S, E356D, L360P, A383V, Y385C, D388Y, S397R, M401T, N410S, K450R, P451T, G452V, E484A, H523L, H524N, G542V, E565K, K577E, K577M, N601S, S684Y, L699I, K713E, N748D, Q754R, E775K, A827V, D851N or L864F. As another non-limiting example, T7 RNA polymerase variants may encode at least mutation as described in U.S. Pub. Nos. 20100120024 and 20070117112; herein incorporated by reference in their entireties. Variants of RNA polymerase may also include, but are not limited to, substitutional variants, conservative amino acid substitution, insertional variants, deletional variants and/or covalent derivatives.

[000268] In one embodiment, the primary construct may be designed to be recognized by the wild type or variant RNA polymerases. In doing so, the primary construct may be modified to contain sites or regions of sequence changes from the wild type or parent primary construct.

[000269] In one embodiment, the primary construct may be designed to include at least one substitution and/or insertion upstream of an RNA polymerase binding or recognition site, downstream of the RNA polymerase binding or recognition site, upstream of the TATA box sequence, downstream of the TATA box sequence of the primary construct but upstream of the coding region of the primary construct, within the 5'UTR, before the 5'UTR and/or after the 5'UTR.

[000270] In one embodiment, the 5'UTR of the primary construct may be replaced by the insertion of at least one region and/or string of nucleotides of the same base. The

region and/or string of nucleotides may include, but is not limited to, at least 3, at least 4, at least 5, at least 6, at least 7 or at least 8 nucleotides and the nucleotides may be natural and/or unnatural. As a non-limiting example, the group of nucleotides may include 5-8 adenine, cytosine, thymine, a string of any of the other nucleotides disclosed herein and/or combinations thereof.

[000271] In one embodiment, the 5'UTR of the primary construct may be replaced by the insertion of at least two regions and/or strings of nucleotides of two different bases such as, but not limited to, adenine, cytosine, thymine, any of the other nucleotides disclosed herein and/or combinations thereof. For example, the 5'UTR may be replaced by inserting 5-8 adenine bases followed by the insertion of 5-8 cytosine bases. In another example, the 5'UTR may be replaced by inserting 5-8 cytosine bases followed by the insertion of 5-8 adenine bases.

[000272] In one embodiment, the primary construct may include at least one substitution and/or insertion downstream of the transcription start site which may be recognized by an RNA polymerase. As a non-limiting example, at least one substitution and/or insertion may occur downstream the transcription start site by substituting at least one nucleic acid in the region just downstream of the transcription start site (such as, but not limited to, +1 to +6). Changes to region of nucleotides just downstream of the transcription start site may affect initiation rates, increase apparent nucleotide triphosphate (NTP) reaction constant values, and increase the dissociation of short transcripts from the transcription complex curing initial transcription (Brieba et al, Biochemistry (2002) 41: 5144-5149; herein incorporated by reference in its entirety). The modification, substitution and/or insertion of at least one nucleic acid may cause a silent mutation of the nucleic acid sequence or may cause a mutation in the amino acid sequence.

[000273] In one embodiment, the primary construct may include the substitution of at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12 or at least 13 guanine bases downstream of the transcription start site.

[000274] In one embodiment, the primary construct may include the substitution of at least 1, at least 2, at least 3, at least 4, at least 5 or at least 6 guanine bases in the region

just downstream of the transcription start site. As a non-limiting example, if the nucleotides in the region are GGGAGA the guanine bases may be substituted by at least 1, at least 2, at least 3 or at least 4 adenine nucleotides. In another non-limiting example, if the nucleotides in the region are GGGAGA the guanine bases may be substituted by at least 1, at least 2, at least 3 or at least 4 cytosine bases. In another non-limiting example, if the nucleotides in the region are GGGAGA the guanine bases may be substituted by at least 1, at least 2, at least 3 or at least 4 thymine, and/or any of the nucleotides described herein.

[000275] In one embodiment, the primary construct may include at least one substitution and/or insertion upstream of the start codon. For the purpose of clarity, one of skill in the art would appreciate that the start codon is the first codon of the protein coding region whereas the transcription start site is the site where transcription begins. The primary construct may include, but is not limited to, at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7 or at least 8 substitutions and/or insertions of nucleotide bases. The nucleotide bases may be inserted or substituted at 1, at least 1, at least 2, at least 3, at least 4 or at least 5 locations upstream of the start codon. The nucleotides inserted and/or substituted may be the same base (e.g., all A or all C or all T or all G), two different bases (e.g., A and C, A and T, or C and T), three different bases (e.g., A, C and T or A, C and T) or at least four different bases. As a non-limiting example, the guanine base upstream of the coding region in the primary construct may be substituted with adenine, cytosine, thymine, or any of the nucleotides described herein. In another non-limiting example the substitution of guanine bases in the primary construct may be designed so as to leave one guanine base in the region downstream of the transcription start site and before the start codon (see Esvelt *et al.* Nature (2011) 472(7344):499-503; herein incorporated by reference in its entirety). As a non-limiting example, at least 5 nucleotides may be inserted at 1 location downstream of the transcription start site but upstream of the start codon and the at least 5 nucleotides may be the same base type.

cDNA Template Removal and Clean-Up

[000276] The cDNA template may be removed using methods known in the art such as, but not limited to, treatment with Deoxyribonuclease I (DNase I). RNA clean-up may

also include a purification method such as, but not limited to, AGENCOURT® CLEANSEQ® system from Beckman Coulter (Danvers, MA), HPLC based purification methods such as, but not limited to, strong anion exchange HPLC, weak anion exchange HPLC, reverse phase HPLC (RP-HPLC), and hydrophobic interaction HPLC (HIC-HPLC) .

Capping and/or Tailing Reactions

[000277] The primary construct or mmRNA may also undergo capping and/or tailing reactions. A capping reaction may be performed by methods known in the art to add a 5′ cap to the 5′ end of the primary construct. Methods for capping include, but are not limited to, using a Vaccinia Capping enzyme (New England Biolabs, Ipswich, MA).

[000278] A poly-A tailing reaction may be performed by methods known in the art, such as, but not limited to, 2′ O-methyltransferase and by methods as described herein. If the primary construct generated from cDNA does not include a poly-T, it may be beneficial to perform the poly-A-tailing reaction before the primary construct is cleaned.

mRNA Purification

[000279] Primary construct or mmRNA purification may include, but is not limited to, mRNA or mmRNA clean-up, quality assurance and quality control. mRNA or mmRNA clean-up may be performed by methods known in the arts such as, but not limited to, AGENCOURT® beads (Beckman Coulter Genomics, Danvers, MA), poly-T beads, LNA™ oligo-T capture probes (EXIQON® Inc, Vedbaek, Denmark) or HPLC based purification methods such as, but not limited to, strong anion exchange HPLC, weak anion exchange HPLC, reverse phase HPLC (RP-HPLC), and hydrophobic interaction HPLC (HIC-HPLC). The term "purified" when used in relation to a polynucleotide such as a "purified mRNA or mmRNA" refers to one that is separated from at least one contaminant. As used herein, a "contaminant" is any substance which makes another unfit, impure or inferior. Thus, a purified polynucleotide (e.g., DNA and RNA) is present in a form or setting different from that in which it is found in nature, or a form or setting different from that which existed prior to subjecting it to a treatment or purification method.

[000280] A quality assurance and/or quality control check may be conducted using methods such as, but not limited to, gel electrophoresis, UV absorbance, or analytical HPLC.

[000281] In another embodiment, the mRNA or mmRNA may be sequenced by methods including, but not limited to reverse-transcriptase-PCR.

[000282] In one embodiment, the mRNA or mmRNA may be quantified using methods such as, but not limited to, ultraviolet visible spectroscopy (UV/Vis). A non-limiting example of a UV/Vis spectrometer is a NANODROP® spectrometer (ThermoFisher, Waltham, MA). The quantified mRNA or mmRNA may be analyzed in order to determine if the mRNA or mmRNA may be of proper size, check that no degradation of the mRNA or mmRNA has occurred. Degradation of the mRNA and/or mmRNA may be checked by methods such as, but not limited to, agarose gel electrophoresis, HPLC based purification methods such as, but not limited to, strong anion exchange HPLC, weak anion exchange HPLC, reverse phase HPLC (RP-HPLC), and hydrophobic interaction HPLC (HIC-HPLC), liquid chromatography-mass spectrometry (LCMS), capillary electrophoresis (CE) and capillary gel electrophoresis (CGE).

Signal Sequences

[000283] The primary constructs or mmRNA may also encode additional features which facilitate trafficking of the polypeptides to therapeutically relevant sites. One such feature which aids in protein trafficking is the signal sequence. As used herein, a "signal sequence" or "signal peptide" is a polynucleotide or polypeptide, respectively, which is from about 9 to 200 nucleotides (3-60 amino acids) in length which is incorporated at the 5′ (or N-terminus) of the coding region or polypeptide encoded, respectively. Addition of these sequences result in trafficking of the encoded polypeptide to the endoplasmic reticulum through one or more secretory pathways. Some signal peptides are cleaved from the protein by signal peptidase after the proteins are transported.

[000284] Table 5 is a representative listing of protein signal sequences which may be incorporated for encoding by the polynucleotides, primary constructs or mmRNA of the invention.

Table 5. Signal Sequences

ID	Description	NUCLEOTIDE SEQUENCE (5′-3′)	SEQ ID NO.	ENCODED PEPTIDE	SEQ ID NO.
SS-001	α-1-antitrypsin	ATGATGCCATCCTCAGTCTCATGGGGTATTTTGCTCTTGGCGGGTCTGTGCTGTCTCGTGCCGGTGTCGCTCGCA	32	MMPSSVSWGILLAGLCCLVPVSLA	94
SS-002	G-CSF	ATGGCCGGACCGGCGACTCAGTCGCCCATGAAACTCATGGCCCTGCAGTTGTTGCTTTGGCACTCAGCCCTCTGGACCGTCCAAGAGGCG	33	MAGPATQSPMKLMALQLLLWHSALWTVQEA	95
SS-003	Factor IX	ATGCAGAGAGTGAACATGATTATGGCCGAGTCCCCATCGCTCATCACAATCTGCCTGCTTGGTACCTGCTTTCCGCCGAATGCACTGTCTTTCTGGATCACGAGAATGCGAATAAGATCTTGAACCGACCCAAACGG	34	MQRVNMIMAESPSLITICLLGYLLSAECTVFLDHENANKILNRPKR	96
SS-004	Prolactin	ATGAAAGGATCATTGCTGTTGCTCCTCGTGTCGAACCTTCTGCTTTGCCAGTCCGTAGCCCCC	35	MKGSLLLLLVSNLLLCQSVAP	97
SS-005	Albumin	ATGAAATGGGTGACGTTCATCTCACTGTTGTTTTTGTTCTCGTCCGCCTACTCCAGGGGAGTATTCCGCCGA	36	MKWVTFISLLFLFSSAYSRGVFRR	98
SS-006	HMMSP38	ATGTGGTGGCGGCTCTGGTGGCTGCTCCTGTTGCTCCTCTTGCTGTGGCCCATGGTGTGGGCA	37	MWWRLWWLLLLLLLLPMWA	99
MLS-001	ornithine carbamoyltransferase	TGCTCTTTAACCTCCGCATCCTGTTGAATAACGCTGCGTTCCGAAATGGGCATAACTTCATGGTACGCAACTTCAGATGCGGCCAGCCACTCCAG	38	MLFNLRILLNNAAFRNGHNFMVRNFRCGQPLQ	100
MLS-002	Cytochrome C Oxidase subunit 8A	ATGTCCGTCTTGACACCCCTGCTCTTGAGAGGGCTGACGGGGTCCGCTAGACGCCTGCCGGTACCGCGAGCGAAGATCCACTCCCTG	39	MSVLTPLLLRGLTGSARRLPVPRAKIHSL	101
MLS-003	Cytochrome C Oxidase subunit 8A	ATGAGCGTGCTCACTCCGTTGCTTCTTCGAGGGCTTACGGGATCGGCTCGGAGGTTGCCCGTCCCGAGAGCGAAGATCCATTCGTTG	40	MSVLTPLLLRGLTGSARRLPVPRAKIHSL	102
SS-007	Type III, bacterial	TGACAAAAATAACTTTATCTCCCCAGAATTTTAGAATCCAAAAACAGGAAACCACACTACTAAAAGAAAAATCAACCGAGAAAAATTCTTTAGCAAAAAGTAT	41	MVTKITLSPQNFRIQKQETTLLKEKSTEKNSLAKKSILAVKNHFIELRSKLSERFIS	103

		TCTCGCAGTAAAAATCACTTC ATCGAATTAAGGTCAAAATTA TCGGAACGTTTTATTTCGCAT AAGAACACT		HKNT	
SS-008	Viral	ATGCTGAGCTTTGTGGATACC CGCACCCTGCTGCTGCTGGCG GTGACCAGCTGCCTGGCGACC TGCCAG	42	MLSFVDTRTLL LLAVTSCLATC Q	104
SS-009	viral	ATGGGCAGCAGCCAGGCGCC GCGCATGGGCAGCGTGGGCG GCCATGGCCTGATGGCGCTGC TGATGGCGGGCCTGATTCTGC CGGGCATTCTGGCG	43	MGSSQAPRMG SVGGHGLMAL LMAGLILPGIL A	105
SS-010	Viral	ATGGCGGGCATTTTTTATTTTC TGTTTAGCTTTCTGTTTGGCAT TTGCGAT	44	MAGIFYFLFSF LFGICD	106
SS-011	Viral	ATGGAAAACCGCCTGCTGCGC GTGTTTCTGGTGTGGGCGGCG CTGACCATGGATGGCGCGAGC GCG	45	MENRLLRVFL VWAALTMDG ASA	107
SS-012	Viral	ATGGCGCGCCAGGGCTGCTTT GGCAGCTATCAGGTGATTAGC CTGTTTACCTTTGCGATTGGC GTGAACCTGTGCCTGGGC	46	MARQGCFGSY QVISLFTFAIGV NLCLG	108
SS-013	*Bacillus*	ATGAGCCGCCTGCCGGTGCTG CTGCTGCTGCAGCTGCTGGTG CGCCCGGGCCTGCAG	47	MSRLPVLLLLQ LLVRPGLQ	109
SS-014	*Bacillus*	ATGAAACAGCAGAAACGCCT GTATGCGCGCCTGCTGACCCT GCTGTTTGCGCTGATTTTTCTG CTGCCGCATAGCAGCGCGAGC GCG	48	MKQQKRLYAR LLTLLFALIFLL PHSSASA	110
SS-015	Secretion signal	ATGGCGACGCCGCTGCCTCCG CCCTCCCCGCGGCACCTGCGG CTGCTGCGGCTGCTGCTCTCC GCCCTCGTCCTCGGC	49	MATPLPPPSPR HLRLLRLLLSG	111
SS-016	Secretion signal	ATGAAGGCTCCGGGTCGGCTC GTGCTCATCATCCTGTGCTCC GTGGTCTTCTCT	50	MKAPGRLVLII LCSVVFS	112
SS-017	Secretion signal	ATGCTTCAGCTTTGGAAACTT GTTCTCCTGTGCGGCGTGCTC ACT	51	MLQLWKLLCG VLT	113
SS-018	Secretion signal	ATGCTTTATCTCCAGGGTTGG AGCATGCCTGCTGTGGCA	52	MLYLQGWSM PAVA	114
SS-019	Secretion signal	ATGGATAACGTGCAGCCGAA AATAAAACATCGCCCCTTCTG CTTCAGTGTGAAAGGCCACGT GAAGATGCTGCGGCTGGATAT TATCAACTCACTGGTAACAAC AGTATTCATGCTCATCGTATC	53	MDNVQPKIKH RPFCFSVKGHV KMLRLDIINSL VTTVFMLIVSV LALIP	115

		TGTGTTGGCACTGATACCA			
SS-020	Secretion signal	ATGCCCTGCCTAGACCAACAG CTCACTGTTCATGCCCTACCCT GCCCTGCCCAGCCCTCCTCTC TGGCCTTCTGCCAAGTGGGGT TCTTAACAGCA	54	MPCLDQQLTV HALPCPAQPSS LAFCQVGFLT A	116
SS-021	Secretion signal	ATGAAAACCTTGTTCAATCCA GCCCCTGCCATTGCTGACCTG GATCCCCAGTTCTACACCCTC TCAGATGTGTTCTGCTGCAAT GAAAGTGAGGCTGAGATTTTA ACTGGCCTCACGGTGGGCAGC GCTGCAGATGCT	55	MKTLFNPAPAI ADLDPQFYTLS DVFCCNESEAE ILTGLTVGSAA DA	117
SS-022	Secretion signal	ATGAAGCCTCTCCTTGTTGTG TTTGTCTTTCTTTTCCTTTGGG ATCCAGTGCTGGCA	56	MKPLLVVFVF LFLWDPVLA	118
SS-023	Secretion signal	ATGTCCTGTTCCCTAAAGTTT ACTTTGATTGTAATTTTTTTT ACTGTTGGCTTTCATCCAGC	57	MSCSLKFTLIVI FFTCTLSSS	119
SS-024	Secretion signal	ATGGTTCTTACTAAACCTCTTC AAAGAAATGGCAGCATGATG AGCTTTGAAAATGTGAAAGAA AAGAGCAGAGAAGGAGGGCC CCATGCACACACACCCGAAGA AGAATTGTGTTTCGTGGTAAC ACACTACCCTCAGGTTCAGAC CACACTCAACCTGTTTTTCCAT ATATTCAAGGTTCTTACTCAA CCACTTTCCCTTCTGTGGGGT	58	MVLTKPLQRN GSMMSFENVK EKSREGGPHA HTPEEELCFVV THTPQVQTTL NLFFHIFKVLT QPLSLLWG	120
SS-025	Secretion signal	ATGGCCACCCCGCCATTCCGG CTGATAAGGAAGATGTTTTCC TTCAAGGTGAGCAGATGGATG GGGCTTGCCTGCTTCCGGTCC CTGGCGGCATCC	59	MATPPFRLIRK MFSFKVSRWM GLACFRSLAAS	121
SS-026	Secretion signal	ATGAGCTTTTTCCAACTCCTG ATGAAAAGGAAGGAACTCAT TCCCTTGGTGGTGTTCATGAC TGTGGCGGCGGGTGGAGCCTC ATCT	60	MSFFQLLMKR KELIPLVVFMT VAAGGASS	122
SS-027	Secretion signal	ATGGTCTCAGCTCTGCGGGGA GCACCCCTGATCAGGGTGCAC TCAAGCCCTGTTTCTTCTCCTT CTGTGAGTGGACCACGGAGGC TGGTGAGCTGCCTGTCATCCC AAAGCTCAGCTCTGAGC	61	MVSALRGAPLI RVHSSPVSSPS VSGPAALVSCL SSQSSALS	123
SS-028	Secretion signal	ATGATGGGGTCCCCAGTGAGT CATCTGCTGGCCGGCTTCTGT GTGTGGGTCGTCTTGGGC	62	MMGSPVSHLL AGFCVWVVLG	124
SS-029	Secretion signal	ATGGCAAGCATGGCTGCCGTG CTCACCTGGGCTCTGGCTCTT	63	MASMAAVLT WALALLSAFS	125

		CTTTCAGCGTTTTCGGCCACC CAGGCA		ATQA	
SS-030	Secretion signal	ATGGTGCTCATGTGGACCAGT GGTGACGCCTTCAAGACGGCC TACTTCCTGCTGAAGGGTGCC CCTCTGCAGTTCTCCGTGTGC GGCCTGCTGCAGGTGCTGGTG GACCTGGCCATCCTGGGGCAG GCCTACGCC	64	MVLMWTSGD AFKTAYFLLK GAPLQFSVCGL LQVLVDLAILG QATA	126
SS-031	Secretion signal	ATGGATTTTGTCGCTGGAGCC ATCGGAGGCGTCTGCGGTGTT GCTGTGGGCTACCCCCTGGAC ACGGTGAAGGTCAGGATCCA GACGGAGCCAAAGTACACAG GCATCTGGCACTGCGTCCGGG ATACGTATCACCGAGAGCGCG TGTGGG GCTTCTACCGGGGCCTCTCGC TGCCCGTGTGCACGGTGTCCC TGGTATCTTCC	65	MDFVAGAIGG VCGVAVGYPL DTVKVRIQTEP LYTGIWHCVR DTYHRERVWG FYRGLSLPVCT VSLVSS	127
SS-032	Secretion signal	ATGGAGAAGCCCCTCTTCCCA TTAGTGCCTTTGCATTGGTTTG GCTTTGGCTACACAGCACTGG TTGTTTCTGGTGGGATCGTTG GCTATGTAAAAACAGGCAGC GTGCCGTCCCTGGCTGCAGGG CTGCTCTTCGGCAGTCTAGCC	66	MEKPLFPLVPL HWFGFGYTAL VVSGGIVGYV KTGSVPSLAA GLLFGSLA	128
SS-033	Secretion signal	ATGGGTCTGCTCCTTCCCCTG GCACTCTGCATCCTAGTCCTG TGC	67	MGLLLPLALCI LVLC	129
SS-034	Secretion signal	ATGGGGATCCAGACGAGCCCC GTCCTGCTGGCCTCCCTGGGG GTGGGGCTGGTCACTCTGCTC GGCCTGGCTGTGGGC	68	MGIQTSPVLLA SLGVGLVTLL GLAVG	130
SS-035	Secretion signal	ATGTCGGACCTGCTACTACTG GGCCTGATTGGGGGCCTGACT CTCTTACTGCTGCTGACGCTG CTAGCCTTTGCC	69	MSDLLLLGLIG GLTLLLLLTLL AFA	131
SS-036	Secretion signal	ATGGAGACTGTGGTGATTGTT GCCATAGGTGTGCTGGCCACC ATGTTTCTGGCTTCGTTTGCAG CCTTGGTGCTGGTTTGCAGGC AG	70	METVVIVAIGV LATIFLASFAA LVLVCRQ	132
SS-037	Secretion signal	ATGCGCGGCTCTGTGGAGTGC ACCTGGGGTTGGGGGCACTGT GCCCCCAGCCCCCTGCTCCTT TGGACTCTACTTCTGTTTGCA GCCCCATTTGGCCTGCTGGGG	71	MAGSVECTWG WGHCAPSPLL LWTLLLFAAPF GLLG	133
SS-038	Secretion signal	ATGATGCCGTCCCGTACCAAC CTGGCTACTGGAATCCCCAGT	72	MMPSRTNLAT GIPSSKVKYSR	134

		AGTAAAGTGAAATATTCAAGGCTCTCCAGCACAGACGATGGCTACATTGACCTTCAGTTTAAGAAAACCCCTCCTAAGATCCCTTATAAGGCCATCGCACTTGCCACTGTGCTGTTTTTGATTGGCGCC		LSSTDDGYIDLQFKKTPPKIPYKAIALATVLFLIGA	
SS-039	Secretion signal	ATGGCCCTGCCCCAGATGTGTGACGGGAGCCACTTGGCCTCCACCCTCCGCTATTGCATGACAGTCAGCGGCACAGTGGTTCTGGTGGCCGGGACGCTCTGCTTCGCT	73	MALPQMCDGSHLASTLRYCMTVSGTVVLVAGTLCFA	135
SS-041	Vrg-6	TGAAAAAGTGGTTCGTTGCTGCCGGCATCGGCGCTGCCGGACTCATGCTCTCCAGCGCCGCCA	74	MKKWFVAAGIGAGLLMLSSAA	136
SS-042	PhoA	ATGAAACAGAGCACCATTGCGCTGGCGCTGCTGCCGCTGCTGTTTACCCCGGTGACCAAAGCG	75	MKQSTIALALLPLLFTPVTKA	137
SS-043	OmpA	ATGAAAAAAACCGCGATTGCGATTGCGGTGGCGCTGGCGGGCTTTGCGACCGTGGCGCAGGCG	76	MKKTAIAIAVALAGFATVAQA	138
SS-044	STI	ATGAAAAAACTGATGCTGGCGATTTTTTTTAGCGTGCTGAGCTTTCCGAGCTTTAGCCAGAGC	77	MKKLMLAIFFSVLSFPSFSQS	139
SS-045	STII	ATGAAAAAAACATTGCGTTTCTGCTGGCGAGCATGTTTGTGTTTAGCATTGCGACCAACGCGTATGCG	78	MKKNIAFLLASMFVFSIATNAYA	140
SS-046	Amylase	ATGTTTGCGAAACGCTTTAAAACCAGCCTGCTGCCGCTGTTTGCGGGCTTTCTGCTGCTGTTTCATCTGGTGCTGGCGGGCCCGGCGGCGGCGAGC	79	MFAKRFKTSLLPLFAGFLLLFHLVLAGPAAAS	141
SS-047	Alpha Factor	ATGCGCTTTCCGAGCATTTTTACCGCGGTGCTGTTTGCGGCGAGCAGCGCGCTGGCG	80	MRFPSIFTAVLFAASSALA	142
SS-048	Alpha Factor	ATGCGCTTTCCGAGCATTTTTACCACCGTGCTGTTTGCGGCGAGCAGCGCGCTGGCG	81	MRFPSIFTTVLFAASSALA	143
SS-049	Alpha Factor	ATGCGCTTTCCGAGCATTTTTACCAGCGTGCTGTTTGCGGCGAGCAGCGCGCTGGCG	82	MRFPSIFTSVLFAASSALA	144
SS-050	Alpha Factor	ATGCGCTTTCCGAGCATTTTTACCCATGTGCTGTTTGCGGCGAGCAGCGCGCTGGCG	83	MRFPSIFTHVLFAASSALA	145
SS-051	Alpha Factor	ATGCGCTTTCCGAGCATTTTTACCATTGTGCTGTTTGCGGCGAGCAGCGCGCTGGCG	84	MRFPSIFTIVLFAASSALA	146

SS-052	Alpha Factor	ATGCGCTTTCCGAGCATTTTT ACCTTTGTGCTGTTTGCGGCG AGCAGCGCGCTGGCG	85	MRFPSIFTFVLF AASSALA	147
SS-053	Alpha Factor	ATGCGCTTTCCGAGCATTTTT ACCGAAGTGCTGTTTGCGGCG AGCAGCGCGCTGGCG	86	MRFPSIFTEVL FAASSALA	148
SS-054	Alpha Factor	ATGCGCTTTCCGAGCATTTTT ACCGGCGTGCTGTTTGCGGCG AGCAGCGCGCTGGCG	87	MRFPSIFTGVL FAASSALA	149
SS-055	Endoglucanase V	ATGCGTTCCTCCCCCCTCCTCC GCTCCGCCGTTGTGGCCGCCC TGCCGGTGTTGGCCCTTGCC	88	MRSSPLLRSAV VAALPVLALA	150
SS-056	Secretion signal	ATGGGCGCGGCGGCCGTGCGC TGGCACTTGTGCGTGCTGCTG GCCCTGGGCACACGCGGGCG GCTG	89	MGAAAVRWH LCVLLALGTR GRL	151
SS-057	Fungal	ATGAGGAGCTCCCTTGTGCTG TTCTTTGTCTCTGCGTGGACG GCCTTGGCCAG	90	MRSSLVLFFVS AWTALA	152
SS-058	Fibronectin	ATGCTCAGGGGTCCGGGACCC GGGCGGCTGCTGCTGCTAGCA GTCCTGTGCCTGGGGACATCG GTGCGCTGCACCGAAACCGGG AAGAGCAAGAGG	91	MLRGPGPGRL LLLAVLCLGTS VRCTETGKSK R	153
SS-059	Fibronectin	ATGCTTAGGGGTCCGGGGCCC GGGCTGCTGCTGCTGGCCGTC CAGCTGGGGACAGCGGTGCCC TCCACG	92	MLRGPGPGLL LLAVQCLGTA VPSTGA	154
SS-060	Fibronectin	ATGCGCCGGGGGGCCCTGACC GGGCTGCTCCTGGTCCTGTGC CTGAGTGTTGTGCTACGTGCA GCCCCCTCTGCAACAAGCAAG AAGCGCAGG	93	MRRGALTGLL LVLCLSVVLR AAPSATSKKR R	155

[000285] In the table, SS is secretion signal and MLS is mitochondrial leader signal. The primary constructs or mmRNA of the present invention may be designed to encode any of the signal sequences of SEQ ID NOs 94-155, or fragments or variants thereof. These sequences may be included at the beginning of the polypeptide coding region, in the middle or at the terminus or alternatively into a flanking region. Further, any of the polynucleotide primary constructs of the present invention may also comprise one or more of the sequences defined by SEQ ID NOs 32-93. These may be in the first region or either flanking region.

[000286] Additional signal sequences which may be utilized in the present invention include those taught in, for example, databases such as those found at

http://www.signalpeptide.de/ or http://proline.bic.nus.edu.sg/spdb/. Those described in US Patents 8,124,379; 7,413,875 and 7,385,034 are also within the scope of the invention and the contents of each are incorporated herein by reference in their entirety.

Target Selection

[000287] According to the present invention, the primary constructs comprise at least a first region of linked nucleosides encoding at least one polypeptide of interest. The polypeptides of interest or "Targets" of the present invention are listed in Table 6. Shown in Table 6, in addition to the name and description of the gene encoding the polypeptide of interest are the ENSEMBL Transcript ID (ENST), the ENSEMBL Protein ID (ENSP) and when available the optimized transcript sequence ID (Optim Trans SEQ ID) or optimized open reading frame sequence ID (Optim ORF SEQ ID). For any particular gene there may exist one or more variants or isoforms. Where these exist, they are shown in the table as well. It will be appreciated by those of skill in the art that disclosed in the Table are potential flanking regions. These are encoded in each ENST transcript either to the 5′ (upstream) or 3′ (downstream) of the ORF or coding region. The coding region is definitively and specifically disclosed by teaching the ENSP sequence. Consequently, the sequences taught flanking that encoding the protein are considered flanking regions. It is also possible to further characterize the 5′ and 3′ flanking regions by utilizing one or more available databases or algorithms. Databases have annotated the features contained in the flanking regions of the ENST transcripts and these are available in the art.

Table 6. Targets

Target No.	Target Description	ENST	Trans SEQ ID NO	ENSP	Peptide SEQ ID NO	Optim ORF SEQ ID	Optim Trans SEQ ID NO
1	4-hydroxyphenylpyruvate dioxygenase	289004	156	289004	769	1673, 2293, 2913, 3533, 4153, 5433-5519	
2	4-hydroxyphenylpyruvate dioxygenase	543163	157	441677	770	1674, 2294, 2914, 3534, 4154	
3	4-hydroxyphenylpyruvate dioxygenase	545969	158	437419	771	1675, 2295, 2915, 3535, 4155	
4	6-pyruvoyltetrahydropterin synthase	280362	159	280362	772	1676, 2296, 2916, 3536, 4156, 4773, 5103, 5520-	1609

						5606	
5	A disintegrin and metalloproteinase with thrombospondin motifs 13 isoform 1 preproprotein	563979	160	456186	773	1677, 2297, 2917, 3537, 4157, 4774, 5104, 5607-5693	1609
6	activity-dependent neuroprotector homeobox	349014	161	342905	774	1678, 2298, 2918, 3538, 4158, 4775, 5105, 5694-5780	
7	activity-dependent neuroprotector homeobox	371602	162	360662	775	1679, 2299, 2919, 3539, 4159, 4776, 5106	
8	activity-dependent neuroprotector homeobox	396029	163	379346	776	1680, 2300, 2920, 3540, 4160, 4777, 5107	
9	activity-dependent neuroprotector homeobox	396032	164	379349	777	1681, 2301, 2921, 3541, 4161, 4778, 5108	
10	activity-dependent neuroprotector homeobox	534467	165	436181	778	1682, 2302, 2922, 3542, 4162, 4779, 5109	
11	ADAM metallopeptidase with thrombospondin type 1 motif, 13	338351	166	345120	779	1683, 2303, 2923, 3543, 4163, 4780, 5110	
12	ADAM metallopeptidase with thrombospondin type 1 motif, 13	355699	167	347927	780	1393, 1684, 2304, 2924, 3544, 4164, 4781, 5111	
13	ADAM metallopeptidase with thrombospondin type 1 motif, 13	356589	168	348997	781	1394, 1685, 2305, 2925, 3545, 4165, 4782, 5112	
14	ADAM metallopeptidase with thrombospondin type 1 motif, 13	371910	169	360978	782	1395, 1686, 2306, 2926, 3546, 4166, 4783, 5113	
15	ADAM metallopeptidase with thrombospondin	371911	170	360979	783	1396, 1687, 2307, 2927, 3547, 4167, 4784, 5114	

	type 1 motif, 13						
16	ADAM metallopeptidase with thrombospondin type 1 motif, 13	371916	171	360984	784	1397, 1688, 2308, 2928, 3548, 4168, 4785, 5115	
17	ADAM metallopeptidase with thrombospondin type 1 motif, 13	371929	172	360997	785	1398, 1689, 2309, 2929, 3549, 4169, 4786, 5116	1610
18	ADAM metallopeptidase with thrombospondin type 1 motif, 13	536611	173	444504	786	1399, 1690, 2310, 2930, 3550, 4170, 4787, 5117	
19	AIFsh						
20	Albiglutide; albiglutide						
21	aldolase A fructose-bisphosphate	563060	174	455800	787	1691, 2311, 2931, 3551, 4171	
22	aldolase A fructose-bisphosphate	564546	175	455917	788	1692, 2312, 2932, 3552, 4172	
23	aldolase A fructose-bisphosphate	564595	176	457468	789	1693, 2313, 2933, 3553, 4173	
24	aldolase A, fructose-bisphosphate	338110	177	336927	790	1694, 2314, 2934, 3554, 4174	
25	aldolase A, fructose-bisphosphate	395240	178	378661	791	1695, 2315, 2935, 3555, 4175	
26	aldolase A, fructose-bisphosphate	395248	179	378669	792	1696, 2316, 2936, 3556, 4176, 5781-5867	
27	aldolase A, fructose-bisphosphate	412304	180	400452	793	1697, 2317, 2937, 3557, 4177	
28	ameloblastin (enamel matrix protein)	322937	181	313809	794	1698, 2318, 2938, 3558, 4178, 4788, 5118, 5868-5954	1611
29	ameloblastin (enamel matrix protein)	449493	182	391234	795	1699, 2319, 2939, 3559, 4179, 4789, 5119	1611
30	ameloblastin (enamel matrix protein)	538728	183	445605	796	1700, 2320, 2940, 3560, 4180, 4790,	1611

						5120	
31	amelogenin, X-linked	348912	184	335312	797	1701, 2321, 2941, 3561, 4181, 4791, 5121	
32	amelogenin, X-linked	380712	185	370088	798	1702, 2322, 2942, 3562, 4182, 4792, 5122, 5955-6041	
33	amelogenin, X-linked	380714	186	370090	799	1703, 2323, 2943, 3563, 4183, 4793, 5123	
34	amelogenin, Y-linked	215479	187	215479	800	1704, 2324, 2944, 3564, 4184	1612
35	amelogenin, Y-linked	383036	188	372505	801	1705, 2325, 2945, 3565, 4185	1612
36	amelogenin, Y-linked	383037	189	372506	802	1706, 2326, 2946, 3566, 4186	1612
37	amylo-alpha-1, 6-glucosidase, 4-alpha-glucanotransferase	294724	190	294724	803	1707, 2327, 2947, 3567, 4187	
38	amylo-alpha-1, 6-glucosidase, 4-alpha-glucanotransferase	361302	191	354971	804	1708, 2328, 2948, 3568, 4188	
39	amylo-alpha-1, 6-glucosidase, 4-alpha-glucanotransferase	361522	192	354635	805	1709, 2329, 2949, 3569, 4189	
40	amylo-alpha-1, 6-glucosidase, 4-alpha-glucanotransferase	361915	193	355106	806	1710, 2330, 2950, 3570, 4190, 6042-6128	
41	amylo-alpha-1, 6-glucosidase, 4-alpha-glucanotransferase	370161	194	359180	807	1711, 2331, 2951, 3571, 4191	
42	amylo-alpha-1, 6-glucosidase, 4-alpha-glucanotransferase	370163	195	359182	808	1712, 2332, 2952, 3572, 4192	
43	amylo-alpha-1, 6-glucosidase, 4-alpha-glucanotransferase	370165	196	359184	809	1713, 2333, 2953, 3573, 4193	
44	amyloid P component, serum	255040	197	255040	810	1714, 2334, 2954, 3574,	

						4194, 6129-6215	
45	angiotensin I converting enzyme (peptidyl-dipeptidase A) 2	252519	198	252519	811	1715, 2335, 2955, 3575, 4195, 6216-6302	
46	angiotensin I converting enzyme (peptidyl-dipeptidase A) 2	427411	199	389326	812	1716, 2336, 2956, 3576, 4196	
47	Anti-TNFalpha (TNFRFusion; Enbrel)						
48	APOA1 Milano; apo A-I(R173C)Milano , ETC-216				813	1400, 1717, 2337, 2957, 3577, 4197, 4794, 5124	1613
49	APOA1 Paris; apo A-I(R151C)Paris				814	1401, 1718, 2338, 2958, 3578, 4198, 4795, 5125	
50	ApoA-a optimized mRNA						1614
51	apolipoprotein A-I	236850	200	236850	815	1402, 1719, 2339, 2959, 3579, 4199, 4796, 5126, 6303-5363	1615
52	apolipoprotein A-I	359492	201	352471	816	1720, 2340, 2960, 3580, 4200, 4797, 5127	1615
53	apolipoprotein A-I	375320	202	364469	817	1721, 2341, 2961, 3581, 4201, 4798, 5128	1615
54	apolipoprotein A-I	375323	203	364472	818	1722, 2342, 2962, 3582, 4202, 4799, 5129	1615
55	apolipoprotein B (including Ag(x) antigen)	233242	204	233242	819	1723, 2343, 2963, 3583, 4203	
56	apolipoprotein B (including Ag(x) antigen)	535079	205	439731	820	1724, 2344, 2964, 3584, 4204	
57	aquaporin 5	293599	206	293599	821	1725, 2345, 2965, 3585, 4205, 4800, 5130, 6564-6650	1616
58	arginase, liver	356962	207	349446	822	1726, 2346, 2966, 3586, 4206,	

59	arginase, liver	368087	208	357066	823	1727, 2347, 2967, 3587, 4207, 6651-6737	
60	arginase, liver	476845	209	417694	824	1728, 2348, 2968, 3588, 4208	
61	argininosuccinate lyase	304874	210	307188	825	1729, 2349, 2969, 3589, 4209, 6738-6876	
62	argininosuccinate lyase	380839	211	370219	826	1730, 2350, 2970, 3590, 4210	
63	argininosuccinate lyase	395331	212	378740	827	1731, 2351, 2971, 3591, 4211	
64	argininosuccinate lyase	395332	213	378741	828	1732, 2352, 2972, 3592, 4212	
65	argininosuccinate lyase	502022	214	441778	829	1733, 2353, 2973, 3593, 4213	
66	argininosuccinate synthase 1	334909	215	361470	830	1403, 1734, 2354, 2974, 3594, 4214	
67	argininosuccinate synthase 1	352480	216	253004	831	1735, 2355, 2975, 3595, 4215, 6877-6963	
68	argininosuccinate synthase 1	372386	217	361461	832	1404, 1736, 2356, 2976, 3596, 4216	
69	argininosuccinate synthase 1	372393	218	361469	833	1737, 2357, 2977, 3597, 4217	
70	argininosuccinate synthase 1	372394	219	361471	834	1738, 2358, 2978, 3598, 4218	
71	argininosuccinate synthase 1	422569	220	394212	835	1405, 1739, 2359, 2979, 3599, 4219	
72	argininosuccinate synthase 1	443588	221	397785	836	1406, 1740, 2360, 2980, 3600, 4220	
73	artemin	372354	222	361429	837	1741, 2361, 2981, 3601, 4221, 4801, 5131, 6964-7050	
74	artemin	372359	223	361434	838	1742, 2362, 2982, 3602, 4222, 4802, 5132	

75	artemin	414809	224	387435	839	1743, 2363, 2983, 3603, 4223, 4803, 5133	
76	artemin	471394	225	435804	840	1744, 2364, 2984, 3604, 4224, 4804, 5134	
77	artemin	474592	226	434856	841	1745, 2365, 2985, 3605, 4225, 4805, 5135	
78	artemin	477048	227	434784	842	1746, 2366, 2986, 3606, 4226, 4806, 5136	
79	artemin	491846	228	436149	843	1747, 2367, 2987, 3607, 4227, 4807, 5137	
80	artemin	498139	229	436727	844	1748, 2368, 2988, 3608, 4228, 4808, 5138	
81	arylsulfatase B	264914	230	264914	845	1749, 2369, 2989, 3609, 4229, 4809, 5139, 7051-7137	1617
82	arylsulfatase B	264914	231	264914	846	1750, 2370, 2990, 3610, 4230, 4810, 5140	1617
83	arylsulfatase B	396151	232	379455	847	1751, 2371, 2991, 3611, 4231, 4811, 5141	1617
84	arylsulfatase B	521117	233	428611	848	1752, 2372, 2992, 3612, 4232, 4812, 5142	1617
85	asparaginase homolog (S. cerevisiae)	299234	234	299234	849	1753, 2373, 2993, 3613, 4233, 4813, 5143	
86	asparaginase homolog (S. cerevisiae)	455920	235	389003	850	1754, 2374, 2994, 3614, 4234, 4814, 5144	
87	asparaginase homolog (S. cerevisiae)	550583	236	446856	851	1755, 2375, 2995, 3615, 4235, 4815, 5145	

88	asparaginase homolog (S. cerevisiae)	551177	237	450040	852	1756, 2376, 2996, 3616, 4236, 4816, 5146	
89	aspartoacylase	263080	238	263080	853	1757, 2377, 2997, 3617, 4237	
90	aspartoacylase	456349	239	409976	854	1758, 2378, 2998, 3618, 4238, 7138-7224	
91	ATPase, aminophospholipid transporter, class I, type 8B, member 1	283684	240	283684	855	1407, 1759, 2379, 2999, 3619, 4239, 7225-7398	
92	ATPase, aminophospholipid transporter, class I, type 8B, member 1	536015	241	445359	856	1760, 2380, 3000, 3620, 4240	
93	ATPase, Cu++ transporting, beta polypeptide	242839	242	242839	857	1761, 2381, 3001, 3621, 4241, 4817, 5147, 7399-7485	1618
94	ATPase, Cu++ transporting, beta polypeptide	344297	243	342559	858	1762, 2382, 3002, 3622, 4242, 4818, 5148	1618
95	ATPase, Cu++ transporting, beta polypeptide	400366	244	383217	859	1763, 2383, 3003, 3623, 4243, 4819, 5149	1618
96	ATPase, Cu++ transporting, beta polypeptide	400370	245	383221	860	1764, 2384, 3004, 3624, 4244, 4820, 5150	1618
97	ATPase, Cu++ transporting, beta polypeptide	417240	246	390360	861	1765, 2385, 3005, 3625, 4245, 4821, 5151	
98	ATPase, Cu++ transporting, beta polypeptide	418097	247	393343	862	1766, 2386, 3006, 3626, 4246, 4822, 5152	1618
99	ATPase, Cu++ transporting, beta polypeptide	448424	248	416738	863	1767, 2387, 3007, 3627, 4247, 4823, 5153	1618
100	ATPase, Cu++ transporting, beta polypeptide	542656	249	443128	864	1768, 2388, 3008, 3628, 4248, 4824, 5154	

101	ATP-binding cassette, sub-family B (MDR/TAP), member 11	263817	250	263817	865	1408, 1769, 2389, 3009, 3629, 4249	
102	ATP-binding cassette, sub-family B (MDR/TAP), member 4	265723	251	265723	866	1409, 1770, 2390, 3010, 3630, 4250, 7486-7659	
103	ATP-binding cassette, sub-family B (MDR/TAP), member 4	358400	252	351172	867	1410, 1771, 2391, 3011, 3631, 4251	
104	ATP-binding cassette, sub-family B (MDR/TAP), member 4	359206	253	352135	868	1411, 1772, 2392, 3012, 3632, 4252	
105	ATP-binding cassette, sub-family B (MDR/TAP), member 4	417608	254	394511	869	1412, 1773, 2393, 3013, 3633, 4253	
106	ATP-binding cassette, sub-family B (MDR/TAP), member 4	453593	255	392983	870	1774, 2394, 3014, 3634, 4254	
107	ATP-binding cassette, sub-family B (MDR/TAP), member 4	545634	256	437465	871	1775, 2395, 3015, 3635, 4255	
108	bactericidal/permeability-increasing protein	262865	257	262865	872	1776, 2396, 3016, 3636, 4256, 7660-7746	
109	basic helix-loop-helix family, member e41	242728	258	242728	873	1413, 1777, 2397, 3017, 3637, 4257, 7747-7833	
110	basic helix-loop-helix family, member e41	540731	259	437369	874	1778, 2398, 3018, 3638, 4258	
111	bone morphogenetic protein 2	378827	260	368104	875	1414, 1779, 2399, 3019, 3639, 4259, 7834-7920	
112	bone morphogenetic protein 7	395863	261	379204	876	1415, 1780, 2400, 3020, 3640, 4260, 4825, 5155, 7921-8007	

113	bone morphogenetic protein 7	395864	262	379205	877	1416, 1781, 2401, 3021, 3641, 4261, 4826, 5156	
114	Cap18						
115	carbamoyl-phosphate synthase 1, mitochondrial	233072	263	233072	878	1417, 1782, 2402, 3022, 3642, 4262	
116	carbamoyl-phosphate synthase 1, mitochondrial	417946	264	388496	879	1418, 1783, 2403, 3023, 3643, 4263	
117	carbamoyl-phosphate synthase 1, mitochondrial	430249	265	402608	880	1419, 1784, 2404, 3024, 3644, 4264, 8008-8146	
118	carbamoyl-phosphate synthase 1, mitochondrial	451903	266	406136	881	1420, 1785, 2405, 3025, 3645, 4265	
119	carbamoyl-phosphate synthase 1, mitochondrial	518043	267	430697	882	1786, 2406, 3026, 3646, 4266,	
120	carbamoyl-phosphate synthase 1, mitochondrial	536125	268	445539	883	1787, 2407, 3027, 3647, 4267	
121	carbamoyl-phosphate synthase 1, mitochondrial	539150	269	444139	884	1788, 2408, 3028, 3648, 4268	
122	carbamoyl-phosphate synthase 1, mitochondrial	544169	270	442790	885	1789, 2409, 3029, 3649, 4269	
123	cardiotrophin 1	279804	271	279804	886	1790, 2410, 3030, 3650, 4270, 8147-8233	
124	cardiotrophin 1	395019	272	378465	887	1791, 2411, 3031, 3651, 4271	
125	cathelicidin antimicrobial peptide	296435	273	296435	888	1792, 2412, 3032, 3652, 4272, 4827, 5157, 8234-8407	1619
126	cathelicidin antimicrobial peptide	576243	274	458149	889	1793, 2413, 3033, 3653, 4273, 4828, 5158	

127	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 4	372638	275	361721	890	1421, 1794, 2414, 3034, 3654, 4274, 8408-8494	
128	ceruloplasmin (ferroxidase)	264613	276	264613	891	1422, 1795, 2415, 3035, 3655, 4275, 4829, 5159, 8495-8581	1620
129	Cheetah Program 2; hyaluronidase (human); insulin lispro						
130	ciliary neurotrophic factor	361987	277	355370	892	1796, 2416, 3036, 3656, 4276	
131	coagulation factor II (thrombin)	311907	278	308541	893	1423, 1797, 2417, 3037, 3657, 4277, 4830, 5160, 8582-8668	
132	coagulation factor II (thrombin)	446804	279	406403	894	1798, 2418, 3038, 3658, 4278, 4831, 5161	
133	coagulation factor II (thrombin)	530231	280	433907	895	1424, 1799, 2419, 3039, 3659, 4279, 4832, 5162	
134	coagulation factor III (thromboplastin, tissue factor)	334047	281	334145	896	1425, 1800, 2420, 3040, 3660, 4280, 8669-8755	
135	coagulation factor III (thromboplastin, tissue factor)	370207	282	359226	897	1426, 1801, 2421, 3041, 3661, 4281, 8756-8842	
136	coagulation factor IX	218099	283	218099	898	1427, 1802, 2422, 3042, 3662, 4282, 8843-9016	1621
137	coagulation factor IX	394090	284	377650	899	1428, 1803, 2423, 3043, 3663, 4283, 4833, 5163	1621
138	coagulation factor V (proaccelerin, labile factor)	367796	285	356770	900	1429, 1804, 2424, 3044, 3664, 4284	
139	coagulation factor V (proaccelerin, labile factor)	367797	286	356771	901	1430, 1805, 2425, 3045, 3665, 4285, 9017-9155	

140	coagulation factor V (proaccelerin, labile factor)	546081	287	439664	902	1431, 1806, 2426, 3046, 3666, 4286	
141	coagulation factor VII (serum prothrombin conversion accelerator)	346342	288	329546	903	1432, 1807, 2427, 3047, 3667, 4287, 4834, 5164	1622
142	coagulation factor VII (serum prothrombin conversion accelerator)	375581	289	364731	904	1433, 1808, 2428, 3048, 3668, 4288, 4835, 5165, 9156-9242	1622
143	coagulation factor VII (serum prothrombin conversion accelerator)	541084	290	442051	905	1434, 1809, 2429, 3049, 3669, 4289, 4836, 5166	1622
144	coagulation factor VIII procoagulant component	330287	291	327895	906	1810, 2430, 3050, 3670, 4290, 4837, 5167	
145	coagulation factor VIII, procoagulant component	360256	292	353393	907	1435, 1811, 2431, 3051, 3671, 4291, 4838, 5168	1623
146	coagulation factor X	375551	293	364701	908	1436, 1812, 2432, 3052, 3672, 4292, 4839, 5169	
147	coagulation factor X	375559	294	364709	909	1437, 1813, 2433, 3053, 3673, 4293, 4840, 5170, 9243-9381	
148	coagulation factor X	409306	295	387092	910	1438, 1814, 2434, 3054, 3674, 4294, 4841, 5171	
149	coagulation factor XI	264692	296	264692	911	1439, 1815, 2435, 3055, 3675, 4295, 4842, 5172	1624
150	coagulation factor XI	403665	297	384957	912	1440, 1816, 2436, 3056, 3676, 4296, 4843, 5173, 9382-9468	1624
151	coagulation factor XI	452239	298	397401	913	1441, 1817, 2437, 3057, 3677, 4297, 4844, 5174,	
152	coagulation factor XIII, A1 polypeptide	264870	299	264870	914	1442, 1818, 2438, 3058, 3678, 4298,	

						9469-9555	
153	coagulation factor XIII, A1 polypeptide	414279	300	413334	915	1443, 1819, 2439, 3059, 3679, 4299	
154	coagulation factor XIII, A1 polypeptide	441301	301	416127	916	1820, 2440, 3060, 3680, 4300	
155	collagen, type VII, alpha 1	328333	302	332371	917	1821, 2441, 3061, 3681, 4301, 4845, 5175, 9556-9642	1625
156	collagen, type VII, alpha 1	454817	303	412569	918	1822, 2442, 3062, 3682, 4302, 4846, 5176,	
157	colony stimulating factor 2 (granulocyte-macrophage)	296871	304	296871	919	1823, 2443, 3063, 3683, 4303, 4847, 5177, 9643-9781	1626
158	colony stimulating factor 3 (granulocyte)	225474	305	225474	920	1824, 2444, 3064, 3684, 4304, 9782-9868	1627
159	colony stimulating factor 3 (granulocyte)	331769	306	327766	921	1825, 2445, 3065, 3685, 4305, 4848, 5178, 9869-9955	
160	colony stimulating factor 3 (granulocyte)	394148	307	377704	922	1826, 2446, 3066, 3686, 4306, 4849, 5179,	1627
161	colony stimulating factor 3 (granulocyte)	394149	308	377705	923	1827, 2447, 3067, 3687, 4307, 4850, 5180, 9956-10042	1627
162	complement factor H	367429	309	356399	924	1444, 1828, 2448, 3068, 3688, 4308, 4851, 5181	1627
163	complement factor H	391986	310	375846	925	1829, 2449, 3069, 3689, 4309, 4852, 5182	
164	complement factor H	439155	311	402656	926	1445, 1830, 2450, 3070, 3690, 4310, 4853, 5183	
165	copper metabolism (Murr1) domain	311832	312	308236	927	1446, 1831, 2451, 3071,	

	containing 1					3691, 4311, 10043-10129	
166	copper metabolism (Murr1) domain containing 1	427417	313	413207	928	1447, 1832, 2452, 3072, 3692, 4312	
167	copper metabolism (Murr1) domain containing 1	444166	314	410050	929	1448, 1833, 2453, 3073, 3693, 4313	
168	copper metabolism (Murr1) domain containing 1	458337	315	401236	930	1834, 2454, 3074, 3694, 4314	
169	copper metabolism (Murr1) domain containing 1	538736	316	438961	931	1449, 1835, 2455, 3075, 3695, 4315	
170	corticotropin releasing hormone	276571	317	276571	932	1836, 2456, 3076, 3696, 4316, 4854, 5184	
171	CTLA4-Ig (Orencia)						
172	cystic fibrosis transmembrane conductance regulator (ATP-binding cassette sub-family C, member 7)	3084	318	3084	933	1450, 1837, 2457, 3077, 3697, 4317, 4855, 5185, 10130-10216	1628
173	cystic fibrosis transmembrane conductance regulator (ATP-binding cassette sub-family C, member 7)	426809	319	389119	934	1451, 1838, 2458, 3078, 3698, 4318, 4856, 5186	
174	cystic fibrosis transmembrane conductance regulator (ATP-binding cassette sub-family C, member 7)	454343	320	403677	935	1452, 1839, 2459, 3079, 3699, 4319, 4857, 5187	1628
175	cystic fibrosis transmembrane conductance regulator (ATP-binding cassette sub-family C, member 7)	468795	321	419254	936	1453, 1840, 2460, 3080, 3700, 4320, 4858, 5188	
176	cystinosin lysosomal cystine transporter	381870	322	371294	937	1841, 2461, 3081, 3701, 4321	

177	cystinosin, lysosomal cystine transporter	46640	323	46640	938	1454, 1842, 2462, 3082, 3702, 4322, 4859, 5189, 10217-10355	
178	cystinosin, lysosomal cystine transporter	414524	324	395471	939	1455, 1843, 2463, 3083, 3703, 4323	
179	cystinosin, lysosomal cystine transporter	441220	325	411465	940	1456, 1844, 2464, 3084, 3704, 4324	
180	cystinosin, lysosomal cystine transporter	452111	326	408652	941	1457, 1845, 2465, 3085, 3705, 4325	
181	defensin, beta 103A	314357	327	320951	942	1846, 2466, 3086, 3706, 4326, 4860, 5190	1629
182	defensin, beta 103B	318124	328	324633	943	1847, 2467, 3087, 3707, 4327, 4861, 5191, 10356-10442	1630
183	defensin, beta 4A	302247	329	303532	944	1848, 2468, 3088, 3708, 4328, 4862, 5192, 10443-10529	1631
184	defensin, beta 4B	318157	330	424598	945	1849, 2469, 3089, 3709, 4329, 4863, 5193	1632
185	DegludecPlus; insulin aspart; insulin degludec						
186	deoxyribonuclease I	246949	331	246949	946	1850, 2470, 3090, 3710, 4330, 4864, 5194, 10530-10616	1633
187	deoxyribonuclease I	407479	332	385905	947	1851, 2471, 3091, 3711, 4331, 4865, 5195	1633
188	deoxyribonuclease I	414110	333	416699	948	1852, 2472, 3092, 3712, 4332, 4866, 5196	
189	dysferlin, limb girdle muscular dystrophy 2B (autosomal recessive)	258104	334	258104	949	1458, 1853, 2473, 3093, 3713, 4333, 4867, 5197	

190	dysferlin, limb girdle muscular dystrophy 2B (autosomal recessive)	394120	335	377678	950	1459, 1854, 2474, 3094, 3714, 4334, 4868, 5198, 10617-10703	1634
191	dysferlin, limb girdle muscular dystrophy 2B (autosomal recessive)	409366	336	386512	951	1460, 1855, 2475, 3095, 3715, 4335, 4869, 5199	
192	dysferlin, limb girdle muscular dystrophy 2B (autosomal recessive)	409582	337	386547	952	1461, 1856, 2476, 3096, 3716, 4336, 4870, 5200	
193	dysferlin, limb girdle muscular dystrophy 2B (autosomal recessive)	409651	338	386683	953	1462, 1857, 2477, 3097, 3717, 4337, 4871, 5201	
194	dysferlin, limb girdle muscular dystrophy 2B (autosomal recessive)	409744	339	386285	954	1463, 1858, 2478, 3098, 3718, 4338, 4872, 5202	
195	dysferlin, limb girdle muscular dystrophy 2B (autosomal recessive)	409762	340	387137	955	1464, 1859, 2479, 3099, 3719, 4339, 4873, 5203	
196	dysferlin, limb girdle muscular dystrophy 2B (autosomal recessive)	410020	341	386881	956	1465, 1860, 2480, 3100, 3720, 4340, 4874, 5204	
197	dysferlin, limb girdle muscular dystrophy 2B (autosomal recessive)	410041	342	386617	957	1466, 1861, 2481, 3101, 3721, 4341, 4875, 5205	
198	dysferlin, limb girdle muscular dystrophy 2B (autosomal recessive)	413539	343	407046	958	1467, 1862, 2482, 3102, 3722, 4342, 4876, 5206	
199	dysferlin, limb girdle muscular dystrophy 2B (autosomal recessive)	429174	344	398305	959	1468, 1863, 2483, 3103, 3723, 4343, 4877, 5207	
200	ectodysplasin A	338901	345	340611	960	1864, 2484, 3104, 3724, 4344, 4878, 5208	

201	ectodysplasin A	374552	346	363680	961	1865, 2485, 3105, 3725, 4345, 4879, 5209	
202	ectodysplasin A	374553	347	363681	962	1866, 2486, 3106, 3726, 4346, 4880, 5210, 10704-10790	1635
203	ectodysplasin A	525810	348	434195	963	1867, 2487, 3107, 3727, 4347, 4881, 5211	
204	ectodysplasin A	524573	349	432585	964	1868, 2488, 3108, 3728, 4348, 4882, 5212	
205	ectodysplasin A	527388	350	434861	965	1869, 2489, 3109, 3729, 4349, 4883, 5213	
206	enamelin	396073	351	379383	966	1870, 2490, 3110, 3730, 4350, 4884, 5214	1636
207	erythropoietin	252723	352	252723	967	1871, 2491, 3111, 3731, 4351, 4885, 5215, 10791-11051	1637
208	Exenatide				968	1872, 2492, 3112, 3732, 4352, 4886, 5216	
209	family with sequence similarity 98, member C	252530	353	252530	969	1873, 2493, 3113, 3733, 4353	
210	family with sequence similarity 98, member C	343358	354	340348	970	1874, 2494, 3114, 3734, 4354	
211	fibrinogen alpha chain	302053	355	306361	971	1469, 1875, 2495, 3115, 3735, 4355, 11052-11190	
212	fibrinogen alpha chain	403106	356	385981	972	1470, 1876, 2496, 3116, 3736, 4356	
213	fibrinogen alpha chain	457487	357	407891	973	1877, 2497, 3117, 3737, 4357	
214	fibrinogen beta chain	302068	358	306099	974	1471, 1878, 2498, 3118, 3738, 4358,	

						11191-11329	
215	fibrinogen beta chain	537843	359	437727	975	1879, 2499, 3119, 3739, 4359	
216	fibrinogen gamma chain	336098	360	336829	976	1472, 1880, 2500, 3120, 3740, 4360, 11330-11468	
217	fibrinogen gamma chain	404648	361	384860	977	1473, 1881, 2501, 3121, 3741, 4361	
218	fibroblast growth factor 21	222157	362	222157	978	1474, 1882, 2502, 3122, 3742, 4362	
219	fibroblast growth factor 21	593756	363	471477	979	1883, 2503, 3123, 3743, 4363, 11469-11555	
220	fibronectin type III domain containing 5	373470	364	362569	980	1884, 2504, 3124, 3744, 4364,	
221	fibronectin type III domain containing 5	373471	365	362570	981	1885, 2505, 3125, 3745, 4365, 11556-11642	
222	follicle stimulating hormone, beta polypeptide	254122	366	254122	982	1886, 2506, 3126, 3746, 4366, 4887, 5217, 11643-11729	
223	follicle stimulating hormone, beta polypeptide	417547	367	416606	983	1887, 2507, 3127, 3747, 4367, 4888, 5218	
224	follicle stimulating hormone, beta polypeptide	533718	368	433424	984	1888, 2508, 3128, 3748, 4368, 4889, 5219	
225	fumarylacetoacetate hydrolase (fumarylacetoacetase)	261755	369	261755	985	1475 , 1889, 2509, 3129, 3749, 4369	
226	fumarylacetoacetate hydrolase (fumarylacetoacetase)	407106	370	385080	986	1890, 2510, 3130, 3750, 4370, 11730-11868	
227	fumarylacetoacetate hydrolase (fumarylacetoacetase)	561421	371	453347	987	1891, 2511, 3131, 3751, 4371	
228	galactokinase 1	225614	372	225614	988	1892, 2512, 3132, 3752, 4372	

229	galactokinase 1	375188	373	364334	989	1893, 2513, 3133, 3753, 4373	
230	galactokinase 1	437911	374	406305	990	1894, 2514, 3134, 3754, 4374	
231	galactokinase 1	588479	375	465930	991	1895, 2515, 3135, 3755, 4375, 11869-11955	
232	galactosamine (N-acetyl)-6-sulfate sulfatase	268695	376	268695	992	1476, 1896, 2516, 3136, 3756, 4376, 4890, 5220, 11956-12042	1638
233	galactosamine (N-acetyl)-6-sulfate sulfatase	439266	377	402127	993	1897, 2517, 3137, 3757, 4377, 4891, 5221	
234	galactosamine (N-acetyl)-6-sulfate sulfatase	542788	378	438197	994	1477, 1898, 2518, 3138, 3758, 4378, 4892, 5222	
235	galactose-1-phosphate uridylyltransferase	378842	379	368119	995	1899, 2519, 3139, 3759, 4379, 12043-12181	
236	galactose-1-phosphate uridylyltransferase	450095	380	401956	996	1900, 2520, 3140, 3760, 4380	
237	galactose-1-phosphate uridylyltransferase	554550	381	451435	997	1901, 2521, 3141, 3761, 4381	
238	galactosidase, alpha	218516	382	218516	998	1902, 2522, 3142, 3762, 4382, 12182-12268	1639
239	Galectin 3 inhibitor (A9)		383		999		1640
240	Galectin 3 inhibitor (C12)		384		1000		1641
241	glial cell derived neurotrophic factor	326524	385	317145	1001	1903, 2523, 3143, 3763, 4383, 4893, 5223	
242	glial cell derived neurotrophic factor	344622	386	339703	1002	1904, 2524, 3144, 3764, 4384, 4894, 5224	
243	glial cell derived neurotrophic factor	427982	387	409007	1003	1905, 2525, 3145, 3765, 4385, 4895, 5225, 12269-12355	1642

244	glial cell derived neurotrophic factor	502572	388	423557	1004	1906, 2526, 3146, 3766, 4386, 4896, 5226	
245	glial cell derived neurotrophic factor	510177	389	424592	1005	1907, 2527, 3147, 3767, 4387, 4897, 5227	
246	glial cell derived neurotrophic factor	515058	390	425928	1006	1908, 2528, 3148, 3768, 4388, 4898, 5228	
247	glial cell derived neurotrophic factor	381826	391	371248	1007	1909, 2529, 3149, 3769, 4389, 4899, 5229	
248	GLP-1 Fc; dulaglutide						
249	glucagon	375497	392	364647	1008	1910, 2530, 3150, 3770, 4390, 4900, 5230	1643
250	glucagon	418842	393	387662	1009	1911, 2531, 3151, 3771, 4391, 4901, 5231, 12356-12703	1643
251	glucan (1,4-alpha-), branching enzyme 1	264326	394	264326	1010	1912, 2532, 3152, 3772, 4392	
252	glucan (1,4-alpha-), branching enzyme 1	429644	395	410833	1011	1478, 1913, 2533, 3153, 3773, 4393, 12704-12842	
253	glucan (1,4-alpha-), branching enzyme 1	536832	396	445365	1012	1914, 2534, 3154, 3774, 4394	
254	glucose-6-phosphatase, catalytic subunit	253801	397	253801	1013	1915, 2535, 3155, 3775, 4395, 12843-12981	
255	glucosidase, alpha; acid	302262	398	305692	1014	1916, 2536, 3156, 3776, 4396, 4902, 5232, 12982-13155	1644
256	glucosidase, alpha; acid	390015	399	374665	1015	1917, 2537, 3157, 3777, 4397, 4903, 5233	1644
257	glucosidase, beta, acid	327247	400	314508	1016	1918, 2538, 3158, 3778, 4398, 4904, 5234	1645

258	glucosidase, beta, acid	368373	401	357357	1017	1919, 2539, 3159, 3779, 4399, 4905, 5235	1645
259	glucosidase, beta, acid	402928	402	385813	1018	1920, 2540, 3160, 3780, 4400, 4906, 5236	1645
260	glucosidase, beta, acid	427500	403	402577	1019	1921, 2541, 3161, 3781, 4401, 4907, 5237	1645
261	glucosidase, beta, acid	428024	404	397986	1020	1922, 2542, 3162, 3782, 4402, 4908, 5238	1645
262	glucosidase, beta, acid	536555	405	446457	1021	1923, 2543, 3163, 3783, 4403, 4909, 5239	1645
263	glucosidase, beta, acid	536770	406	445560	1022	1924, 2544, 3164, 3784, 4404, 4910, 5240	1645
264	glycogen synthase 2 (liver)	261195	407	261195	1023	1925, 2545, 3165, 3785, 4405, 13156-13242	
265	glycoprotein hormones, alpha polypeptide	369582	408	358595	1024	1926, 2546, 3166, 3786, 4406, 4911, 5241, 13243-13329	1646
266	GP2013; rituximab						
267	Granulysin 9 kDa (non-secreted)					1479	
268	Granulysin 9 kDa (secreted)					1480	
269	growth hormone 1	323322	409	312673	1025	1927, 2547, 3167, 3787, 4407, 4912, 5242, 13330-13416	1647
270	growth hormone 1	342364	410	339278	1026	1928, 2548, 3168, 3788, 4408, 4913, 5243	1647
271	growth hormone 1	351388	411	343791	1027	1929, 2549, 3169, 3789, 4409, 4914, 5244	1647
272	growth hormone 1	458650	412	408486	1028	1930, 2550, 3170, 3790, 4410, 4915,	1647

						5245	
273	growth hormone 2	332800	413	333157	1029	1931, 2551, 3171, 3791, 4411, 4916, 5246	
274	growth hormone 2	423893	414	409294	1030	1932, 2552, 3172, 3792, 4412, 4917, 5247	
275	growth hormone 2	449787	415	410618	1031	1933, 2553, 3173, 3793, 4413, 4918, 5248	
276	growth hormone 2	456543	416	394122	1032	1934, 2554, 3174, 3794, 4414, 4919, 5249	
277	GTP cyclohydrolase 1	395514	417	378890	1033	1935, 2555, 3175, 3795, 4415, 4920, 5250, 13417-13503	1648
278	GTP cyclohydrolase 1	395524	418	378895	1034	1936, 2556, 3176, 3796, 4416, 4921, 5251	
279	GTP cyclohydrolase 1	491895	419	419045	1035	1937, 2557, 3177, 3797, 4417, 4922, 5252	1649
280	GTP cyclohydrolase 1	536224	420	445246	1036	1938, 2558, 3178, 3798, 4418, 4923, 5253	
281	GTP cyclohydrolase 1	543643	421	444011	1037	1939, 2559, 3179, 3799, 4419, 4924, 5254	
282	hemochromatosis	309234	422	311698	1038	1940, 2560, 3180, 3800, 4420	
283	hemochromatosis	317896	423	313776	1039	1941, 2561, 3181, 3801, 4421	
284	hemochromatosis	336625	424	337819	1040	1942, 2562, 3182, 3802, 4422	
285	hemochromatosis	349999	425	259699	1041	1943, 2563, 3183, 3803, 4423	
286	hemochromatosis	352392	426	315936	1042	1944, 2564, 3184, 3804, 4424	

287	hemochromatosis	353147	427	312342	1043	1945, 2565, 3185, 3805, 4425	
288	hemochromatosis	357618	428	417404	1044	1946, 2566, 3186, 3806, 4426	
289	hemochromatosis	397022	429	380217	1045	1947, 2567, 3187, 3807, 4427	
290	hemochromatosis	461397	430	420802	1046	1948, 2568, 3188, 3808, 4428	
291	hemochromatosis	470149	431	419725	1047	1949, 2569, 3189, 3809, 4429	
292	hemochromatosis	488199	432	420559	1048	1950, 2570, 3190, 3810, 4430	
293	hemochromatosis	535098	433	445872	1049	1951, 2571, 3191, 3811, 4431	
294	hemochromatosis	539147	434	445098	1050	1952, 2572, 3192, 3812, 4432	
295	hemochromatosis type 2 (juvenile)	336751	435	337014	1051	1481, 1953, 2573, 3193, 3813, 4433, 4925, 5255, 13504-13590	
296	hemochromatosis type 2 (juvenile)	357836	436	350495	1052	1482, 1954, 2574, 3194, 3814, 4434, 4926, 5256	
297	hemochromatosis type 2 (juvenile)	421822	437	411863	1053	1483, 1955, 2575, 3195, 3815, 4435, 4927, 5257	
298	hemochromatosis type 2 (juvenile)	475797	438	425716	1054	1484, 1956, 2576, 3196, 3816, 4436, 4928, 5258	
299	hemochromatosis type 2 (juvenile)	497365	439	421820	1055	1957, 2577, 3197, 3817, 4437, 4929, 5259	
300	hemochromatosis type 2 (juvenile)	577520	440	463276	1056	1958, 2578, 3198, 3818, 4438	
301	hemochromatosis type 2 (juvenile)	580693	441	464413	1057	1959, 2579, 3199, 3819, 4439	
302	hepatocyte growth factor (hepapoietin A; scatter factor)	222390	442	222390	1058	1960, 2580, 3200, 3820, 4440, 13591-13677	

303	hepatocyte growth factor (hepapoietin A; scatter factor)	354224	443	346164	1059	1961, 2581, 3201, 3821, 4441	
304	hepatocyte growth factor (hepapoietin A; scatter factor)	394769	444	378250	1060	1962, 2582, 3202, 3822, 4442	
305	hepatocyte growth factor (hepapoietin A; scatter factor)	412881	445	396307	1061	1963, 2583, 3203, 3823, 4443	
306	hepatocyte growth factor (hepapoietin A; scatter factor)	421558	446	388592	1062	1964, 2584, 3204, 3824, 4444	
307	hepatocyte growth factor (hepapoietin A; scatter factor)	423064	447	413829	1063	1965, 2585, 3205, 3825, 4445	
308	hepatocyte growth factor (hepapoietin A; scatter factor)	444829	448	389854	1064	1966, 2586, 3206, 3826, 4446	
309	hepatocyte growth factor (hepapoietin A; scatter factor)	453411	449	408270	1065	1967, 2587, 3207, 3827, 4447	
310	hepatocyte growth factor (hepapoietin A; scatter factor)	457544	450	391238	1066	1968, 2588, 3208, 3828, 4448	
311	hepcidin antimicrobial peptide	222304	451	222304	1067	14851969, 2589, 3209, 3829, 4449	
312	hepcidin antimicrobial peptide	598398	452	471894	1068	1970, 2590, 3210, 3830, 4450, 13678-13764	
313	Herceptin Heavy Chain				1069	1971, 2591, 3211, 3831, 4451, 4930, 5260	
314	Herceptin Light Chain				1070	1972, 2592, 3212, 3832, 4452, 4931, 5261	
315	hypoxanthine phosphoribosyltransferase 1	298556	453	298556	1071	1486, 1973, 2593, 3213, 3833, 4453, 4932, 5262, 13765-13851	1650
316	hypoxanthine phosphoribosyltransferase 1	370796	454	359832	1072	1974, 2594, 3214, 3834, 4454, 4933, 5263	
317	IDegLira; insulin degludec; liraglutide						
318	iduronate 2-sulfatase	340855	455	339801	1073	1975, 2595, 3215, 3835, 4455, 4934, 5264	1651

319	iduronate 2-sulfatase	370441	456	359470	1074	1976, 2596, 3216, 3836, 4456, 4935, 5265	1651
320	iduronate 2-sulfatase	370443	457	359472	1075	1977, 2597, 3217, 3837, 4457, 4936, 5266	1651
321	iduronate 2-sulfatase	428056	458	390241	1076	1978, 2598, 3218, 3838, 4458, 4937, 5267	1651
322	iduronate 2-sulfatase	521702	459	429745	1077	1979, 2599, 3219, 3839, 4459, 4938, 5268	1651
323	iduronate 2-sulfatase	537071	460	440324	1078	1980, 2600, 3220, 3840, 4460, 4939, 5269	1651
324	iduronate 2-sulfatase	541269	461	441261	1079	1981, 2601, 3221, 3841, 4461, 4940, 5270	1651
325	iduronidase, alpha-L-	247933	462	247933	1080	1982, 2602, 3222, 3842, 4462, 4941, 5271, 13852-13938	1652
326	iduronidase, alpha-L-	453894	463	396458	1081	1983, 2603, 3223, 3843, 4463, 4942, 5272	1652
327	IgM Heavy						
328	IgM Light						
329	INS-IGF2 readthrough	397270	464	380440	1082	1984, 2604, 3224, 3844, 4464, 4943, 5273	
330	insulin	250971	465	250971	1083	1985, 2605, 3225, 3845, 4465, 4944, 5274, 13939-14286	1653
331	insulin	381330	466	370731	1084	1986, 2606, 3226, 3846, 4466, 4945, 5275	1653
332	insulin	397262	467	380432	1085	1987, 2607, 3227, 3847, 4467, 4946, 5276, 14287-14373	1653

333	insulin	512523	468	424008	1086	1988, 2608, 3228, 3848, 4468, 4947, 5277	
334	Insulin Aspart				1087	1989, 2609, 3229, 3849, 4469, 4948, 5278	
335	Insulin Glargine				1088	1990, 2610, 3230, 3850, 4470, 4949, 5279	
336	Insulin Glulisine				1089	1991, 2611, 3231, 3851, 4471, 4950, 5280	
337	Insulin Lispro				1090	1992, 2612, 3232, 3852, 4472, 4951, 5281	
338	interferon, alpha 10	357374	469	369566	1091	1993, 2613, 3233, 3853, 4473, 4952, 5282	
339	interferon, alpha 13	449498	470	394494	1092	1994, 2614, 3234, 3854, 4474, 4953, 5283	
340	interferon, alpha 14	380222	471	369571	1093	1995, 2615, 3235, 3855, 4475, 4954, 5284	
341	interferon, alpha 16	380216	472	369564	1094	1996, 2616, 3236, 3856, 4476, 4955, 5285	
342	interferon, alpha 17	413767	473	411940	1095	1997, 2617, 3237, 3857, 4477, 4956, 5286	
343	interferon, alpha 2	380206	474	369554	1096	1998, 2618, 3238, 3858, 4478, 4957, 5287, 14374-14460	1654
344	interferon, alpha 21	380225	475	369574	1097	1999, 2619, 3239, 3859, 4479, 4958, 5288	
345	interferon, alpha 4	421715	476	412897	1098	2000, 2620, 3240, 3860, 4480, 4959, 5289	

346	interferon, alpha 5	259555	477	259555	1099	2001, 2621, 3241, 3861, 4481, 4960, 5290	
347	interferon, alpha 6	380210	478	369558	1100	2002, 2622, 3242, 3862, 4482, 4961, 5291	
348	interferon, alpha 7	239347	479	239347	1101	2003, 2623, 3243, 3863, 4483, 4962, 5292	
349	interferon, alpha 8	380205	480	369553	1102	2004, 2624, 3244, 3864, 4484, 4963, 5293	
350	interferon, beta 1, fibroblast	380232	481	369581	1103	2005, 2625, 3245, 3865, 4485, 4964, 5294, 14461-14547	1655
351	interleukin 10	423557	482	412237	1104	1487, 2006, 2626, 3246, 3866, 4486, 14548-14634	
352	interleukin 21	264497	483	264497	1105	2007, 2627, 3247, 3867, 4487, 14635-14721	
353	interleukin 7	263851	484	263851	1106	1488, 2008, 2628, 3248, 3868, 4488, 4965, 5295, 14722-14860	1656
354	interleukin 7	379113	485	368408	1107	1489, 2009, 2629, 3249, 3869, 4489, 4966, 5296	1656
355	interleukin 7	379114	486	368409	1108	2010, 2630, 3250, 3870, 4490, 4967, 5297	
356	interleukin 7	518982	487	430272	1109	2011, 2631, 3251, 3871, 4491, 4968, 5298	
357	interleukin 7	520215	488	428364	1110	1490, 2012, 2632, 3252, 3872, 4492, 4969, 5299	
358	interleukin 7	520269	489	427750	1111	1491, 2013, 2633, 3253, 3873, 4493, 4970, 5300	

359	interleukin 7	520317	490	427800	1112	2014, 2634, 3254, 3874, 4494, 4971, 5301	
360	interleukin 7	541183	491	438922	1113	1492, 2015, 2635, 3255, 3875, 4495, 4972, 5302	
361	KIT ligand	228280	492	228280	1114	2016, 2636, 3256, 3876, 4496, 14861-14947	
362	KIT ligand	347404	493	54216	1115	2017, 2637, 3257, 3877, 4497	
363	KIT ligand	537835	494	438889	1116	2018, 2638, 3258, 3878, 4498	
364	klotho	380099	495	369442	1117	1493, 2019, 2639, 3259, 3879, 4499, 14948-15347	
365	klotho	426690	496	399513	1118	14942020, 2640, 3260, 3880, 4500	
366	Kruppel-like factor 4 (gut)	374672	497	363804	1119	2021, 2641, 3261, 3881, 4501	
367	Kruppel-like factor 4 (gut)	411706	498	399921	1120	2022, 2642, 3262, 3882, 4502	
368	Kruppel-like factor 4 (gut)	420475	499	404922	1121	2023, 2643, 3263, 3883, 4503	
369	Kruppel-like factor 4 (gut)	439281	500	396294	1122	2024, 2644, 3264, 3884, 4504	
370	lecithin-cholesterol acyltransferase	264005	501	264005	1123	1495, 2025, 2645, 3265, 3885, 4505, 15348-15434	
371	lectin, galactoside-binding, soluble, 3	254301	502	254301	1124	2026, 2646, 3266, 3886, 4506, 15435-15521	
372	lin-28 homolog A (C. elegans)	254231	503	254231	1125	2027, 2647, 3267, 3887, 4507	
373	lin-28 homolog A (C. elegans)	326279	504	363314	1126	2028, 2648, 3268, 3888, 4508, 15522-15608	
374	lipase A, lysosomal acid, cholesterol	282673	505	282673	1127	1496 , 2029, 2649, 3269, 3889, 4509,	

	esterase					4973, 5303	
375	lipase A, lysosomal acid, cholesterol esterase	336233	506	337354	1128	1497, 2030, 2650, 3270, 3890, 4510, 4974, 5304, 15609-15782	1657
376	lipase A, lysosomal acid, cholesterol esterase	354621	507	360895	1129	2031, 2651, 3271, 3891, 4511, 4975, 5305	
377	lipase A, lysosomal acid, cholesterol esterase	371829	508	360894	1130	2032, 2652, 3272, 3892, 4512, 4976, 5306	1657
378	lipase A, lysosomal acid, cholesterol esterase	371837	509	360903	1131	1498, 2033, 2653, 3273, 3893, 4513, 4977, 5307	
379	lipase A, lysosomal acid, cholesterol esterase	425287	510	392037	1132	1499, 2034, 2654, 3274, 3894, 4514, 4978, 5308	
380	lipase A, lysosomal acid, cholesterol esterase	428800	511	388415	1133	1500, 2035, 2655, 3275, 3895, 4515, 4979, 5309	1657
381	lipase A, lysosomal acid, cholesterol esterase	456827	512	413019	1134	2036, 2656, 3276, 3896, 4516, 4980, 5310	1657
382	lipase A, lysosomal acid, cholesterol esterase	540050	513	439727	1135	2037, 2657, 3277, 3897, 4517, 4981, 5311	
383	lipase A, lysosomal acid, cholesterol esterase	541980	514	438127	1136	2038, 2658, 3278, 3898, 4518, 4982, 5312	1657
384	lipase A, lysosomal acid, cholesterol esterase	542307	515	437564	1137	1501, 2039, 2659, 3279, 3899, 4519, 4983, 5313	1657
385	lipoprotein lipase	311322	516	309757	1138	1502, 2040, 2660, 3280, 3900, 4520, 4984, 5314, 15783-15869	
386	lipoprotein lipase	520959	517	428496	1139	1503, 2041, 2661, 3281, 3901, 4521, 4985, 5315	
387	lipoprotein lipase	522701	518	428557	1140	1504, 2042, 2662, 3282,	

						3902, 4522, 4986, 5316	
388	lipoprotein lipase	524029	519	428237	1141	1505, 2043, 2663, 3283, 3903, 4523, 4987, 5317	
389	lipoprotein lipase	535763	520	438606	1142	2044, 2664, 3284, 3904, 4524, 4988, 5318	
390	lipoprotein lipase	538071	521	439407	1143	2045, 2665, 3285, 3905, 4525, 4989, 5319	
391	liraglutide native 7-37					1506	
392	liraglutide native 7-37-A8G					1507	
393	liraglutide native 7-37-A8G-10X					1508	
394	liraglutide; GLP1; Victoza					1509	
395	LL-37; Cathelicidin anti-microbial peptide				1144		1620
396	low density lipoprotein receptor	455727	522	397829	1145	1510, 2046, 2666, 3286, 3906, 4526	
397	low density lipoprotein receptor	535915	523	440520	1146	1511, 2047, 2667, 3287, 3907, 4527	
398	low density lipoprotein receptor	545707	524	437639	1147	1512, 2048, 2668, 3288, 3908, 4528	
399	low density lipoprotein receptor	558013	525	453346	1148	1513, 2049, 2669, 3289, 3909, 4529	
400	low density lipoprotein receptor	558518	526	454071	1149	1514 , 2050, 2670, 3290, 3910, 4530, 15870-16478	
401	low density lipoprotein receptor	561343	527	454147	1150	2051, 2671, 3291, 3911, 4531	
402	low density lipoprotein receptor	252444	528	252444	1151	2052, 2672, 3292, 3912, 4532	
403	luteinizing hormone beta polypeptide	221421	529	221421	1152	2053, 2673, 3293, 3913, 4533	
404	luteinizing hormone beta polypeptide	391870	530	375743	1153	2054, 2674, 3294, 3914, 4534, 4990, 5320,	

405	LY2963016; insulin glargine						
406	Ly6/neurotoxin 1	317543	531	319846	1154	2055, 2675, 3295, 3915, 4535	
407	MabThera SC; rituximab						
408	mannosidase alpha class 2B member 1	221363	532	221363	1155	2056, 2676, 3296, 3916, 4536	
409	mannosidase, alpha, class 2B, member 1	433513	533	390583	1156	1515, 2057, 2677, 3297, 3917, 4537	
410	mannosidase, alpha, class 2B, member 1	456935	534	395473	1157	1516, 2058, 2678, 3298, 3918, 4538, 16479-16565	
411	mannosidase, alpha, class 2B, member 1	536796	535	438266	1158	2059, 2679, 3299, 3919, 4539	
412	meteorin glial cell differentiation regulator	568223	536	455068	1159	2060, 2680, 3300, 3920, 4540, 16566-16652	
413	meteorin, glial cell differentiation regulator	219542	537	219542	1160	2061, 2681, 3301, 3921, 4541	
414	methylmalonyl CoA mutase	274813	538	274813	1161	1517, 2062, 2682, 3302, 3922, 4542, 4991, 5321, 16653-16739	1658
415	methylmalonyl CoA mutase	540138	539	445535	1162	2063, 2683, 3303, 3923, 4543, 4992, 5322,	
416	microsomal triglyceride transfer protein	265517	540	265517	1163	1518, 2064, 2684, 3304, 3924, 4544, 16740-16878	
417	microsomal triglyceride transfer protein	457717	541	400821	1164	2065, 2685, 3305, 3925, 4545	
418	microsomal triglyceride transfer protein	506883	542	426755	1165	1519, 2066, 2686, 3306, 3926, 4546	
419	microsomal triglyceride transfer protein	538053	543	437358	1166	2067, 2687, 3307, 3927, 4547	
420	N-acetylglucosamine -1-phosphate transferase, alpha and beta subunits	299314	544	299314	1167	1520, 2068, 2688, 3308, 3928, 4548, 16879-17017	

421	N-acetylglucosamine-1-phosphate transferase, alpha and beta subunits	549940	545	449150	1168	1521, 2069, 2689, 3309, 3929, 4549	
422	N-acetylglutamate synthase	293404	546	293404	1169	2070, 2690, 3310, 3930, 4550, 17018-17156	
423	N-acetylglutamate synthase	541745	547	441262	1170	2071, 2691, 3311, 3931, 4551	
424	N-acylsphingosine amidohydrolase (acid ceramidase) 1	262097	548	262097	1171	1522, 2072, 2692, 3312, 3932, 455217157-17295	
425	N-acylsphingosine amidohydrolase (acid ceramidase) 1	314146	549	326970	1172	1523, 2073, 2693, 3313, 3933, 4553	
426	N-acylsphingosine amidohydrolase (acid ceramidase) 1	381733	550	371152	1173	1524 , 2074, 2694, 3314, 3934, 4554	
427	N-acylsphingosine amidohydrolase (acid ceramidase) 1	417108	551	394125	1174	1525 , 2075, 2695, 3315, 3935, 4555	
428	N-acylsphingosine amidohydrolase (acid ceramidase) 1	520781	552	427751	1175	1526, 2076, 2696, 3316, 3936, 4556	
429	natriuretic peptide B	376468	553	365651	1176	2077, 2697, 3317, 3937, 4557, 4993, 5323,	
430	nerve growth factor (beta polypeptide)	369512	554	358525	1177	2078, 2698, 3318, 3938, 4558, 4994, 5324, 17296-17382	
431	neuregulin 1	287840	555	287840	1178	2079, 2699, 3319, 3939, 4559	
432	neuregulin 1	287842	556	287842	1179	2080, 2700, 3320, 3940, 4560, 17383-17469	
433	neuregulin 1	287845	557	287845	1180	2081, 2701, 3321, 3941, 4561	
434	neuregulin 1	338921	558	343395	1181	2082, 2702, 3322, 3942, 4562	

435	neuregulin 1	341377	559	340497	1182	2083, 2703, 3323, 3943, 4563	
436	neuregulin 1	356819	560	349275	1183	2084, 2704, 3324, 3944, 4564	
437	neuregulin 1	405005	561	384620	1184	2085, 2705, 3325, 3945, 4565	
438	neuregulin 1	519301	562	429582	1185	2086, 2706, 3326, 3946, 4566	
439	neuregulin 1	520407	563	434640	1186	2087, 2707, 3327, 3947, 4567, 17470-17556	
440	neuregulin 1	520502	564	433289	1187	2088, 2708, 3328, 3948, 4568	
441	neuregulin 1	521670	565	428828	1188	2089, 2709, 3329, 3949, 4569	
442	neuregulin 1	539990	566	439276	1189	2090, 2710, 3330, 3950, 4570	
443	neuregulin 1	523079	567	430120	1190	2091, 2711, 3331, 3951, 4571	
444	OMOMYC				1191	1527, 17557-17643	
445	OMOMYC90 (90AA truncated domain)					1528, 17644-17784	
446	Orencia S.C.; abatacept						
447	ornithine carbamoyltransferase	39007	568	39007	1192	1529, 2092, 2712, 3332, 3952, 4572, 4995, 5325, 17785-17871	1659
448	parathyroid hormone	282091	569	282091	1193	2093, 2713, 3333, 3953, 4573, 4996, 5326	
449	parathyroid hormone	529816	570	433208	1194	2094, 2714, 3334, 3954, 4574, 4997, 5327	
450	PBO-326; rituximab						
451	phenylalanine hydroxylase	546844	571	446658	1195	1530, 2095, 2715, 3335, 3955, 4575, 4998, 5328	

452	phenylalanine hydroxylase	551337	572	447620	1196	1531, 2096, 2716, 3336, 3956, 4576, 4999, 5329	
453	phenylalanine hydroxylase	553106	573	448059	1197	1532, 2097, 2717, 3337, 3957, 4577, 5000, 5330, 17872-17958	1660
454	phosphorylase kinase beta	566044	574	456729	1198	2098, 2718, 3338, 3958, 4578	
455	phosphorylase kinase gamma 2 (testis)	563588	575	455607	1199	2099, 2719, 3339, 3959, 4579, 17959-18045	
456	phosphorylase kinase, alpha 2 (liver)	379942	576	369274	1200	2100, 2720, 3340, 3960, 4580, 18046-18132	
457	phosphorylase kinase, beta	299167	577	299167	1201	2101, 2721, 3341, 3961, 4581	
458	phosphorylase kinase, beta	323584	578	313504	1202	2102, 2722, 3342, 3962, 4582, 18133-18219	
459	phosphorylase kinase, beta	455779	579	414345	1203	2103, 2723, 3343, 3963, 4583	
460	phosphorylase kinase, gamma 2 (testis)	328273	580	329968	1204	2104, 2724, 3344, 3964, 4584	
461	phosphorylase kinase, gamma 2 (testis)	424889	581	388571	1205	2105, 2725, 3345, 3965, 4585	
462	phosphorylase, glycogen, liver	216392	582	216392	1206	2106, 2726, 3346, 3966, 4586	
463	phosphorylase, glycogen, liver	544180	583	443787	1207	2107, 2727, 3347, 3967, 4587	
464	plasminogen	308192	584	308938	1208	1533, 2108, 2728, 3348, 3968, 4588, 18220-18271	
465	plasminogen	316325	585	321466	1209	2109, 2729, 3349, 3969, 4589	
466	plasminogen	366924	586	355891	1210	1534, 2110, 2730, 3350, 3970, 4590	
467	plasminogen activator, tissue	220809	587	220809	1211	1535, 2111, 2731, 3351, 3971, 4591,	1661

						18272-18358	
468	plasminogen activator, tissue	270189	588	270189	1212	1536, 2112, 2732, 3352, 3972, 4592, 5001, 5331	1661
469	plasminogen activator, tissue	352041	589	270188	1213	1537, 2113, 2733, 3353, 3973, 4593, 5002, 5332	1661
470	plasminogen activator, tissue	429089	590	392045	1214	2114, 2734, 3354, 3974, 4594, 5003, 5333	1661
471	plasminogen activator, tissue	429710	591	407861	1215	1538, 2115, 2735, 3355, 3975, 4595, 5004, 5334	
472	plasminogen activator, tissue	519510	592	428886	1216	1539, 2116, 2736, 3356, 3976, 4596, 5005, 5335	
473	plasminogen activator, tissue	520523	593	428797	1217	1540, 2117, 2737, 3357, 3977, 4597, 5006, 5336	1661
474	plasminogen activator, tissue	521694	594	429801	1218	1541, 2118, 2738, 3358, 3978, 4598, 5007, 5337	1661
475	plasminogen activator, tissue	524009	595	429401	1219	1542, 2119, 2739, 3359, 3979, 4599, 5008, 5338	
476	POU class 5 homeobox 1	259915	596	259915	1220	2120, 2740, 3360, 3980, 4600	
477	POU class 5 homeobox 1	376243	597	365419	1221	2121, 2741, 3361, 3981, 4601	
478	POU class 5 homeobox 1	383524	598	373016	1222	2122, 2742, 3362, 3982, 4602	
479	POU class 5 homeobox 1	412166	599	387646	1223	2123, 2743, 3363, 3983, 4603	
480	POU class 5 homeobox 1	419095	600	413622	1224	2124, 2744, 3364, 3984, 4604	
481	POU class 5 homeobox 1	429603	601	392877	1225	2125, 2745, 3365, 3985, 4605	
482	POU class 5 homeobox 1	433063	602	405041	1226	2126, 2746, 3366, 3986,	

						4606	
483	POU class 5 homeobox 1	433348	603	412665	1227	2127, 2747, 3367, 3987, 4607	
484	POU class 5 homeobox 1	434616	604	388842	1228	2128, 2748, 3368, 3988, 4608, 18359-18445	
485	POU class 5 homeobox 1	448657	605	416165	1229	2129, 2749, 3369, 3989, 4609	
486	POU class 5 homeobox 1	451077	606	391507	1230	2130, 2750, 3370, 3990, 4610	
487	POU class 5 homeobox 1	454714	607	400047	1231	2131, 2751, 3371, 3991, 4611	
488	POU class 5 homeobox 1	546505	608	448154	1232	2132, 2752, 3372, 3992, 4612	
489	POU class 5 homeobox 1	547234	609	449442	1233	2133, 2753, 3373, 3993, 4613	
490	POU class 5 homeobox 1	547658	610	446962	1234	2134, 2754, 3374, 3994, 4614	
491	POU class 5 homeobox 1	548682	611	446815	1235	2135, 2755, 3375, 3995, 4615	
492	POU class 5 homeobox 1	550059	612	447874	1236	2136, 2756, 3376, 3996, 4616	
493	POU class 5 homeobox 1	550521	613	447969	1237	2137, 2757, 3377, 3997, 4617	
494	POU class 5 homeobox 1	550572	614	448254	1238	2138, 2758, 3378, 3998, 4618	
495	POU class 5 homeobox 1	553069	615	448231	1239	2139, 2759, 3379, 3999, 4619	
496	POU class 5 homeobox 1	553206	616	446757	1240	2140, 2760, 3380, 4000, 4620	
497	proprotein convertase subtilisin/kexin type 9	302118	617	303208	1241	2141, 2761, 3381, 4001, 4621	
498	proprotein convertase subtilisin/kexin type 9	452118	618	401598	1242	2142, 2762, 3382, 4002, 4622	

499	proprotein convertase subtilisin/kexin type 9	543384	619	441859	1243	2143, 2763, 3383, 4003, 4623	
500	protein C (inactivator of coagulation factors Va and VIIIa)	234071	620	234071	1244	2144, 2764, 3384, 4004, 4624	
501	protein C (inactivator of coagulation factors Va and VIIIa)	409048	621	386679	1245	2145, 2765, 3385, 4005, 4625, 5009, 5339	
502	protein C (inactivator of coagulation factors Va and VIIIa)	422777	622	409543	1246	2146, 2766, 3386, 4006, 4626, 5010, 5340	
503	protein C (inactivator of coagulation factors Va and VIIIa)	427769	623	406295	1247	2147, 2767, 3387, 4007, 4627, 5011, 5341	
504	protein C (inactivator of coagulation factors Va and VIIIa)	429925	624	412697	1248	2148, 2768, 3388, 4008, 4628, 5012, 5342	
505	protein C (inactivator of coagulation factors Va and VIIIa)	453608	625	404030	1249	2149, 2769, 3389, 4009, 4629, 5013, 5343	
506	protein C (inactivator of coagulation factors Va and VIIIa)	537436	626	442106	1250	2150, 2770, 3390, 4010, 4630, 5014, 5344	
507	relaxin 2	308420	627	308018	1251	2151, 2771, 3391, 4011, 4631	
508	relaxin 2	381627	628	371040	1252	2152, 2772, 3392, 4012, 4632, 18446-18532	
509	Reverse Caspase 3 (cleavable)					1543	
510	Reverse Caspase 3 (non-cleavable)					1544	
511	Reverse Caspase 6					1545	
512	rhodopsin	296271	629	296271	1253	2153, 2773, 3393, 4013, 4633, 5015, 5345, 18533-18619	
513	Rituximab; rituximab						
514	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase,	355814	630	348068	1254	2154, 2774, 3394, 4014, 4634, 5016, 5346, 18620-	1662

	antitrypsin), member 1					18706	
515	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	393087	631	376802	1255	2155, 2775, 3395, 4015, 4635, 5017, 5347	1662
516	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	393088	632	376803	1256	2156, 2776, 3396, 4016, 4636, 5018, 5348	1662
517	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	402629	633	386094	1257	2157, 2777, 3397, 4017, 4637, 5019, 5349	
518	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	404814	634	385960	1258	2158, 2778, 3398, 4018, 4638, 5020, 5350	1662
519	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	437397	635	408474	1259	2159, 2779, 3399, 4019, 4639, 5021, 5351	1662
520	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	440909	636	390299	1260	2160, 2780, 3400, 4020, 4640, 5022, 5352	1662
521	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	448921	637	416066	1261	2161, 2781, 3401, 4021, 4641, 5023, 5353	1662
522	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	449399	638	416354	1262	2162, 2782, 3402, 4022, 4642, 5024, 5354	1662

523	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	553327	639	452480	1263	2163, 2783, 3403, 4023, 4643, 5025, 5355, 18707-18793	
524	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	556091	640	452169	1264	2164, 2784, 3404, 4024, 4644, 5026, 5356	
525	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	556955	641	451098	1265	2165, 2785, 3405, 4025, 4645, 5027, 5357	
526	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	557118	642	451826	1266	2166, 2786, 3406, 4026, 4646, 5028, 5358	
527	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1	557492	643	452452	1267	2167, 2787, 3407, 4027, 4647, 5029, 5359	
528	serpin peptidase inhibitor, clade C (antithrombin), member 1	351522	644	307953	1268	2168, 2788, 3408, 4028, 4648, 5030, 5360	
529	serpin peptidase inhibitor, clade C (antithrombin), member 1	367698	645	356671	1269	1546, 2169, 2789, 3409, 4029, 4649, 5031, 5361, 18794-18932	
530	serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 2	324015	646	321853	1270	1547, 2170, 2790, 3410, 4030, 4650	
531	serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 2	382061	647	371493	1271	2171, 2791, 3411, 4031, 4651, 18933-19071	
532	serpin peptidase inhibitor, clade F	450523	648	403877	1272	1548, 2172, 2792, 3412,	

	(alpha-2 antiplasmin, pigment epithelium derived factor), member 2					4032, 4652	
533	serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 2	453066	649	402286	1273	1549 , 2173, 2793, 3413, 4033, 4653	
534	serpin peptidase inhibitor, clade G (C1 inhibitor), member 1	278407	650	278407	1274	1550, 2174, 2794, 3414, 4034, 4654, 5032, 5362, 19072-19210	
535	serpin peptidase inhibitor, clade G (C1 inhibitor), member 1	378323	651	367574	1275	1551 , 2175, 2795, 3415, 4035, 4655, 5033, 5363	
536	serpin peptidase inhibitor, clade G (C1 inhibitor), member 1	378324	652	367575	1276	1552, 2176, 2796, 3416, 4036, 4656, 5034, 5364	
537	serpin peptidase inhibitor, clade G (C1 inhibitor), member 1	405496	653	384561	1277	1553, 2177, 2797, 3417, 4037, 4657, 5035, 5365	
538	serpin peptidase inhibitor, clade G (C1 inhibitor), member 1	433668	654	399800	1278	2178, 2798, 3418, 4038, 4658, 5036, 5366	
539	sirtuin 1	212015	655	212015	1279	1554, 2179, 2799, 3419, 4039, 4659 , 19211-19297	1663
540	sirtuin 1	403579	656	384063	1280	1555, 2180, 2800, 3420, 4040, 4660, 5037, 5367	
541	sirtuin 1	406900	657	384508	1281	2181, 2801, 3421, 4041, 4661, 5038, 5368	
542	sirtuin 1	432464	658	409208	1282	1556, 2182, 2802, 3422, 4042, 4662, 5039, 5369	1664
543	sirtuin 6	305232	659	305310	1283	1557, 2183, 2803, 3423, 4043, 4663, 5040, 5370	

544	sirtuin 6	337491	660	337332	1284	1558, 2184, 2804, 3424, 4044, 4664, 5041, 5371, 19298-19384	1665
545	sirtuin 6	381935	661	371360	1285	1559, 2185, 2805, 3425, 4045, 4665, 5042, 5372	
546	solute carrier family 2 (facilitated glucose transporter), member 2	314251	662	323568	1286	1560, 2186, 2806, 3426, 4046, 4666, 19385-19523	
547	solute carrier family 2 (facilitated glucose transporter), member 2	382808	663	372258	1287	1561, 2187, 2807, 3427, 4047, 4667	
548	solute carrier family 37 (glucose-6-phosphate transporter) member 4	577093	664	461344	1288	2188, 2808, 3428, 4048, 4668,, 19524-19610	
549	solute carrier family 37 (glucose-6-phosphate transporter) member 4	590663	665	464769	1289	2189, 2809, 3429, 4049, 4669	
550	solute carrier family 40 (iron-regulated transporter), member 1	261024	666	261024	1290	2190, 2810, 3430, 4050, 4670	
551	solute carrier family 40 (iron-regulated transporter), member 1	427241	667	390005	1291	2191, 2811, 3431, 4051, 4671	
552	solute carrier family 40 (iron-regulated transporter), member 1	427419	668	392730	1292	2192, 2812, 3432, 4052, 4672	
553	solute carrier family 40 (iron-regulated transporter), member 1	440626	669	396134	1293	2193, 2813, 3433, 4053, 4673	
554	solute carrier family 40 (iron-regulated transporter),	455320	670	413549	1294	2194, 2814, 3434, 4054, 4674	

	member 1						
555	solute carrier family 40 (iron-regulated transporter), member 1	481497	671	446069	1295	2195, 2815, 3435, 4055, 4675	
556	solute carrier family 40 (iron-regulated transporter), member 1	544056	672	444582	1296	2196, 2816, 3436, 4056, 4676	
557	sortilin 1	256637	673	256637	1297	1562, 2197, 2817, 3437, 4057, 4677, 5043, 5373	
558	sortilin 1	538502	674	438597	1298	1563 , 2198, 2818, 3438, 4058, 4678, 5044, 5374, 19611-19697	
559	sperm adhesion molecule 1 (PH-20 hyaluronidase, zona pellucida binding)	223028	675	223028	1299	2199, 2819, 3439, 4059, 4679	
560	sperm adhesion molecule 1 (PH-20 hyaluronidase, zona pellucida binding)	340011	676	345849	1300	2200, 2820, 3440, 4060, 4680, 5045, 5375	
561	sperm adhesion molecule 1 (PH-20 hyaluronidase, zona pellucida binding)	402183	677	386028	1301	2201, 2821, 3441, 4061, 4681, 5046, 5376	
562	sperm adhesion molecule 1 (PH-20 hyaluronidase, zona pellucida binding)	413927	678	391491	1302	2202, 2822, 3442, 4062, 4682, 5047, 5377	
563	sperm adhesion molecule 1 (PH-20 hyaluronidase, zona pellucida binding)	439500	679	402123	1303	2203, 2823, 3443, 4063, 4683, 5048, 5378	
564	sperm adhesion molecule 1 (PH-20 hyaluronidase, zona pellucida binding)	460182	680	417934	1304	2204, 2824, 3444, 4064, 4684, 5049, 5379	
565	sphingomyelin phosphodiesterase 1, acid lysosomal	299397	681	299397	1305	1564, 2205, 2825, 3445, 4065, 4685,	

						5050, 5380,	
566	sphingomyelin phosphodiesterase 1, acid lysosomal	342245	682	340409	1306	1565, 2206, 2826, 3446, 4066, 4686, 5051, 5381, 19698-19784	1666
567	sphingomyelin phosphodiesterase 1, acid lysosomal	356761	683	349203	1307	1566, 2207, 2827, 3447, 4067, 4687, 5052, 5382	
568	sphingomyelin phosphodiesterase 1, acid lysosomal	527275	684	435350	1308	1567, 2208, 2828, 3448, 4068, 4688, 5053, 5383	
569	sphingomyelin phosphodiesterase 1, acid lysosomal	533123	685	435950	1309	1568, 2209, 2829, 3449, 4069, 4689, 5054, 5384	
570	sphingomyelin phosphodiesterase 1, acid lysosomal	534405	686	434353	1310	1569, 2210, 2830, 3450, 4070, 4690, 5055, 5385	
571	SRY (sex determining region Y)-box 2	325404	687	323588	1311	2211, 2831, 3451, 4071, 4691	
572	SRY (sex determining region Y)-box 2	431565	688	439111	1312	2212, 2832, 3452, 4072, 4692, 19785-19871	
573	Subcutaneous Herceptin; trastuzumab						
574	surfactant protein B	393822	689	377409	1313	2213, 2833, 3453, 4073, 4693	
575	surfactant protein B	409383	690	386346	1314	2214, 2834, 3454, 4074, 4694	
576	surfactant protein B	441838	691	395757	1315	2215, 2835, 3455, 4075, 4695	
577	surfactant protein B	519937	692	428719	1316	2216, 2836, 3456, 4076, 4696	
578	Synagis						
579	thrombomodulin	377103	693	366307	1317	1570, 2217, 2837, 3457, 4077, 4697, 19872-19958	
580	thrombomodulin	503590	694	440119	1318	2218, 2838, 3458, 4078, 4698	

581	thrombopoietin	204615	695	204615	1319	1571, 2219, 2839, 3459, 4079, 4699, 5056, 5386, 19959-20045	1667
582	thrombopoietin	353488	696	341335	1320	2220, 2840, 3460, 4080, 4700, 5057, 5387	
583	thrombopoietin	421442	697	411704	1321	1572, 2221, 2841, 3461, 4081, 4701, 5058, 5388	
584	thrombopoietin	445696	698	410763	1322	1573, 2222, 2842, 3462, 4082, 4702, 5059, 5389	
585	TL011; rituximab						
586	transferrin receptor 2	223051	699	223051	1323	2223, 2843, 3463, 4083, 4703	
587	transferrin receptor 2	431692	700	413905	1324	2224, 2844, 3464, 4084, 4704	
588	transferrin receptor 2	462107	701	420525	1325	2225, 2845, 3465, 4085, 4705	
589	transferrin receptor 2	544242	702	443656	1326	2226, 2846, 3466, 4086, 4706	
590	transforming growth factor, beta 1	221930	703	221930	1327	2227, 2847, 3467, 4087, 4707 , 20046-20132	1668
591	transforming growth factor, beta 2	366929	704	355896	1328	2228, 2848, 3468, 4088, 4708	
592	transforming growth factor, beta 2	366930	705	355897	1329	2229, 2849, 3469, 4089, 4709	
593	transforming growth factor, beta 3	238682	706	238682	1330	2230, 2850, 3470, 4090, 4710, 5060, 5390, 20133-20219	
594	transforming growth factor, beta 3	556285	707	451110	1331	2231, 2851, 3471, 4091, 4711, 5061, 5391	
595	transthyretin	237014	708	237014	1332	2232, 2852, 3472, 4092, 4712, 20220-20358	

596	transthyretin	432547	709	396762	1333	2233, 2853, 3473, 4093, 4713	
597	transthyretin	541025	710	442134	1334	2234, 2854, 3474, 4094, 4714	
598	tuftelin 1	353024	711	343781	1335	2235, 2855, 3475, 4095, 4715, 5062, 5392	1669
599	tuftelin 1	368848	712	357841	1336	2236, 2856, 3476, 4096, 4716, 5063, 5393	1669
600	tuftelin 1	368849	713	357842	1337	2237, 2857, 3477, 4097, 4717, 5064, 5394, 20359-20445	1669
601	tuftelin 1	507671	714	443643	1338	2238, 2858, 3478, 4098, 4718, 5065, 5395	1669
602	tuftelin 1	538902	715	437997	1339	2239, 2859, 3479, 4099, 4719, 5066, 5396	
603	tuftelin 1	544350	716	441557	1340	2240, 2860, 3480, 4100, 4720, 5067, 5397	1669
604	tumor protein p53	269305	717	269305	1341	1574, 2241, 2861, 3481, 4101, 4721, 5068, 5398, 20446-20532	1670
605	tumor protein p53	359597	718	352610	1342	1575, 2242, 2862, 3482, 4102, 4722, 5069, 5399	
606	tumor protein p53	396473	719	379735	1343	2243, 2863, 3483, 4103, 4723, 5070, 5400	
607	tumor protein p53	413465	720	410739	1344	1576, 2244, 2864, 3484, 4104, 4724, 5071, 5401	
608	tumor protein p53	414315	721	394195	1345	2245, 2865, 3485, 4105, 4725, 5072, 5402	
609	tumor protein p53	419024	722	402130	1346	2246, 2866, 3486, 4106, 4726, 5073,	

						5403	
610	tumor protein p53	420246	723	391127	1347	1577, 2247, 2867, 3487, 4107, 4727, 5074, 5404	
611	tumor protein p53	445888	724	391478	1348	2248, 2868, 3488, 4108, 4728, 5075, 5405	1670
612	tumor protein p53	455263	725	398846	1349	1578, 2249, 2869, 3489, 4109, 4729, 5076, 5406	
613	tumor protein p53	503591	726	426252	1350	1579 , 2250, 2870, 3490, 4110, 4730, 5077, 5407	
614	tumor protein p53	508793	727	424104	1351	1580, 2251, 2871, 3491, 4111, 4731, 5078, 5408	
615	tumor protein p53	509690	728	425104	1352	1581,, 2252, 2872, 3492, 4112, 4732, 5079, 5409	
616	tumor protein p53	514944	729	423862	1353	1582, 2253, 2873, 3493, 4113, 4733, 5080, 5410	
617	tumor protein p53	545858	730	437792	1354	2254, 2874, 3494, 4114, 4734, 5081, 5411	
618	tyrosinase (oculocutaneous albinism IA)	263321	731	263321	1355	2255, 2875, 3495, 4115, 4735, , , 20533-20619	
619	tyrosine aminotransferase	355962	732	348234	1356	1583, 2256, 2876, 3496, 4116, 4736, , , 20620-20758	
620	UDP glucuronosyltransferase 1 family, polypeptide A1	305208	733	304845	1357	1584, 2257, 2877, 3497, 4117, 4737, , , 20759-20845	
621	UDP glucuronosyltransferase 1 family, polypeptide A1	360418	734	353593	1358	1585, 2258, 2878, 3498, 4118, 4738	
622	UDP glucuronosyltransferase 1 family, polypeptide A10	344644	735	343838	1359	2259, 2879, 3499, 4119, 4739	

623	UDP glucuronosyltransferase 1 family, polypeptide A3	482026	736	418532	1360	2260, 2880, 3500, 4120, 4740	
624	UDP glycosyltransferase 3 family polypeptide A2	513300	737	427404	1361	2261, 2881, 3501, 4121, 4741, 5082, 5412	
625	UDP glycosyltransferase 3 family, polypeptide A2	282507	738	282507	1362	2262, 2882, 3502, 4122, 4742, 5083, 5413	1671
626	UDP glycosyltransferase 3 family, polypeptide A2	515131	739	420865	1363	2263, 2883, 3503, 4123, 4743, 5084, 5414	
627	UDP glycosyltransferase 3 family, polypeptide A2	545528	740	445367	1364	2264, 2884, 3504, 4124, 4744, 5085, 5415	
628	vascular endothelial growth factor A	230480	741	230480	1365	1586, 2265, 2885, 3505, 4125, 4745	
629	vascular endothelial growth factor A	324450	742	317598	1366	1587, 2266, 2886, 3506, 4126, 4746, 5086, 5416	
630	vascular endothelial growth factor A	372055	743	361125	1367	1588, 2267, 2887, 3507, 4127, 4747, 5087, 5417	
631	vascular endothelial growth factor A	372064	744	361134	1368	1589, 2268, 2888, 3508, 4128, 4748, 5088, 5418	
632	vascular endothelial growth factor A	372067	745	361137	1369	1590, 2269, 2889, 3509, 4129, 4749, 5089, 5419, 20846-20932	1672
633	vascular endothelial growth factor A	372077	746	361148	1370	1591, 2270, 2890, 3510, 4130, 4750, 5090, 5420	
634	vascular endothelial growth factor A	413642	747	389864	1371	1592, 2271, 2891, 3511, 4131, 4751, 5091, 5421	
635	vascular endothelial growth factor A	417285	748	388663	1372	1593, 2272, 2892, 3512, 4132, 4752, 5092, 5422	
636	vascular endothelial growth factor A	425836	749	388465	1373	1594, 2273, 2893, 3513, 4133, 4753,	

						5093, 5423	
637	vascular endothelial growth factor A	457104	750	409911	1374	1595, 2274, 2894, 3514, 4134, 4754, 5094, 5424	
638	vascular endothelial growth factor A	482630	751	421561	1375	1596, 2275, 2895, 3515, 4135, 4755, 5095, 5425	
639	vascular endothelial growth factor A	518689	752	430829	1376	1597, 2276, 2896, 3516, 4136, 4756, 5096, 5426	
640	vascular endothelial growth factor A	518824	753	430002	1377	1598, 2277, 2897, 3517, 4137, 4757, 5097, 5427	
641	vascular endothelial growth factor A	519767	754	430594	1378	1599, 2278, 2898, 3518, 4138, 4758, 5098, 5428	
642	vascular endothelial growth factor A	520948	755	428321	1379	1600, 2279, 2899, 3519, 4139, 4759, 5099, 5429	
643	vascular endothelial growth factor A	523125	756	429008	1380	1601, 2280, 2900, 3520, 4140, 4760, 5100, 5430	
644	vascular endothelial growth factor A	523873	757	430479	1381	1602, 2281, 2901, 3521, 4141, 4761, 5101, 5431	
645	vascular endothelial growth factor A	523950	758	429643	1382	1603, 2282, 2902, 3522, 4142, 4762, 5102, 5432	
646	vascular endothelial growth factor C	280193	759	280193	1383	1604, 2283, 2903, 3523, 4143, 4763, 20933-21019	
647	vasoactive intestinal peptide	367243	760	356212	1384	1605, 2284, 2904, 3524, 4144, 4764	
648	vasoactive intestinal peptide	367244	761	356213	1385	1606, 2285, 2905, 3525, 4145, 4765, 21020-21106	
649	vasoactive intestinal peptide	431366	762	410356	1386	1607, 2286, 2906, 3526, 4146, 4766	
650	v-myc myelocytomatosis	259523	763	259523	1387	2287, 2907, 3527, 4147,	

	viral oncogene homolog (avian)					4767	
651	v-myc myelocytomatosis viral oncogene homolog (avian)	377970	764	367207	1388	2288, 2908, 3528, 4148, 4768, 21107-21110	
652	v-myc myelocytomatosis viral oncogene homolog (avian)	454617	765	405312	1389	2289, 2909, 3529, 4149, 4769	
653	v-myc myelocytomatosis viral oncogene homolog (avian)	524013	766	430235	1390	2290, 2910, 3530, 4150, 4770, 21111-21284	
654	von Willebrand factor	261405	767	261405	1391	1608, 2291, 2911, 3531, 4151, 4771, 21285-21423	
655	zinc finger, GATA-like protein 1	403903	768	384434	1392	2292, 2912, 3532, 4152, 4772	

Protein Cleavage Signals and Sites

[000288] In one embodiment, the polypeptides of the present invention may include at least one protein cleavage signal containing at least one protein cleavage site. The protein cleavage site may be located at the N-terminus, the C-terminus, at any space between the N- and the C- termini such as, but not limited to, half-way between the N- and C-termini, between the N-terminus and the half way point, between the half way point and the C-terminus, and combinations thereof.

[000289] The polypeptides of the present invention may include, but is not limited to, a proprotein convertase (or prohormone convertase), thrombin or Factor Xa protein cleavage signal. Proprotein convertases are a family of nine proteinases, comprising seven basic amino acid-specific subtilisin-like serine proteinases related to yeast kexin, known as prohormone convertase 1/3 (PC1/3), PC2, furin, PC4, PC5/6, paired basic amino-acid cleaving enzyme 4 (PACE4) and PC7, and two other subtilases that cleave at non-basic residues, called subtilisin kexin isozyme 1 (SKI-1) and proprotein convertase subtilisin kexin 9 (PCSK9). Non-limiting examples of protein cleavage signal amino acid sequences are listing in Table 7. In Table 7, "X" refers to any amino acid, "n" may be 0, 2, 4 or 6 amino acids and "*" refers to the protein cleavage site. In Table 7, SEQ ID NO: 21426 refers to when n=4 and SEQ ID NO: 21427 refers to when n=6.

Table 7. Protein Cleavage Site Sequences

Protein Cleavage Signal	Amino Acid Cleavage Sequence	SEQ ID NO
Proprotein convertase	R-X-X-R*	21424
	R-X-K/R-R*	21425
	K/R-Xn-K/R*	21426 or 21427
Thrombin	L-V-P-R*-G-S	21428
	L-V-P-R*	21429
	A/F/G/I/L/T/V/M-A/F/G/I/L/T/V/W-P-R*	21430
Factor Xa	I-E-G-R*	21431
	I-D-G-R*	21432
	A-E-G-R*	21433
	A/F/G/I/L/T/V/M-D/E-G-R*	21434

[000290] In one embodiment, the primary constructs and the mmRNA of the present invention may be engineered such that the primary construct or mmRNA contains at least one encoded protein cleavage signal. The encoded protein cleavage signal may be located before the start codon, after the start codon, before the coding region, within the coding region such as, but not limited to, half way in the coding region, between the start codon and the half way point, between the half way point and the stop codon, after the coding region, before the stop codon, between two stop codons, after the stop codon and combinations thereof.

[000291] In one embodiment, the primary constructs or mmRNA of the present invention may include at least one encoded protein cleavage signal containing at least one protein cleavage site. The encoded protein cleavage signal may include, but is not limited to, a proprotein convertase (or prohormone convertase), thrombin and/or Factor Xa protein cleavage signal. One of skill in the art may use Table 1 above or other known methods to determine the appropriate encoded protein cleavage signal to include in the primary constructs or mmRNA of the present invention. For example, starting with the signal of Table 7 and considering the codons of Table 1 one can design a signal for the primary construct which can produce a protein signal in the resulting polypeptide.

[000292] In one embodiment, the polypeptides of the present invention include at least one protein cleavage signal and/or site.

[000293] As a non-limiting example, U.S. Pat. No. 7,374,930 and U.S. Pub. No. 20090227660, herein incorporated by reference in their entireties, use a furin cleavage site to cleave the N-terminal methionine of GLP-1 in the expression product from the Golgi apparatus of the cells. In one embodiment, the polypeptides of the present invention include at least one protein cleavage signal and/or site with the proviso that the polypeptide is not GLP-1.

[000294] In one embodiment, the primary constructs or mmRNA of the present invention includes at least one encoded protein cleavage signal and/or site.

[000295] In one embodiment, the primary constructs or mmRNA of the present invention includes at least one encoded protein cleavage signal and/or site with the proviso that the primary construct or mmRNA does not encode GLP-1.

[000296] In one embodiment, the primary constructs or mmRNA of the present invention may include more than one coding region. Where multiple coding regions are present in the primary construct or mmRNA of the present invention, the multiple coding regions may be separated by encoded protein cleavage sites. As a non-limiting example, the primary construct or mmRNA may be signed in an ordered pattern. On such pattern follows AXBY form where A and B are coding regions which may be the same or different coding regions and/or may encode the same or different polypeptides, and X and Y are encoded protein cleavage signals which may encode the same or different protein cleavage signals. A second such pattern follows the form AXYBZ where A and B are coding regions which may be the same or different coding regions and/or may encode the same or different polypeptides, and X, Y and Z are encoded protein cleavage signals which may encode the same or different protein cleavage signals. A third pattern follows the form ABXCY where A, B and C are coding regions which may be the same or different coding regions and/or may encode the same or different polypeptides, and X and Y are encoded protein cleavage signals which may encode the same or different protein cleavage signals.

[000297] In one embodiment, the polypeptides, primary constructs and mmRNA can also contain sequences that encode protein cleavage sites so that the polypeptides, primary constructs and mmRNA can be released from a carrier region or a fusion partner by treatment with a specific protease for said protein cleavage site.

[000298] In one embodiment, the polypeptides, primary constructs and mmRNA of the present invention may include a sequence encoding the 2A peptide. In one embodiment, this sequence may be used to separate the coding region of two or more polypeptides of interest. As a non-limiting example, the sequence encoding the 2A peptide may be between coding region A and coding region B (A-2Apep-B). The presence of the 2A peptide would result in the cleavage of one long protein into protein A, protein B and the 2A peptide. Protein A and protein B may be the same or different polypeptides of interest. In another embodiment, the 2A peptide may be used in the polynucleotides, primary constructs and/or mmRNA of the present invention to produce two, three, four, five, six, seven, eight, nine, ten or more proteins.

Incorporating Post Transcriptional Control Modulators

[000299] In one embodiment, the polynucleotides, primary constructs and/or mmRNA of the present invention may include at least one post transcriptional control modulator. These post transcriptional control modulators may be, but are not limited to, small molecules, compounds and regulatory sequences. As a non-limiting example, post transcriptional control may be achieved using small molecules identified by PTC Therapeutics Inc. (South Plainfield, NJ) using their GEMS^{TM} (Gene Expression Modulation by Small-Moleclues) screening technology.

[000300] The post transcriptional control modulator may be a gene expression modulator which is screened by the method detailed in or a gene expression modulator described in International Publication No. WO2006022712, herein incorporated by reference in its entirety. Methods identifying RNA regulatory sequences involved in translational control are described in International Publication No. WO2004067728, herein incorporated by reference in its entirety; methods identifying compounds that modulate untranslated region dependent expression of a gene are described in International Publication No. WO2004065561, herein incorporated by reference in its entirety.

[000301] In one embodiment, the polynucleotides, primary constructs and/or mmRNA of the present invention may include at least one post transcriptional control modulator is located in the 5' and/or the 3' untranslated region of the polynucleotides, primary constructs and/or mmRNA of the present invention

[000302] In another embodiment, the polynucleotides, primary constructs and/or mmRNA of the present invention may include at least one post transcription control modulator to modulate premature translation termination. The post transcription control modulators may be compounds described in or a compound found by methods outlined in International Publication Nso. WO2004010106, WO2006044456, WO2006044682, WO2006044503 and WO2006044505, each of which is herein incorporated by reference in its entirety. As a non-limiting example, the compound may bind to a region of the 28S ribosomal RNA in order to modulate premature translation termination (See e.g., WO2004010106, herein incorporated by reference in its entirety).

[000303] In one embodiment, polynucleotides, primary constructs and/or mmRNA of the present invention may include at least one post transcription control modulator to alter protein expression. As a non-limiting example, the expression of VEGF may be regulated using the compounds described in or a compound found by the methods described in International Publication Nos. WO2005118857, WO2006065480, WO2006065479 and WO2006058088, each of which is herein incorporated by reference in its entirety.

[000304] The polynucleotides, primary constructs and/or mmRNA of the present invention may include at least one post transcription control modulator to control translation. In one embodiment, the post transcription control modulator may be a RNA regulatory sequence. As a non-limiting example, the RNA regulatory sequence may be identified by the methods described in International Publication No. WO2006071903, herein incorporated by reference in its entirety.

III. Modifications

[000305] Herein, in a polynucleotide (such as a primary construct or an mRNA molecule), the terms "modification" or, as appropriate, "modified" refer to modification with respect to A, G, U or C ribonucleotides. Generally, herein, these terms are not intended to refer to the ribonucleotide modifications in naturally occurring 5′-terminal mRNA cap moieties. In a polypeptide, the term "modification" refers to a modification as compared to the canonical set of 20 amino acids, moiety)

[000306] The modifications may be various distinct modifications. In some embodiments, the coding region, the flanking regions and/or the terminal regions may contain one, two, or more (optionally different) nucleoside or nucleotide modifications. In some embodiments, a modified polynucleotide, primary construct, or mmRNA introduced to a cell may exhibit reduced degradation in the cell, as compared to an unmodified polynucleotide, primary construct, or mmRNA.

[000307] The polynucleotides, primary constructs, and mmRNA can include any useful modification, such as to the sugar, the nucleobase, or the internucleoside linkage (*e.g.* to a linking phosphate / to a phosphodiester linkage / to the phosphodiester backbone). One or more atoms of a pyrimidine nucleobase may be replaced or substituted with optionally substituted amino, optionally substituted thiol, optionally substituted alkyl (e.g., methyl or ethyl), or halo (e.g., chloro or fluoro). In certain embodiments, modifications (e.g., one or more modifications) are present in each of the sugar and the internucleoside linkage. Modifications according to the present invention may be modifications of ribonucleic acids (RNAs) to deoxyribonucleic acids (DNAs), threose nucleic acids (TNAs), glycol nucleic acids (GNAs), peptide nucleic acids (PNAs), locked nucleic acids (LNAs) or hybrids thereof). Additional modifications are described herein.

[000308] As described herein, the polynucleotides, primary constructs, and mmRNA of the invention do not substantially induce an innate immune response of a cell into which the mRNA is introduced. Featues of an induced innate immune response include 1) increased expression of pro-inflammatory cytokines, 2) activation of intracellular PRRs (RIG-I, MDA5, etc, and/or 3) termination or reduction in protein translation.

[000309] In certain embodiments, it may desirable to intracellularly degrade a modified nucleic acid molecule introduced into the cell. For example, degradation of a modified nucleic acid molecule may be preferable if precise timing of protein production is desired. Thus, in some embodiments, the invention provides a modified nucleic acid molecule containing a degradation domain, which is capable of being acted on in a directed manner within a cell. In another aspect, the present disclosure provides polynucleotides comprising a nucleoside or nucleotide that can disrupt the binding of a major groove interacting, e.g. binding, partner with the polynucleotide (e.g., where the

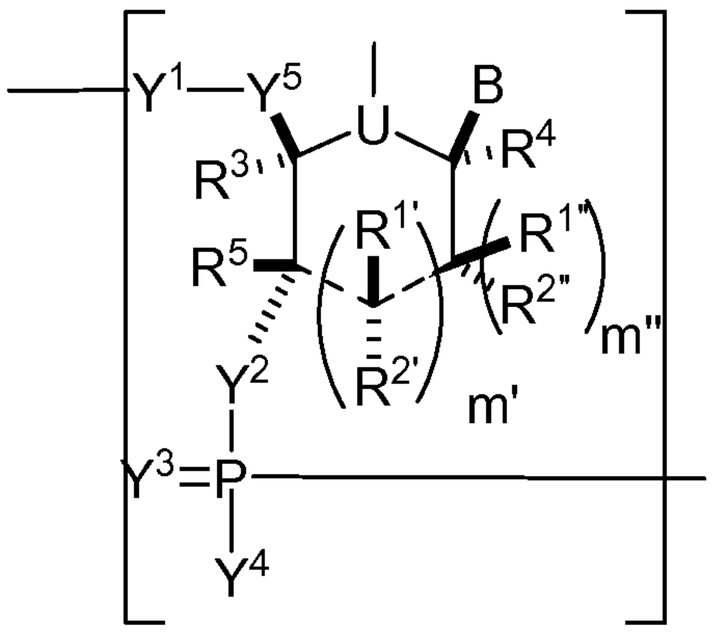
modified nucleotide has decreased binding affinity to major groove interacting partner, as compared to an unmodified nucleotide).

[000310] The polynucleotides, primary constructs, and mmRNA can optionally include other agents (e.g., RNAi-inducing agents, RNAi agents, siRNAs, shRNAs, miRNAs, antisense RNAs, ribozymes, catalytic DNA, tRNA, RNAs that induce triple helix formation, aptamers, vectors, etc.). In some embodiments, the polynucleotides, primary constructs, or mmRNA may include one or more messenger RNAs (mRNAs) and one or more modified nucleoside or nucleotides (e.g., mmRNA molecules). Details for these polynucleotides, primary constructs, and mmRNA follow.

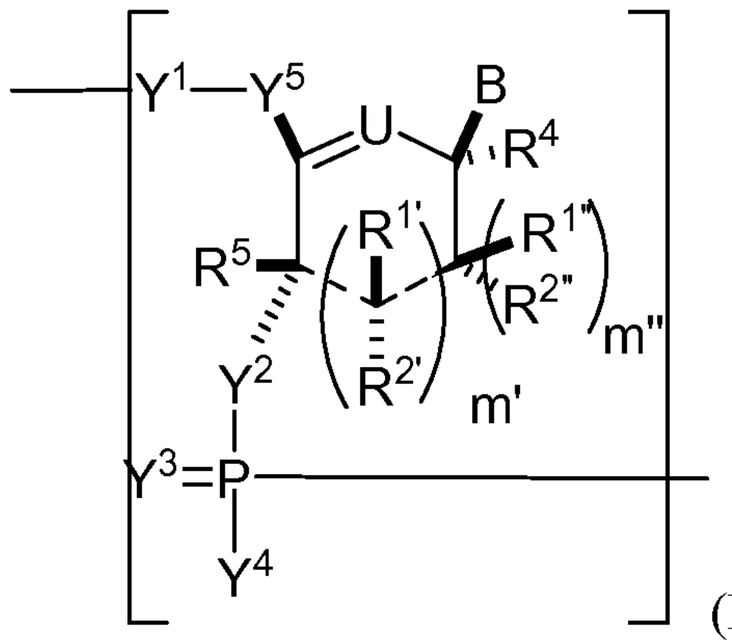
Polynucleotides and Primary Constructs

[000311] The polynucleotides, primary constructs, and mmRNA of the invention includes a first region of linked nucleosides encoding a polypeptide of interest, a first flanking region located at the 5′ terminus of the first region, and a second flanking region located at the 3′ terminus of the first region.

[000312] In some embodiments, the polynucleotide, primary construct, or mmRNA (e.g., the first region, first flanking region, or second flanking region) includes n number of linked nucleosides having Formula (Ia) or Formula (Ia-1):



(Ia)



(Ia-1) or a pharmaceutically acceptable salt or stereoisomer thereof,

[000313] wherein

[000314] U is O, S, $N(R^U)_{nu}$, or $C(R^U)_{nu}$, wherein nu is an integer from 0 to 2 and each R^U is, independently, H, halo, or optionally substituted alkyl;

[000315] --- is a single bond or absent;

[000316] each of $R^{1'}$, $R^{2'}$, $R^{1''}$, $R^{2''}$, R^1, R^2, R^3, R^4, and R^5 is, independently, if present, H, halo, hydroxy, thiol, optionally substituted alkyl, optionally substituted alkoxy,

optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted aminoalkoxy, optionally substituted alkoxyalkoxy, optionally substituted hydroxyalkoxy, optionally substituted amino, azido, optionally substituted aryl, optionally substituted aminoalkyl, optionally substituted aminoalkenyl, optionally substituted aminoalkynyl,or absent; wherein the combination of R3 with one or more of R1', R1", R2′, R2", or R5 (e.g., the combination of R1' and R3, the combination of R1" and R3, the combination of R2' and R3, the combination of R2" and R3, or the combination of R5 and R3) can join together to form optionally substituted alkylene or optionally substituted heteroalkylene and, taken together with the carbons to which they are attached, provide an optionally substituted heterocyclyl (e.g., a bicyclic, tricyclic, or tetracyclic heterocyclyl); wherein the combination of R5 with one or more of R1', R1", R2′, or R2" (e.g., the combination of R1' and R5, the combination of R1" and R5, the combination of R2′ and R5, or the combination of R2" and R5) can join together to form optionally substituted alkylene or optionally substituted heteroalkylene and, taken together with the carbons to which they are attached, provide an optionally substituted heterocyclyl (e.g., a bicyclic, tricyclic, or tetracyclic heterocyclyl); and wherein the combination of R^4 and one or more of $R^{1'}$, $R^{1''}$, $R^{2'}$, $R^{2''}$, R^3, or R^5 can join together to form optionally substituted alkylene or optionally substituted heteroalkylene and, taken together with the carbons to which they are attached, provide an optionally substituted heterocyclyl (e.g., a bicyclic, tricyclic, or tetracyclic heterocyclyl);each of m' and m" is, independently, an integer from 0 to 3 (e.g., from 0 to 2, from 0 to 1, from 1 to 3, or from 1 to 2);

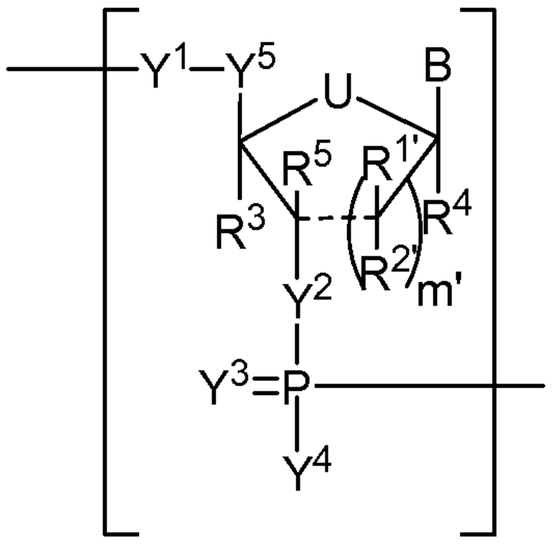
[000317] each Y^1, Y^2, and Y^3, is, independently, O, S, Se, -NR^{N1}-, optionally substituted alkylene, or optionally substituted heteroalkylene, wherein R^{N1} is H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted aryl, or absent;

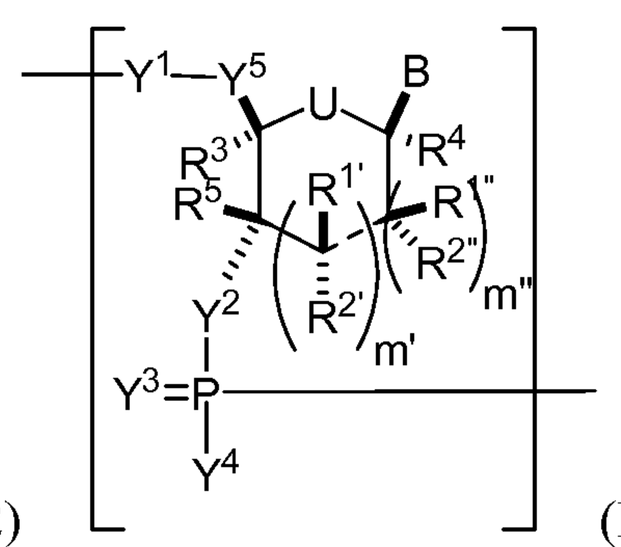
[000318] each Y^4 is, independently, H, hydroxy, thiol, boranyl, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted thioalkoxy, optionally substituted alkoxyalkoxy, or optionally substituted amino;

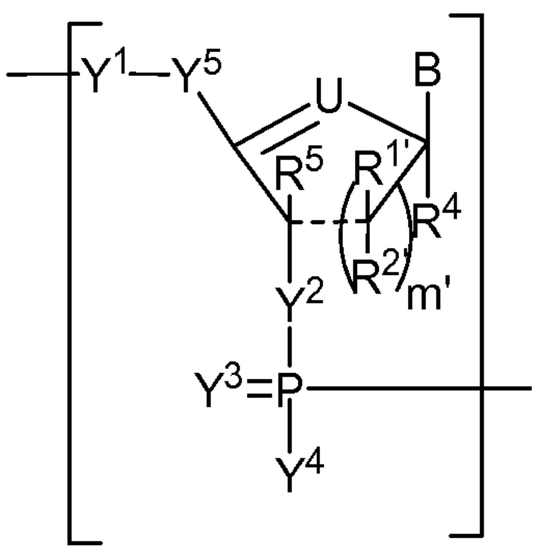
[000319] each Y^5 is, independently, O, S, Se, optionally substituted alkylene (e.g., methylene), or optionally substituted heteroalkylene;

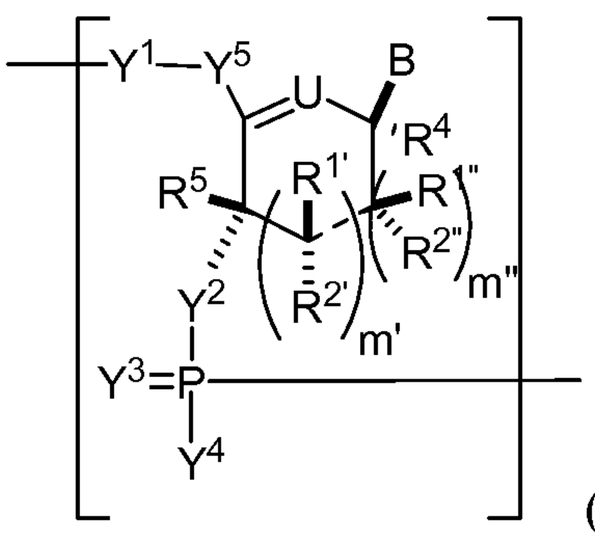
[000320] n is an integer from 1 to 100,000; and

[000321] B is a nucleobase (e.g., a purine, a pyrimidine, or derivatives thereof), wherein the combination of B and $R^{1'}$, the combination of B and $R^{2'}$, the combination of B and $R^{1"}$, or the combination of B and $R^{2"}$ can, taken together with the carbons to which they are attached, optionally form a bicyclic group (e.g., a bicyclic heterocyclyl) or wherein the combination of B, $R^{1"}$, and R^3 or the combination of B, $R^{2"}$, and R^3 can optionally form a tricyclic or tetracyclic group (e.g., a tricyclic or tetracyclic heterocyclyl, such as in Formula (IIo)-(IIp) herein).In some embodiments, the polynucleotide, primary construct, or mmRNA includes a modified ribose. In some embodiments, the polynucleotide, primary construct, or mmRNA (e.g., the first region, the first flanking region, or the second flanking region) includes n number of linked nucleosides having Formula (Ia-2)-(Ia-5) or a pharmaceutically acceptable salt or stereoisomer thereof.

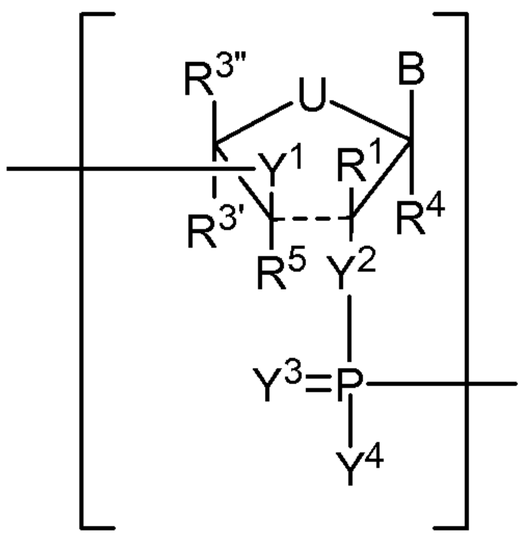

(Ia-2)


(Ia-3)

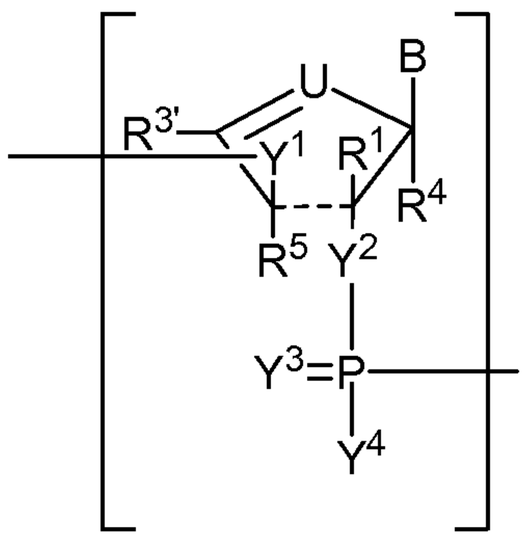

(Ia-4),


(Ia-5).

[000322] In some embodiments, the polynucleotide, primary construct, or mmRNA (e.g., the first region, the first flanking region, or the second flanking region) includes n number of linked nucleosides having Formula (Ib) or Formula (Ib-1):



(Ib),



(Ib-1) or a pharmaceutically acceptable salt or stereoisomer thereof,

[000323] wherein

[000324] U is O, S, $N(R^U)_{nu}$, or $C(R^U)_{nu}$, wherein nu is an integer from 0 to 2 and each R^U is, independently, H, halo, or optionally substituted alkyl;

[000325] --- is a single bond or absent;

[000326] each of R^1, $R^{3'}$, $R^{3"}$, and R^4 is, independently, H, halo, hydroxy, optionally substituted alkyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted aminoalkoxy, optionally substituted alkoxyalkoxy, optionally substituted hydroxyalkoxy, optionally substituted amino, azido, optionally substituted aryl, optionally substituted aminoalkyl, optionally substituted aminoalkenyl, optionally substituted aminoalkynyl, or absent; and wherein the combination of R^1 and $R^{3'}$ or the combination of R^1 and $R^{3"}$ can be taken together to form optionally substituted alkylene or optionally substituted heteroalkylene (e.g., to produce a locked nucleic acid);

[000327] each R^5 is, independently, H, halo, hydroxy, optionally substituted alkyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted aminoalkoxy, optionally substituted alkoxyalkoxy, or absent;

[000328] each of Y^1, Y^2, and Y^3 is, independently, O, S, Se, $-NR^{N1}-$, optionally substituted alkylene, or optionally substituted heteroalkylene, wherein R^{N1} is H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, or optionally substituted aryl;

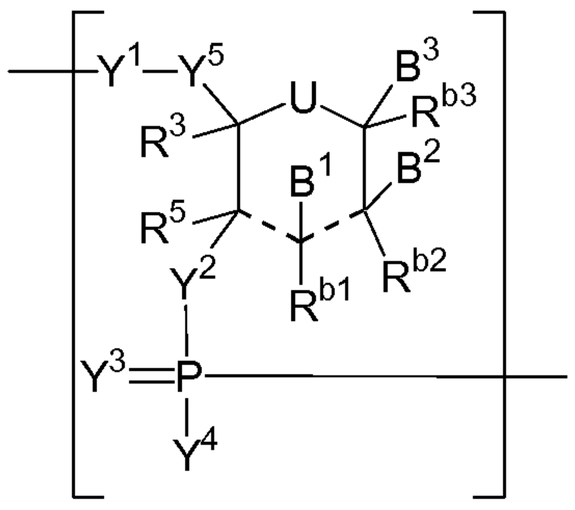
[000329] each Y^4 is, independently, H, hydroxy, thiol, boranyl, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted

alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted alkoxyalkoxy, or optionally substituted amino;

[000330] n is an integer from 1 to 100,000; and

[000331] B is a nucleobase.

[000332] In some embodiments, the polynucleotide, primary construct, or mmRNA (e.g., the first region, first flanking region, or second flanking region) includes n number of linked nucleosides having Formula (Ic):



(Ic), or a pharmaceutically acceptable salt or stereoisomer thereof,

[000333] wherein

[000334] U is O, S, $N(R^U)_{nu}$, or $C(R^U)_{nu}$, wherein nu is an integer from 0 to 2 and each R^U is, independently, H, halo, or optionally substituted alkyl;

[000335] --- is a single bond or absent;

[000336] each of B^1, B^2, and B^3 is, independently, a nucleobase (e.g., a purine, a pyrimidine, or derivatives thereof, as described herein), H, halo, hydroxy, thiol, optionally substituted alkyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted aminoalkoxy, optionally substituted alkoxyalkoxy, optionally substituted hydroxyalkoxy, optionally substituted amino, azido, optionally substituted aryl, optionally substituted aminoalkyl, optionally substituted aminoalkenyl, or optionally substituted aminoalkynyl, wherein one and only one of B^1, B^2, and B^3 is a nucleobase;

[000337] each of R^{b1}, R^{b2}, R^{b3}, R^3, and R^5 is, independently, H, halo, hydroxy, thiol, optionally substituted alkyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted aminoalkoxy, optionally substituted alkoxyalkoxy, optionally substituted hydroxyalkoxy, optionally

substituted amino, azido, optionally substituted aryl, optionally substituted aminoalkyl, optionally substituted aminoalkenyl or optionally substituted aminoalkynyl;

[000338] each Y^1, Y^2, and Y^3, is, independently, O, S, Se, $-NR^{N1}-$, optionally substituted alkylene, or optionally substituted heteroalkylene, wherein R^{N1} is H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, or optionally substituted aryl;

[000339] each Y^4 is, independently, H, hydroxy, thiol, boranyl, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted thioalkoxy, optionally substituted alkoxyalkoxy, or optionally substituted amino;

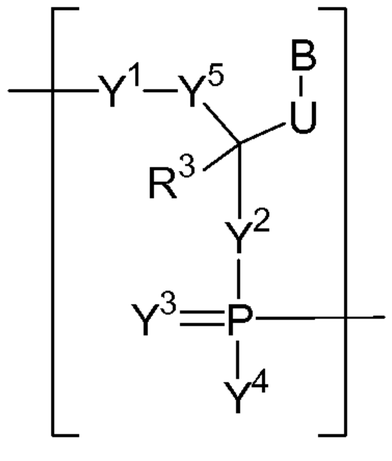
[000340] each Y^5 is, independently, O, S, Se, optionally substituted alkylene (e.g., methylene), or optionally substituted heteroalkylene;

[000341] n is an integer from 1 to 100,000; and

[000342] wherein the ring including U can include one or more double bonds.

[000343] In particular embodiments, the ring including U does not have a double bond between $U\text{-}CB^3R^{b3}$ or between $CB^3R^{b3}\text{-}C^{B2}R^{b2}$.

[000344] In some embodiments, the polynucleotide, primary construct, or mmRNA (e.g., the first region, first flanking region, or second flanking region) includes n number of linked nucleosides having Formula (Id):



(Id), or a pharmaceutically acceptable salt or stereoisomer thereof,

[000345] wherein

[000346] U is O, S, $N(R^U)_{nu}$, or $C(R^U)_{nu}$, wherein nu is an integer from 0 to 2 and each R^U is, independently, H, halo, or optionally substituted alkyl;

[000347] each R^3 is, independently, H, halo, hydroxy, thiol, optionally substituted alkyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted aminoalkoxy, optionally substituted alkoxyalkoxy,

optionally substituted hydroxyalkoxy, optionally substituted amino, azido, optionally substituted aryl, optionally substituted aminoalkyl, optionally substituted aminoalkenyl, or optionally substituted aminoalkynyl;

[000348] each Y^1, Y^2, and Y^3, is, independently, O, S, Se, -NR^{N1}-, optionally substituted alkylene, or optionally substituted heteroalkylene, wherein R^{N1} is H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, or optionally substituted aryl;

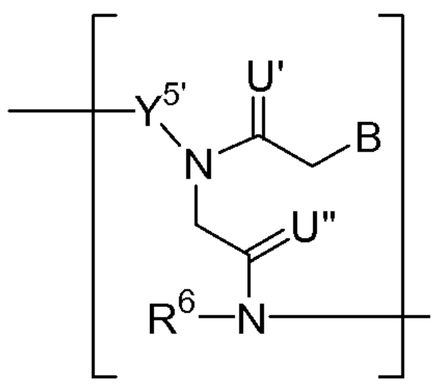
[000349] each Y^4 is, independently, H, hydroxy, thiol, boranyl, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted thioalkoxy, optionally substituted alkoxyalkoxy, or optionally substituted amino;

[000350] each Y^5 is, independently, O, S, optionally substituted alkylene (e.g., methylene), or optionally substituted heteroalkylene;

[000351] n is an integer from 1 to 100,000; and

[000352] B is a nucleobase (e.g., a purine, a pyrimidine, or derivatives thereof).

[000353] In some embodiments, the polynucleotide, primary construct, or mmRNA (e.g., the first region, first flanking region, or second flanking region) includes n number of linked nucleosides having Formula (Ie):



(Ie), or a pharmaceutically acceptable salt or stereoisomer thereof,

[000354] wherein

[000355] each of U' and U" is, independently, O, S, $N(R^U)_{nu}$, or $C(R^U)_{nu}$, wherein nu is an integer from 0 to 2 and each R^U is, independently, H, halo, or optionally substituted alkyl;

[000356] each R^6 is, independently, H, halo, hydroxy, thiol, optionally substituted alkyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted aminoalkoxy, optionally substituted alkoxyalkoxy,

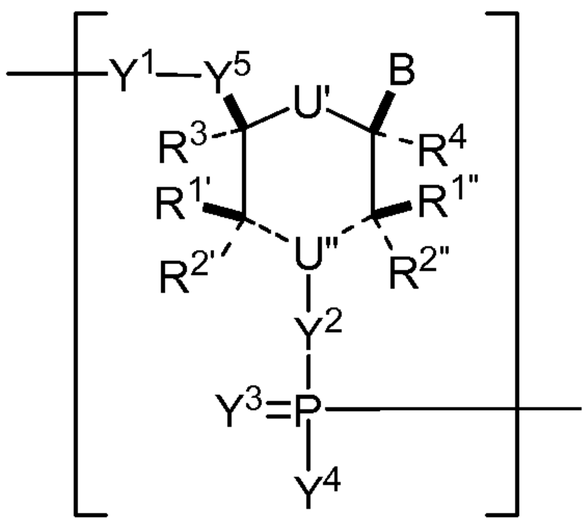
optionally substituted hydroxyalkoxy, optionally substituted amino, azido, optionally substituted aryl, optionally substituted aminoalkyl, optionally substituted aminoalkenyl, or optionally substituted aminoalkynyl;

[000357] each $Y^{5'}$ is, independently, O, S, optionally substituted alkylene (e.g., methylene or ethylene), or optionally substituted heteroalkylene;

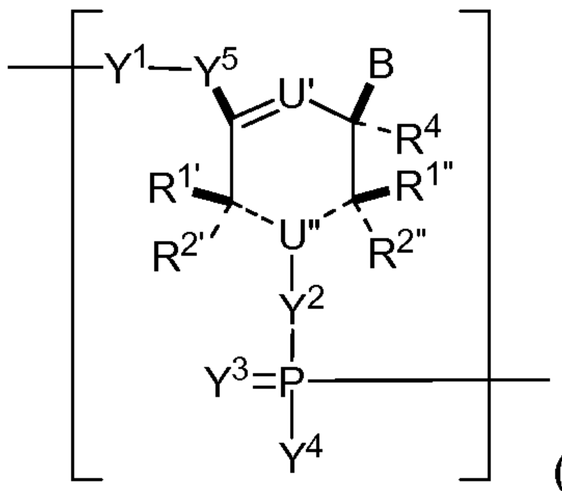
[000358] n is an integer from 1 to 100,000; and

[000359] B is a nucleobase (e.g., a purine, a pyrimidine, or derivatives thereof).

[000360] In some embodiments, the polynucleotide, primary construct, or mmRNA (e.g., the first region, first flanking region, or second flanking region) includes n number of linked nucleosides having Formula (If) or (If-1):



(If),



(If-1), or a pharmaceutically acceptable salt or stereoisomer thereof,

[000361] wherein

[000362] each of U' and U" is, independently, O, S, N, $N(R^U)_{nu}$, or $C(R^U)_{nu}$, wherein nu is an integer from 0 to 2 and each R^U is, independently, H, halo, or optionally substituted alkyl (e.g., U' is O and U" is N);

[000363] --- is a single bond or absent;

[000364] each of $R^{1'}$, $R^{2'}$, $R^{1"}$, $R^{2"}$, R^3, and R^4 is, independently, H, halo, hydroxy, thiol, optionally substituted alkyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted aminoalkoxy, optionally substituted alkoxyalkoxy, optionally substituted hydroxyalkoxy, optionally substituted amino, azido, optionally substituted aryl, optionally substituted aminoalkyl, optionally substituted aminoalkenyl, optionally substituted aminoalkynyl, or absent; and wherein the combination of $R^{1'}$ and R^3, the combination of $R^{1"}$ and R^3, the combination of $R^{2'}$ and R^3, or the combination of $R^{2"}$ and R^3 can be taken together to form optionally substituted alkylene or optionally substituted heteroalkylene (e.g., to produce a locked

nucleic acid);each of m' and m" is, independently, an integer from 0 to 3 (e.g., from 0 to 2, from 0 to 1, from 1 to 3, or from 1 to 2);

[000365] each of Y^1, Y^2, and Y^3, is, independently, O, S, Se, -NR^{N1}-, optionally substituted alkylene, or optionally substituted heteroalkylene, wherein R^{N1} is H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted aryl, or absent;

[000366] each Y^4 is, independently, H, hydroxy, thiol, boranyl, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted thioalkoxy, optionally substituted alkoxyalkoxy, or optionally substituted amino;

[000367] each Y^5 is, independently, O, S, Se, optionally substituted alkylene (e.g., methylene), or optionally substituted heteroalkylene;

[000368] n is an integer from 1 to 100,000; and

[000369] B is a nucleobase (e.g., a purine, a pyrimidine, or derivatives thereof).

[000370] In some embodiments of the polynucleotides, primary constructs, or mmRNA (e.g., Formulas (Ia), (Ia-1)-(Ia-3), (Ib)-(If), and (IIa)-(IIp)), the ring including U has one or two double bonds.

[000371] In some embodiments of the polynucleotides, primary constructs, or mmRNA (e.g., Formulas (Ia)-(Ia-5), (Ib)-(If-1), (IIa)-(IIp), (IIb-1), (IIb-2), (IIc-1)-(IIc-2), (IIn-1), (IIn-2), (IVa)-(IVl), and (IXa)-(IXr)), each of R^1, $R^{1'}$, and $R^{1"}$, if present, is H. In further embodiments, each of R^2, $R^{2'}$, and $R^{2"}$, if present, is, independently, H, halo (e.g., fluoro), hydroxy, optionally substituted alkoxy (e.g., methoxy or ethoxy), or optionally substituted alkoxyalkoxy. In particular embodiments, alkoxyalkoxy is -$(CH_2)_{s2}(OCH_2CH_2)_{s1}(CH_2)_{s3}OR'$, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R' is H or C_{1-20} alkyl). In some embodiments, s2 is 0, s1 is 1 or 2, s3 is 0 or 1, and R' is C_{1-6} alkyl.

[000372] In some embodiments of the polynucleotides, primary constructs, or mmRNA (e.g., Formulas (Ia)-(Ia-5), (Ib)-(If-1), (IIa)-(IIp), (IIb-1), (IIb-2), (IIc-1)-(IIc-2), (IIn-1), (IIn-2), (IVa)-(IVl), and (IXa)-(IXr)), each of R^2, $R^{2'}$, and $R^{2"}$, if present, is H. In further

embodiments, each of R^1, $R^{1'}$, and $R^{1''}$, if present, is, independently, H, halo (e.g., fluoro), hydroxy, optionally substituted alkoxy (e.g., methoxy or ethoxy), or optionally substituted alkoxyalkoxy. In particular embodiments, alkoxyalkoxy is -$(CH_2)_{s2}(OCH_2CH_2)_{s1}(CH_2)_{s3}OR'$, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R' is H or C_{1-20} alkyl). In some embodiments, s2 is 0, s1 is 1 or 2, s3 is 0 or 1, and R' is C_{1-6} alkyl.

[000373] In some embodiments of the polynucleotides, primary constructs, or mmRNA (e.g., Formulas (Ia)-(Ia-5), (Ib)-(If-1), (IIa)-(IIp), (IIb-1), (IIb-2), (IIc-1)-(IIc-2), (IIn-1), (IIn-2), (IVa)-(IVl), and (IXa)-(IXr)), each of R^3, R^4, and R^5 is, independently, H, halo (e.g., fluoro), hydroxy, optionally substituted alkyl, optionally substituted alkoxy (e.g., methoxy or ethoxy), or optionally substituted alkoxyalkoxy. In particular embodiments, R^3 is H, R^4 is H, R^5 is H, or R^3, R^4, and R^5 are all H. In particular embodiments, R^3 is C_{1-6} alkyl, R^4 is C_{1-6} alkyl, R^5 is C_{1-6} alkyl, or R^3, R^4, and R^5 are all C_{1-6} alkyl. In particular embodiments, R^3 and R^4 are both H, and R^5 is C_{1-6} alkyl.

[000374] In some embodiments of the polynucleotides, primary constructs, or mmRNA (e.g., Formulas (Ia)-(Ia-5), (Ib)-(If-1), (IIa)-(IIp), (IIb-1), (IIb-2), (IIc-1)-(IIc-2), (IIn-1), (IIn-2), (IVa)-(IVl), and (IXa)-(IXr)), R^3 and R^5 join together to form optionally substituted alkylene or optionally substituted heteroalkylene and, taken together with the carbons to which they are attached, provide an optionally substituted heterocyclyl (e.g., a bicyclic, tricyclic, or tetracyclic heterocyclyl, such as trans-3′,4' analogs, wherein R^3 and R^5 join together to form heteroalkylene (e.g., -$(CH_2)_{b1}O(CH_2)_{b2}O(CH_2)_{b3}$-, wherein each of b1, b2, and b3 are, independently, an integer from 0 to 3).

[000375] In some embodiments of the polynucleotides, primary constructs, or mmRNA (e.g., Formulas (Ia)-(Ia-5), (Ib)-(If-1), (IIa)-(IIp), (IIb-1), (IIb-2), (IIc-1)-(IIc-2), (IIn-1), (IIn-2), (IVa)-(IVl), and (IXa)-(IXr)), R^3 and one or more of $R^{1'}$, $R^{1''}$, $R^{2'}$, $R^{2''}$, or R^5 join together to form optionally substituted alkylene or optionally substituted heteroalkylene and, taken together with the carbons to which they are attached, provide an optionally substituted heterocyclyl (e.g., a bicyclic, tricyclic, or tetracyclic heterocyclyl, R^3 and one or more of $R^{1'}$, $R^{1''}$, $R^{2'}$, $R^{2''}$, or R^5 join together to form heteroalkylene (e.g., -

$(CH_2)_{b1}O(CH_2)_{b2}O(CH_2)_{b3}$-, wherein each of b1, b2, and b3 are, independently, an integer from 0 to 3).

[000376] In some embodiments of the polynucleotides, primary constructs, or mmRNA (e.g., Formulas (Ia)-(Ia-5), (Ib)-(If-1), (IIa)-(IIp), (IIb-1), (IIb-2), (IIc-1)-(IIc-2), (IIn-1), (IIn-2), (IVa)-(IVl), and (IXa)-(IXr)), R^5 and one or more of $R^{1'}$, $R^{1''}$, $R^{2'}$, or $R^{2''}$ join together to form optionally substituted alkylene or optionally substituted heteroalkylene and, taken together with the carbons to which they are attached, provide an optionally substituted heterocyclyl (e.g., a bicyclic, tricyclic, or tetracyclic heterocyclyl, R^5 and one or more of $R^{1'}$, $R^{1''}$, $R^{2'}$, or $R^{2''}$ join together to form heteroalkylene (e.g., -$(CH_2)_{b1}O(CH_2)_{b2}O(CH_2)_{b3}$-, wherein each of b1, b2, and b3 are, independently, an integer from 0 to 3).

[000377] In some embodiments of the polynucleotides, primary constructs, or mmRNA (e.g., Formulas (Ia)-(Ia-5), (Ib)-(If-1), (IIa)-(IIp), (IIb-1), (IIb-2), (IIc-1)-(IIc-2), (IIn-1), (IIn-2), (IVa)-(IVl), and (IXa)-(IXr)), each Y^2 is, independently, O, S, or -NR^{N1}-, wherein R^{N1} is H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, or optionally substituted aryl. In particular embodiments, Y^2 is NR^{N1}-, wherein R^{N1} is H or optionally substituted alkyl (e.g., C_{1-6} alkyl, such as methyl, ethyl, isopropyl, or n-propyl).

[000378] In some embodiments of the polynucleotides, primary constructs, or mmRNA (e.g., Formulas (Ia)-(Ia-5), (Ib)-(If-1), (IIa)-(IIp), (IIb-1), (IIb-2), (IIc-1)-(IIc-2), (IIn-1), (IIn-2), (IVa)-(IVl), and (IXa)-(IXr)), each Y^3 is, independently, O or S.

[000379] In some embodiments of the polynucleotides, primary constructs, or mmRNA (e.g., Formulas (Ia)-(Ia-5), (Ib)-(If-1), (IIa)-(IIp), (IIb-1), (IIb-2), (IIc-1)-(IIc-2), (IIn-1), (IIn-2), (IVa)-(IVl), and (IXa)-(IXr)), R^1 is H; each R^2 is, independently, H, halo (e.g., fluoro), hydroxy, optionally substituted alkoxy (e.g., methoxy or ethoxy), or optionally substituted alkoxyalkoxy (e.g., -$(CH_2)_{s2}(OCH_2CH_2)_{s1}(CH_2)_{s3}$OR', wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R' is H or C_{1-20} alkyl, such as wherein s2 is 0, s1 is 1 or 2, s3 is 0 or 1, and R' is C_{1-6} alkyl); each Y^2 is, independently, O or -NR^{N1}-, wherein R^{N1} is H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, or

optionally substituted aryl (e.g., wherein R^{N1} is H or optionally substituted alkyl (e.g., C_{1-6} alkyl, such as methyl, ethyl, isopropyl, or n-propyl)); and each Y^3 is, independently, O or S (e.g., S). In further embodiments, R^3 is H, halo (e.g., fluoro), hydroxy, optionally substituted alkyl, optionally substituted alkoxy (e.g., methoxy or ethoxy), or optionally substituted alkoxyalkoxy. In yet further embodiments, each Y^1 is , independently, O or -NR^{N1}-, wherein R^{N1} is H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, or optionally substituted aryl (e.g., wherein R^{N1} is H or optionally substituted alkyl (e.g., C_{1-6} alkyl, such as methyl, ethyl, isopropyl, or n-propyl)); and each Y^4 is, independently, H, hydroxy, thiol, optionally substituted alkyl, optionally substituted alkoxy, optionally substituted thioalkoxy, optionally substituted alkoxyalkoxy, or optionally substituted amino.

[000380] In some embodiments of the polynucleotides, primary constructs, or mmRNA (e.g., Formulas (Ia)-(Ia-5), (Ib)-(If-1), (IIa)-(IIp), (IIb-1), (IIb-2), (IIc-1)-(IIc-2), (IIn-1), (IIn-2), (IVa)-(IVl), and (IXa)-(IXr)), each R^1 is, independently, H, halo (e.g., fluoro), hydroxy, optionally substituted alkoxy (e.g., methoxy or ethoxy), or optionally substituted alkoxyalkoxy (e.g., $-(CH_2)_{s2}(OCH_2CH_2)_{s1}(CH_2)_{s3}OR'$, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R' is H or C_{1-20} alkyl, such as wherein s2 is 0, s1 is 1 or 2, s3 is 0 or 1, and R' is C_{1-6} alkyl); R^2 is H; each Y^2 is, independently, O or -NR^{N1}-, wherein R^{N1} is H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, or optionally substituted aryl (e.g., wherein R^{N1} is H or optionally substituted alkyl (e.g., C_{1-6} alkyl, such as methyl, ethyl, isopropyl, or n-propyl)); and each Y^3 is, independently, O or S (e.g., S). In further embodiments, R^3 is H, halo (e.g., fluoro), hydroxy, optionally substituted alkyl, optionally substituted alkoxy (e.g., methoxy or ethoxy), or optionally substituted alkoxyalkoxy. In yet further embodiments, each Y^1 is , independently, O or -NR^{N1}-, wherein R^{N1} is H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, or optionally substituted aryl (e.g., wherein R^{N1} is H or optionally substituted alkyl (e.g., C_{1-6} alkyl, such as methyl, ethyl, isopropyl, or n-propyl)); and each Y^4 is, independently, H, hydroxy, thiol, optionally substituted alkyl,

optionally substituted alkoxy, optionally substituted thioalkoxy, optionally substituted alkoxyalkoxy, or optionally substituted amino.

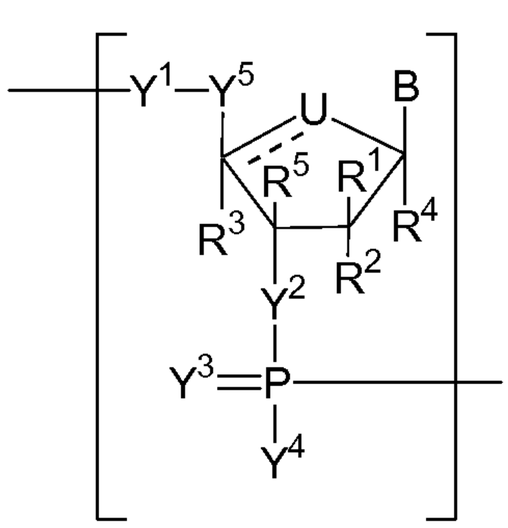
[000381] In some embodiments of the polynucleotides, primary constructs, or mmRNA (e.g., Formulas (Ia)-(Ia-5), (Ib)-(If-1), (IIa)-(IIp), (IIb-1), (IIb-2), (IIc-1)-(IIc-2), (IIn-1), (IIn-2), (IVa)-(IVl), and (IXa)-(IXr)), the ring including U is in the β-D (e.g., β-D-ribo) configuration.

[000382] In some embodiments of the polynucleotides, primary constructs, or mmRNA (e.g., Formulas (Ia)-(Ia-5), (Ib)-(If-1), (IIa)-(IIp), (IIb-1), (IIb-2), (IIc-1)-(IIc-2), (IIn-1), (IIn-2), (IVa)-(IVl), and (IXa)-(IXr)), the ring including U is in the α-L (e.g., α-L-ribo) configuration.

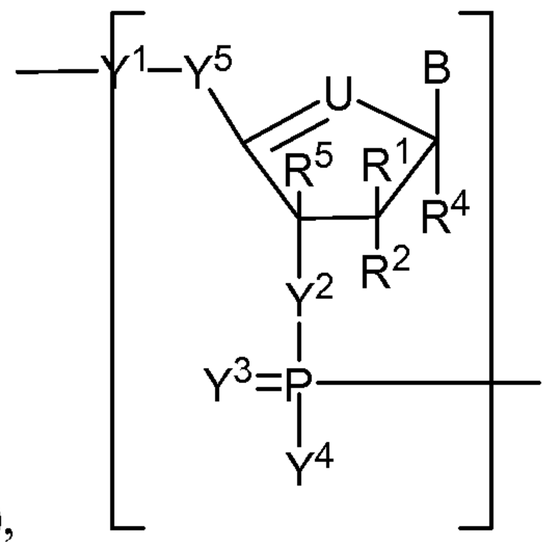
[000383] In some embodiments of the polynucleotides, primary constructs, or mmRNA (e.g., Formulas (Ia)-(Ia-5), (Ib)-(If-1), (IIa)-(IIp), (IIb-1), (IIb-2), (IIc-1)-(IIc-2), (IIn-1), (IIn-2), (IVa)-(IVl), and (IXa)-(IXr)), one or more B is not pseudouridine (ψ) or 5-methyl-cytidine (m^5C). In some embodiments, about 10% to about 100% of n number of B nucleobases is not ψ or m^5C (e.g., from 10% to 20%, from 10% to 35%, from 10% to 50%, from 10% to 60%, from 10% to 75%, from 10% to 90%, from 10% to 95%, from 10% to 98%, from 10% to 99%, from 20% to 35%, from 20% to 50%, from 20% to 60%, from 20% to 75%, from 20% to 90%, from 20% to 95%, from 20% to 98%, from 20% to 99%, from 20% to 100%, from 50% to 60%, from 50% to 75%, from 50% to 90%, from 50% to 95%, from 50% to 98%, from 50% to 99%, from 50% to 100%, from 75% to 90%, from 75% to 95%, from 75% to 98%, from 75% to 99%, and from 75% to 100% of n number of B is not ψ or m^5C). In some embodiments, B is not ψ or m^5C.

[000384] In some embodiments of the polynucleotides, primary constructs, or mmRNA (e.g., Formulas (Ia)-(Ia-5), (Ib)-(If-1), (IIa)-(IIp), (IIb-1), (IIb-2), (IIc-1)-(IIc-2), (IIn-1), (IIn-2), (IVa)-(IVl), and (IXa)-(IXr)), when B is an unmodified nucleobase selected from cytosine, guanine, uracil and adenine, then at least one of Y^1, Y^2, or Y^3 is not O.

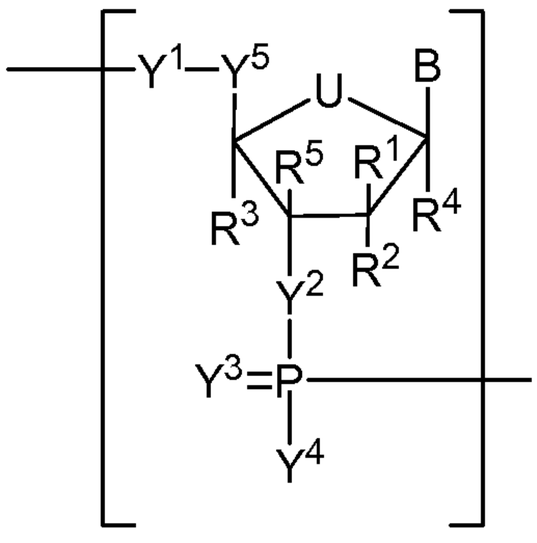
[000385] In some embodiments, the polynucleotide, primary construct, or mmRNA includes a modified ribose. In some embodiments, the polynucleotide, primary construct, or mmRNA (e.g., the first region, the first flanking region, or the second flanking region) includes n number of linked nucleosides having Formula (IIa)-(IIc):



(IIa),

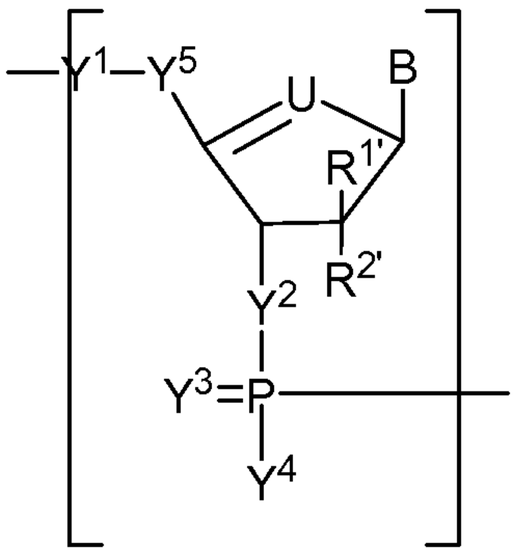


(IIb), or

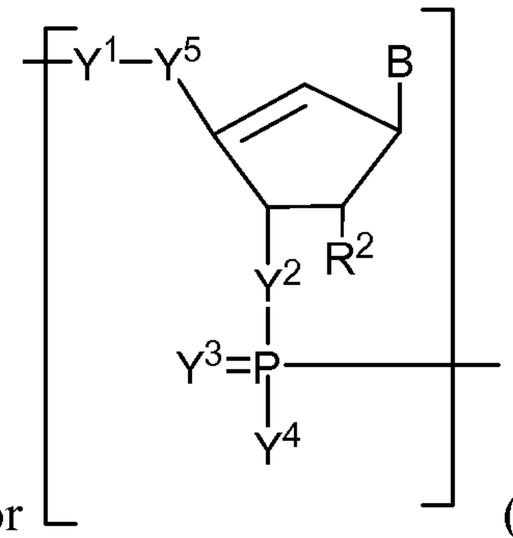


(IIc), or a pharmaceutically acceptable salt or stereoisomer thereof. In particular embodiments, U is O or $C(R^U)_{nu}$, wherein nu is an integer from 0 to 2 and each R^U is, independently, H, halo, or optionally substituted alkyl (e.g., U is $–CH_2–$ or –CH–). In other embodiments, each of R^1, R^2, R^3, R^4, and R^5 is, independently, H, halo, hydroxy, thiol, optionally substituted alkyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted aminoalkoxy, optionally substituted alkoxyalkoxy, optionally substituted hydroxyalkoxy, optionally substituted amino, azido, optionally substituted aryl, optionally substituted aminoalkyl, optionally substituted aminoalkenyl, optionally substituted aminoalkynyl, or absent (e.g., each R^1 and R^2 is, independently, H, halo, hydroxy, optionally substituted alkyl, or optionally substituted alkoxy; each R^3 and R^4 is, independently, H or optionally substituted alkyl; and R^5 is H or hydroxy), and ___ is a single bond or double bond.

[000386] In particular embodiments, the polynucleotidesor mmRNA includes n number of linked nucleosides having Formula (IIb-1)-(IIb-2):

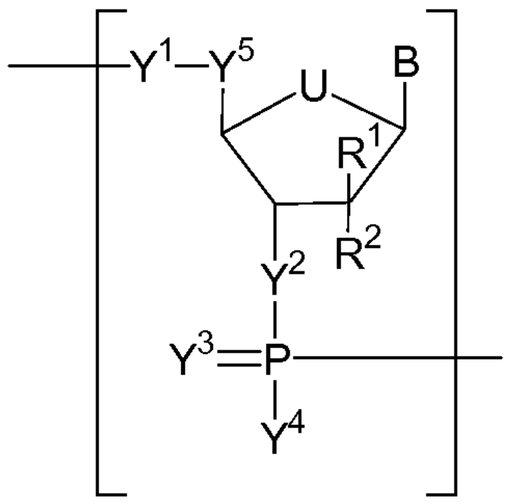


(IIb-1) or

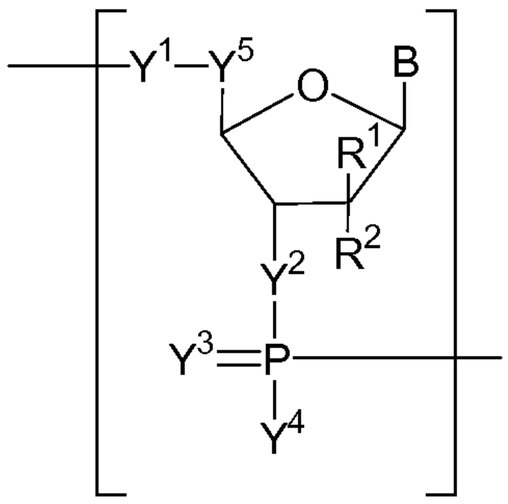


(IIb-2) or a pharmaceutically acceptable salt or stereoisomer thereof. In some embodiments, U is O or $C(R^U)_{nu}$, wherein nu is an integer from 0 to 2 and each R^U is, independently, H, halo, or optionally substituted alkyl (e.g., U is $–CH_2–$ or –CH–). In other embodiments, each of R^1 and R^2 is, independently, H, halo, hydroxy, thiol, optionally substituted alkyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted aminoalkoxy, optionally substituted alkoxyalkoxy, optionally substituted hydroxyalkoxy, optionally substituted amino, azido, optionally substituted aryl, optionally substituted aminoalkyl, optionally substituted aminoalkenyl, optionally substituted aminoalkynyl, or absent (e.g., each R^1 and R^2 is, independently, H, halo, hydroxy, optionally substituted alkyl, or optionally substituted alkoxy, e.g., H, halo, hydroxy, alkyl, or alkoxy). In particular embodiments, R^2 is hydroxy or optionally substituted alkoxy (e.g., methoxy, ethoxy, or any described herein).

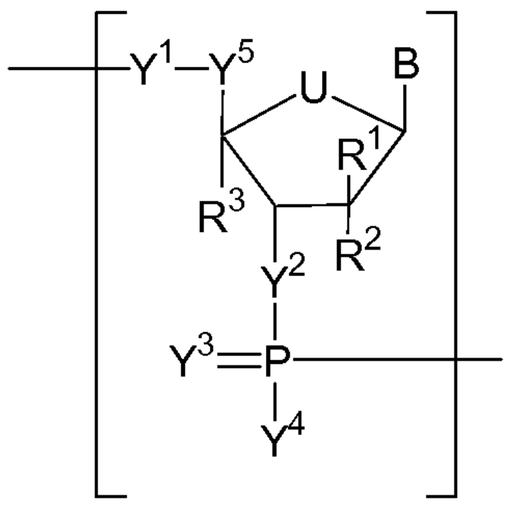
[000387] In particular embodiments, the polynucleotide, primary construct, or mmRNA includes n number of linked nucleosides having Formula (IIc-1)-(IIc-4):



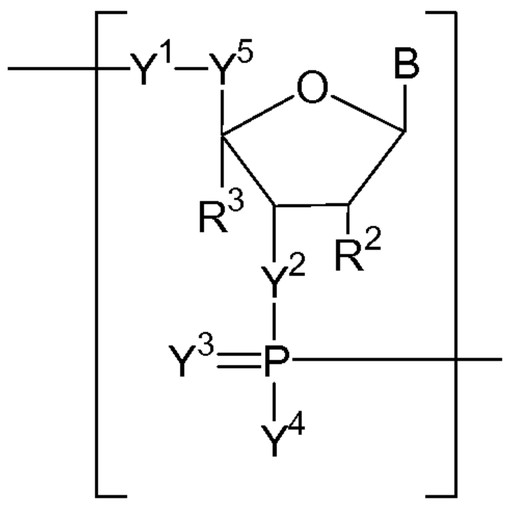
(IIc-1),



(IIc-2),

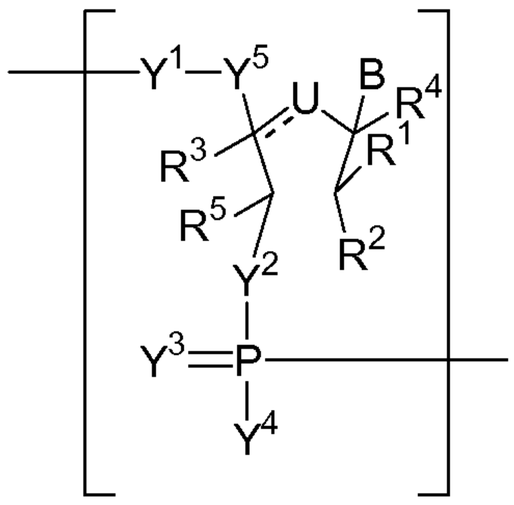


(IIc-3), or

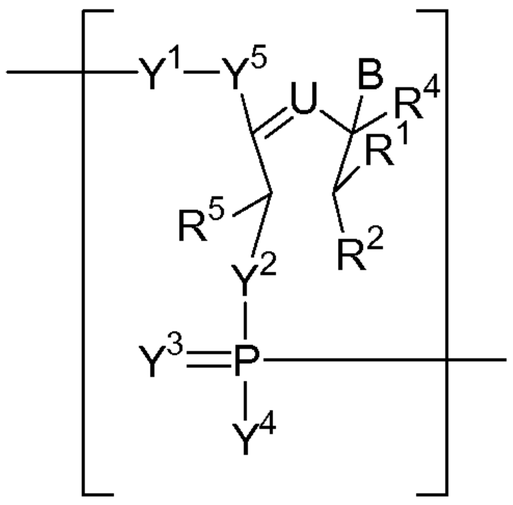


(IIc-4), or a pharmaceutically acceptable salt or stereoisomer thereof. In some embodiments, U is O or $C(R^U)_{nu}$, wherein nu is an integer from 0 to 2 and each R^U is, independently, H, halo, or optionally substituted alkyl (e.g., U is $–CH_2–$ or –CH–). In some embodiments, each of R^1, R^2, and R^3 is, independently, H, halo, hydroxy, thiol, optionally substituted alkyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted aminoalkoxy, optionally substituted alkoxyalkoxy, optionally substituted hydroxyalkoxy, optionally substituted amino, azido, optionally substituted aryl, optionally substituted aminoalkyl, optionally substituted aminoalkenyl, optionally substituted aminoalkynyl, or absent (e.g., each R^1 and R^2 is, independently, H, halo, hydroxy, optionally substituted alkyl, or optionally substituted alkoxy, e.g., H, halo, hydroxy, alkyl, or alkoxy; and each R^3 is, independently, H or optionally substituted alkyl)). In particular embodiments, R^2 is optionally substituted alkoxy (e.g., methoxy or ethoxy, or any described herein). In particular embodiments, R^1 is optionally substituted alkyl, and R^2 is hydroxy. In other embodiments, R^1 is hydroxy, and R^2 is optionally substituted alkyl. In further embodiments, R^3 is optionally substituted alkyl.

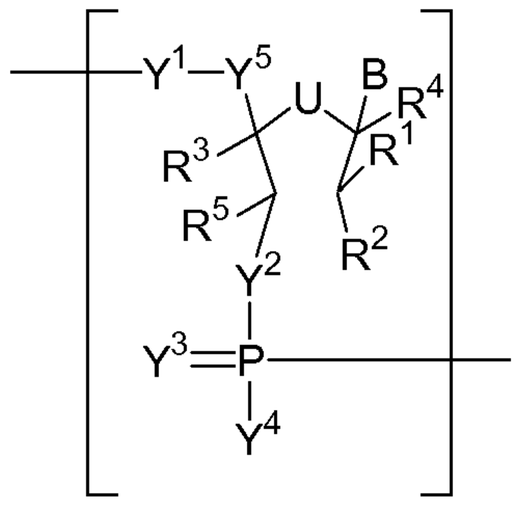
[000388] In some embodiments, the polynucleotide, primary construct, or mmRNA includes an acyclic modified ribose. In some embodiments, the polynucleotide, primary construct, or mmRNA (e.g., the first region, the first flanking region, or the second flanking region) includes n number of linked nucleosides having Formula (IId)-(IIf):



(IId),

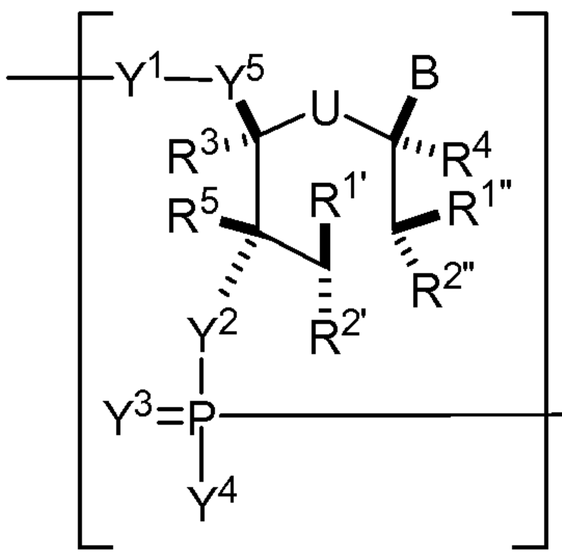


(IIe), or

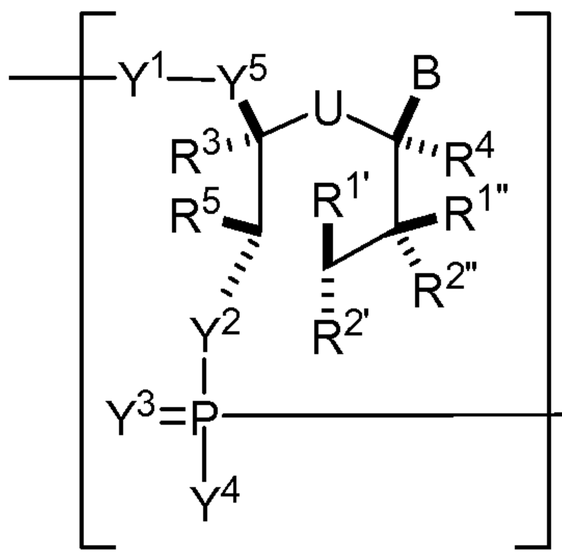


(IIf), or a pharmaceutically acceptable salt or stereoisomer thereof.

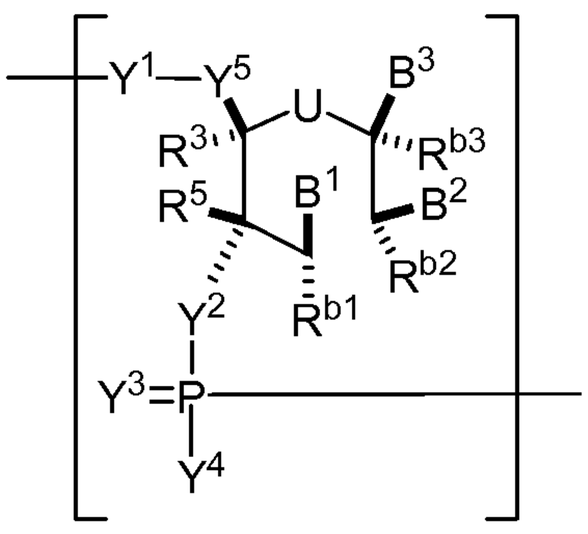
[000389] In some embodiments, the polynucleotide, primary construct, or mmRNA includes an acyclic modified hexitol. In some embodiments, the polynucleotide, primary construct, or mmRNA (e.g., the first region, the first flanking region, or the second flanking region) includes n number of linked nucleosides Formula (IIg)-(IIj):



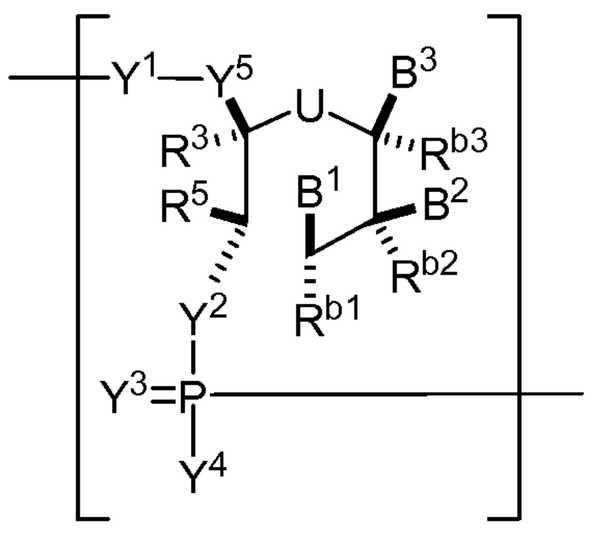
(IIg),



(IIh),



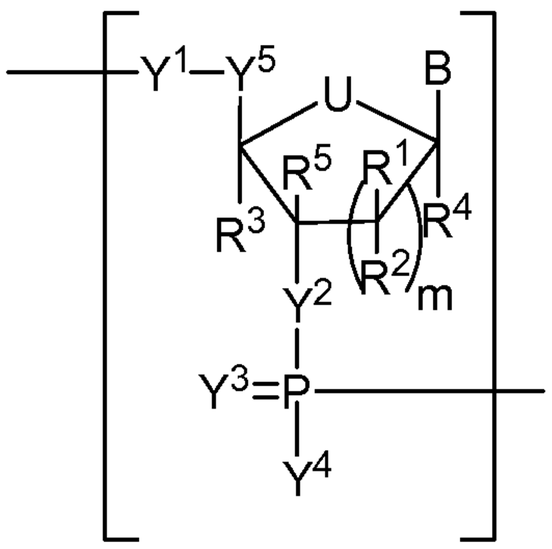
(IIi), or



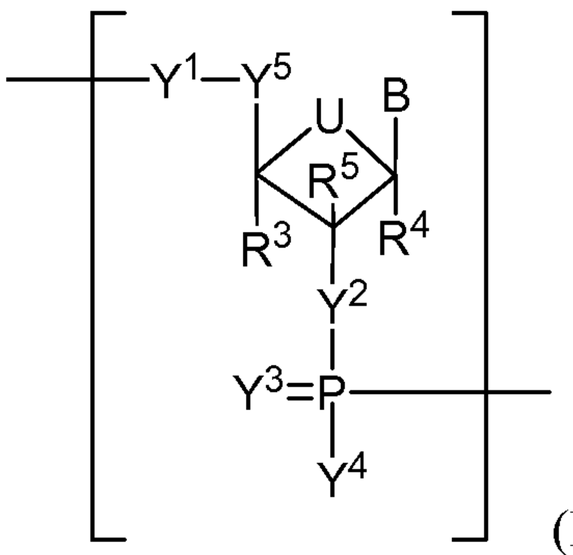
(IIj), or a pharmaceutically acceptable salt or stereoisomer thereof.

[000390] In some embodiments, the polynucleotide, primary construct, or mmRNA includes a sugar moiety having a contracted or an expanded ribose ring. In some embodiments, the polynucleotide, primary construct, or mmRNA (e.g., the first region, the first flanking region, or the second flanking region) includes n number of linked nucleosides having Formula

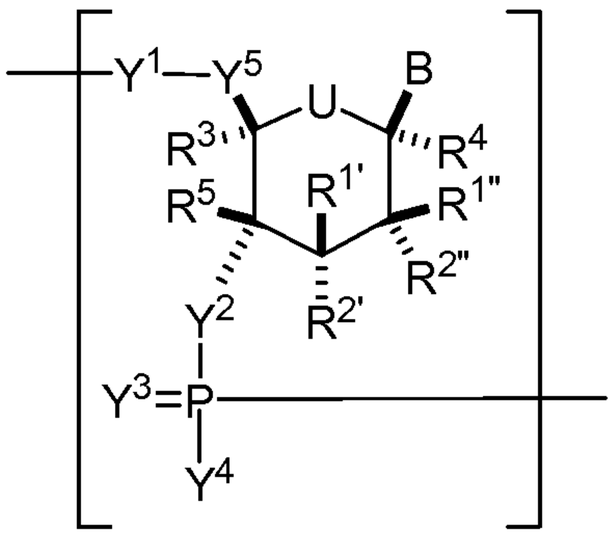
(IIk)-(IIm):



(IIk),

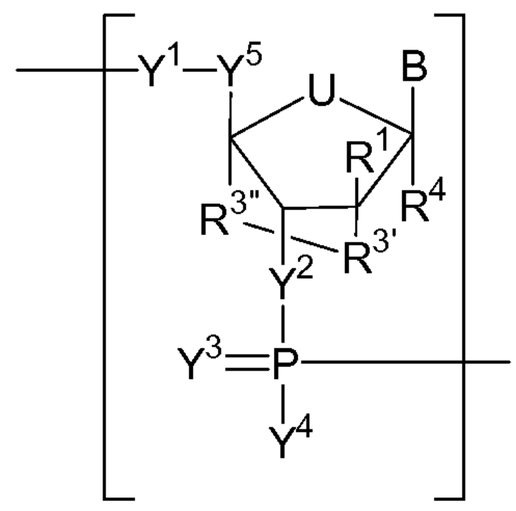


(IIl), or



(IIm),or a pharmaceutically acceptable salt or stereoisomer thereof, wherein each of $R^{1'}$, $R^{1''}$, $R^{2'}$, and $R^{2''}$ is, independently, H, halo, hydroxy, optionally substituted alkyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted aminoalkoxy, optionally substituted alkoxyalkoxy, or absent; and wherein the combination of $R^{2'}$ and R^3 or the combination of $R^{2''}$ and R^3 can be taken together to form optionally substituted alkylene or optionally substituted heteroalkylene.

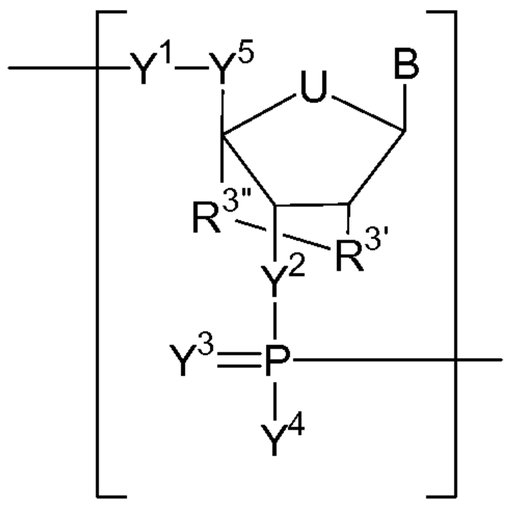
[000391] In some embodiments, the polynucleotide, primary construct, or mmRNA includes a locked modified ribose. In some embodiments, the polynucleotide, primary construct, or mmRNA (e.g., the first region, the first flanking region, or the second flanking region) includes n number of linked nucleosides having Formula (IIn):



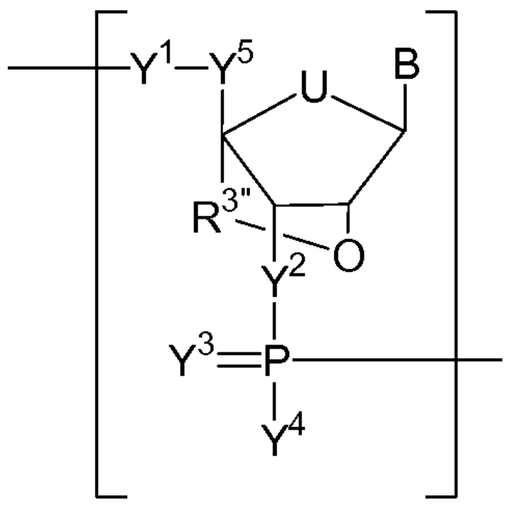
(IIn), or a pharmaceutically acceptable salt or stereoisomer thereof, wherein $R^{3'}$ is O, S, or $-NR^{N1}-$, wherein R^{N1} is H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, or optionally substituted

aryl and $R^{3"}$ is optionally substituted alkylene (e.g., $-CH_2-$, $-CH_2CH_2-$, or $-CH_2CH_2CH_2-$) or optionally substituted heteroalkylene (e.g., $-CH_2NH-$, $-CH_2CH_2NH-$, $-CH_2OCH_2-$, or $-CH_2CH_2OCH_2-$)(e.g., $R^{3'}$ is O and $R^{3"}$ is optionally substituted alkylene (e.g., $-CH_2-$, $-CH_2CH_2-$, or $-CH_2CH_2CH_2-$)).

[000392] In some embodiments, the polynucleotide, primary construct, or mmRNA includes n number of linked nucleosides having Formula (IIn-1)-(II-n2):

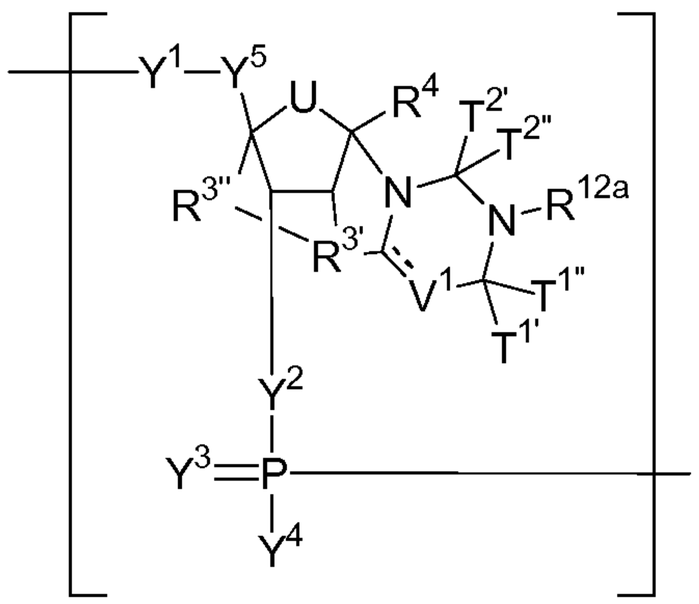


(IIn-1) or

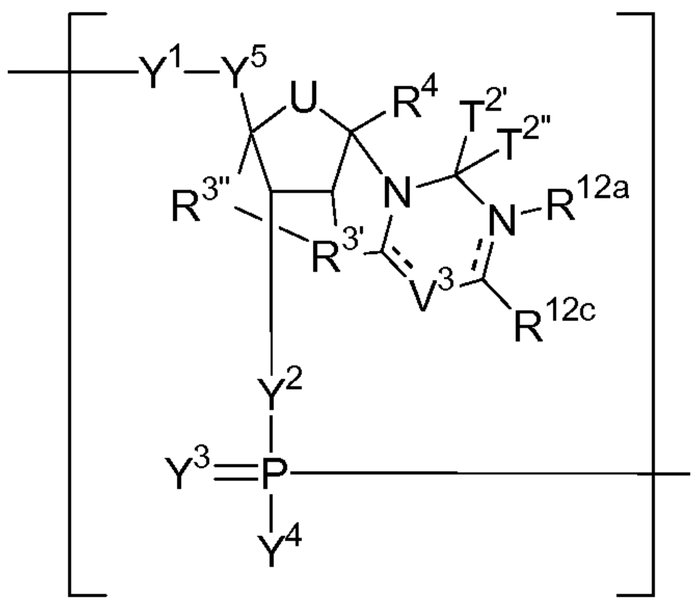


(IIn-2), or a pharmaceutically acceptable salt or stereoisomer thereof, wherein $R^{3'}$ is O, S, or $-NR^{N1}-$, wherein R^{N1} is H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, or optionally substituted aryl and $R^{3"}$ is optionally substituted alkylene (e.g., $-CH_2-$, $-CH_2CH_2-$, or $-CH_2CH_2CH_2-$) or optionally substituted heteroalkylene (e.g., $-CH_2NH-$, $-CH_2CH_2NH-$, $-CH_2OCH_2-$, or $-CH_2CH_2OCH_2-$) (e.g., $R^{3'}$ is O and $R^{3"}$ is optionally substituted alkylene (e.g., $-CH_2-$, $-CH_2CH_2-$, or $-CH_2CH_2CH_2-$)).

[000393] In some embodiments, the polynucleotide, primary construct, or mmRNA includes a locked modified ribose that forms a tetracyclic heterocyclyl. In some embodiments, the polynucleotide, primary construct, or mmRNA (e.g., the first region, the first flanking region, or the second flanking region) includes n number of linked nucleosides having Formula (IIo):



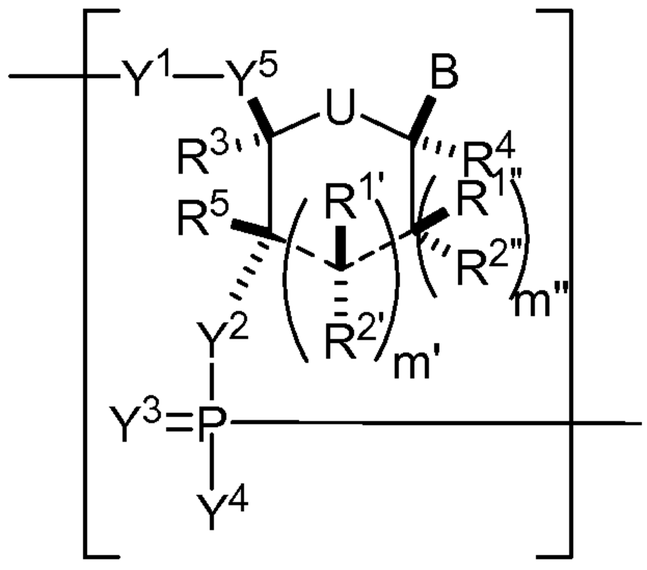
(IIo) or



(IIp), or a pharmaceutically acceptable salt or stereoisomer thereof, wherein R^{12a}, R^{12c}, $T^{1'}$, $T^{1''}$,$T^{2'}$, $T^{2''}$, V^1, and V^3 are as described herein.

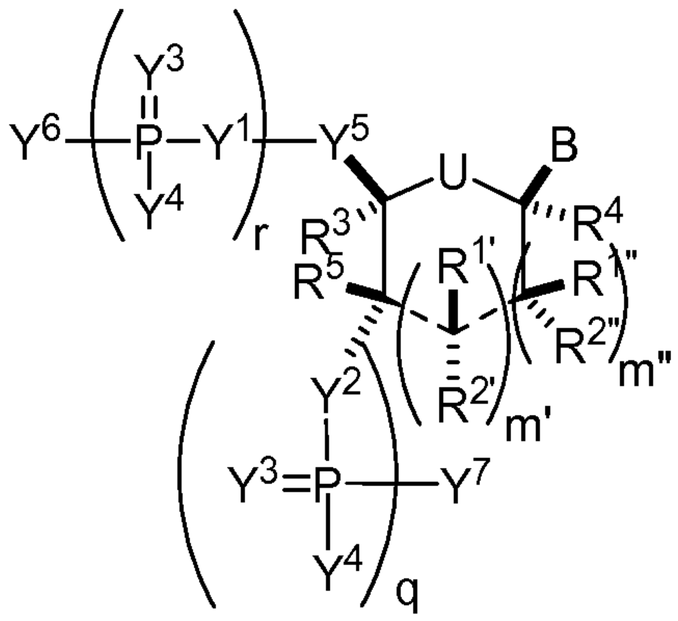
[000394] Any of the formulas for the polynucleotides, primary constructs, or mmRNA can include one or more nucleobases described herein (e.g., Formulas (b1)-(b43)).

[000395] In one embodiment, the present invention provides methods of preparing a polynucleotide, primary construct, or mmRNA, wherein the polynucleotide comprises n number of nucleosides having Formula (Ia), as defined herein:



(Ia),

the method comprising reacting a compound of Formula (IIIa), as defined herein:

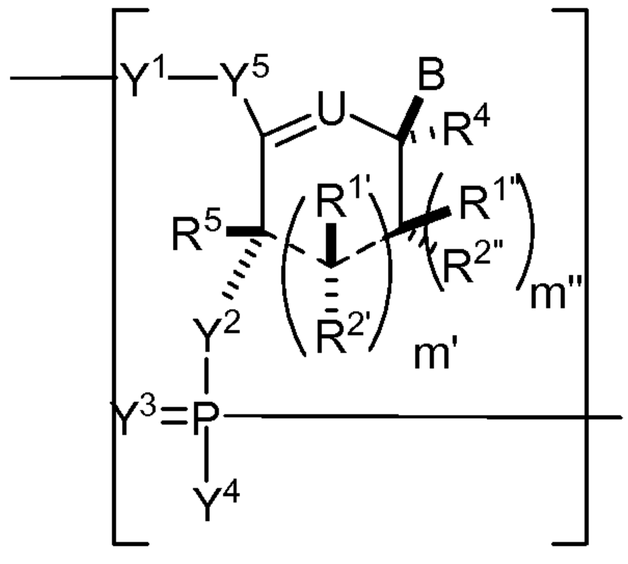


(IIIa),

with an RNA polymerase, and a cDNA template.

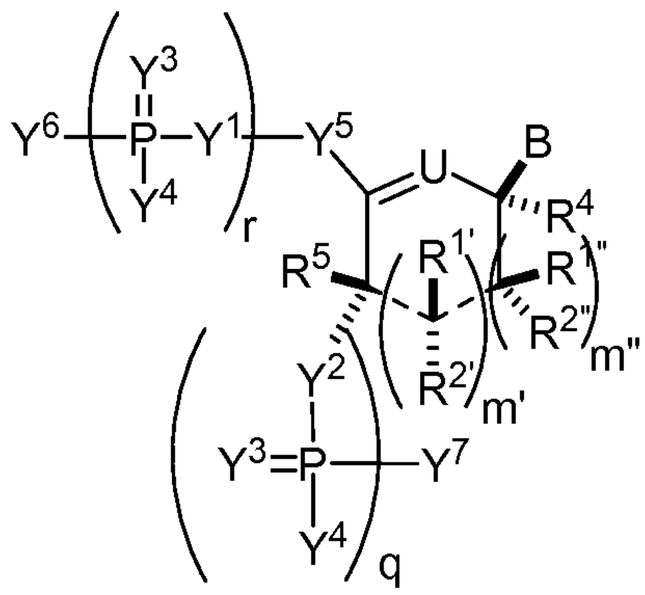
[000396] In a further embodiment, the present invention provides methods of amplifying a polynucleotide, primary construct, or mmRNA comprising at least one nucleotide (e.g., mmRNA molecule), the method comprising: reacting a compound of Formula (IIIa), as defined herein, with a primer, a cDNA template, and an RNA polymerase.

[000397] In one embodiment, the present invention provides methods of preparing a polynucleotide, primary construct, or mmRNA comprising at least one nucleotide (e.g., mmRNA molecule), wherein the polynucleotide comprises n number of nucleosides having Formula (Ia), as defined herein:



(Ia-1),

the method comprising reacting a compound of Formula (IIIa-1), as defined herein:



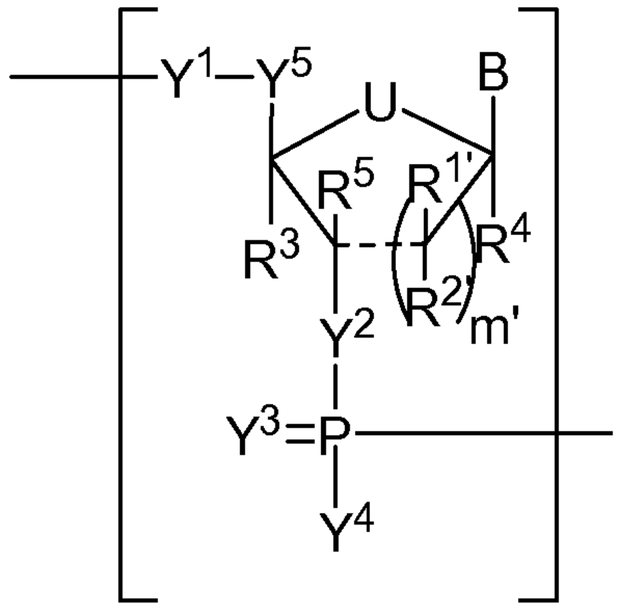
(IIIa-1),

with an RNA polymerase, and a cDNA template.

[000398] In a further embodiment, the present invention provides methods of amplifying a polynucleotide, primary construct, or mmRNA comprising at least one nucleotide (e.g., mmRNA molecule), the method comprising:

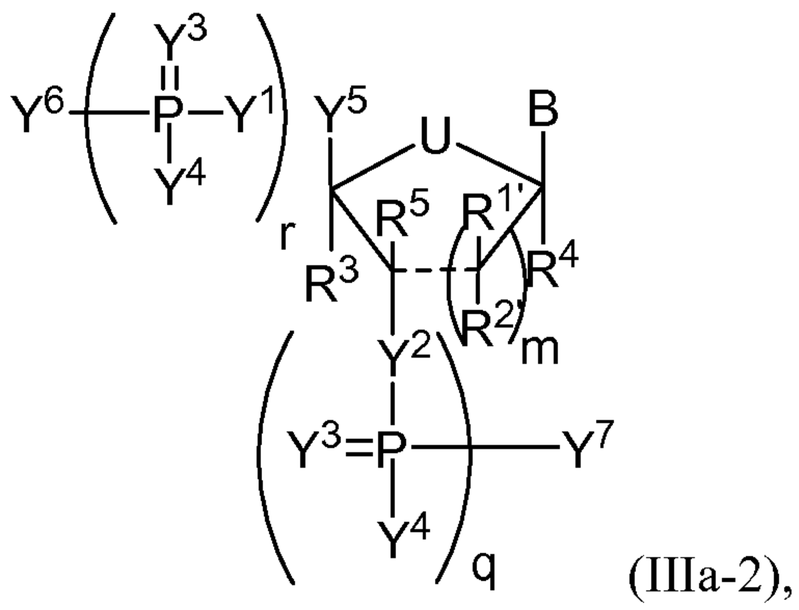
[000399] reacting a compound of Formula (IIIa-1), as defined herein, with a primer, a cDNA template, and an RNA polymerase.

[000400] In one embodiment, the present invention provides methods of preparing a modified mRNA comprising at least one nucleotide (e.g., mmRNA molecule), wherein the polynucleotide comprises n number of nucleosides having Formula (Ia-2), as defined herein:



(Ia-2),

the method comprising reacting a compound of Formula (IIIa-2), as defined herein:



(IIIa-2),

with an RNA polymerase, and a cDNA template.

[000401] In a further embodiment, the present invention provides methods of amplifying a modified mRNA comprising at least one nucleotide (e.g., mmRNA molecule), the method comprising:

[000402] reacting a compound of Formula (IIIa-2), as defined herein, with a primer, a cDNA template, and an RNA polymerase.

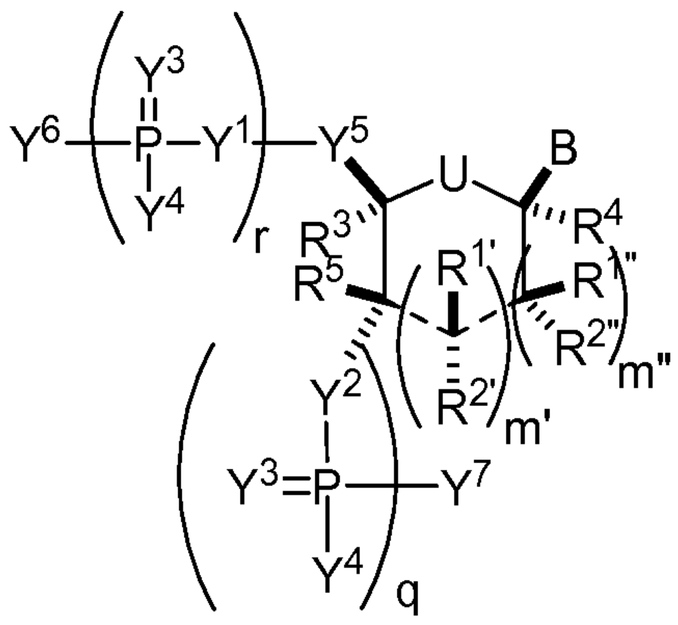
[000403] In some embodiments, the reaction may be repeated from 1 to about 7,000 times. In any of the embodiments herein, B may be a nucleobase of Formula (b1)-(b43).

[000404] The polynucleotides, primary constructs, and mmRNA can optionally include 5′ and/or 3′ flanking regions, which are described herein.

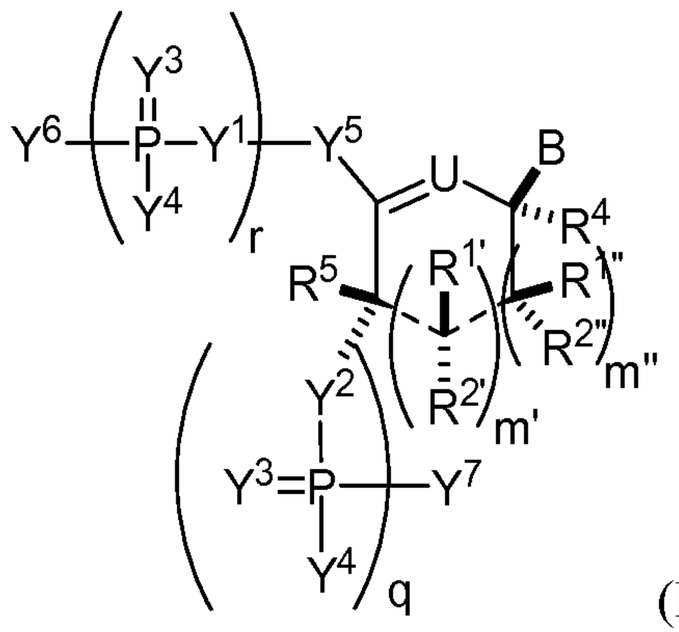
Modified RNA (mmRNA) Molecules

[000405] The present invention also includes building blocks, e.g., modified ribonucleosides, modified ribonucleotides, of modified RNA (mmRNA) molecules. For example, these building blocks can be useful for preparing the polynucleotides, primary constructs, or mmRNA of the invention.

In some embodiments, the building block molecule has Formula (IIIa) or (IIIa-1):

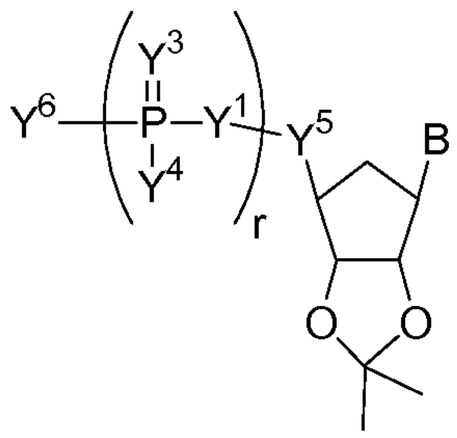


(IIIa),

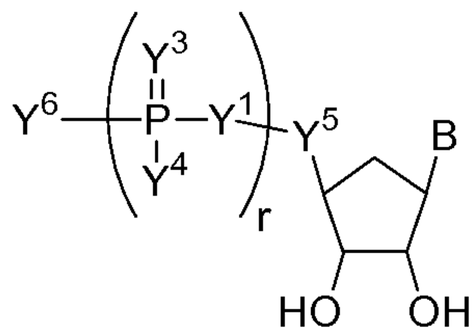


(IIIa-1) or a pharmaceutically acceptable salt or stereoisomer thereof, wherein the substituents are as described herein (e.g., for Formula (Ia) and (Ia-1)), and wherein when B is an unmodified nucleobase selected from cytosine, guanine, uracil and adenine, then at least one of Y^1, Y^2, or Y^3 is not O.

[000406] In some embodiments, the building block molecule, which may be incorporated into a polynucleotide, primary construct, or mmRNA, has Formula (IVa)-(IVb):



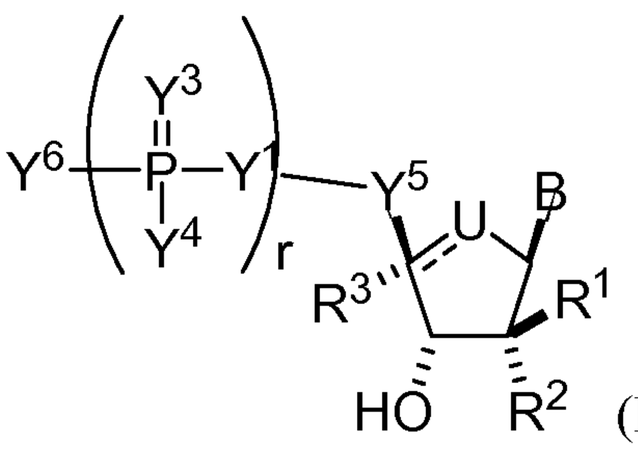
(IVa) or

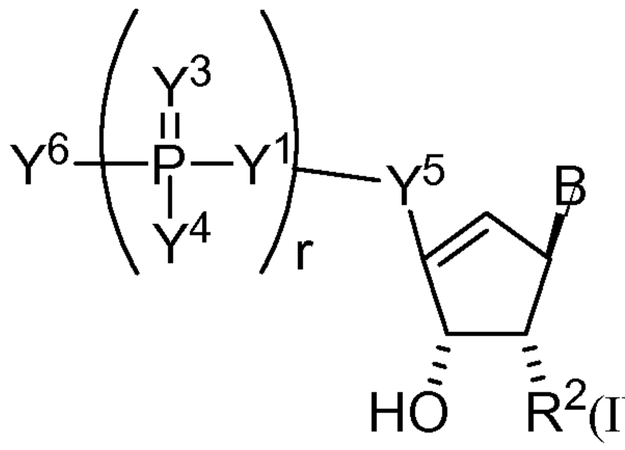


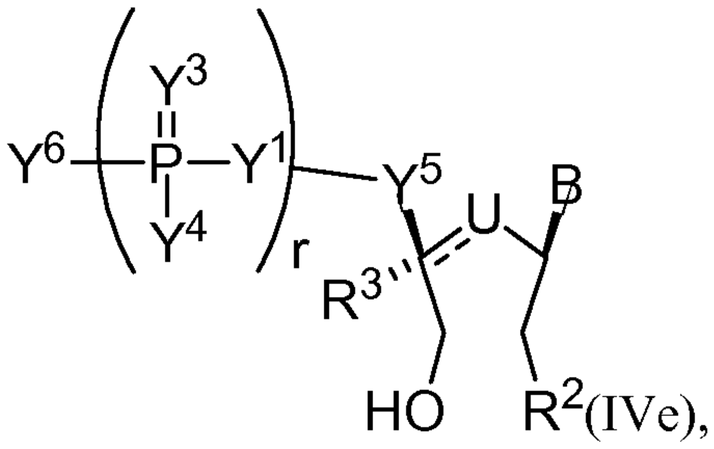
(IVb), or a pharmaceutically acceptable salt or stereoisomer thereof, wherein B is as described herein (e.g., any one of (b1)-(b43)). In particular embodiments, Formula (IVa) or (IVb) is combined with a modified uracil (e.g., any one of formulas (b1)-(b9), (b21)-(b23), and (b28)-(b31), such as formula (b1), (b8), (b28), (b29), or (b30)). In particular embodiments, Formula (IVa) or (IVb) is combined with a modified cytosine (e.g., any one of formulas (b10)-(b14),

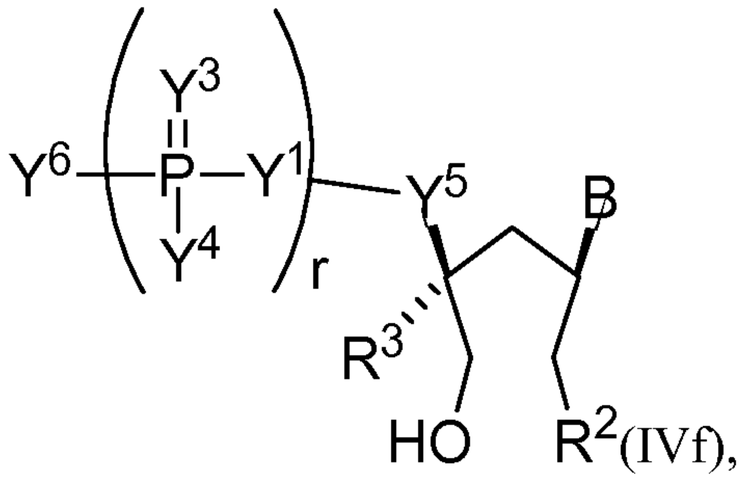
(b24), (b25), and (b32)-(b36), such as formula (b10) or (b32)). In particular embodiments, Formula (IVa) or (IVb) is combined with a modified guanine (e.g., any one of formulas (b15)-(b17) and (b37)-(b40)). In particular embodiments, Formula (IVa) or (IVb) is combined with a modified adenine (e.g., any one of formulas (b18)-(b20) and (b41)-(b43)).

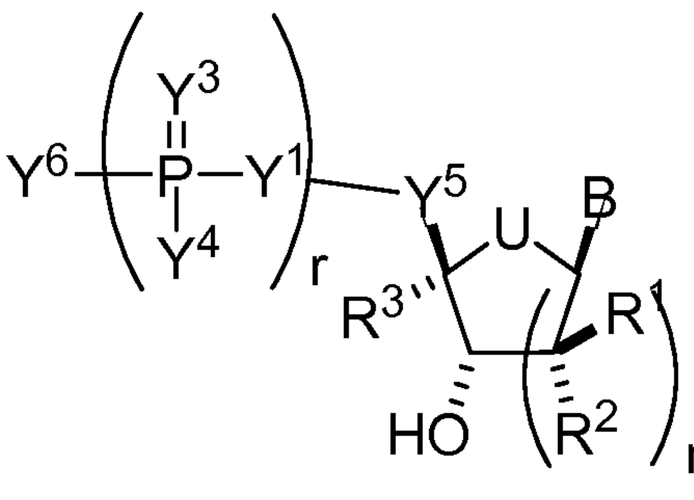
[000407] In some embodiments, the building block molecule, which may be incorporated into a polynucleotide, primary construct, or mmRNA, has Formula (IVc)-(IVk):

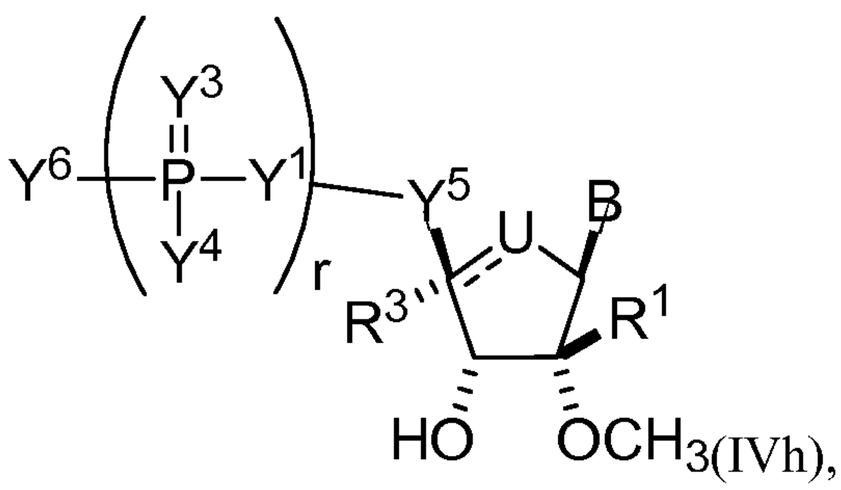

(IVc),

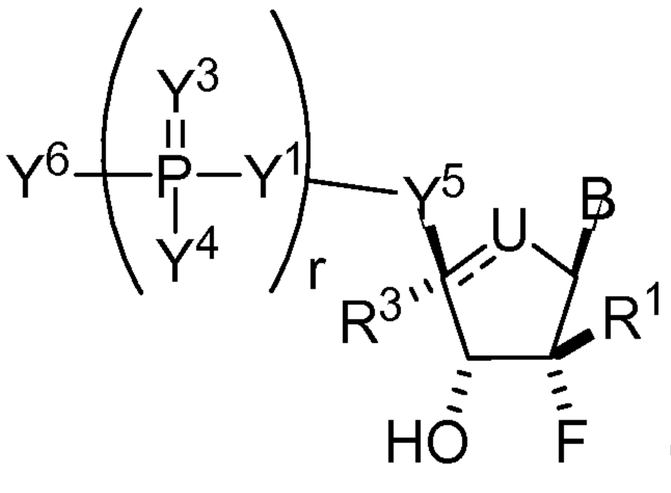

(IVd),

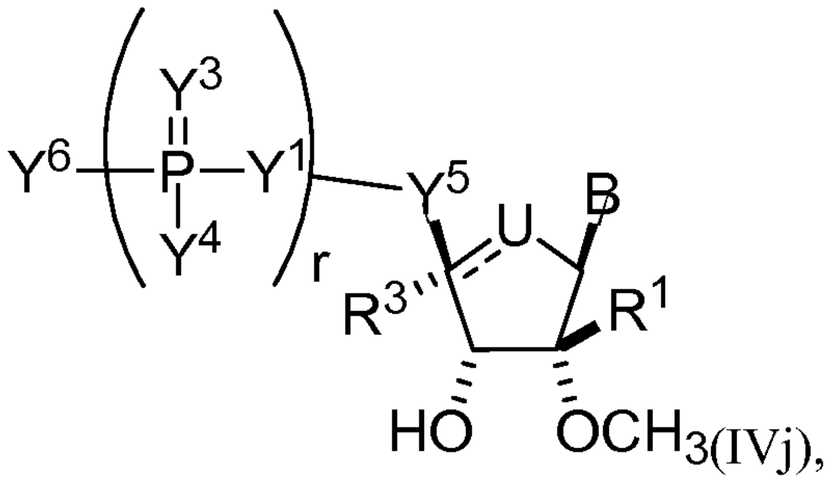

(IVe),

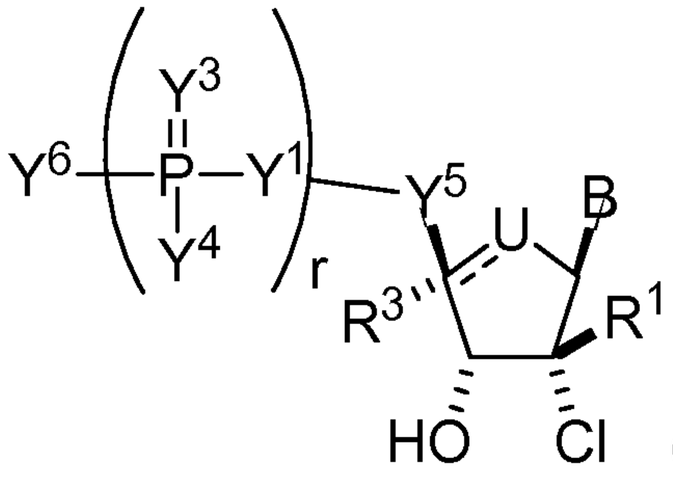

(IVf),


(IVg),

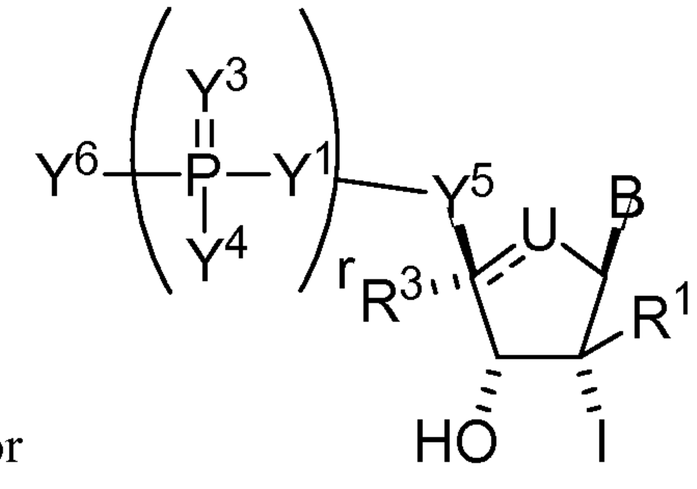

(IVh),


(IVi),


(IVj),

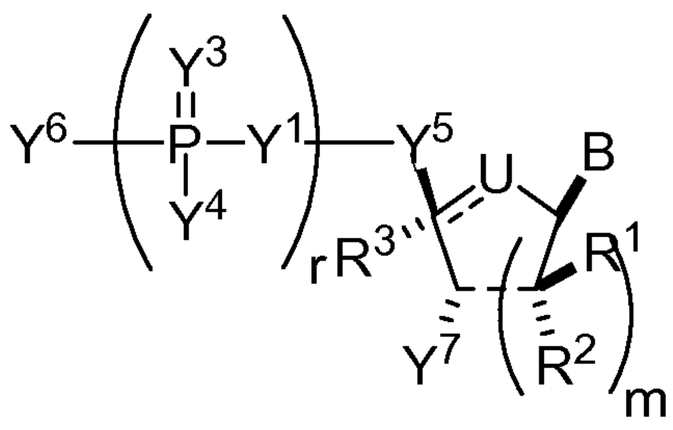


(IVk), or

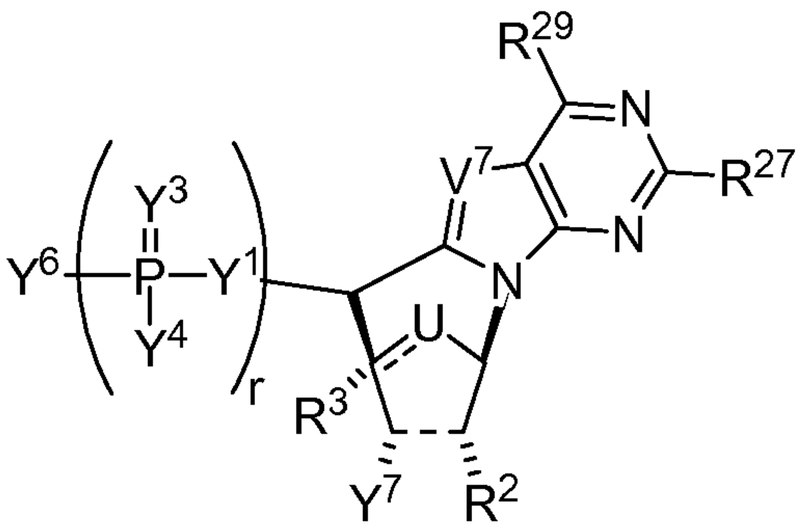


(IVl), or a pharmaceutically acceptable salt or stereoisomer thereof, wherein B is as described herein (e.g., any one of (b1)-(b43)). In particular embodiments, one of Formulas (IVc)-(IVk) is combined with a modified uracil (e.g., any one of formulas (b1)-(b9), (b21)-(b23), and (b28)-(b31), such as formula (b1), (b8), (b28), (b29), or (b30)). In particular embodiments, one of Formulas (IVc)-(IVk) is combined with a modified cytosine (e.g., any one of formulas (b10)-(b14), (b24), (b25), and (b32)-(b36), such as formula (b10) or (b32)). In particular embodiments, one of Formulas (IVc)-(IVk) is combined with a modified guanine (e.g., any one of formulas (b15)-(b17) and (b37)-(b40)). In particular embodiments, one of Formulas (IVc)-(IVk) is combined with a modified adenine (e.g., any one of formulas (b18)-(b20) and (b41)-(b43)).

[000408] In other embodiments, the building block molecule, which may be incorporated into a polynucleotide, primary construct, or mmRNA, has Formula (Va) or (Vb):

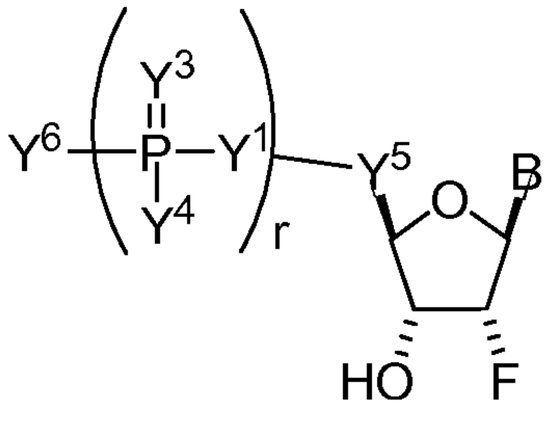
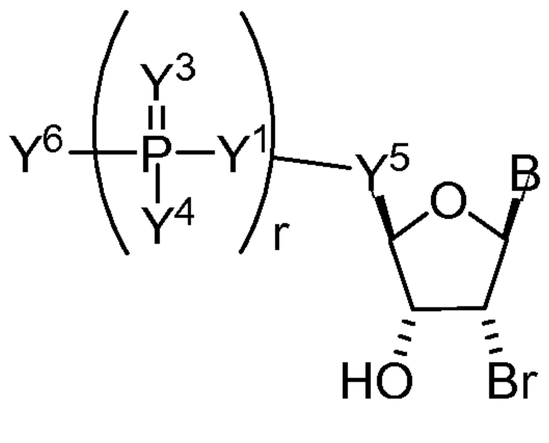
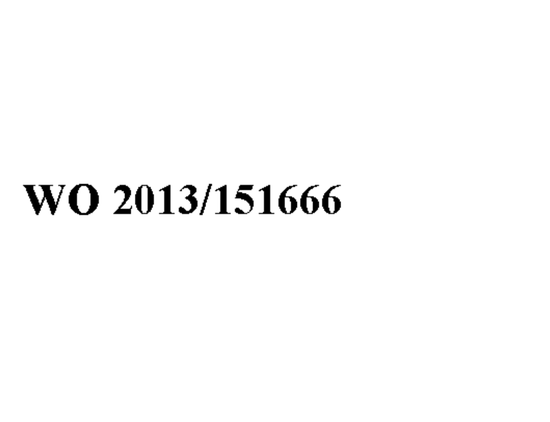


(Va) or



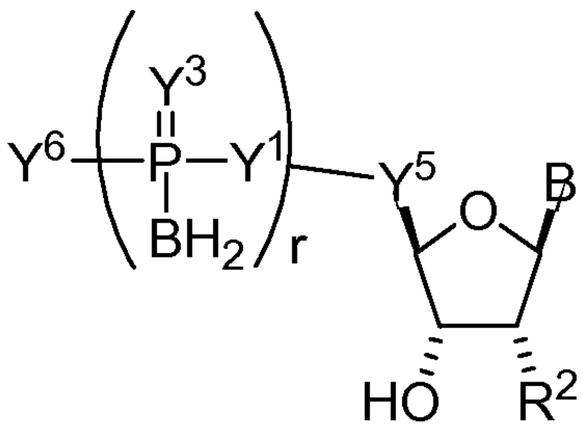
(Vb), or a pharmaceutically acceptable salt or stereoisomer thereof, wherein B is as described herein (e.g., any one of (b1)-(b43)).

[000409] In other embodiments, the building block molecule, which may be incorporated into a polynucleotide, primary construct, or mmRNA, has Formula (IXa)-(IXd):

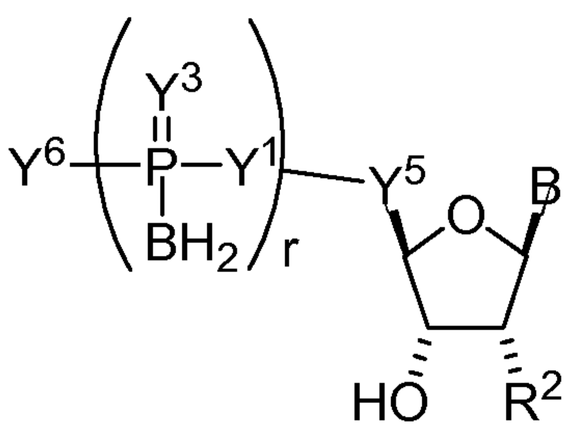

(IXa),

(IXb),

(IXc), or

(IXd), or a pharmaceutically acceptable salt or stereoisomer thereof, wherein B is as described herein (e.g., any one of (b1)-(b43)). In particular embodiments, one of Formulas (IXa)-(IXd) is combined with a modified uracil (e.g., any one of formulas (b1)-(b9), (b21)-(b23), and (b28)-(b31), such as formula (b1), (b8), (b28), (b29), or (b30)). In particular embodiments, one of Formulas (IXa)-(IXd) is combined with a modified cytosine (e.g., any one of formulas (b10)-(b14), (b24), (b25), and (b32)-(b36), such as formula (b10) or (b32)). In particular embodiments, one of Formulas (IXa)-(IXd) is combined with a modified guanine (e.g., any one of formulas (b15)-(b17) and (b37)-(b40)). In particular embodiments, one of Formulas (IXa)-(IXd) is combined with a modified adenine (e.g., any one of formulas (b18)-(b20) and (b41)-(b43)).

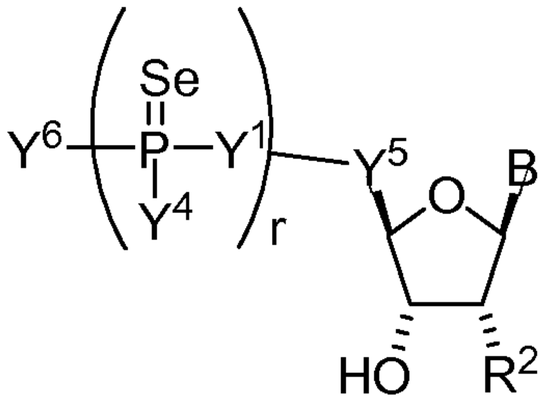
[000410] In other embodiments, the building block molecule, which may be incorporated into a polynucleotide, primary construct, or mmRNA, has Formula (IXe)-(IXg):



(IXe),

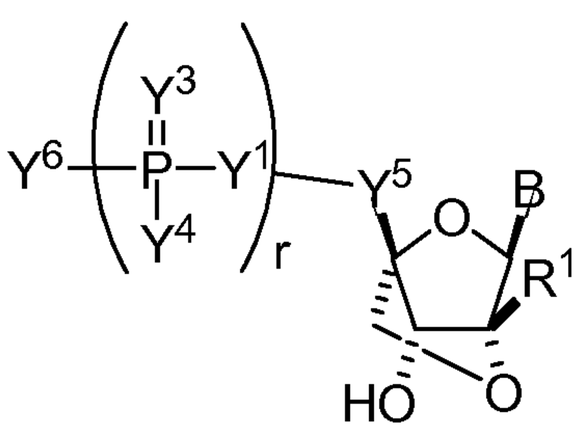


(IXf), or

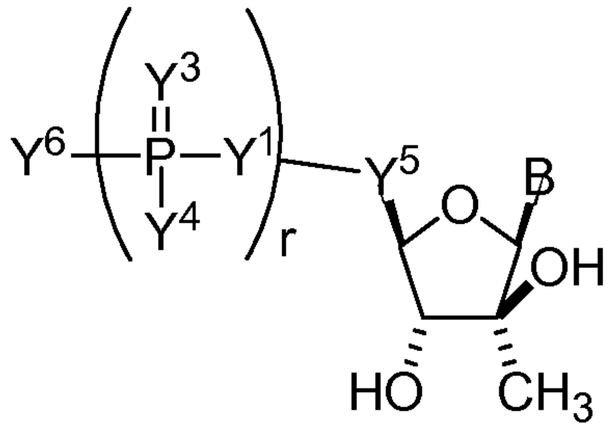


(IXg), or a pharmaceutically acceptable salt or stereoisomer thereof, wherein B is as described herein (e.g., any one of (b1)-(b43)). In particular embodiments, one of Formulas (IXe)-(IXg) is combined with a modified uracil (e.g., any one of formulas (b1)-(b9), (b21)-(b23), and (b28)-(b31), such as formula (b1), (b8), (b28), (b29), or (b30)). In particular embodiments, one of Formulas (IXe)-(IXg) is combined with a modified cytosine (e.g., any one of formulas (b10)-(b14), (b24), (b25), and (b32)-(b36), such as formula (b10) or (b32)). In particular embodiments, one of Formulas (IXe)-(IXg) is combined with a modified guanine (e.g., any one of formulas (b15)-(b17) and (b37)-(b40)). In particular embodiments, one of Formulas (IXe)-(IXg) is combined with a modified adenine (e.g., any one of formulas (b18)-(b20) and (b41)-(b43)).

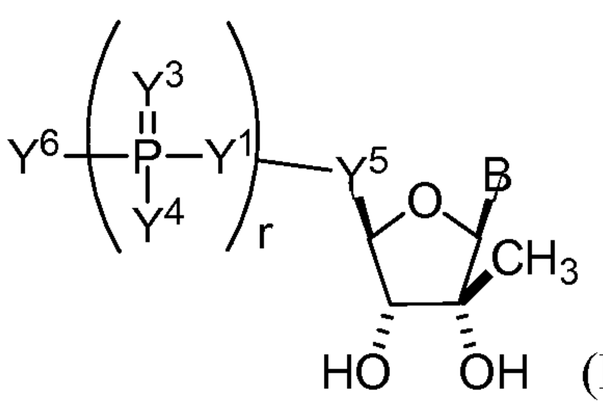
[000411] In other embodiments, the building block molecule, which may be incorporated into a polynucleotide, primary construct, or mmRNA, has Formula (IXh)-(IXk):



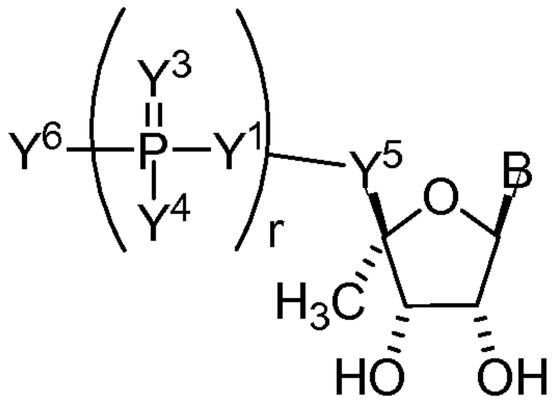
(IXh),



(IXi),

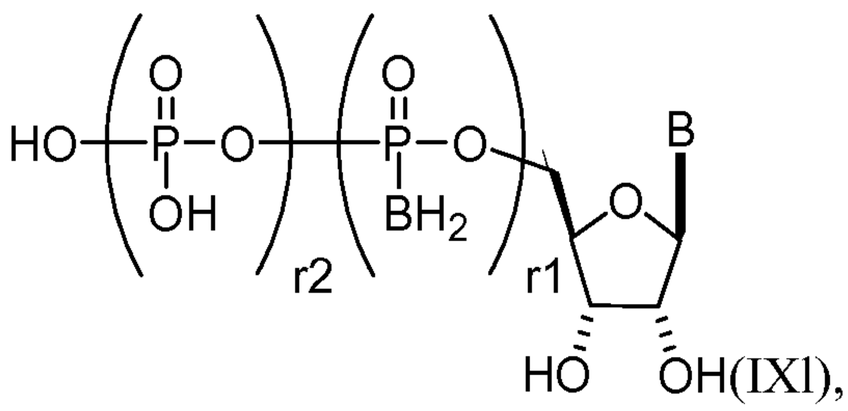


(IXj), or

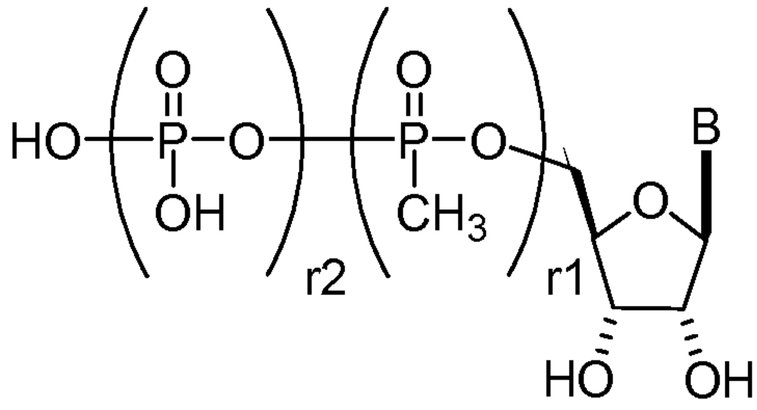


(IXk), or a pharmaceutically acceptable salt or stereoisomer thereof, wherein B is as described herein (e.g., any one of (b1)-(b43)). In particular embodiments, one of Formulas (IXh)-(IXk) is combined with a modified uracil (e.g., any one of formulas (b1)-(b9), (b21)-(b23), and (b28)-(b31), such as formula (b1), (b8), (b28), (b29), or (b30)). In particular embodiments, one of Formulas (IXh)-(IXk) is combined with a modified cytosine (e.g., any one of formulas (b10)-(b14), (b24), (b25), and (b32)-(b36), such as formula (b10) or (b32)). In particular embodiments, one of Formulas (IXh)-(IXk) is combined with a modified guanine (e.g., any one of formulas (b15)-(b17) and (b37)-(b40)). In particular embodiments, one of Formulas (IXh)-(IXk) is combined with a modified adenine (e.g., any one of formulas (b18)-(b20) and (b41)-(b43)).

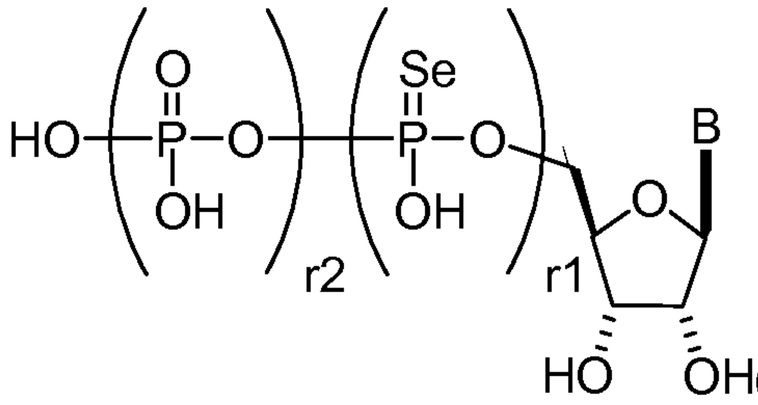
[000412] In other embodiments, the building block molecule, which may be incorporated into a polynucleotide, primary construct, or mmRNA, has Formula (IXl)-(IXr):



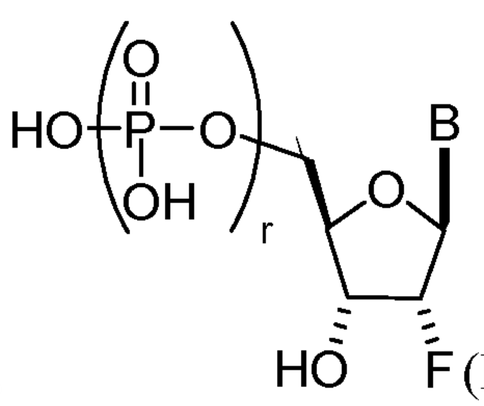
(IXl),



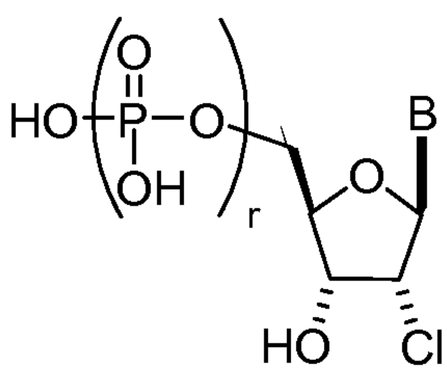
(IXm),



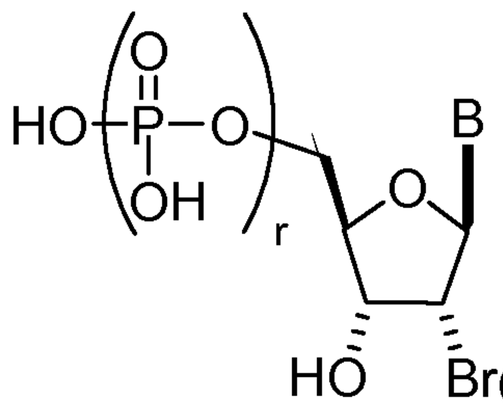
(IXn),



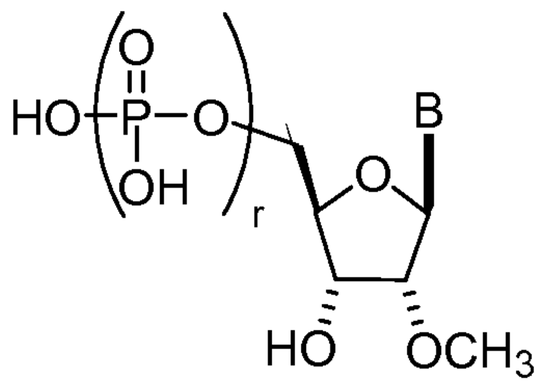
(IXo),



(IXp),

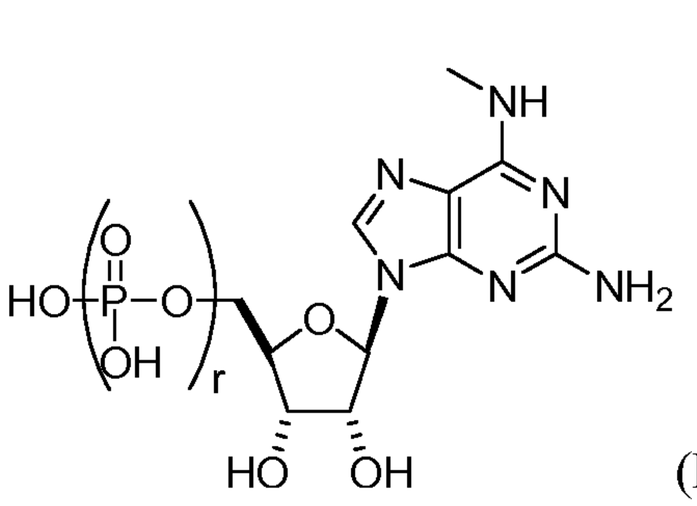


(IXq), or

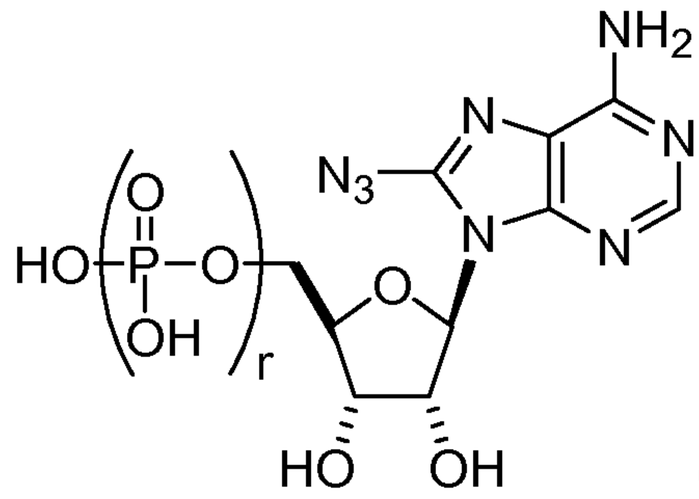


(IXr) or a pharmaceutically acceptable salt or stereoisomer thereof, wherein each r1 and r2 is, independently, an integer from 0 to 5 (e.g., from 0 to 3, from 1 to 3, or from 1 to 5) and B is as described herein (e.g., any one of (b1)-(b43)). In particular embodiments, one of Formulas (IXl)-(IXr) is combined with a modified uracil (e.g., any one of formulas (b1)-(b9), (b21)-(b23), and (b28)-(b31), such as formula (b1), (b8), (b28), (b29), or (b30)). In particular embodiments, one of Formulas (IXl)-(IXr) is combined with a modified cytosine (e.g., any one of formulas (b10)-(b14), (b24), (b25), and (b32)-(b36), such as formula (b10) or (b32)). In particular embodiments, one of Formulas (IXl)-(IXr) is combined with a modified guanine (e.g., any one of formulas (b15)-(b17) and (b37)-(b40)). In particular embodiments, one of Formulas (IXl)-(IXr) is combined with a modified adenine (e.g., any one of formulas (b18)-(b20) and (b41)-(b43)).

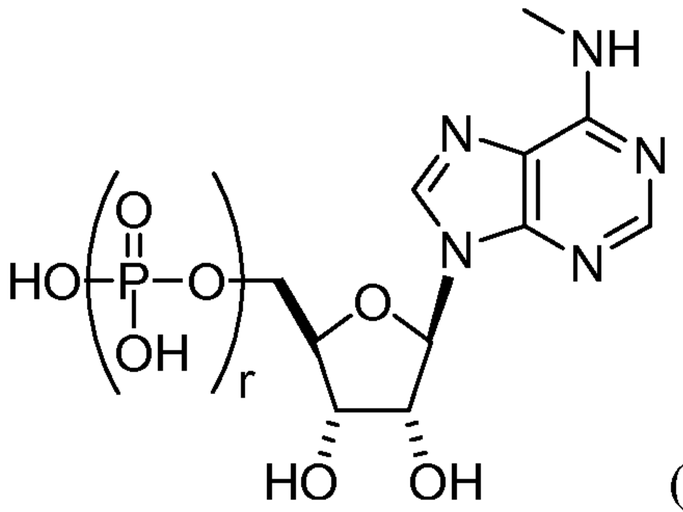
[000413] In some embodiments, the building block molecule, which may be incorporated into a polynucleotide, primary construct, or mmRNA, can be selected from the group consisting of:



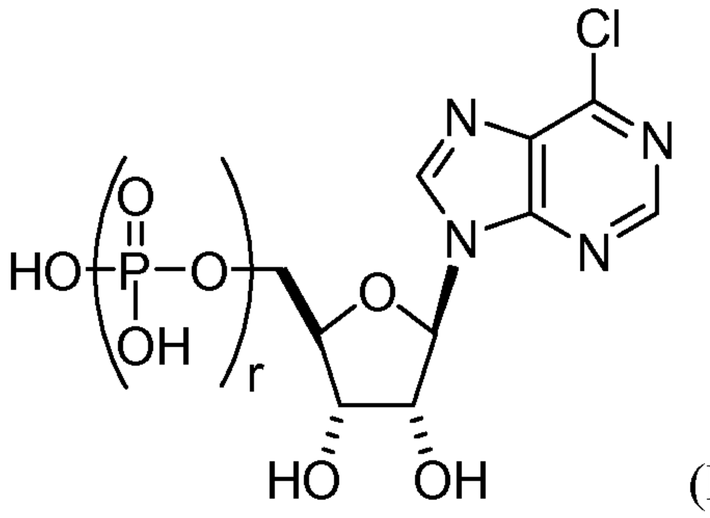
(BB- 1),



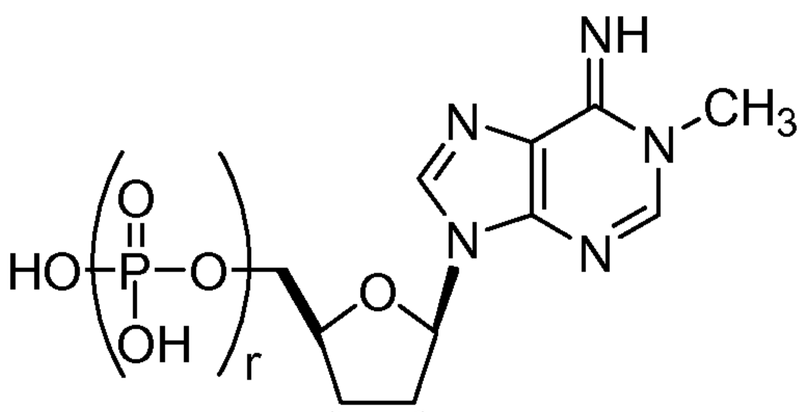
(BB- 2),



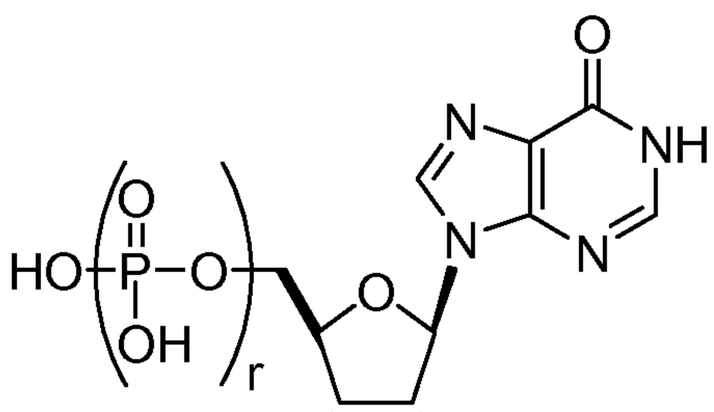
(BB- 3),



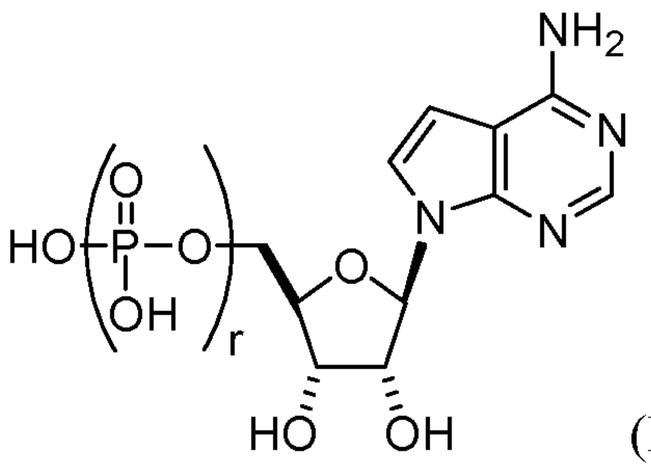
(BB- 4),



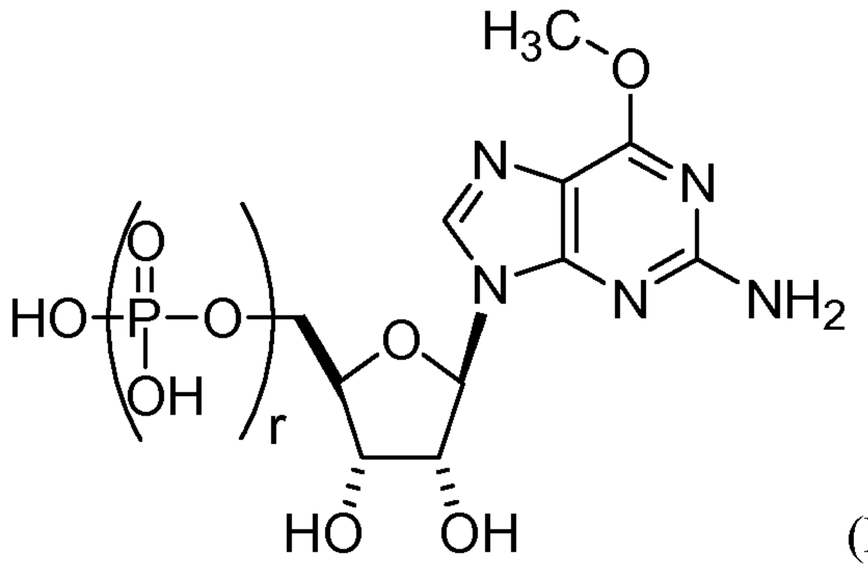
(BB- 5),



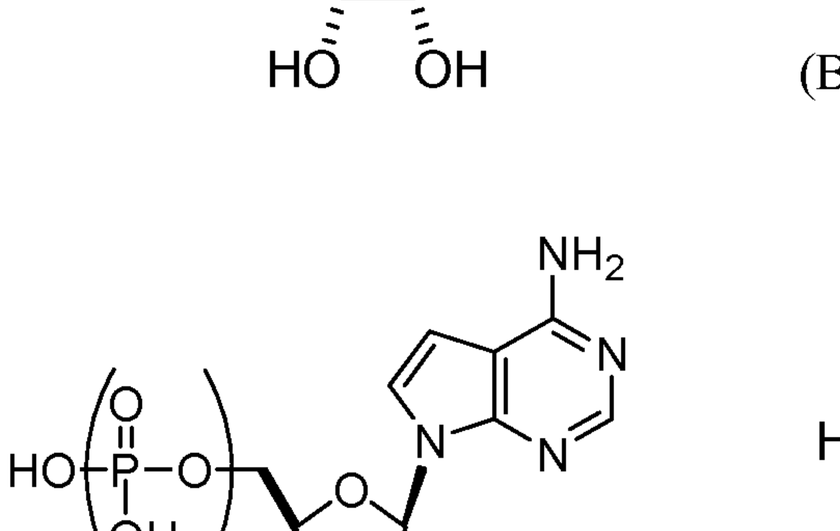
(BB- 6),



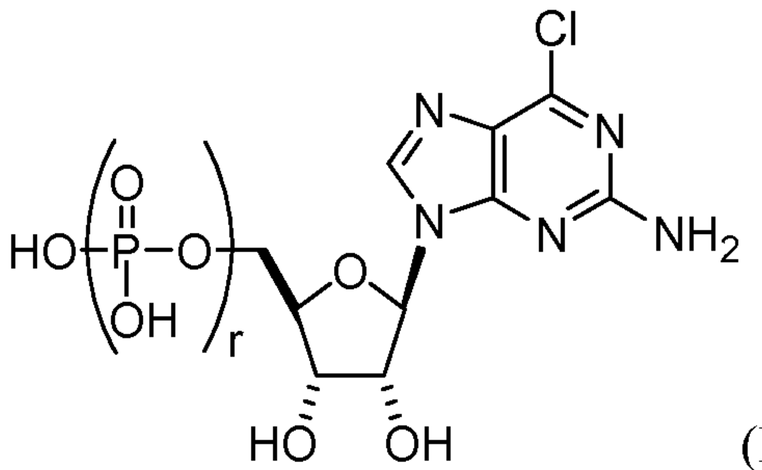
(BB- 7),



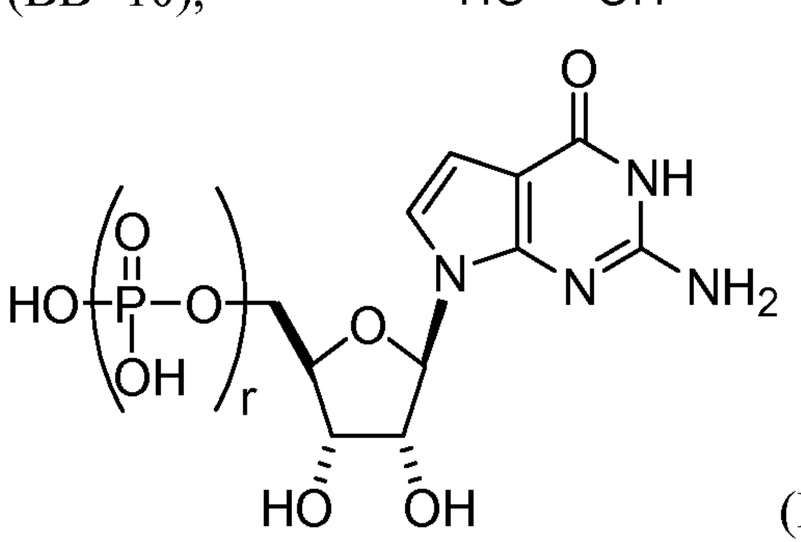
(BB- 8),



(BB- 9),



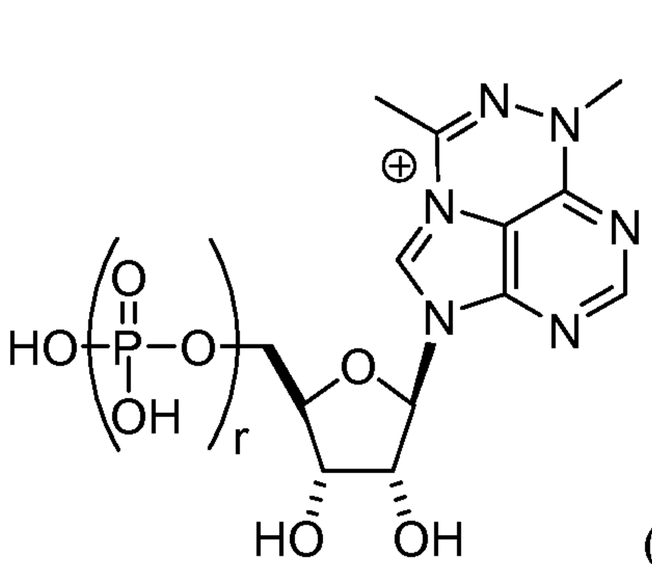
(BB- 10),



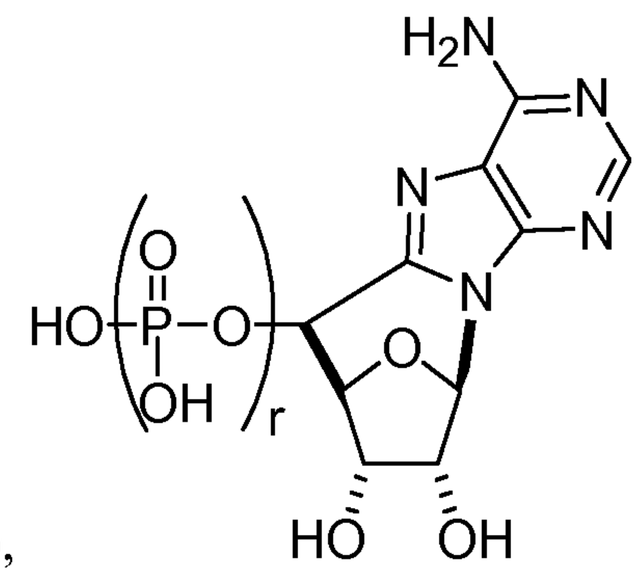
(BB- 11), and

(BB- 12), or a pharmaceutically acceptable salt or stereoisomer thereof, wherein each r is, independently, an integer from 0 to 5 (e.g., from 0 to 3, from 1 to 3, or from 1 to 5).

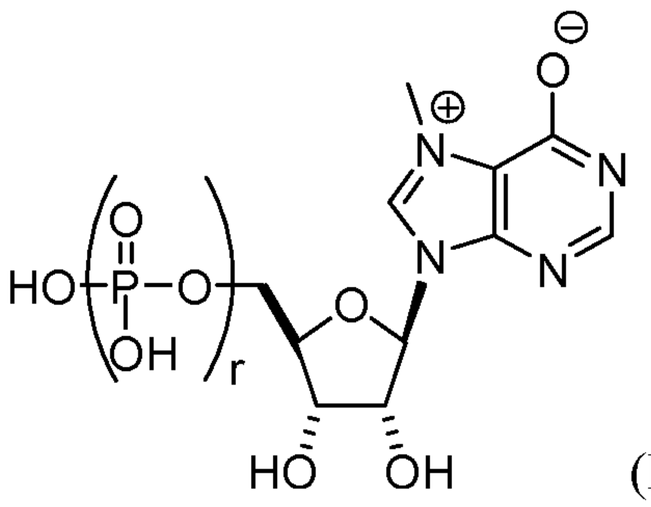
[000414] In some embodiments, the building block molecule, which may be incorporated into a polynucleotide, primary construct, or mmRNA, can be selected from the group consisting of:



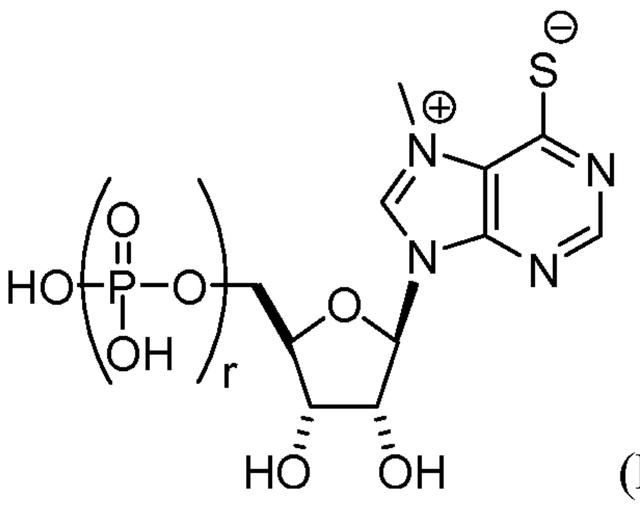
(BB- 13),



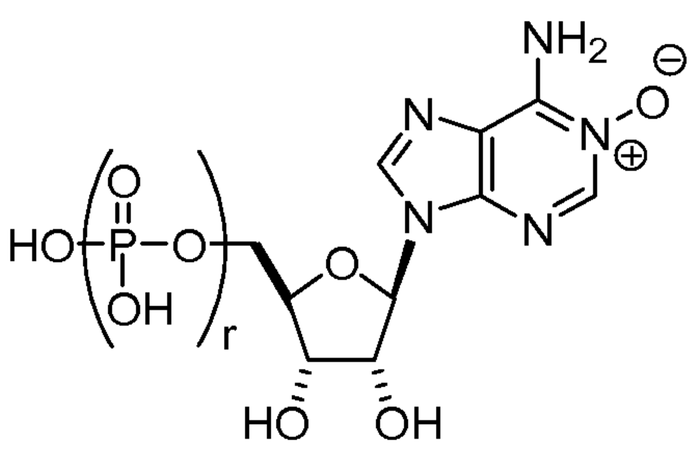
(BB- 14),



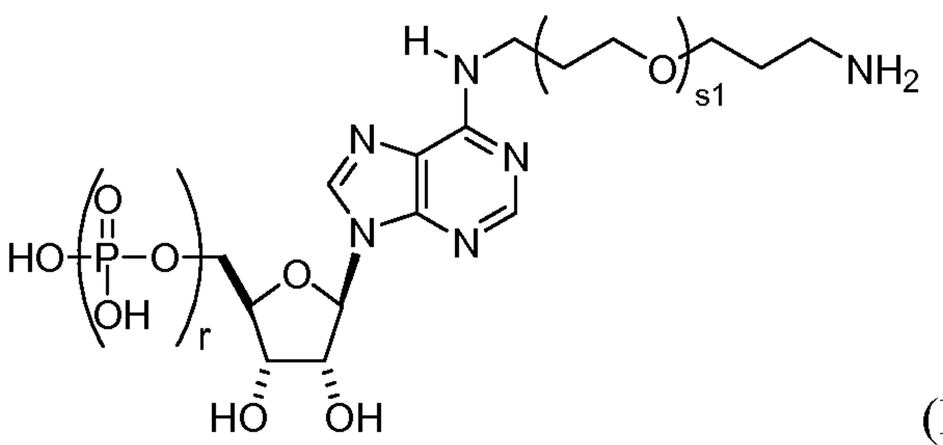
(BB- 15),



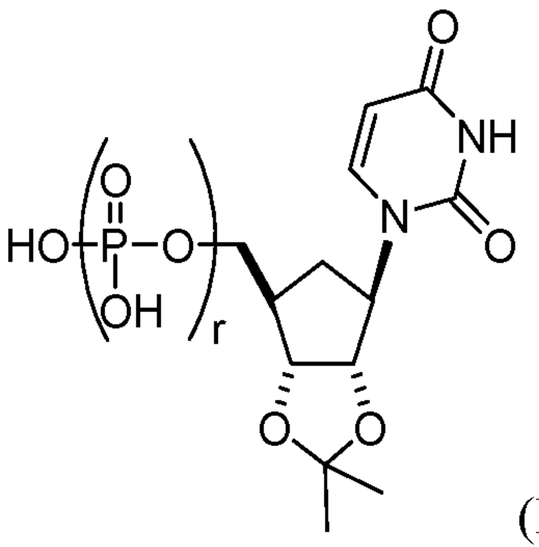
(BB- 16),



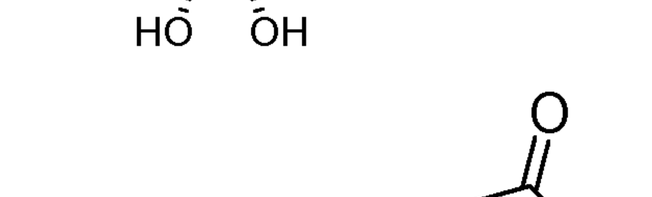
(BB- 17),



(BB- 18),



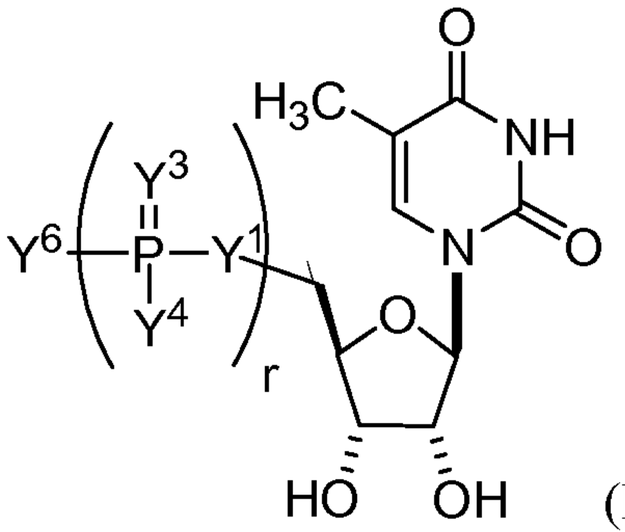
(BB- 19),

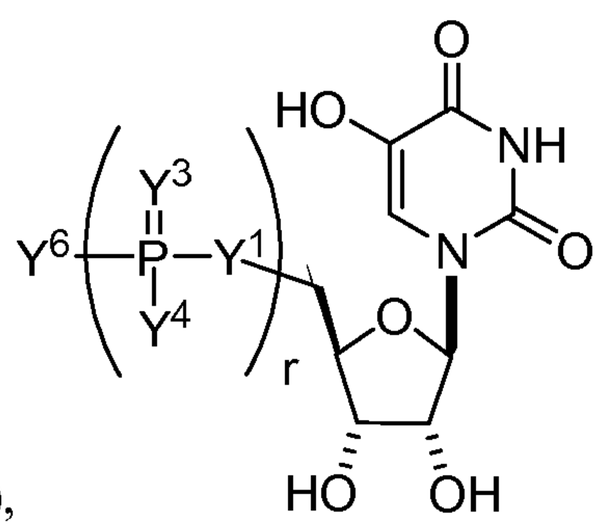


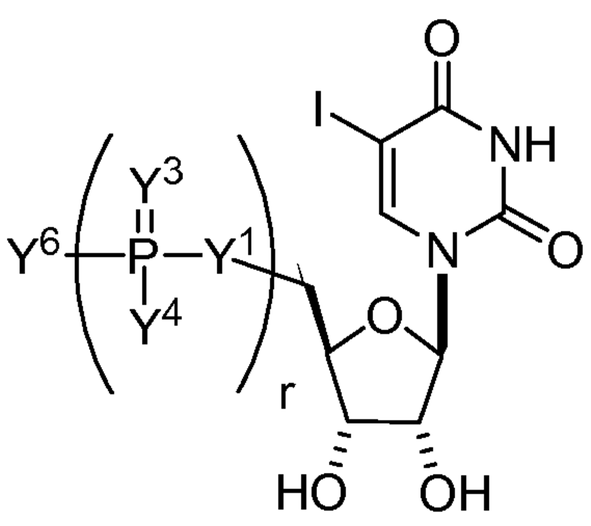
and (BB- 20), or a pharmaceutically acceptable salt or

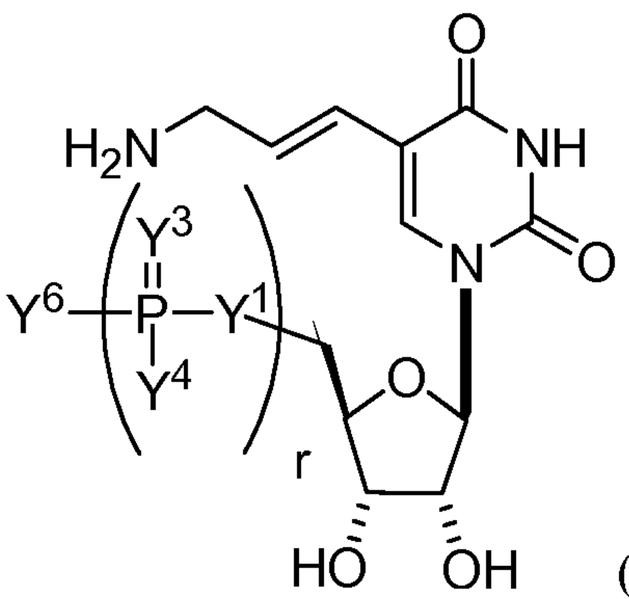
stereoisomer thereof, wherein each r is, independently, an integer from 0 to 5 (e.g., from 0 to 3, from 1 to 3, or from 1 to 5) and s1 is as described herein.

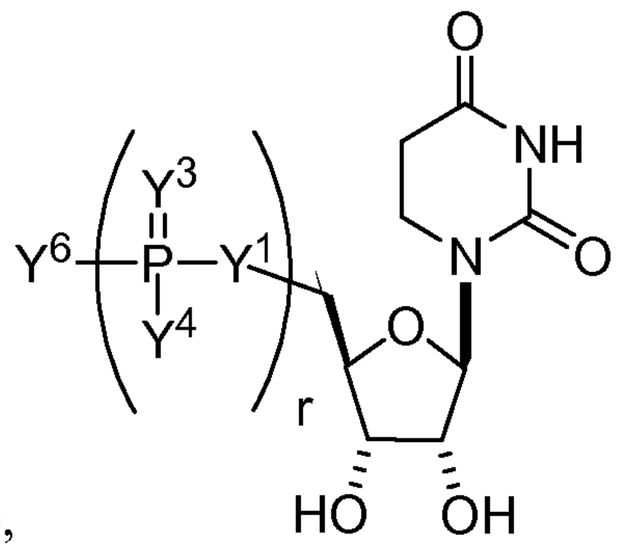
[000415] In some embodiments, the building block molecule, which may be incorporated into a nucleic acid (e.g., RNA, mRNA, polynucleotide, primary construct, or mmRNA), is a modified uridine (e.g., selected from the group consisting of:


(BB- 21),


(BB- 22),

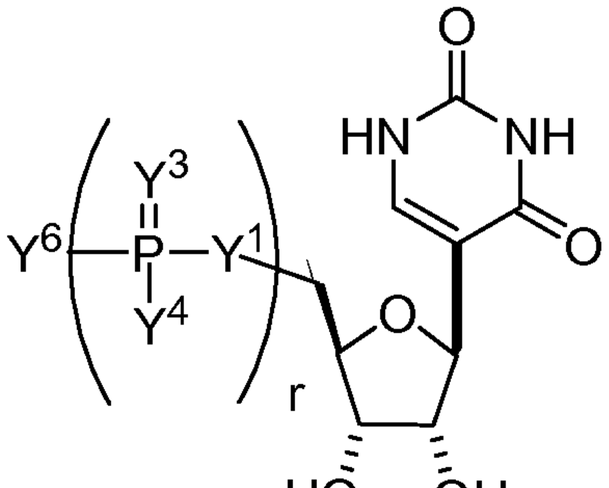

(BB- 23),


(BB- 24),

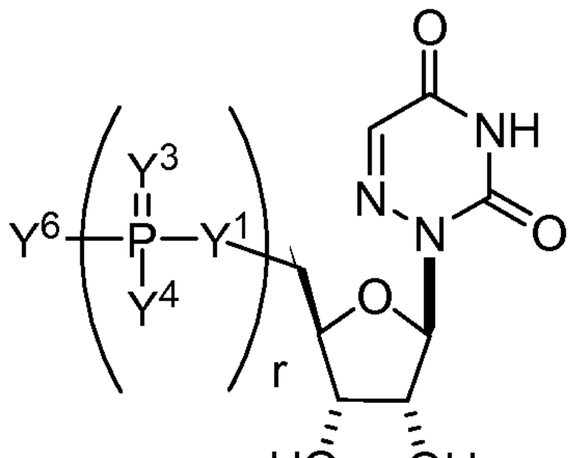

(BB- 25),

(BB- 26),

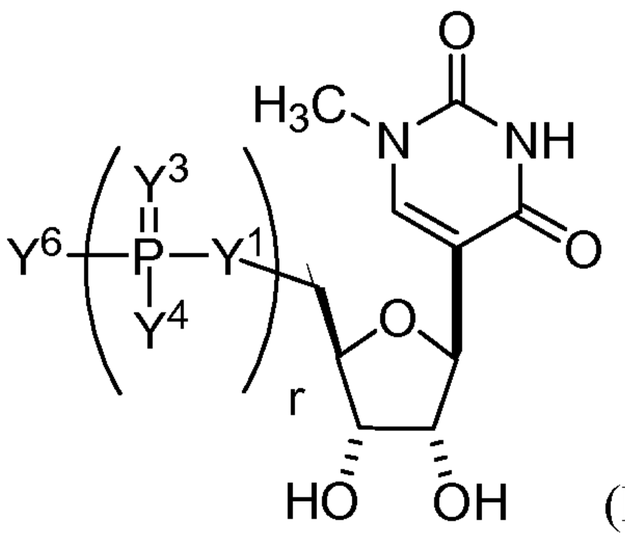
(BB- 27),



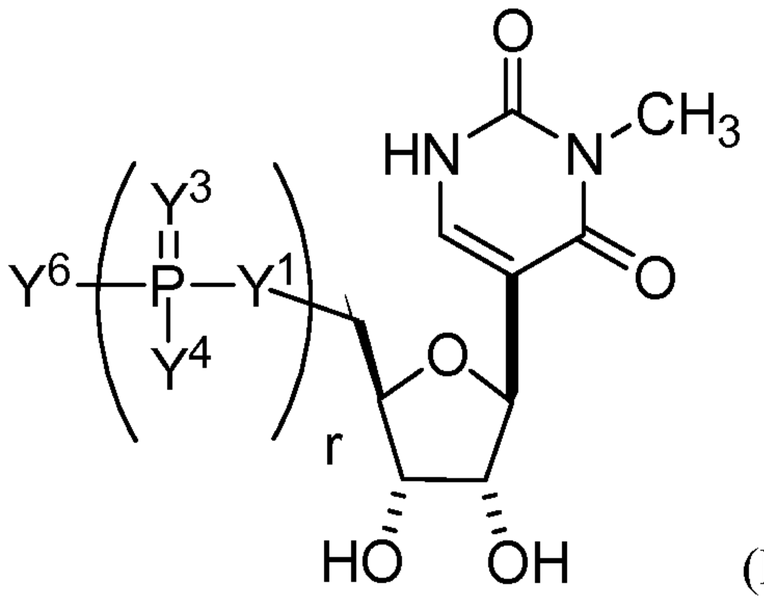
(BB- 28),



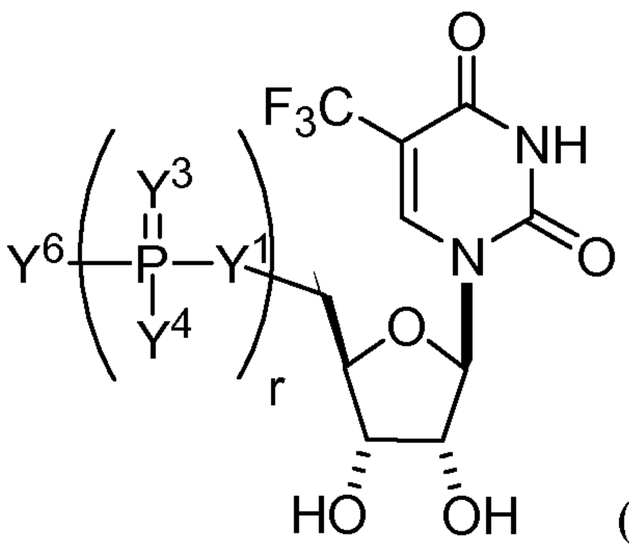
(BB- 29),



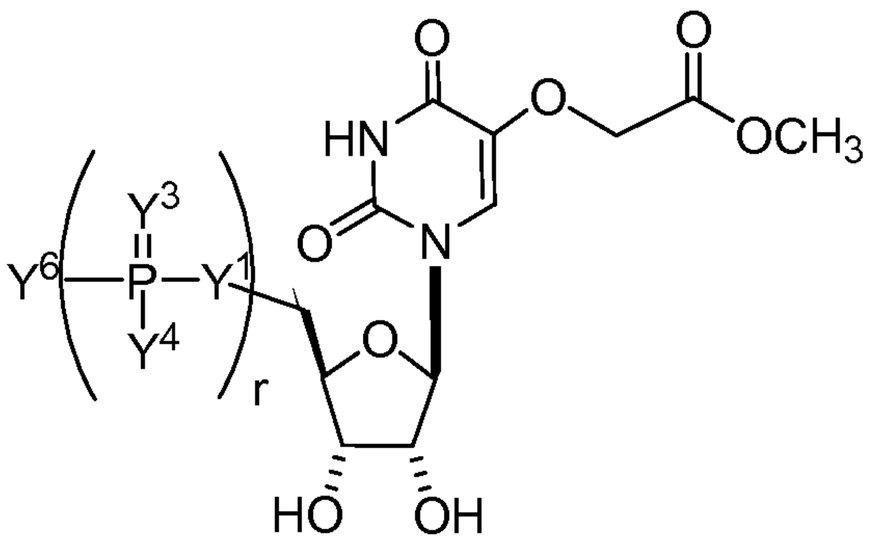
(BB- 30),



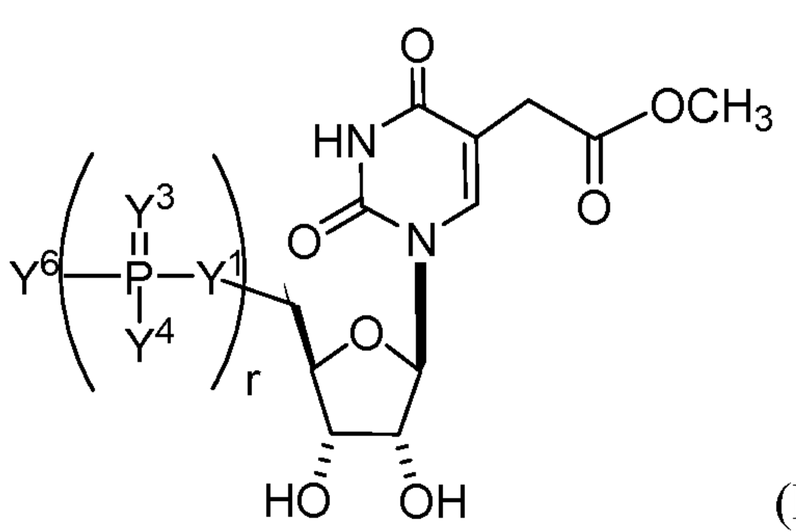
(BB- 31),



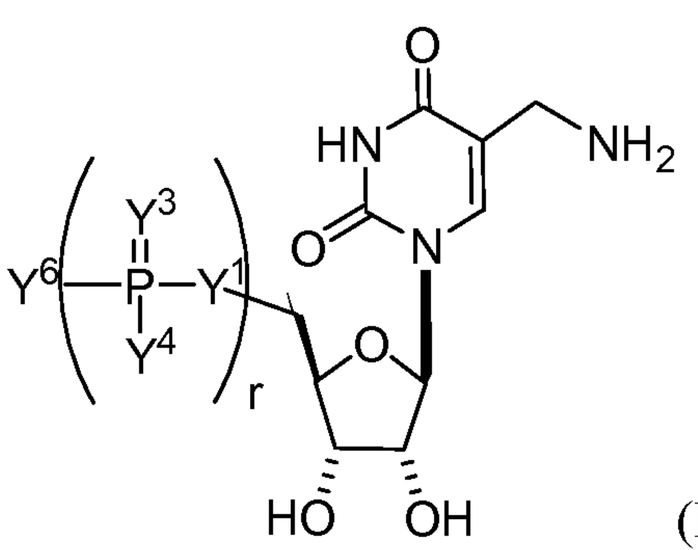
(BB- 32),



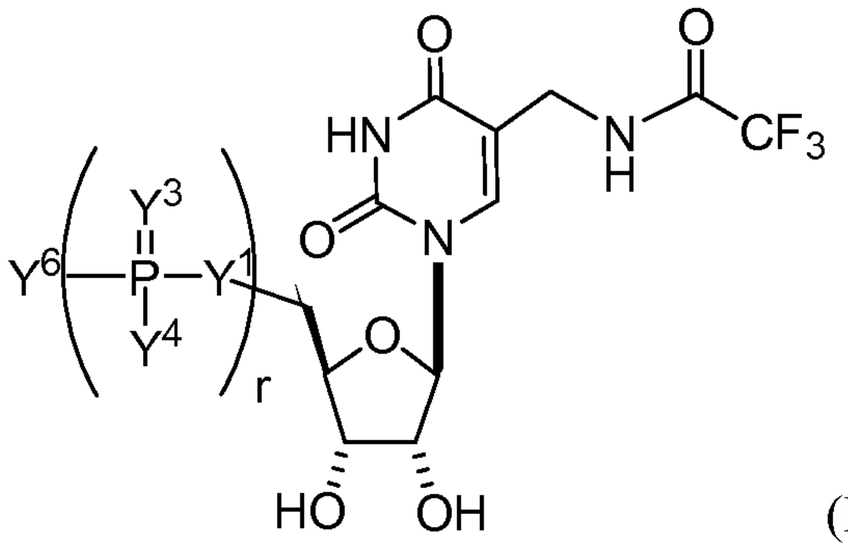
(BB- 33),



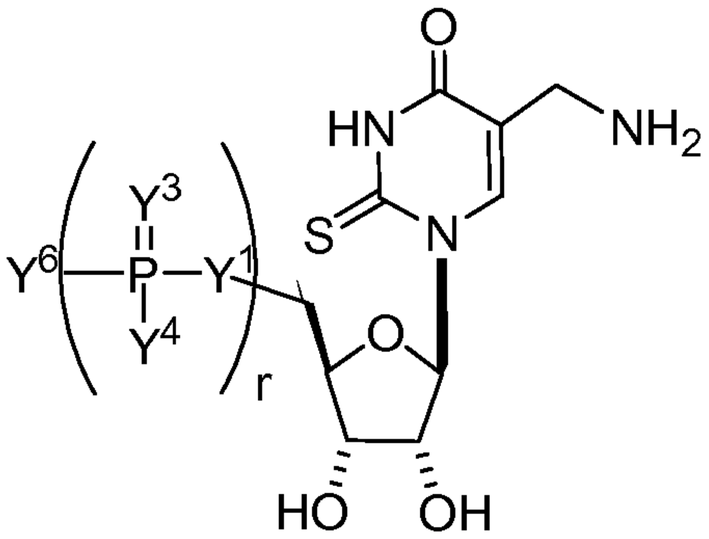
(BB- 34),



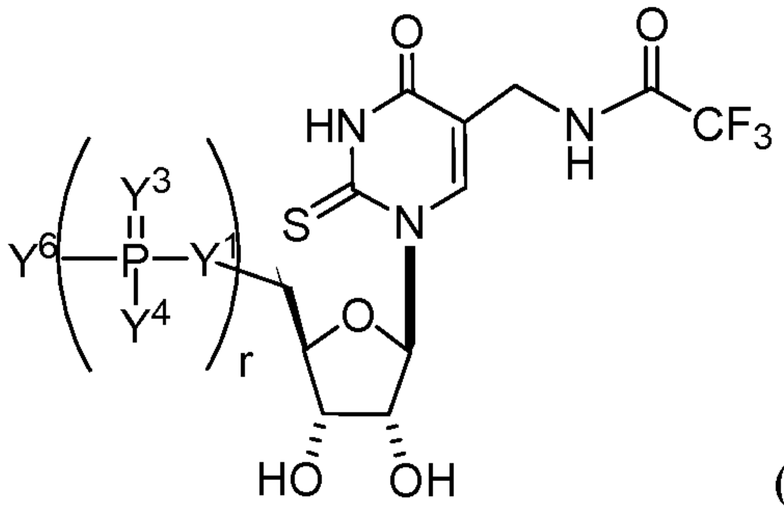
(BB-



(BB- 35),

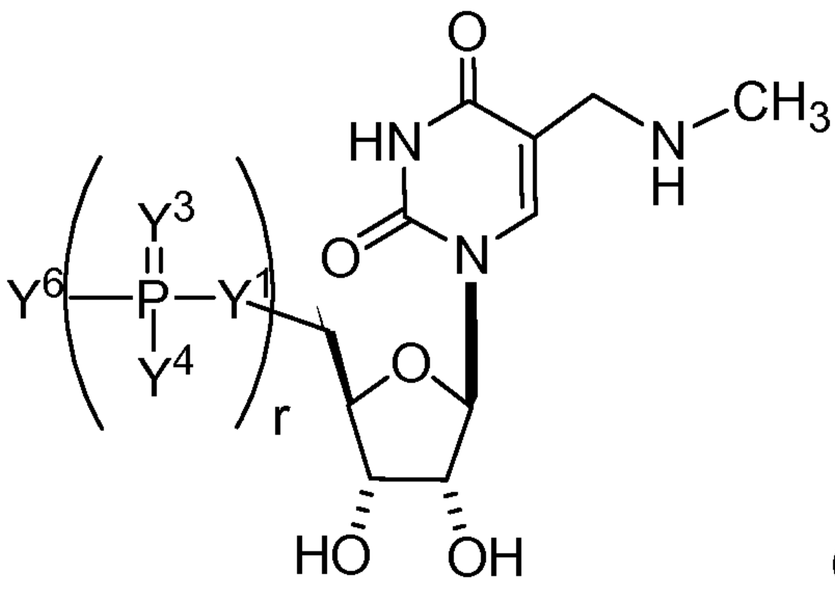


(BB- 36),

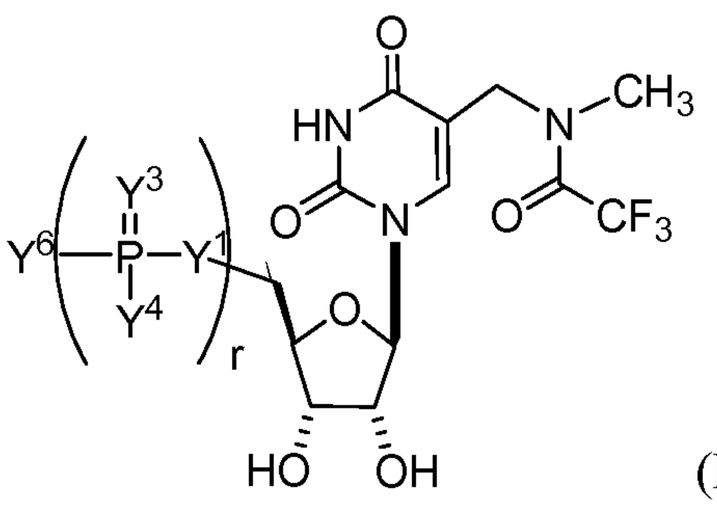


(BB- 37),

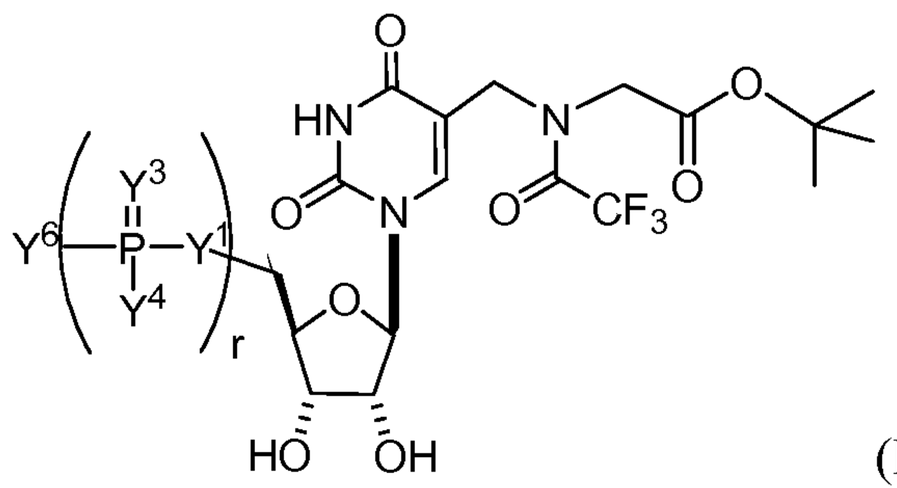
(BB- 38),



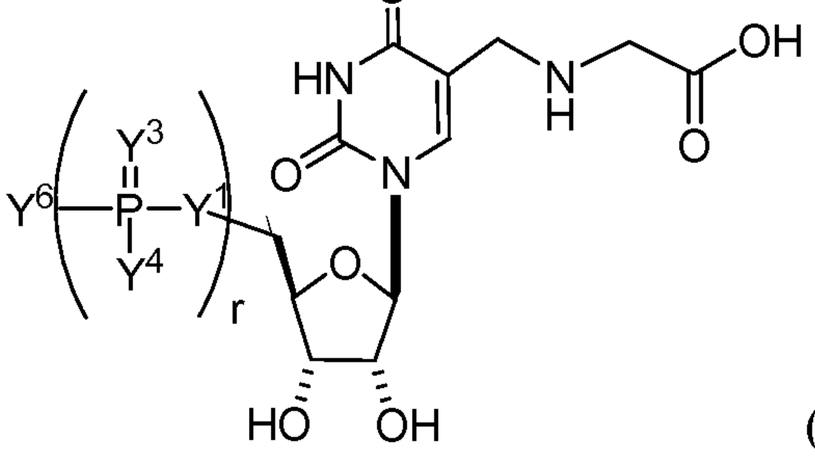
(BB- 39),



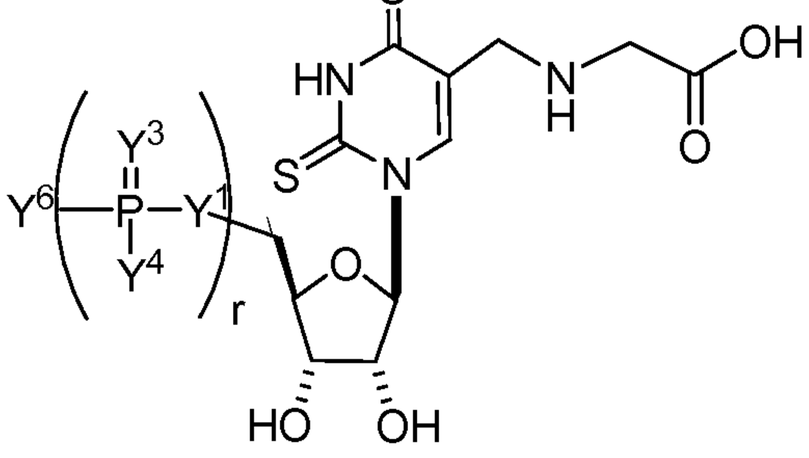
(BB- 40),

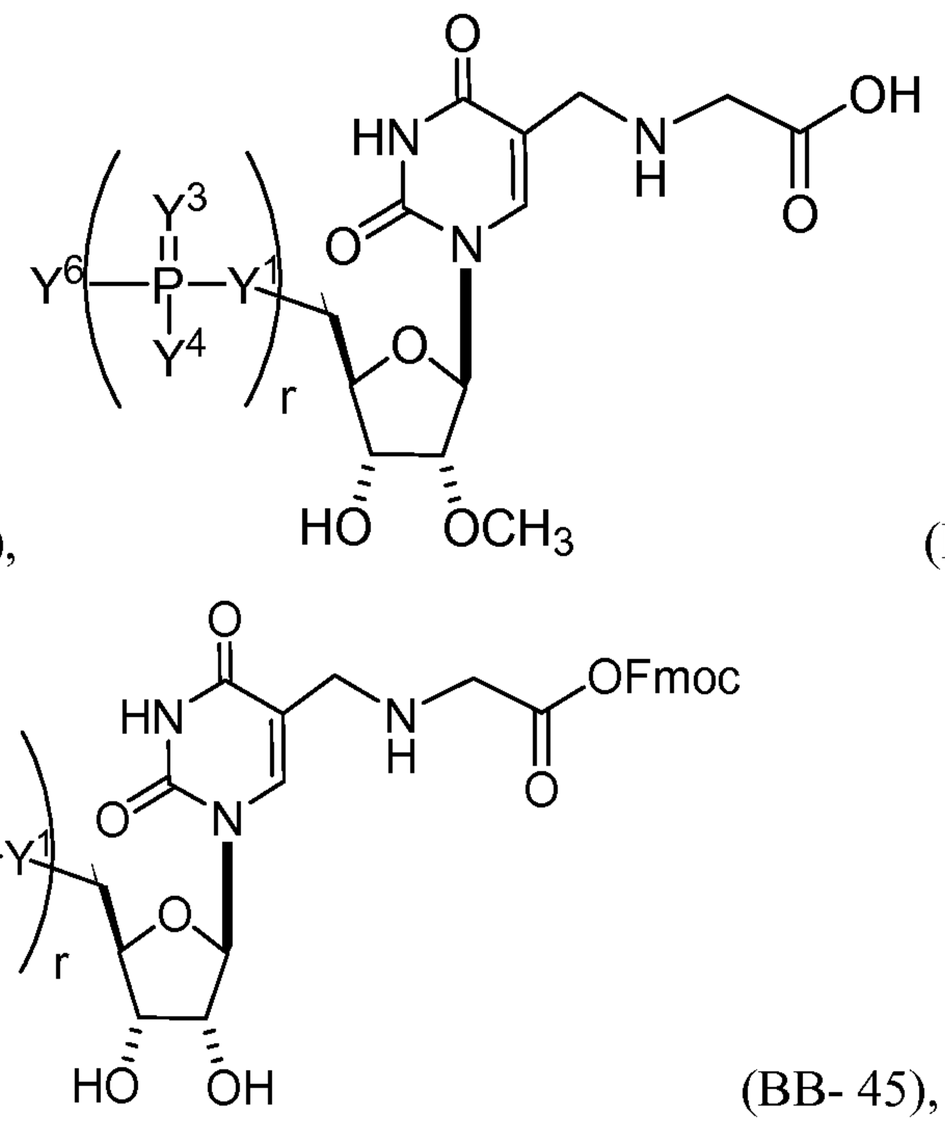


(BB- 41),

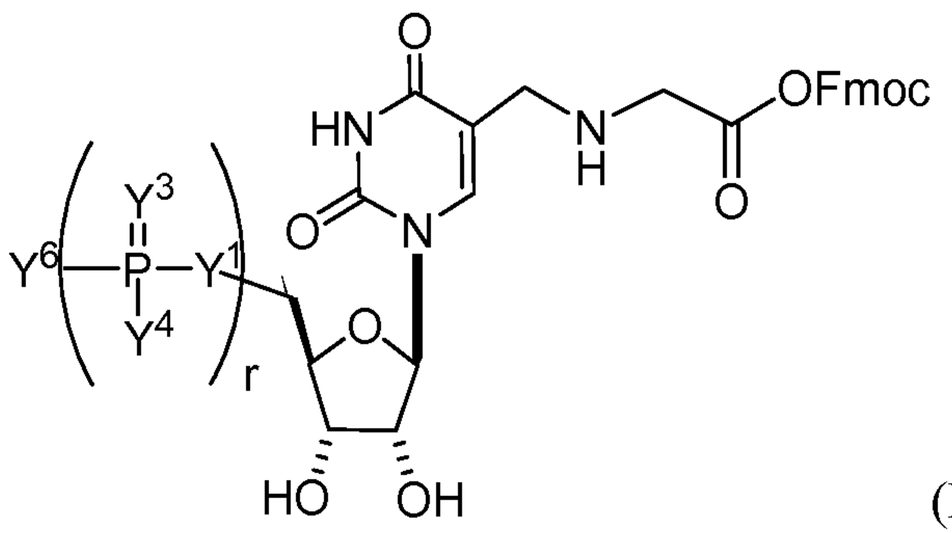


(BB- 42),

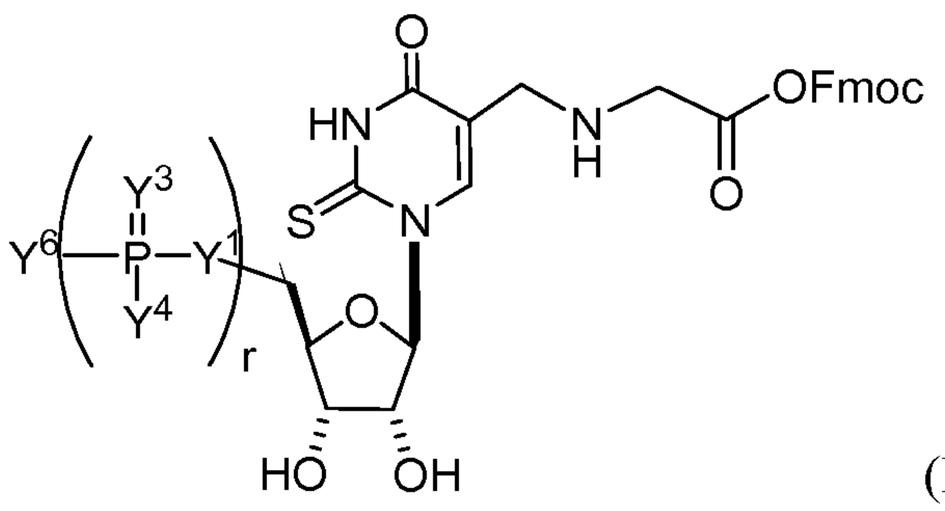




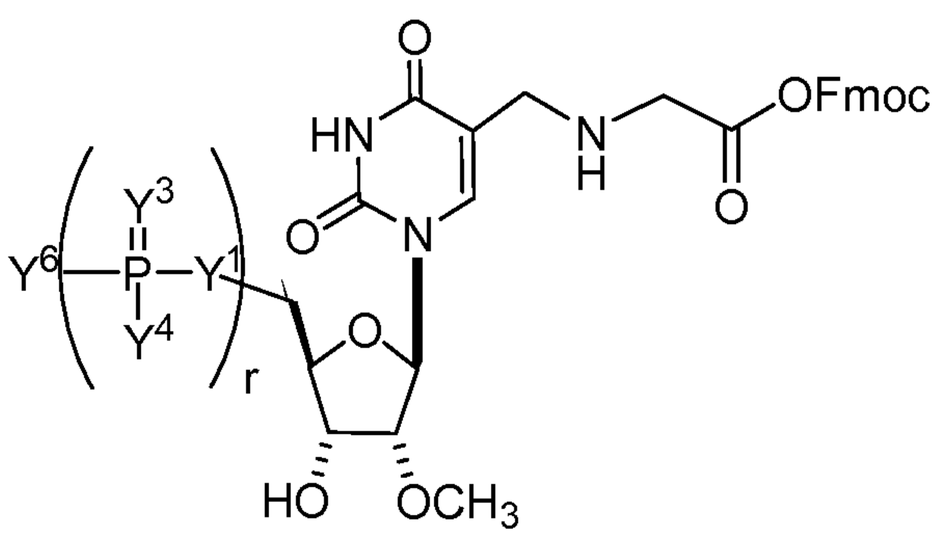
(BB- 43),



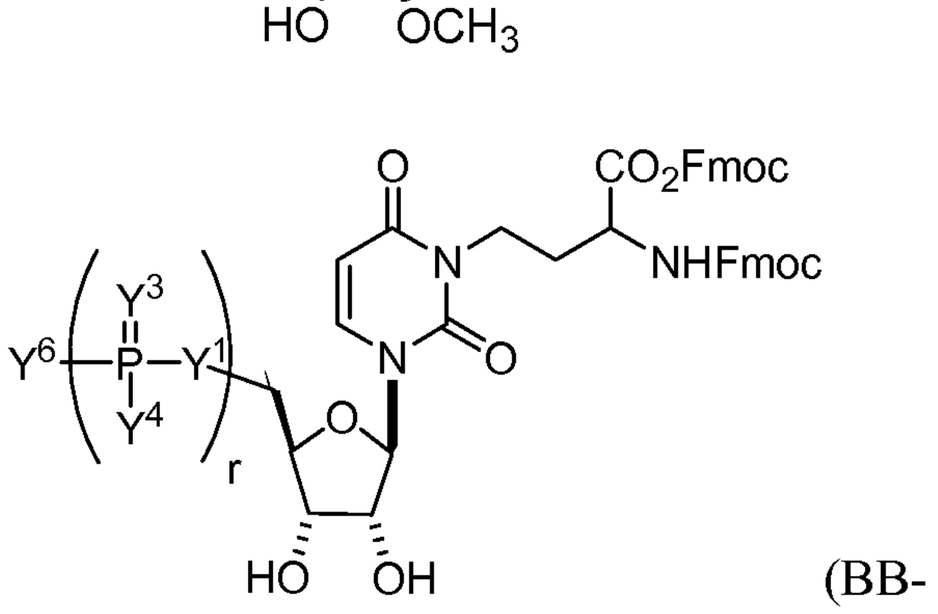
(BB- 44),



(BB- 45),

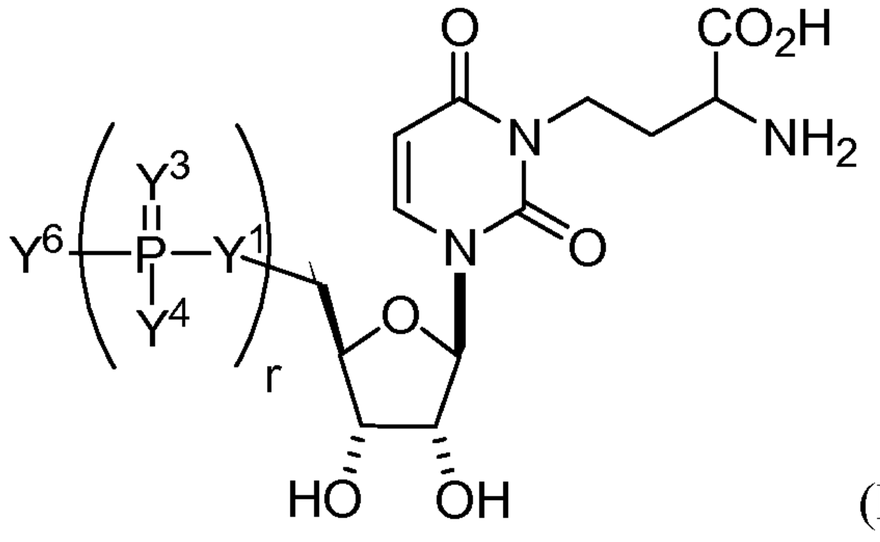


(BB-46),

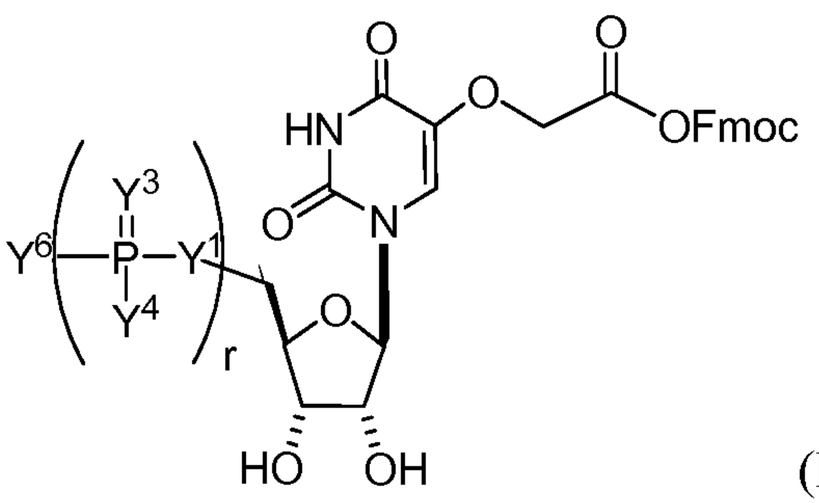


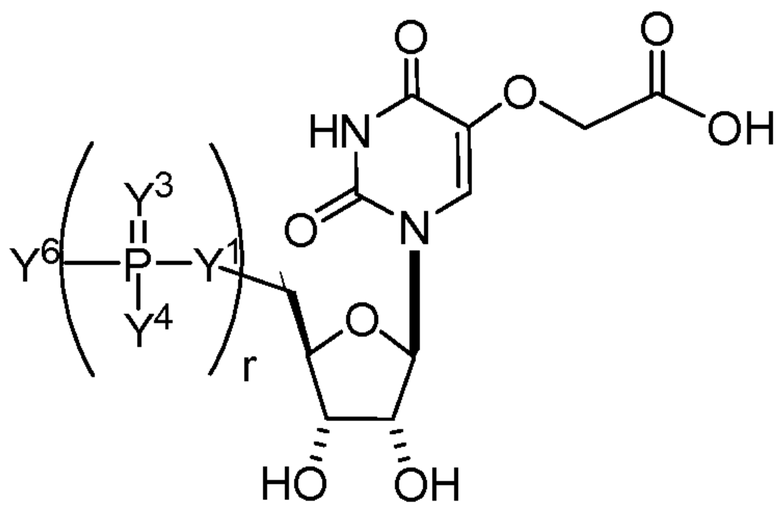
(BB- 47),

(BB- 48),

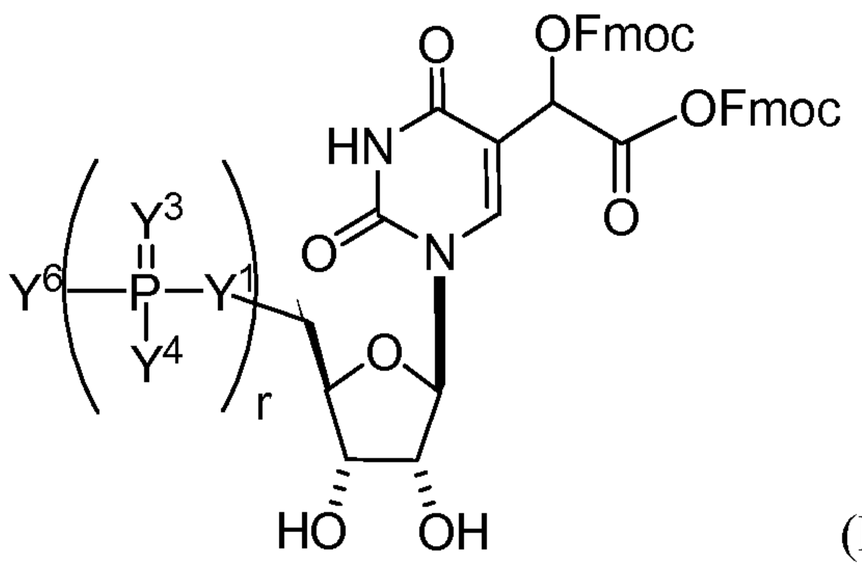


(BB- 49),



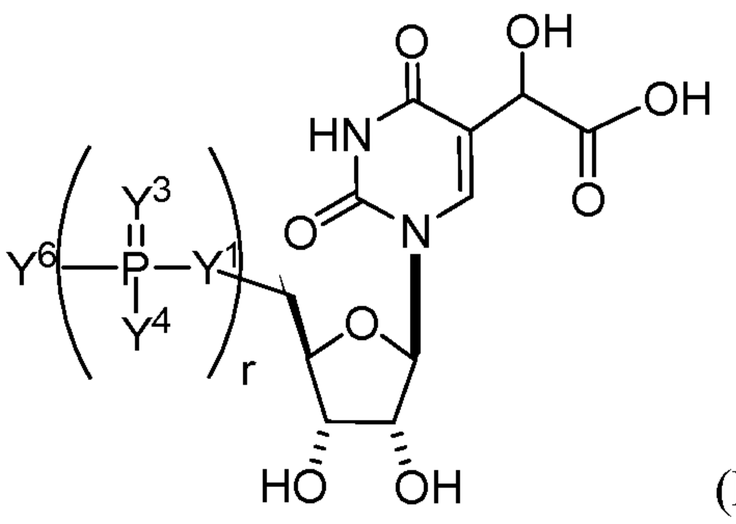


(BB- 50),

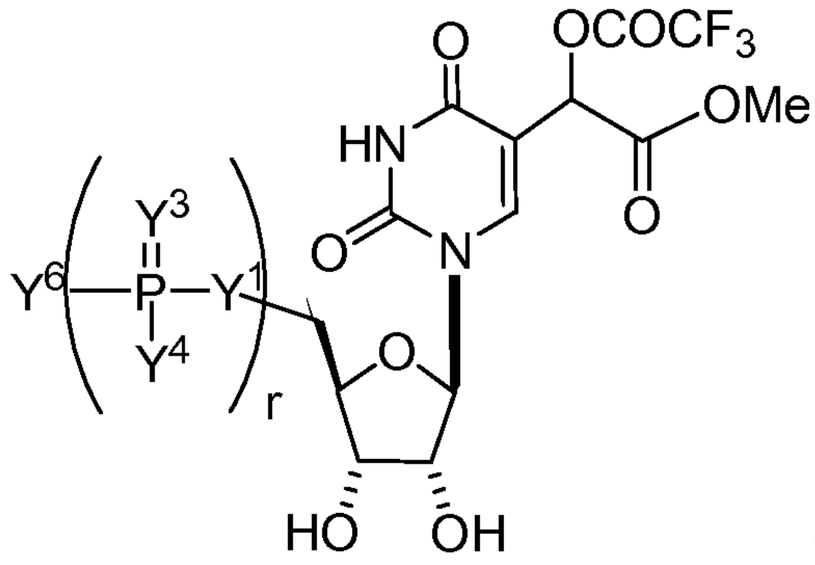


(BB- 51),

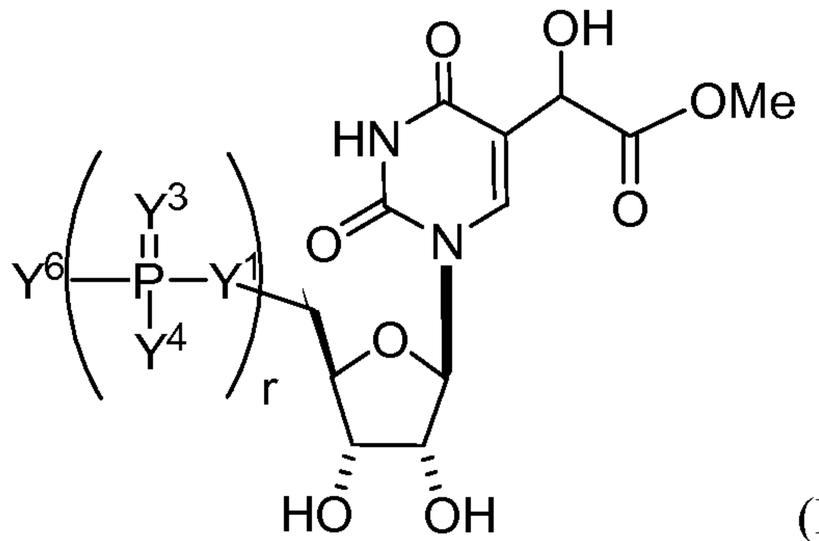
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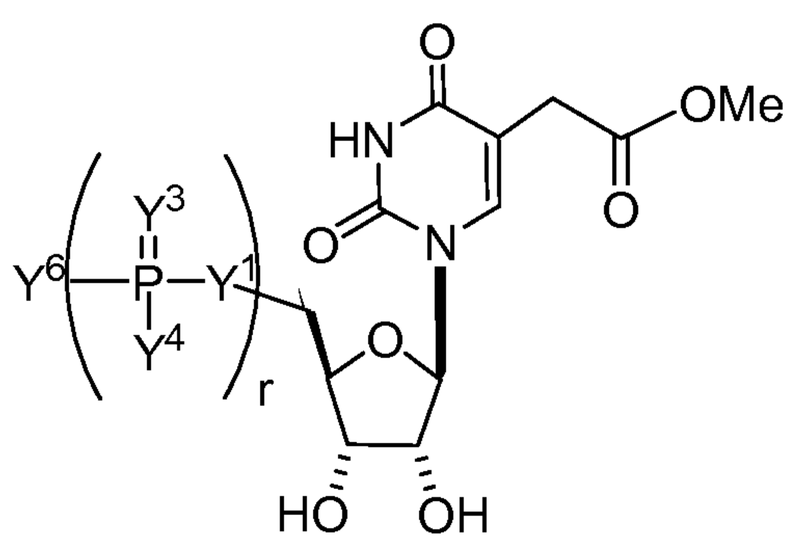
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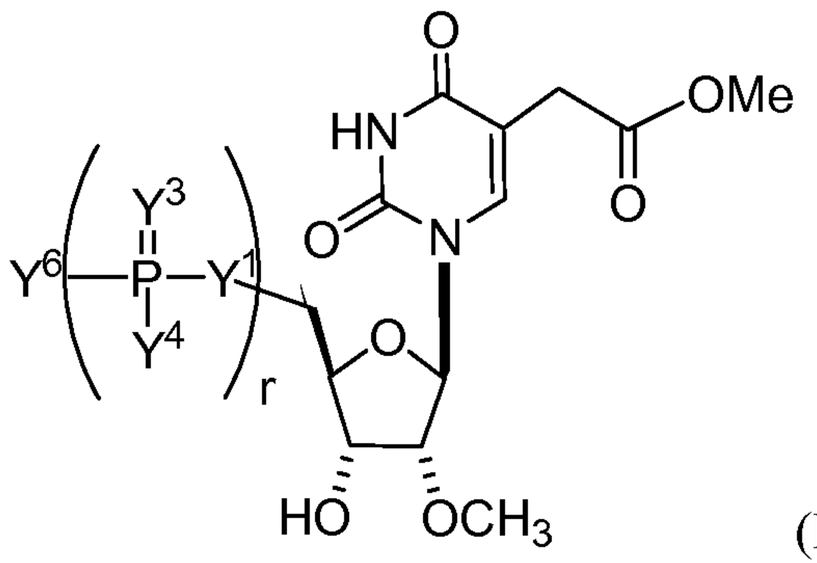
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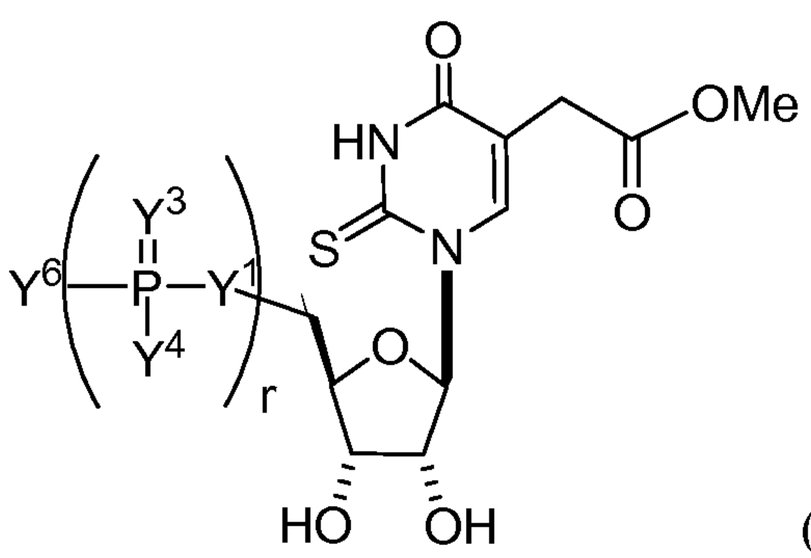


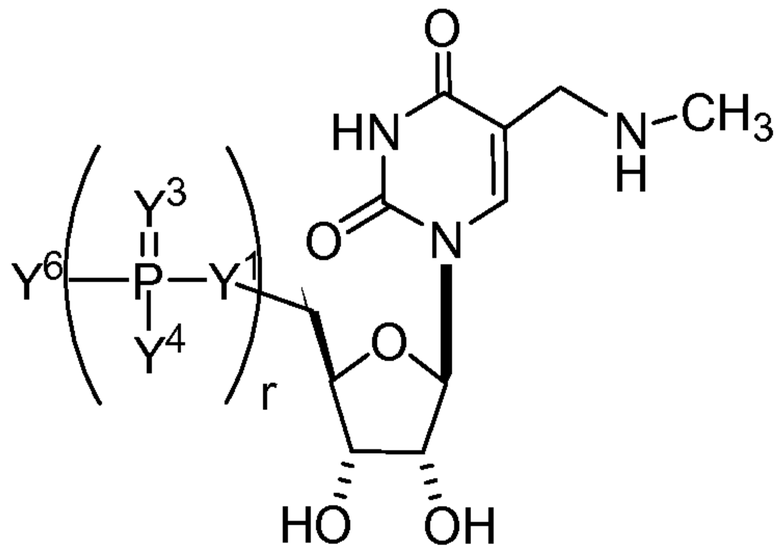
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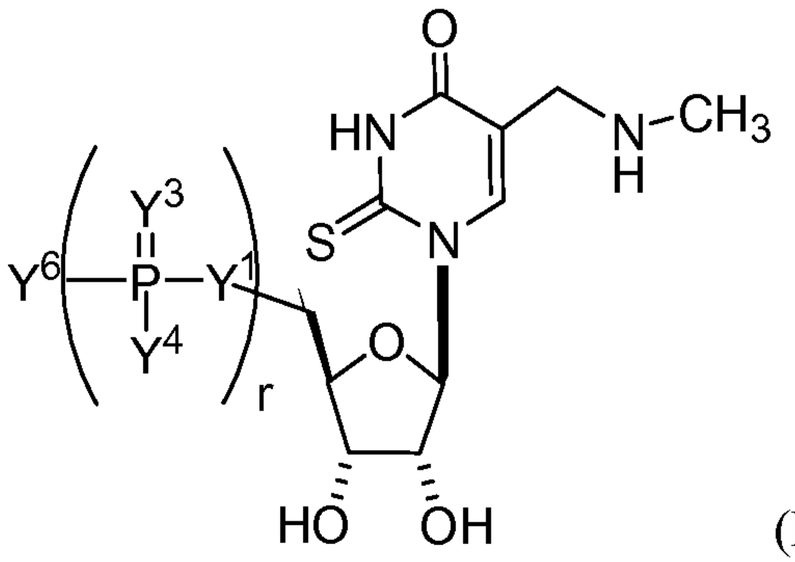
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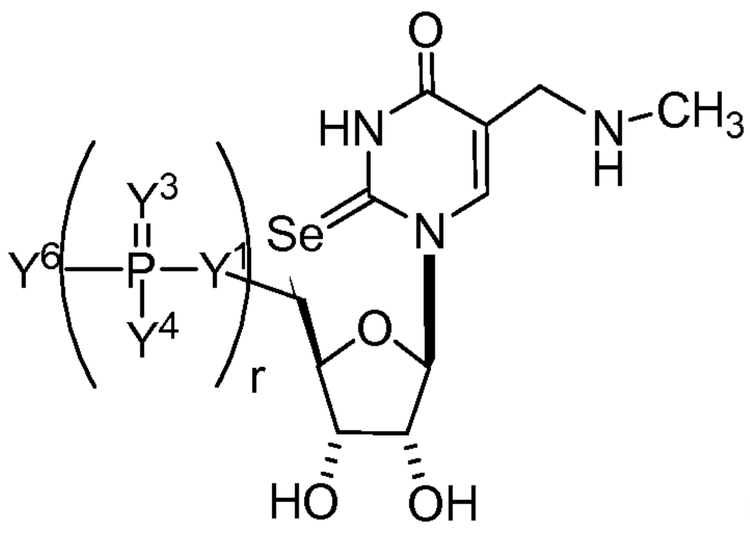




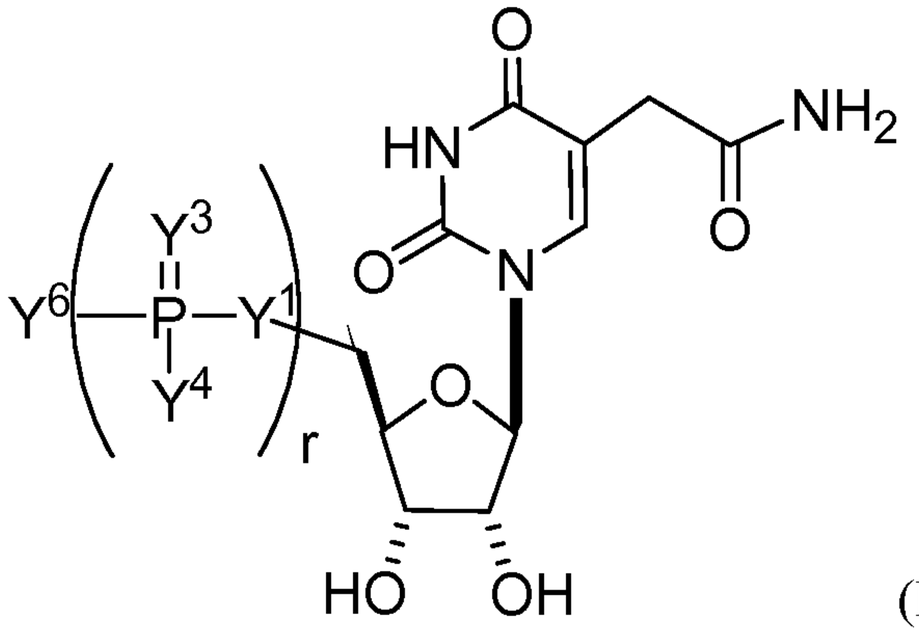
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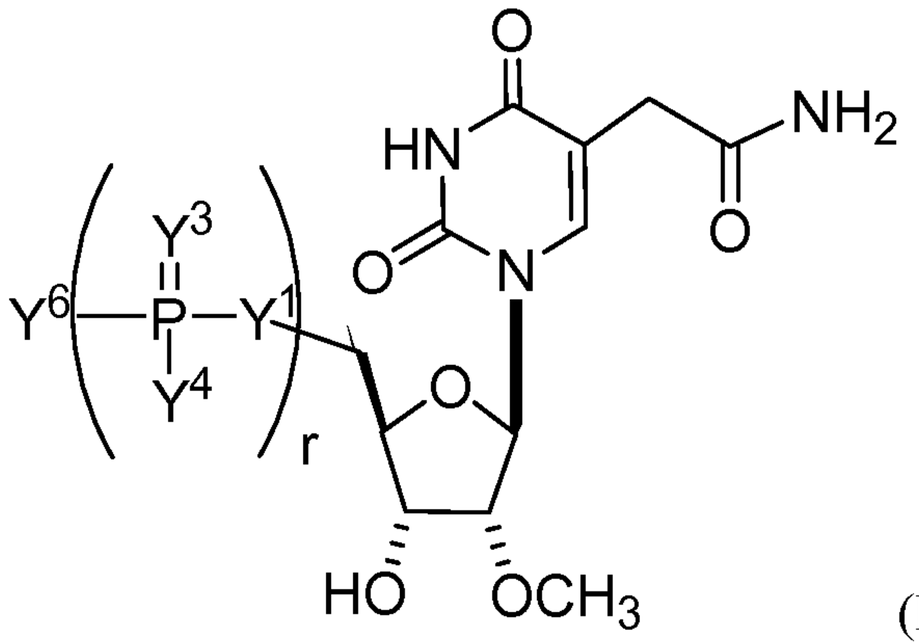
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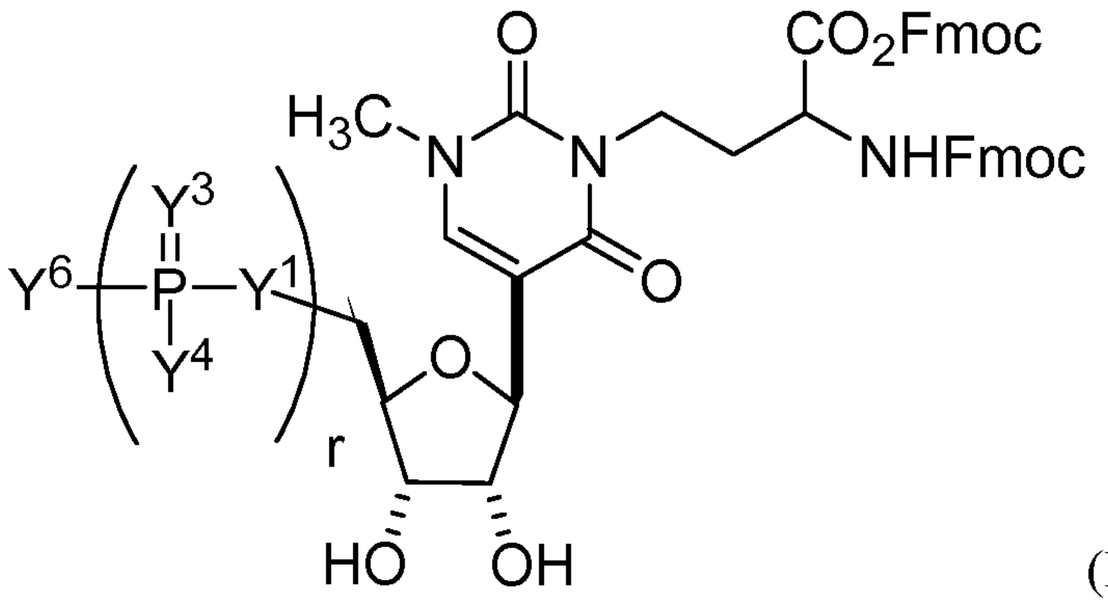


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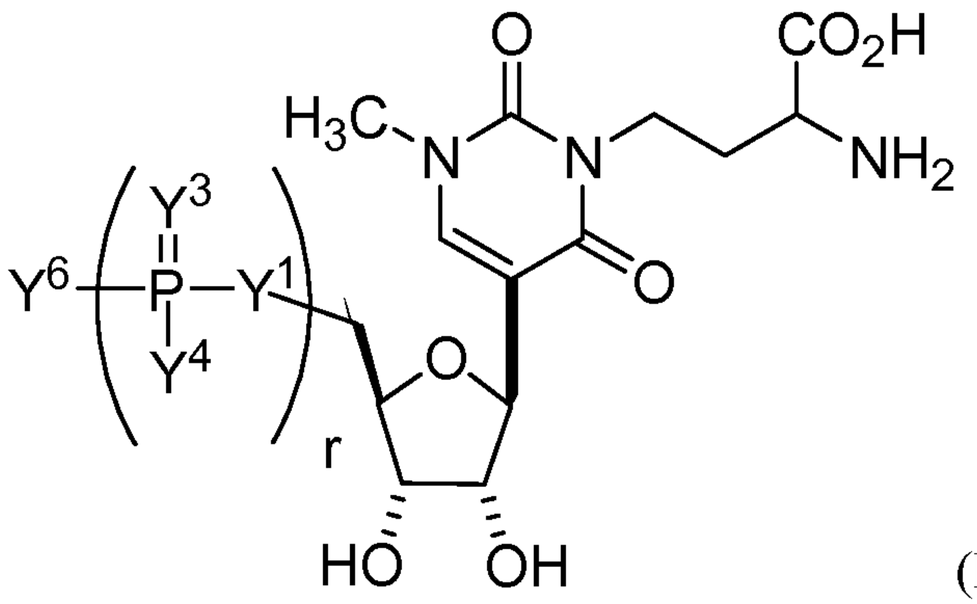


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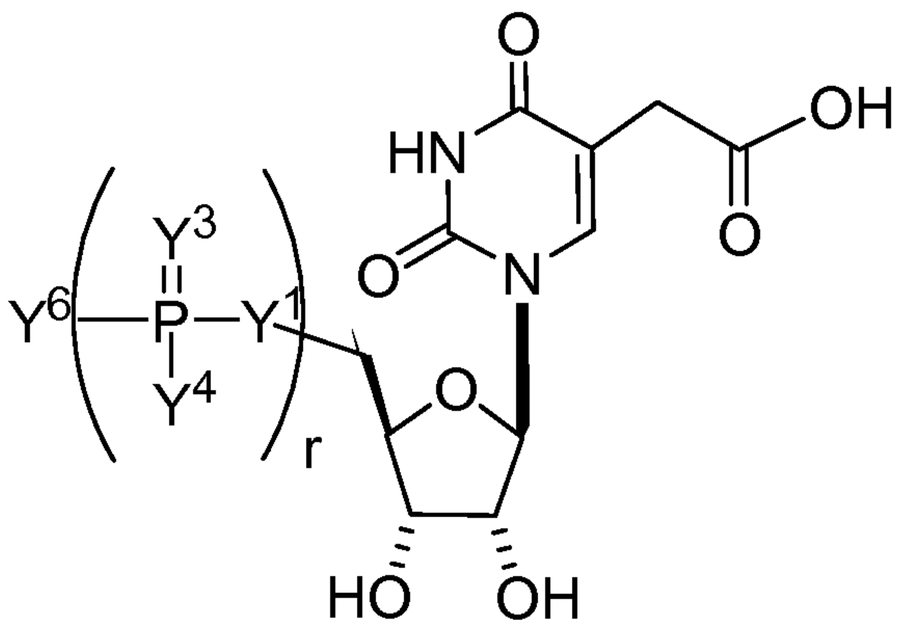
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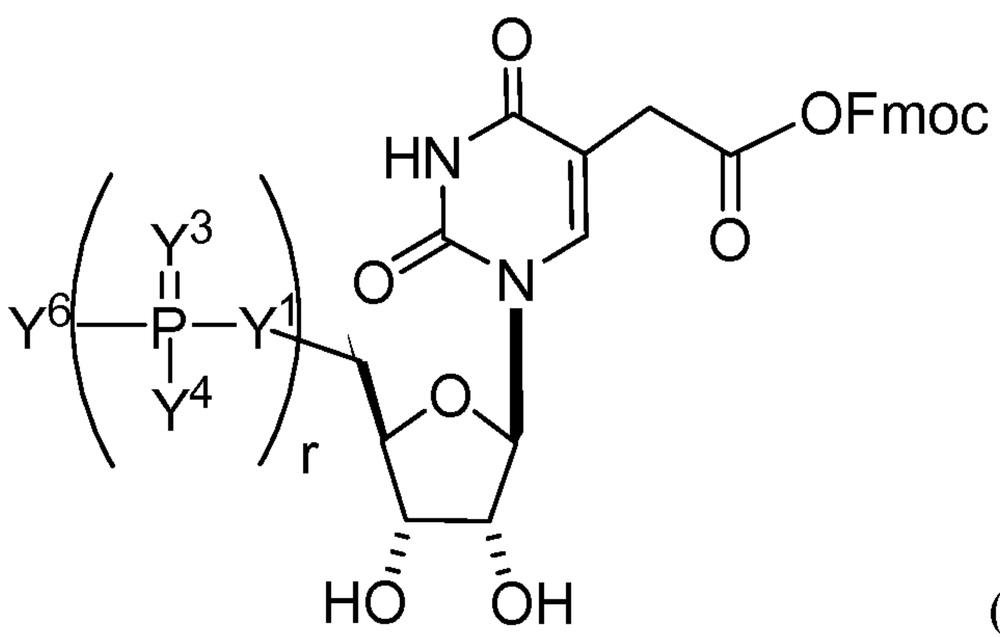
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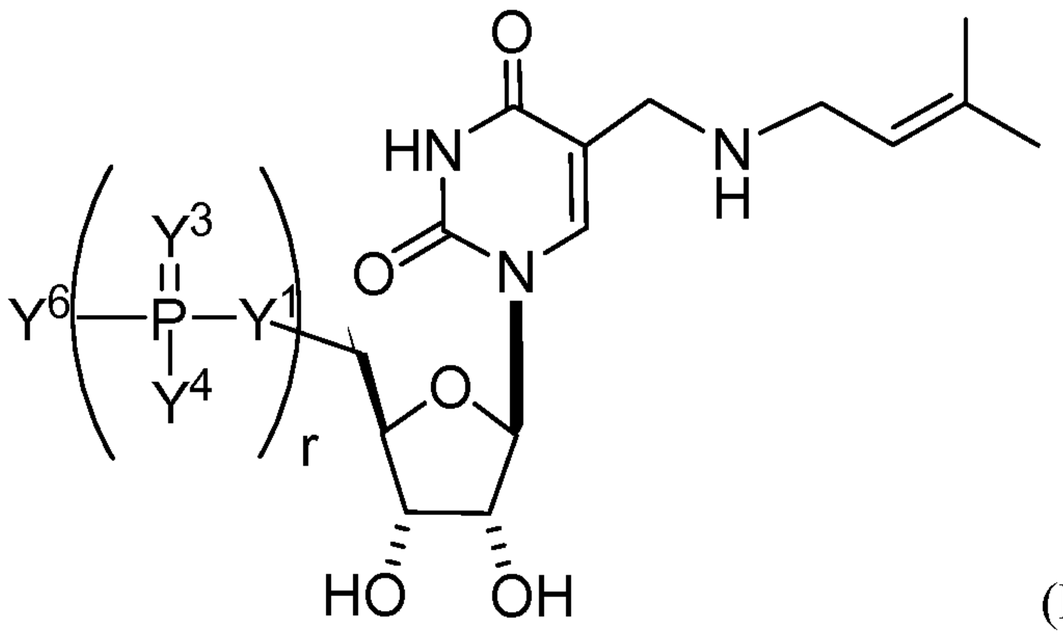
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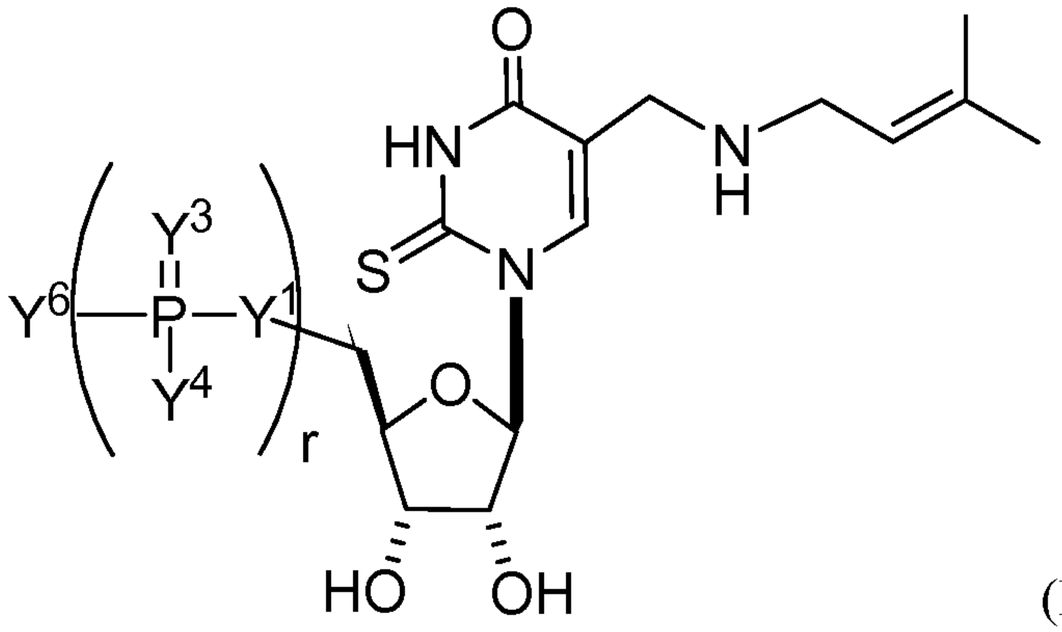
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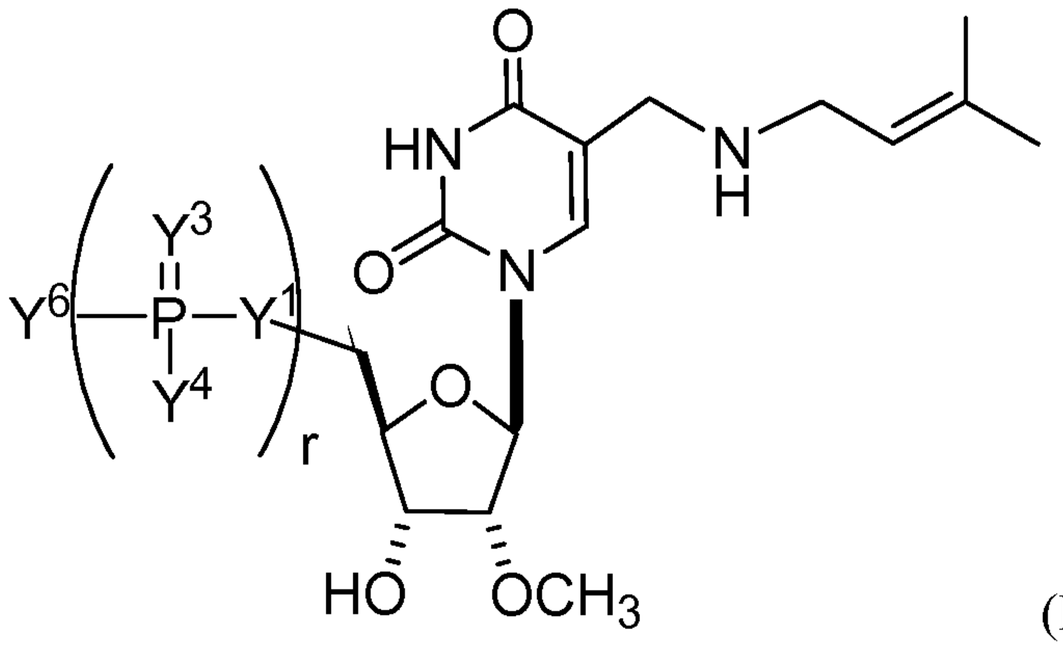
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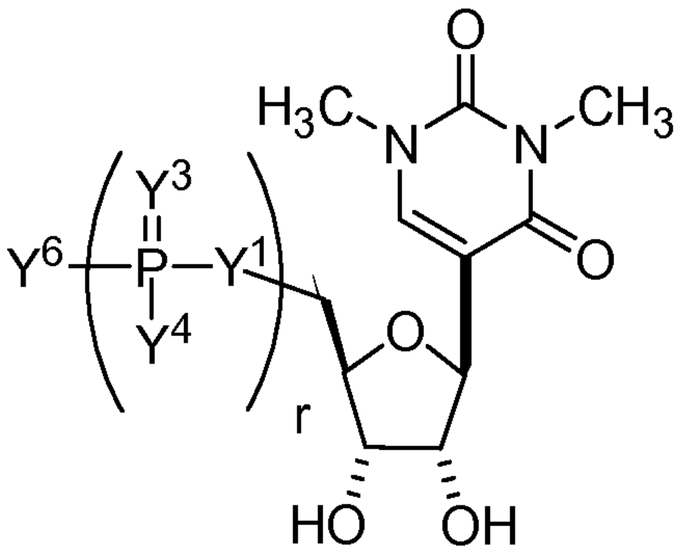
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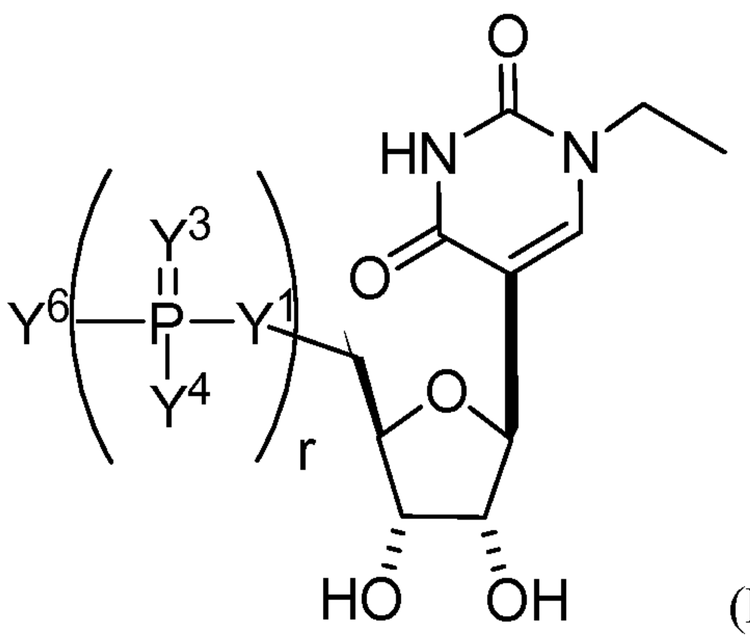
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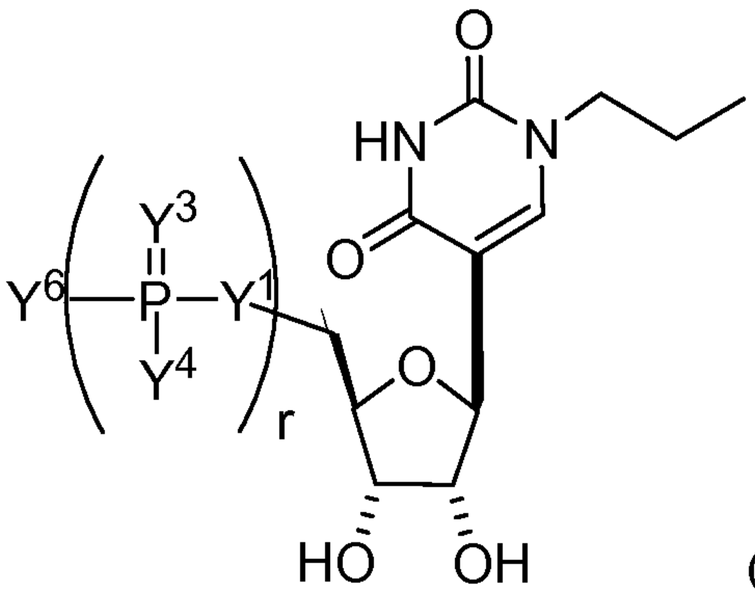
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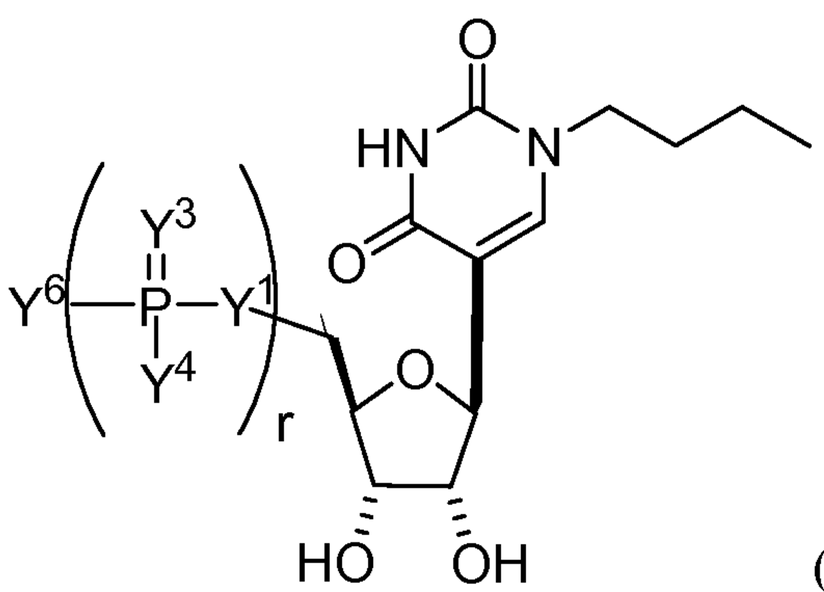
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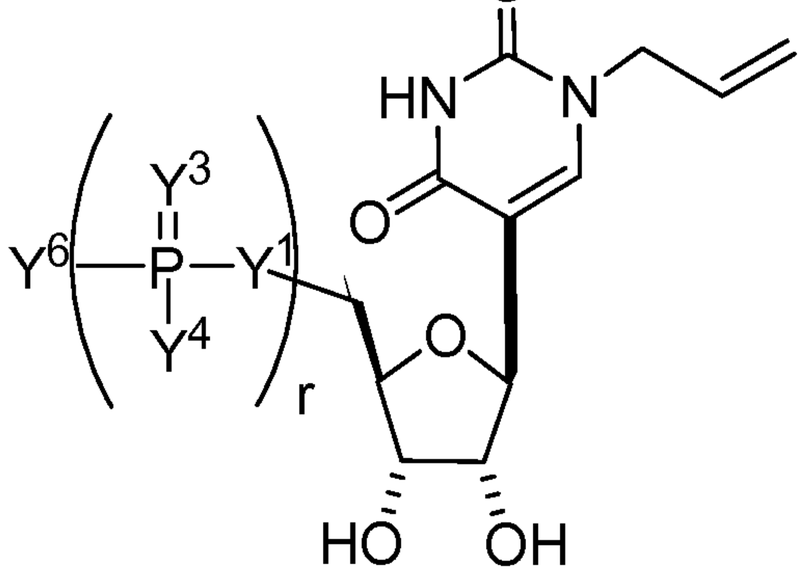


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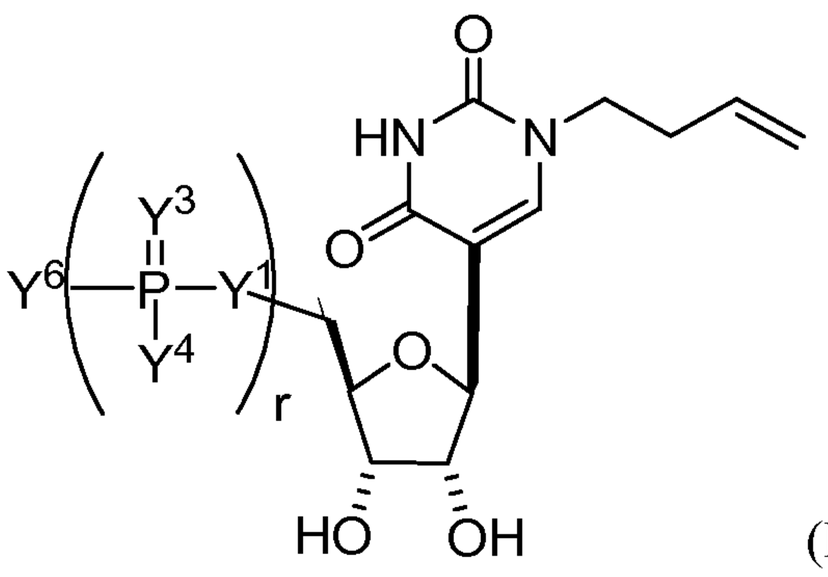


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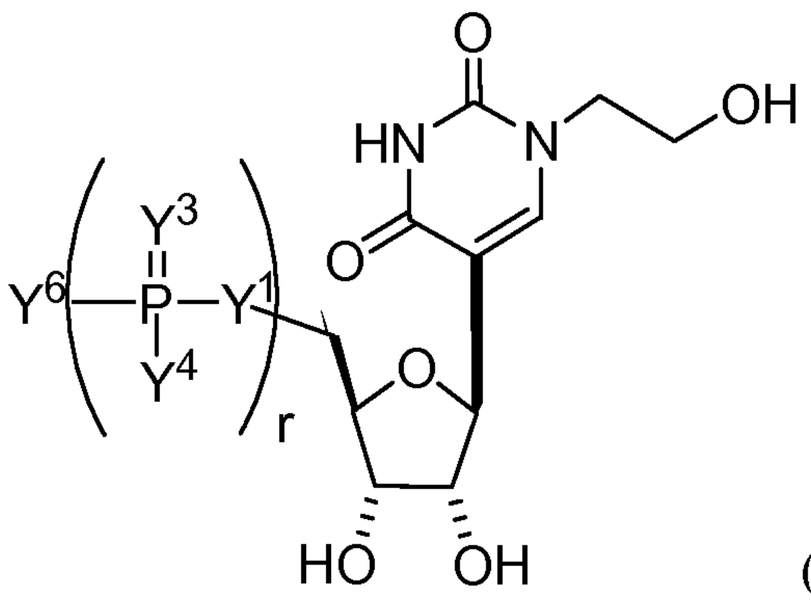
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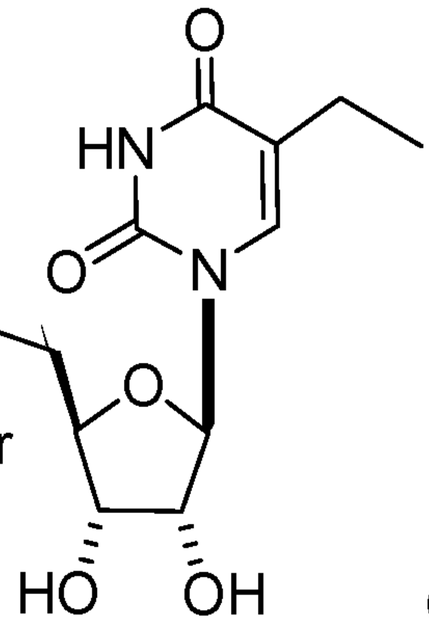
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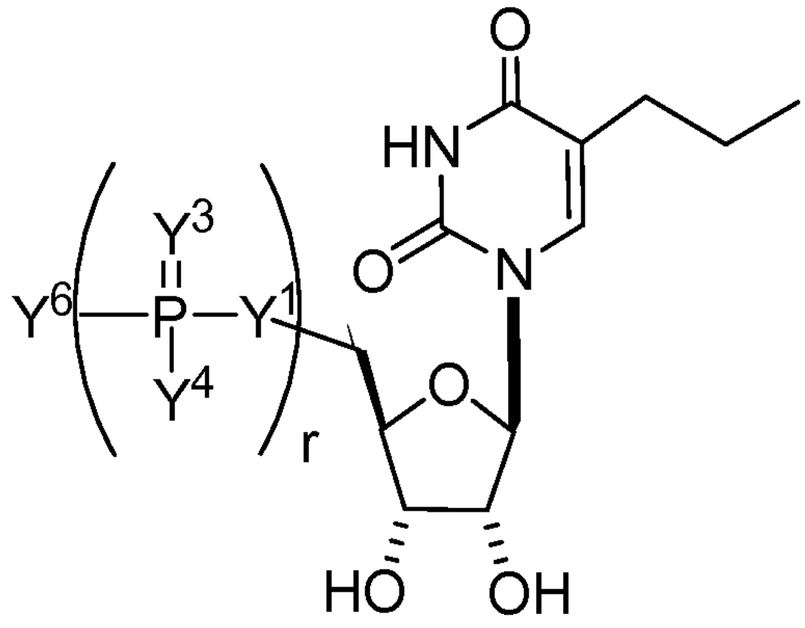
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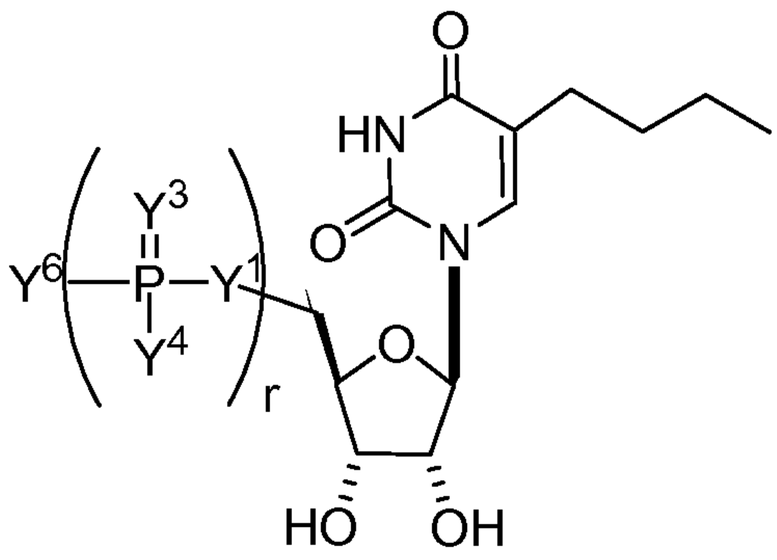
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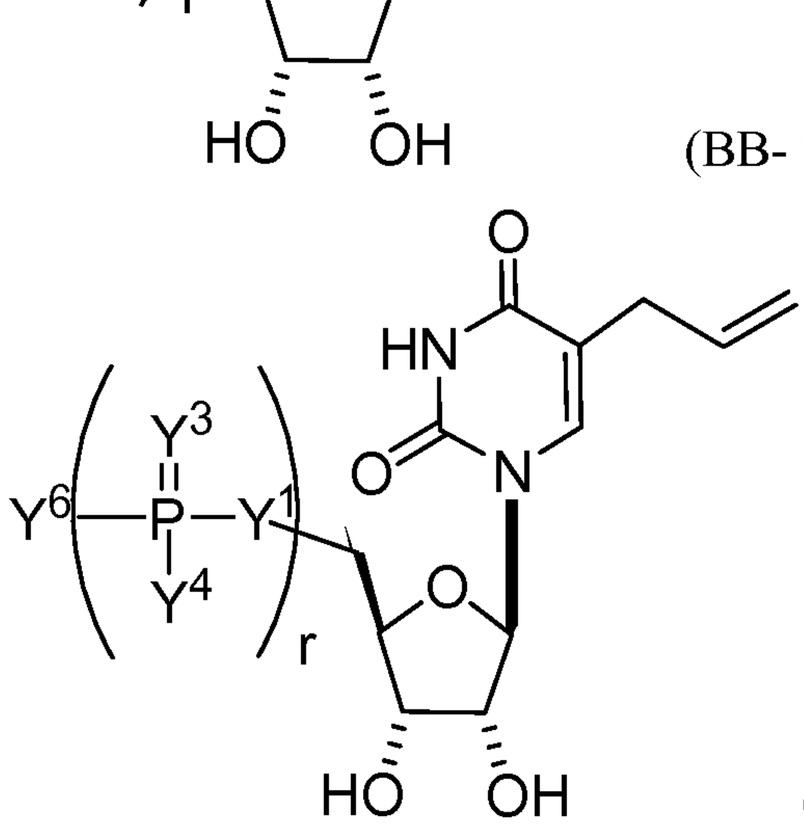
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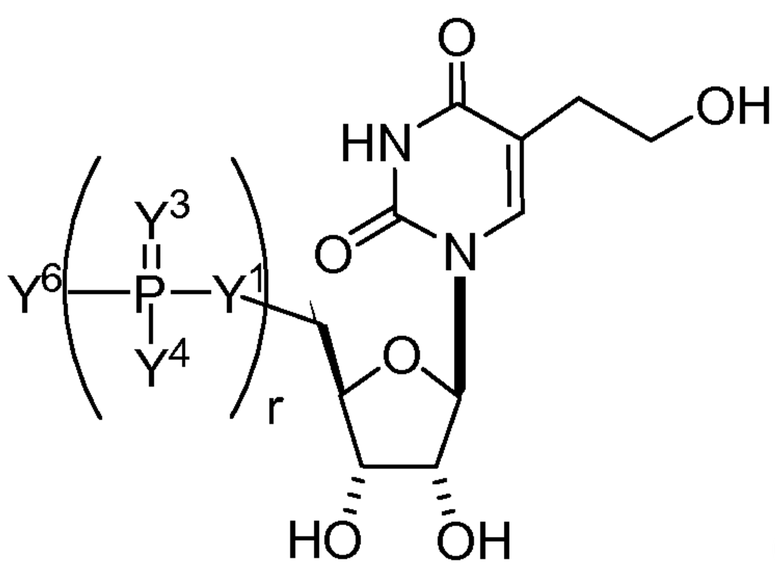
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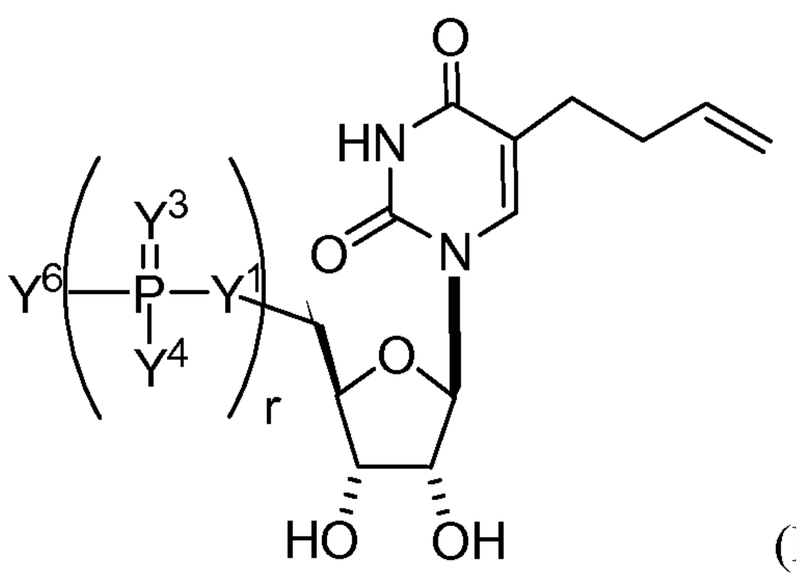
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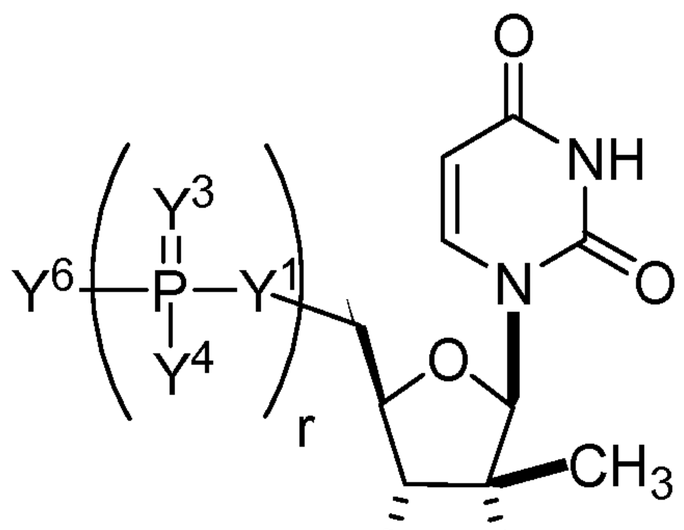
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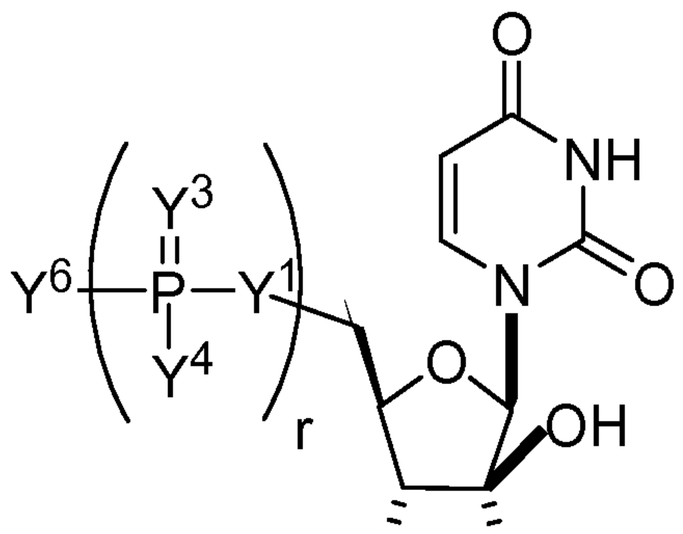
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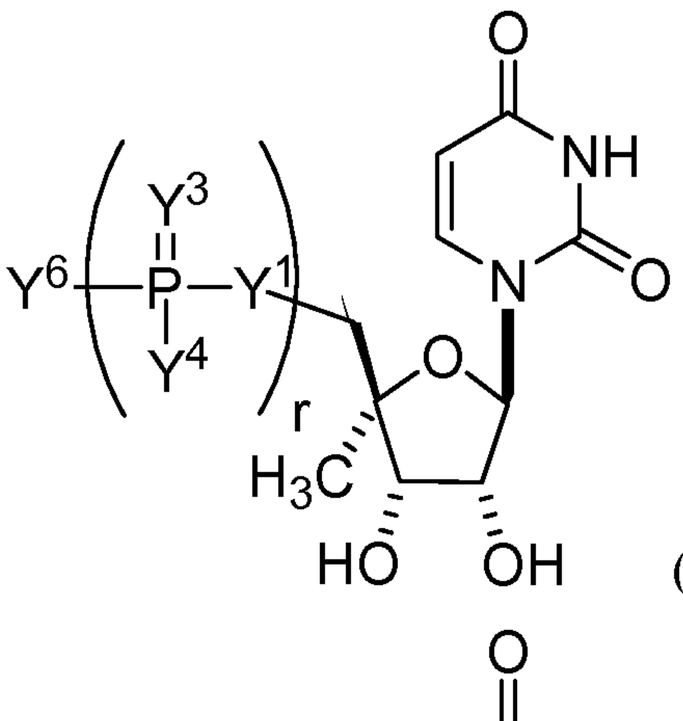
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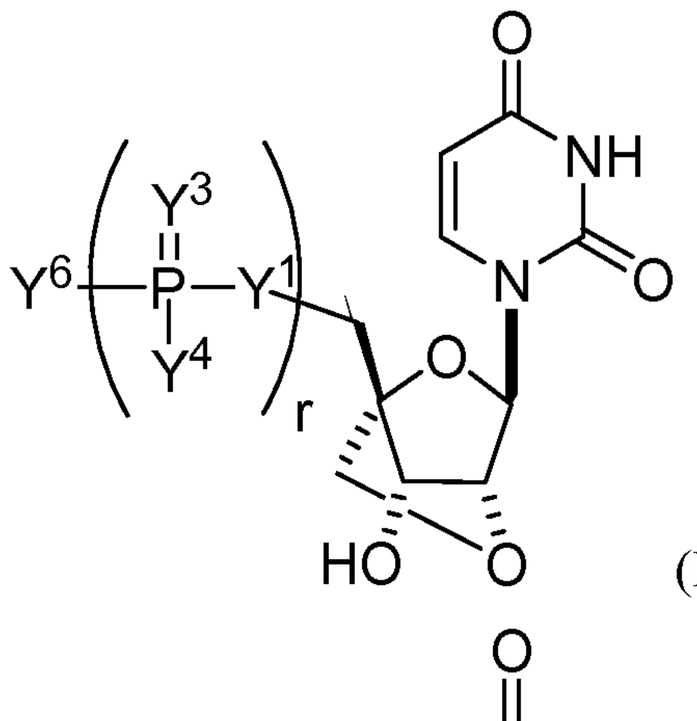
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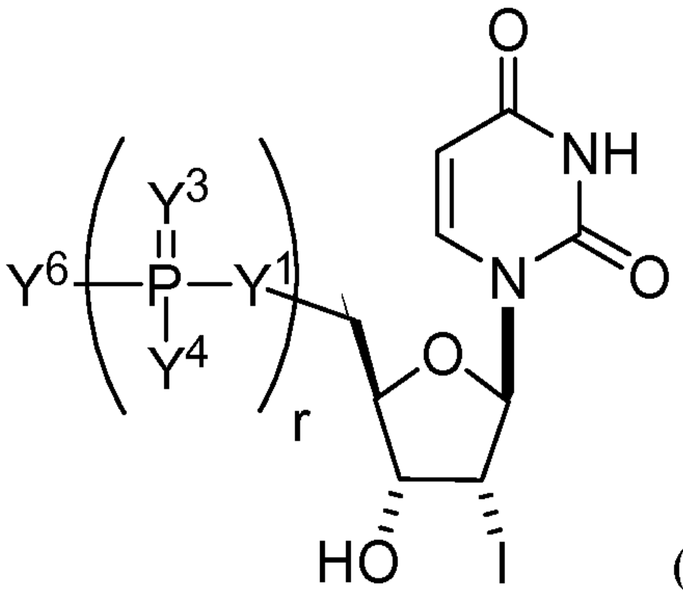
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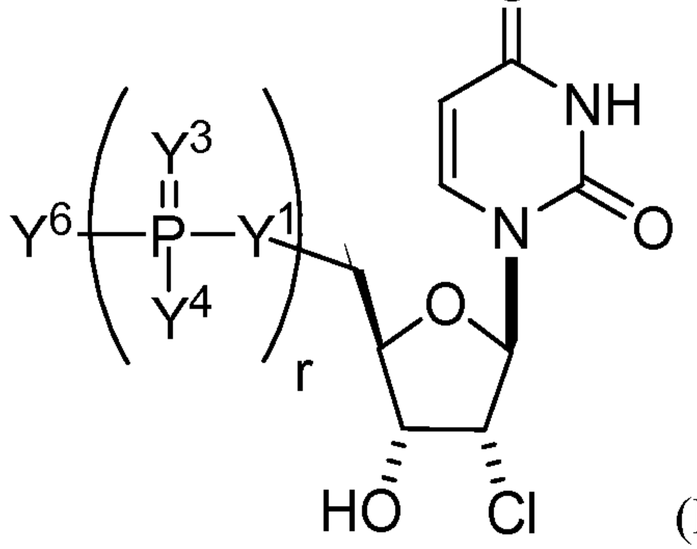


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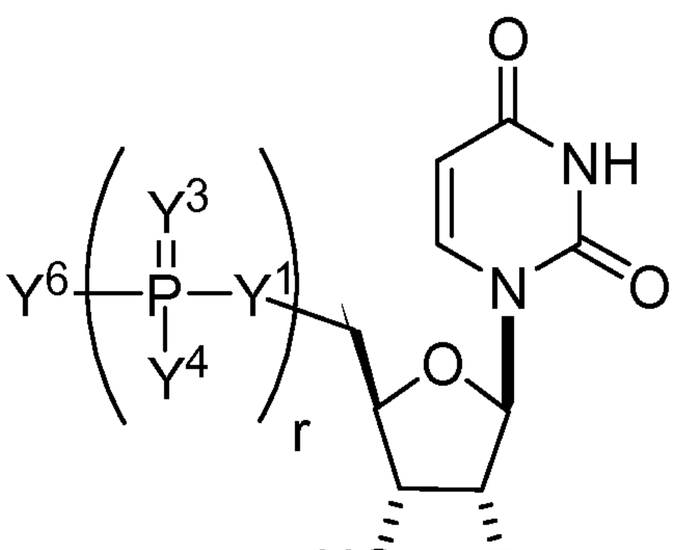


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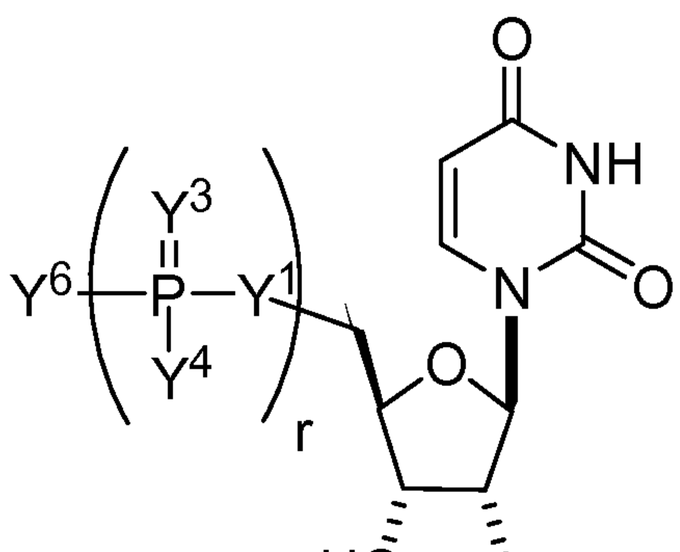
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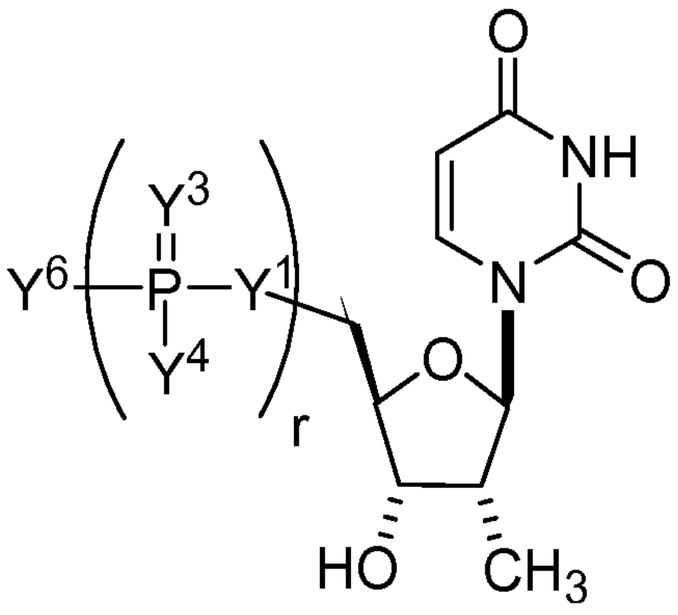
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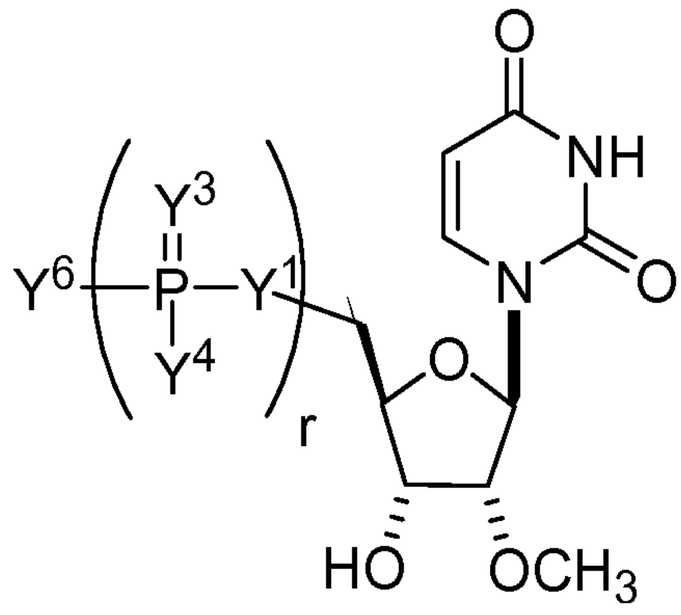
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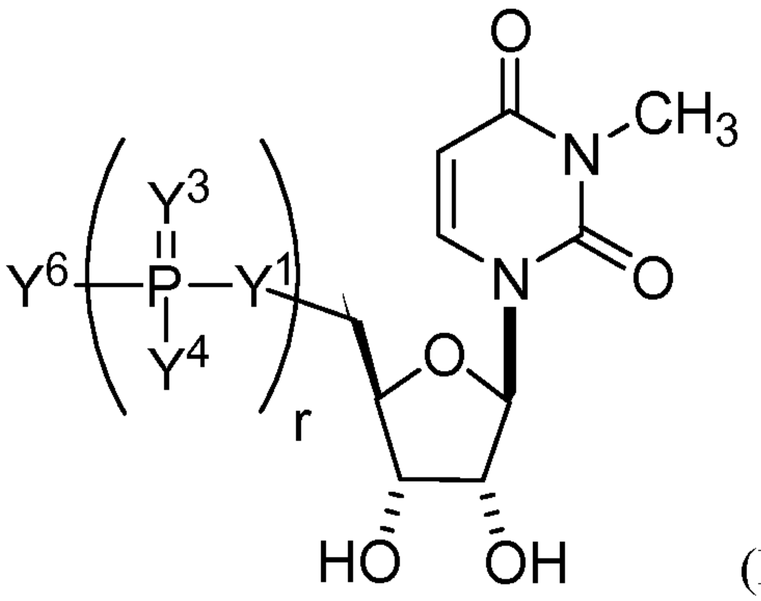
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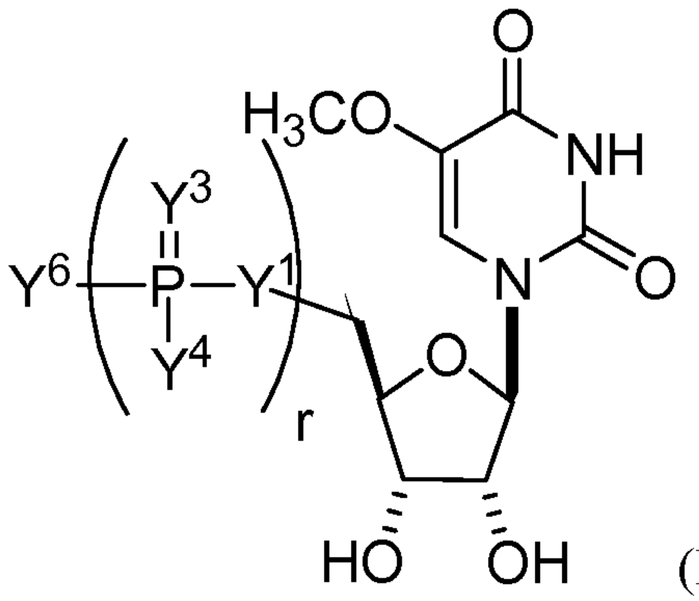
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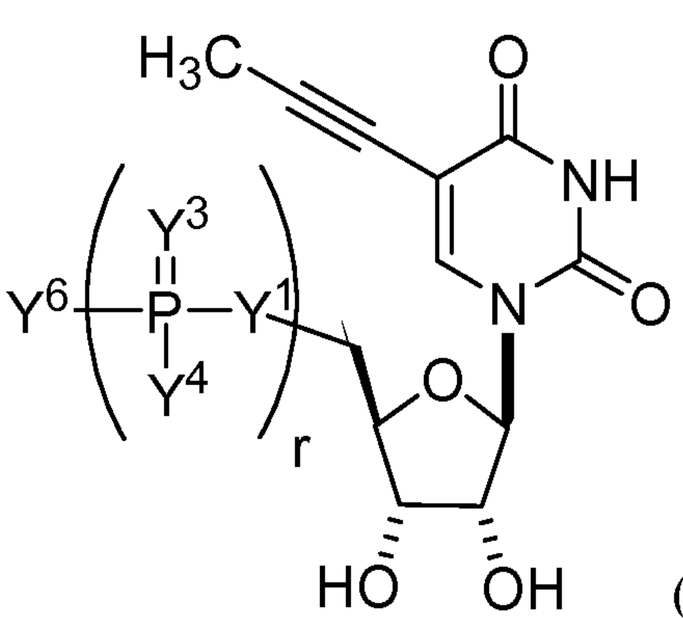
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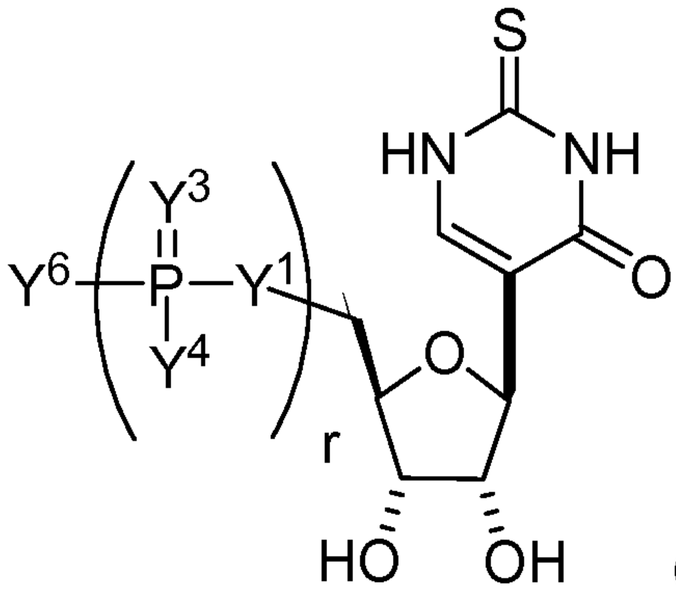
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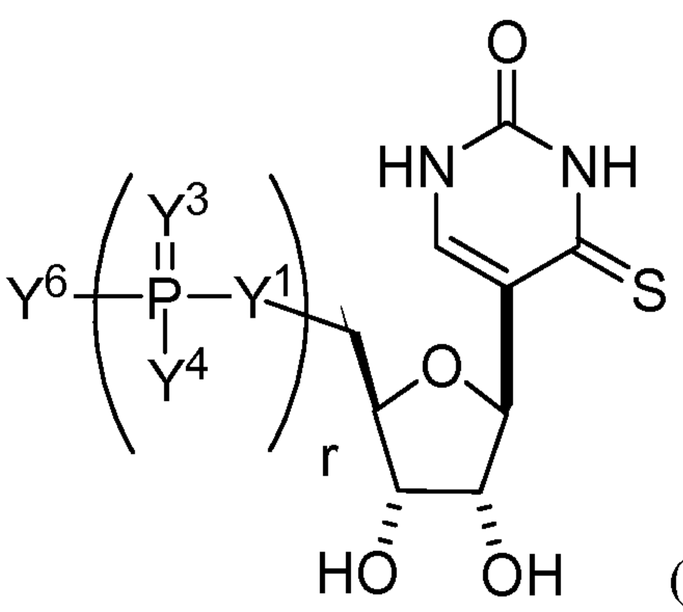
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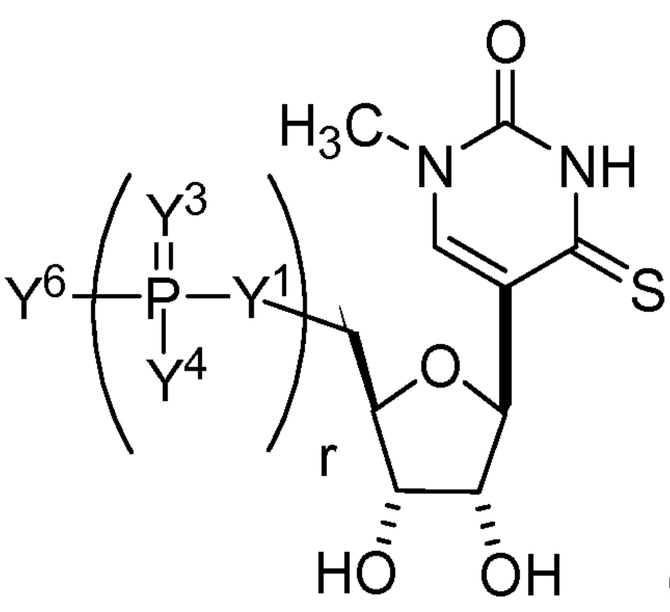
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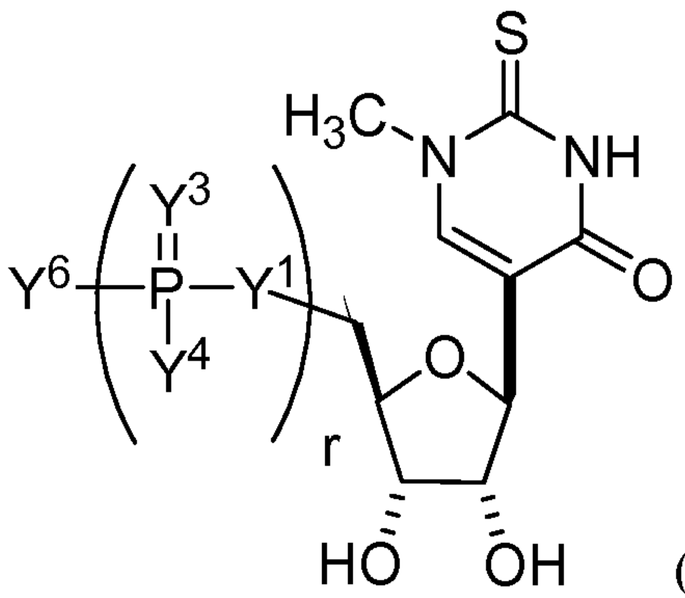
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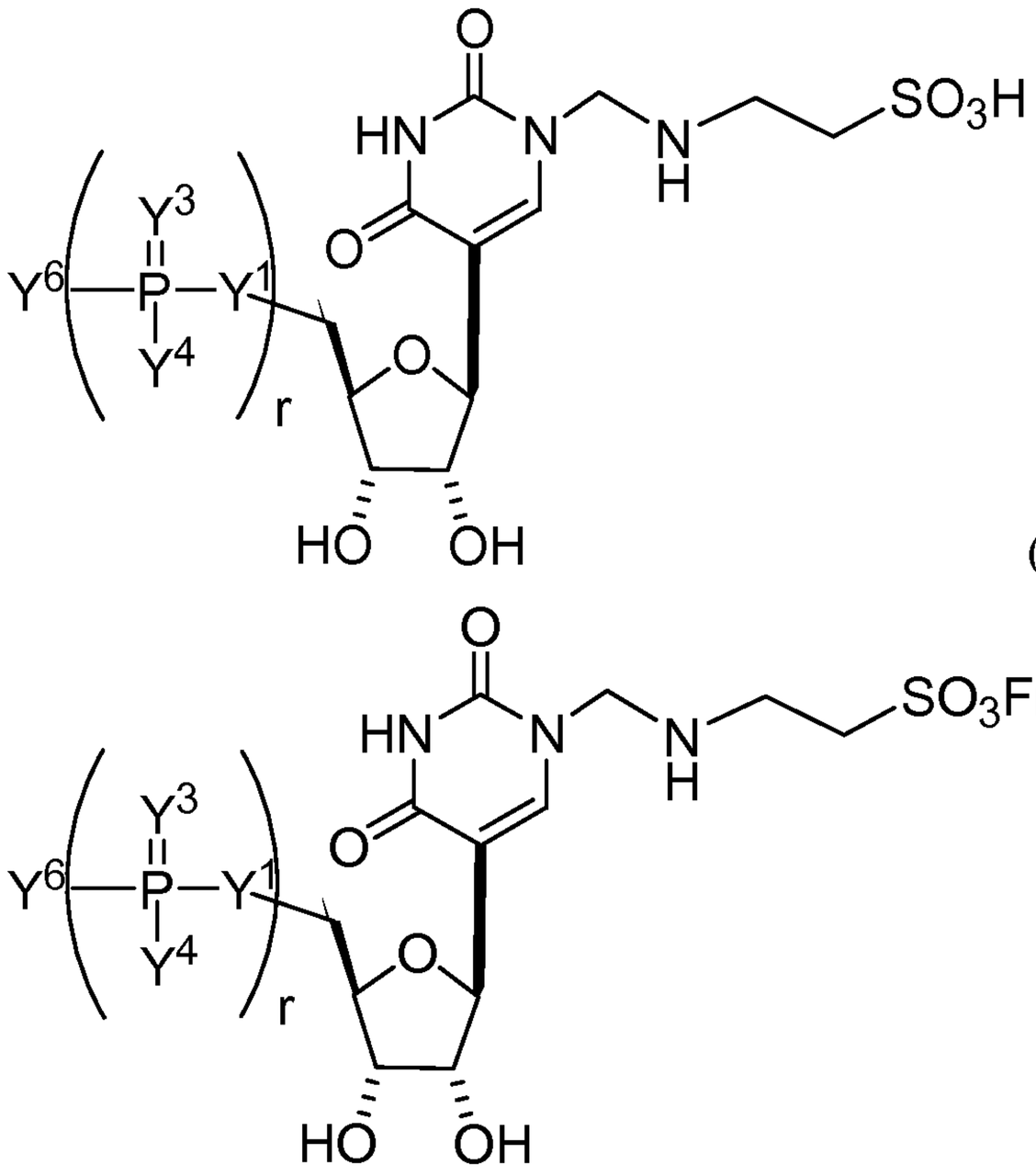
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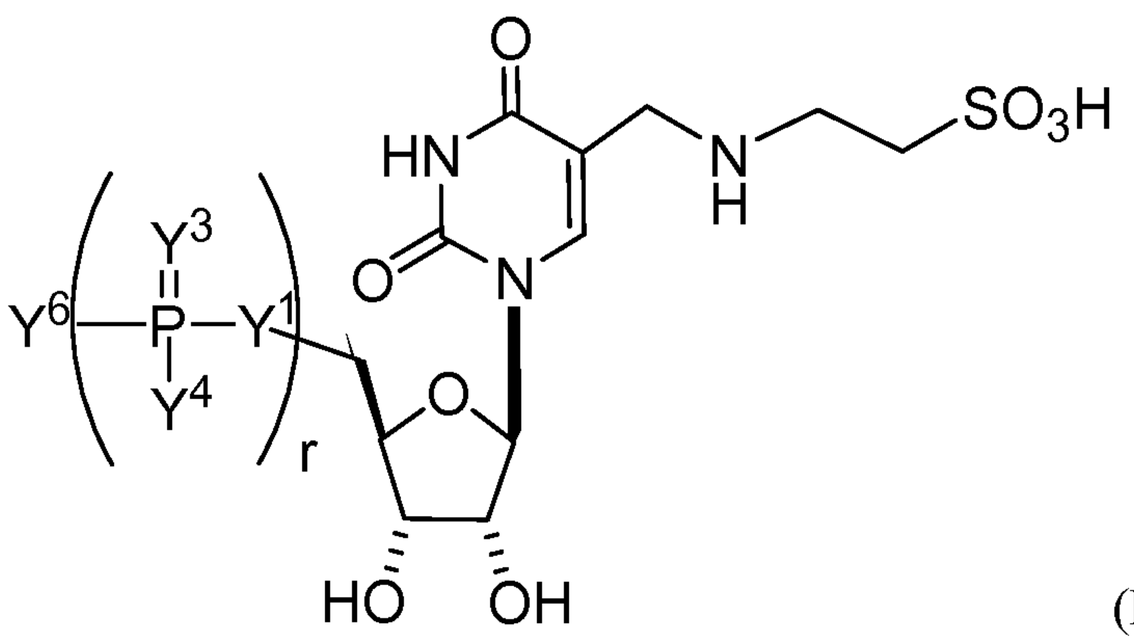


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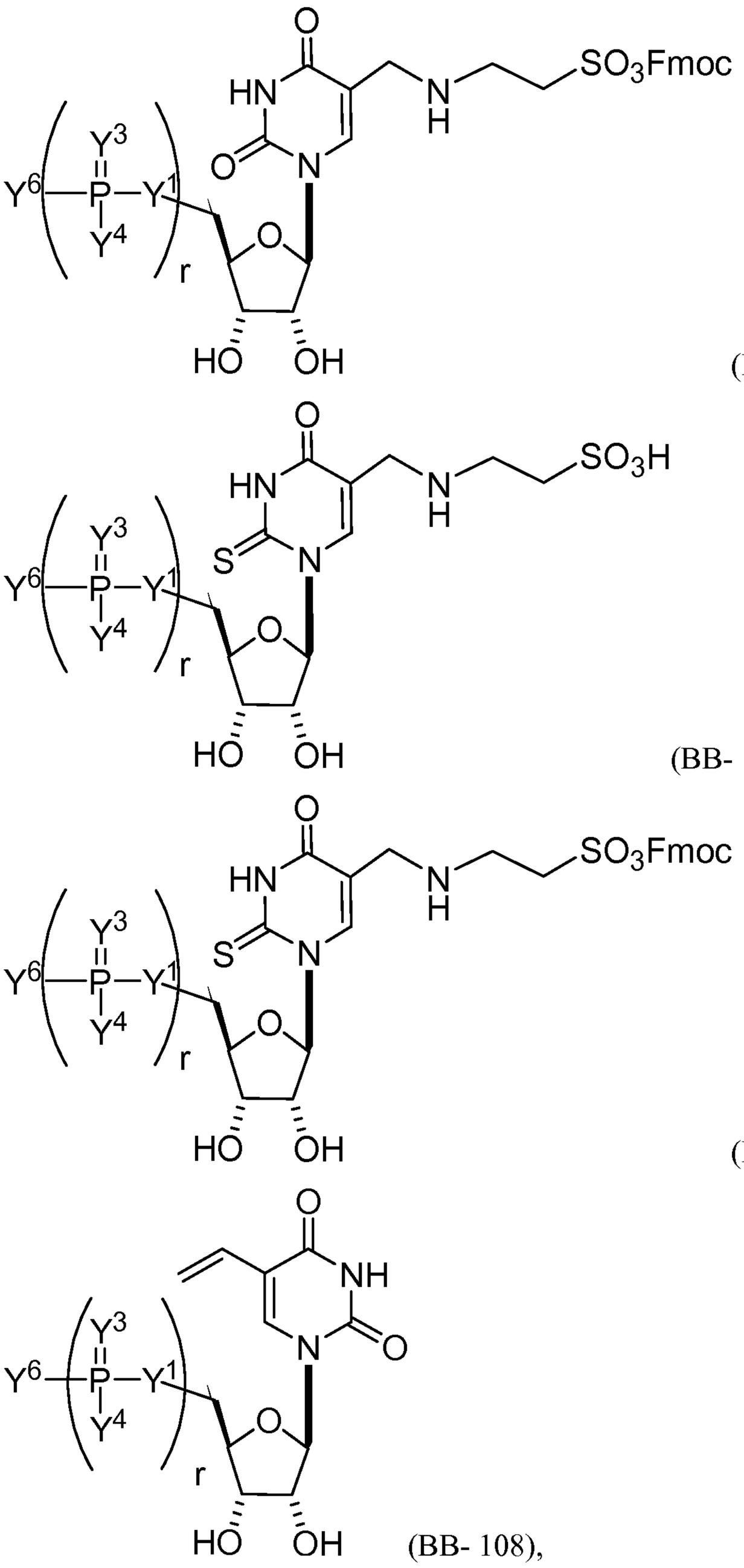


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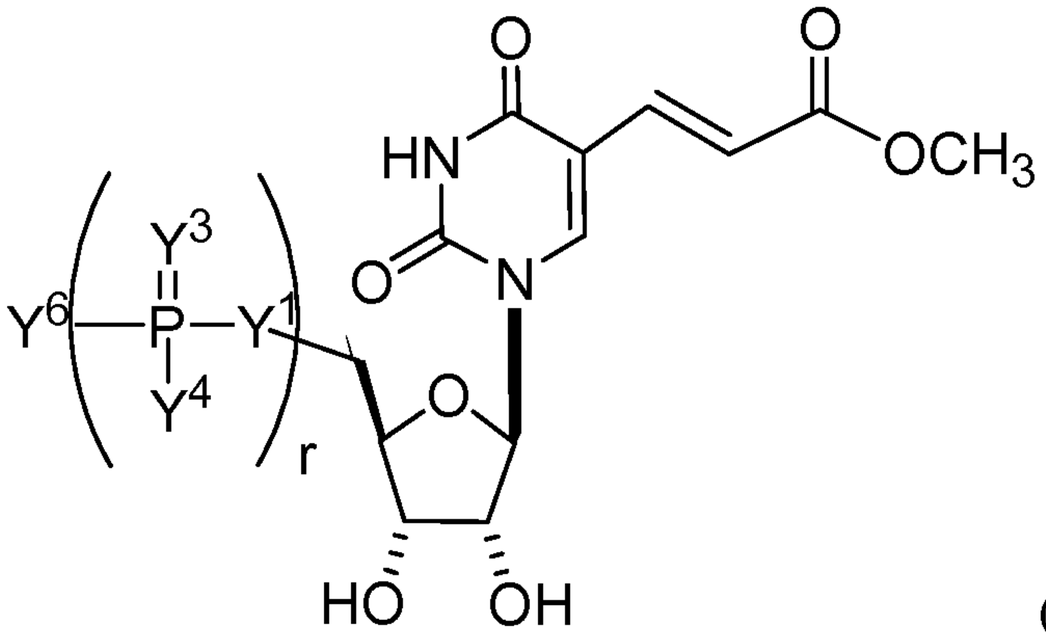


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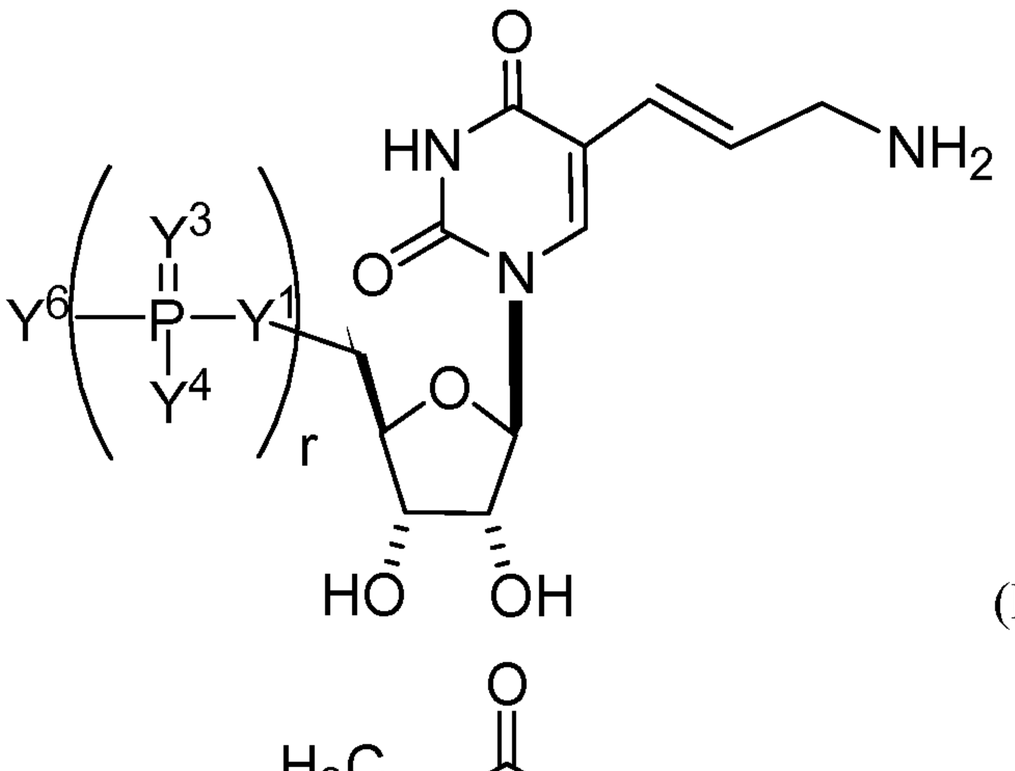
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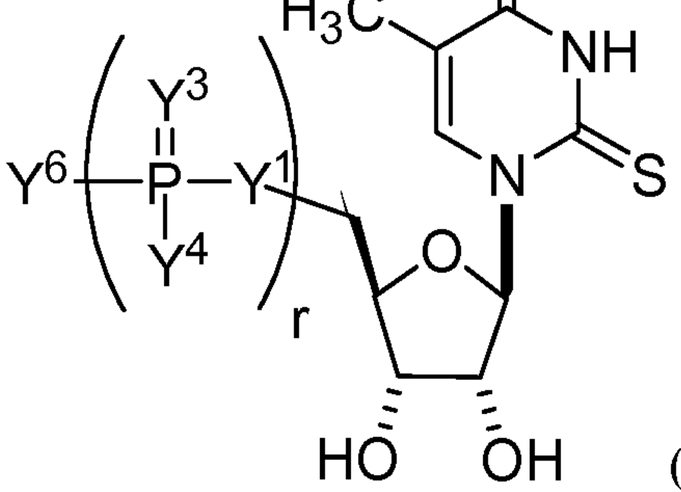
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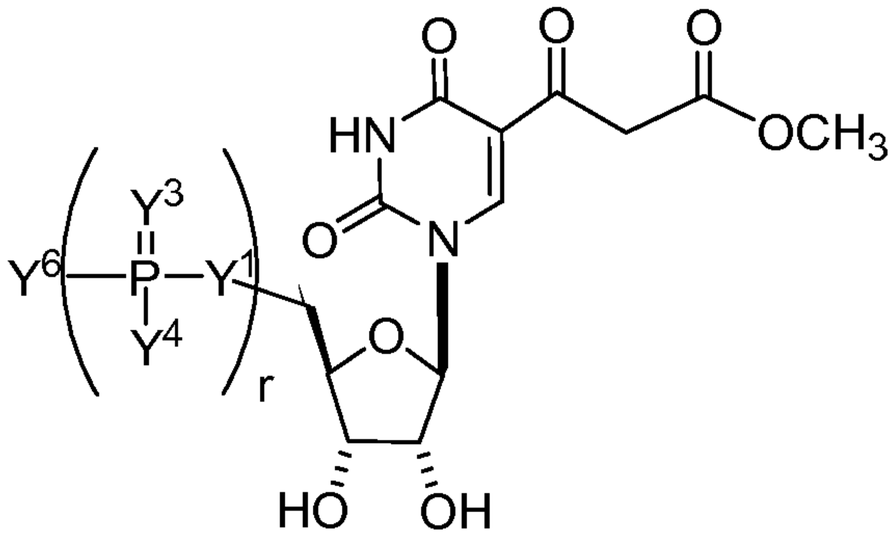
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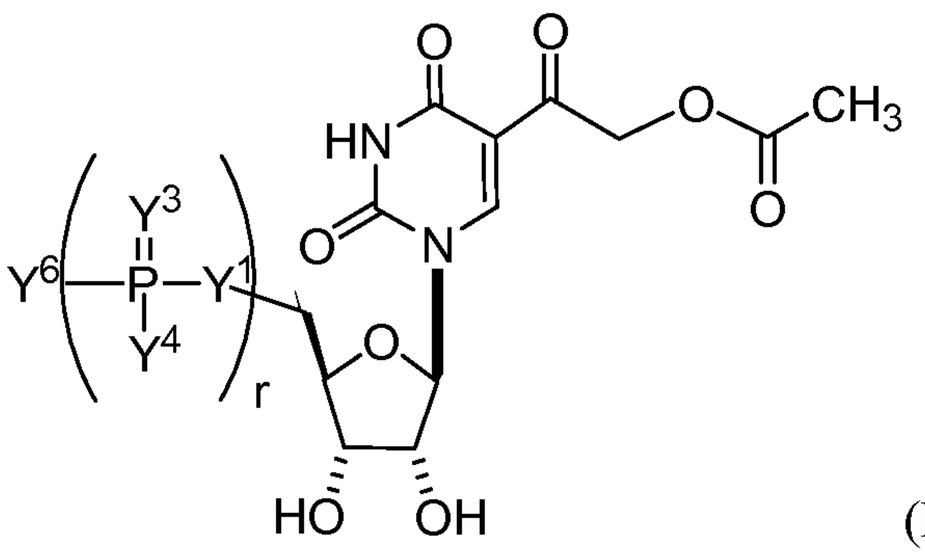
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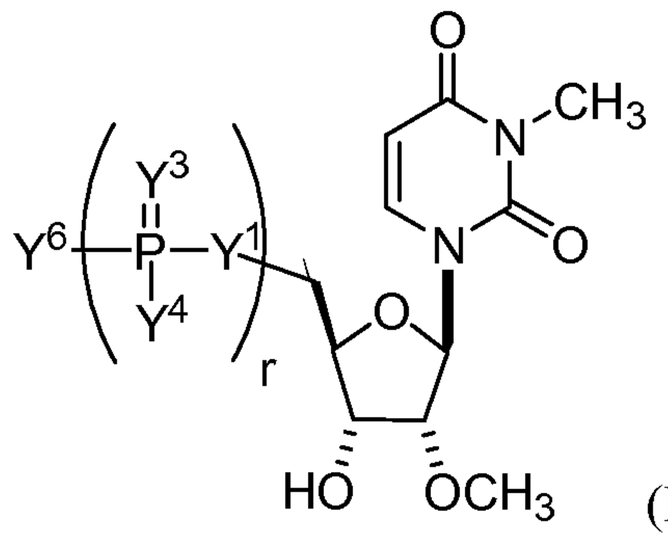
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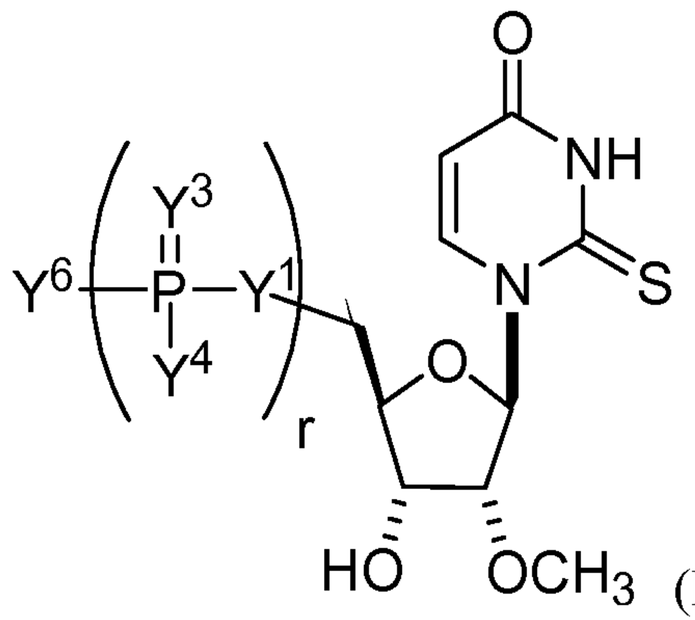
(BB- 112),



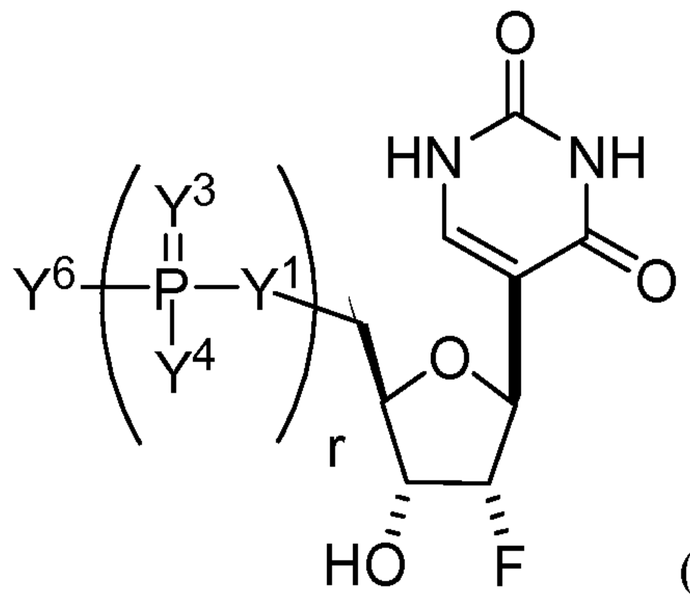
(BB- 113),



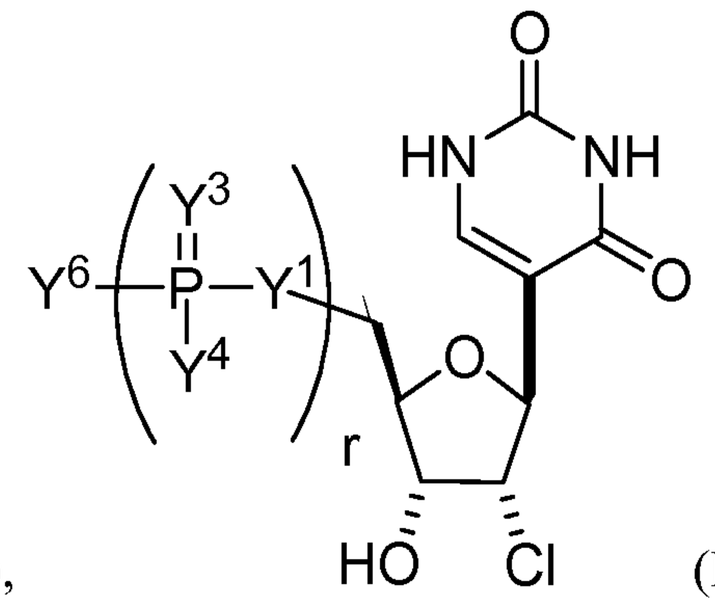
(BB- 114),



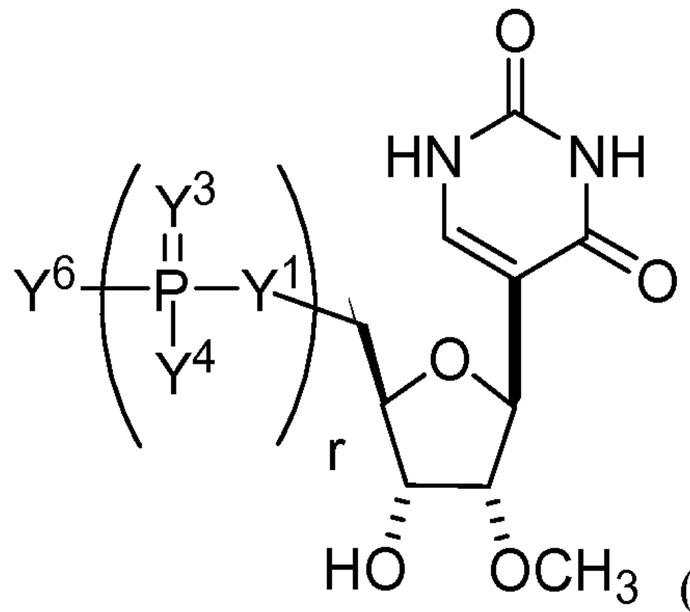
(BB- 115),



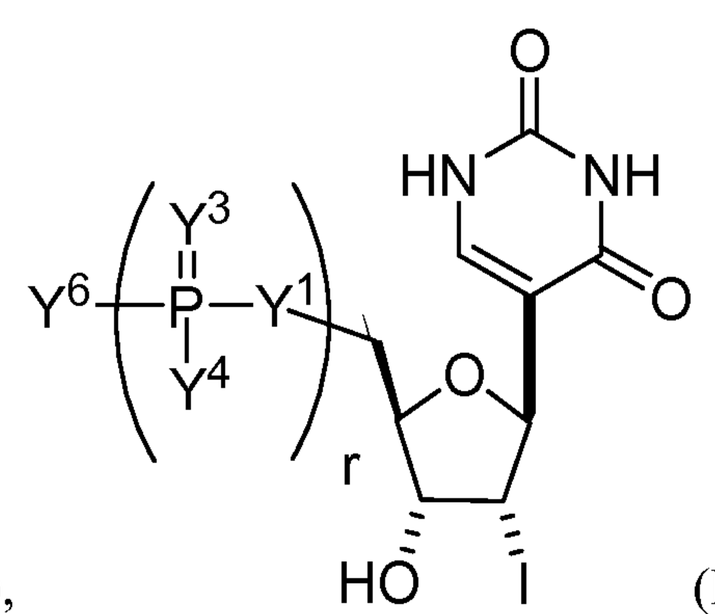
(BB- 116),



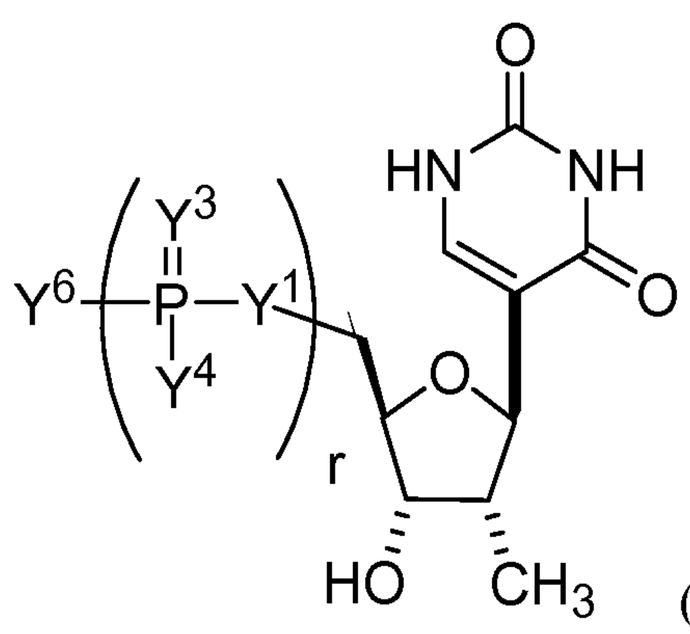
(BB- 117),



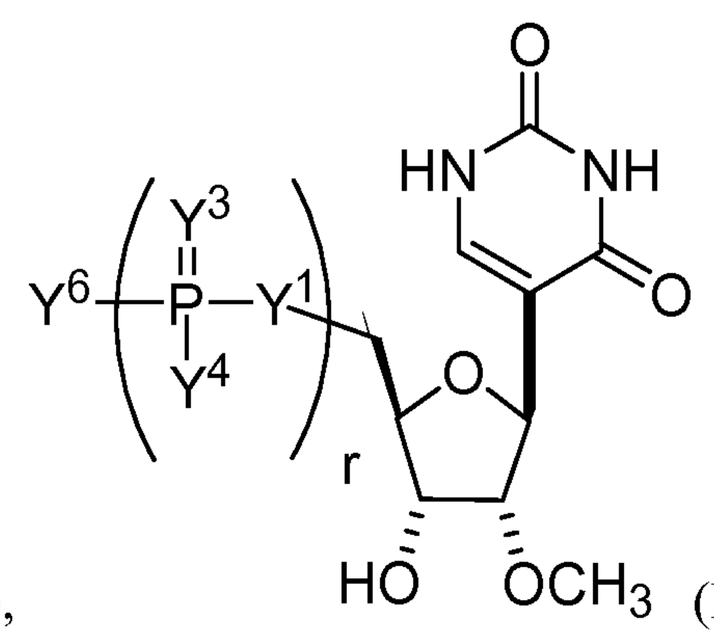
(BB- 118),



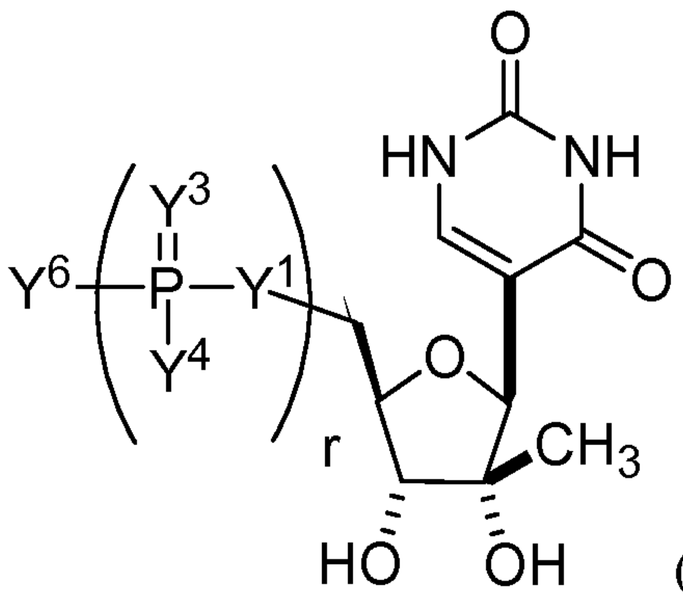
(BB- 119),



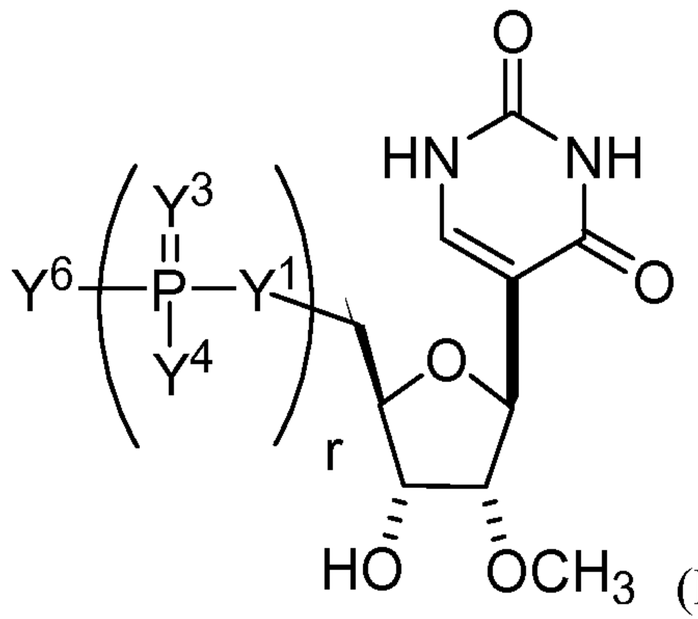
(BB- 120),



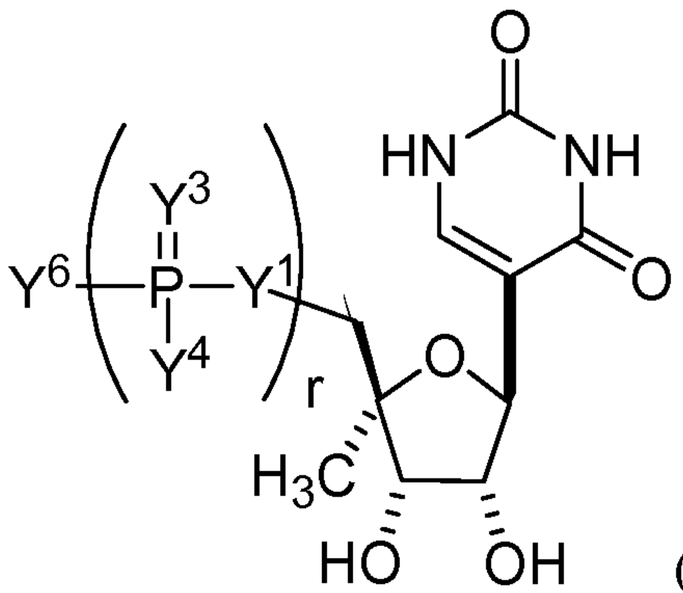
(BB- 121),



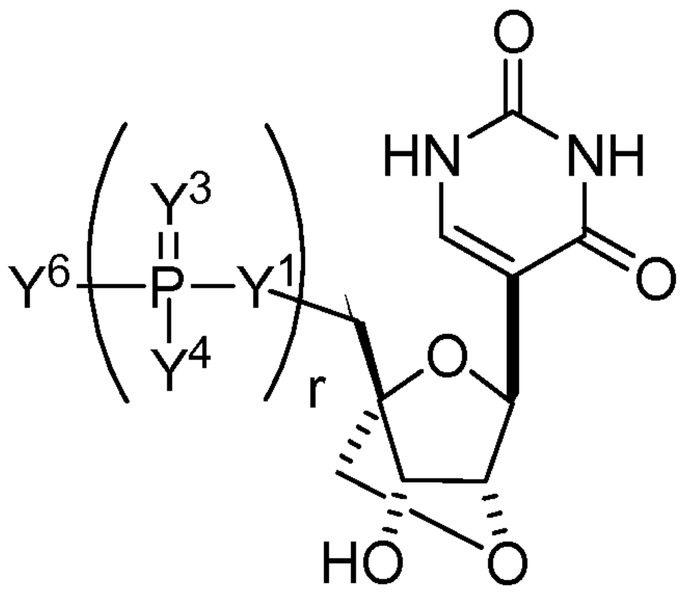
(BB- 122),



(BB- 123),

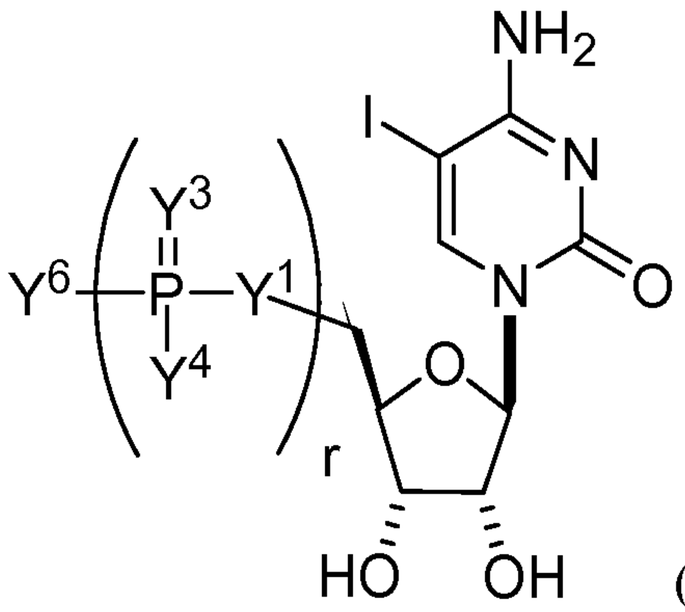


(BB- 124), and

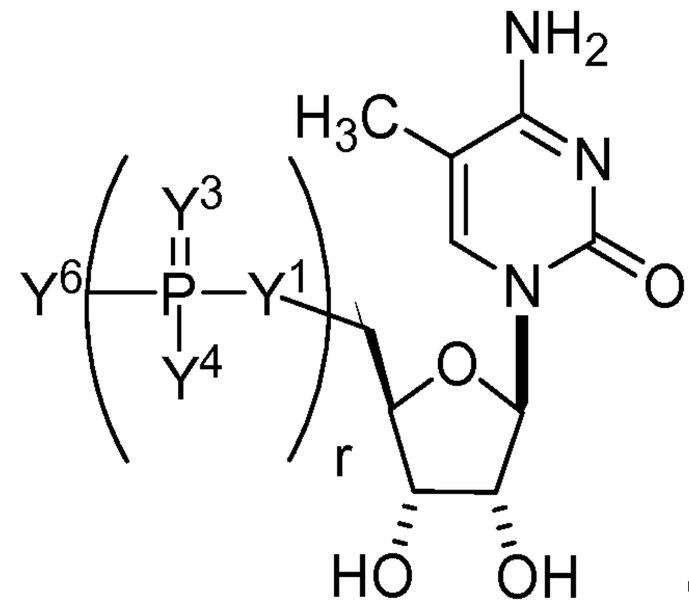


(BB- 125), or a pharmaceutically acceptable salt or stereoisomer thereof, wherein Y^1, Y^3, Y^4, Y^6, and r are as described herein (e.g., each r is, independently, an integer from 0 to 5, such as from 0 to 3, from 1 to 3, or from 1 to 5)).

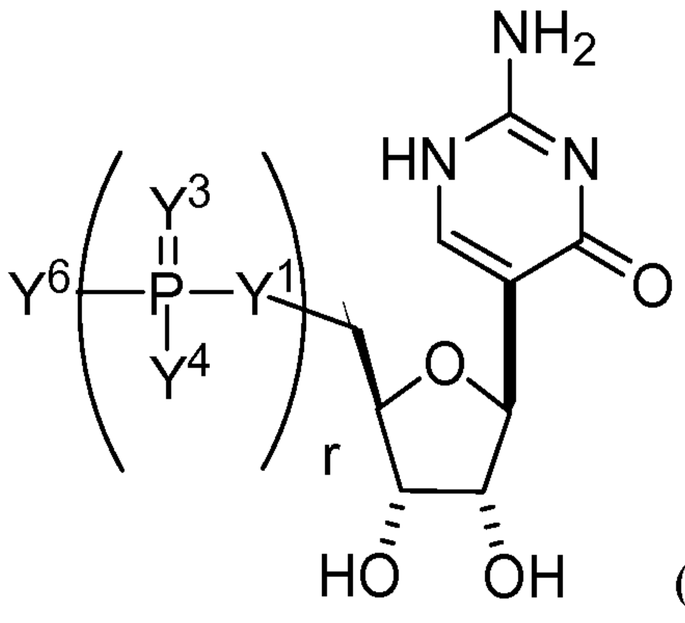
[000416] In some embodiments, the building block molecule, which may be incorporated into a polynucleotide, primary construct, or mmRNA, is a modified cytidine (e.g., selected from the group consisting of:



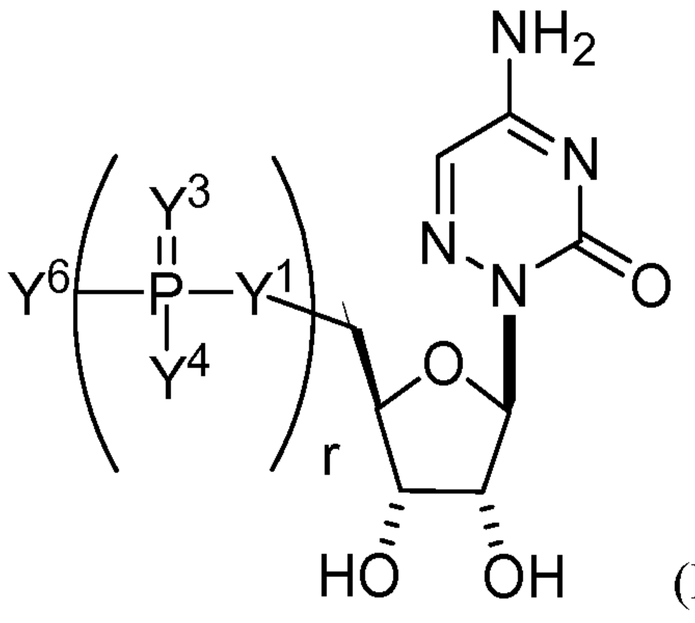
(BB- 126),



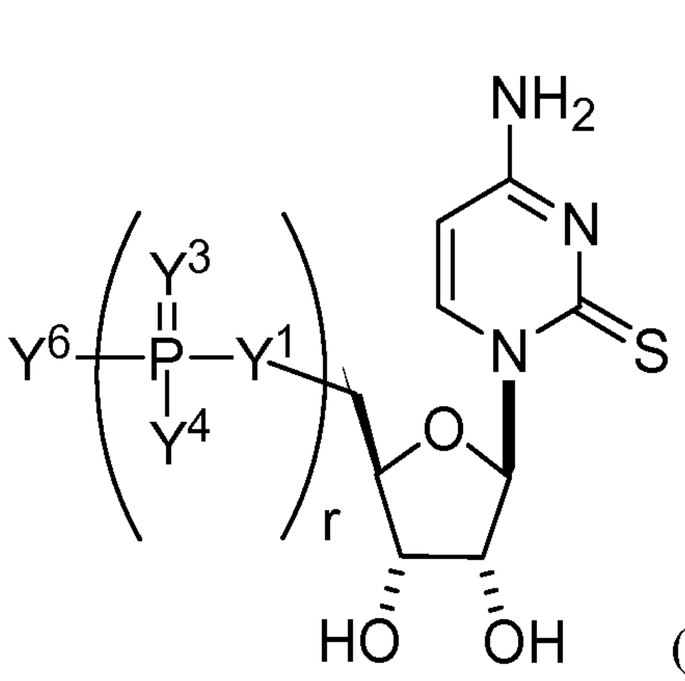
(BB- 127),



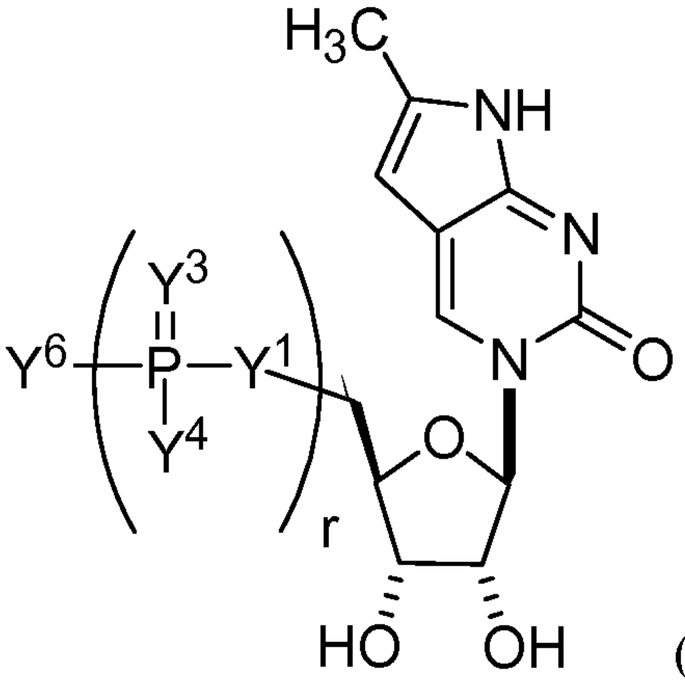
(BB- 128),



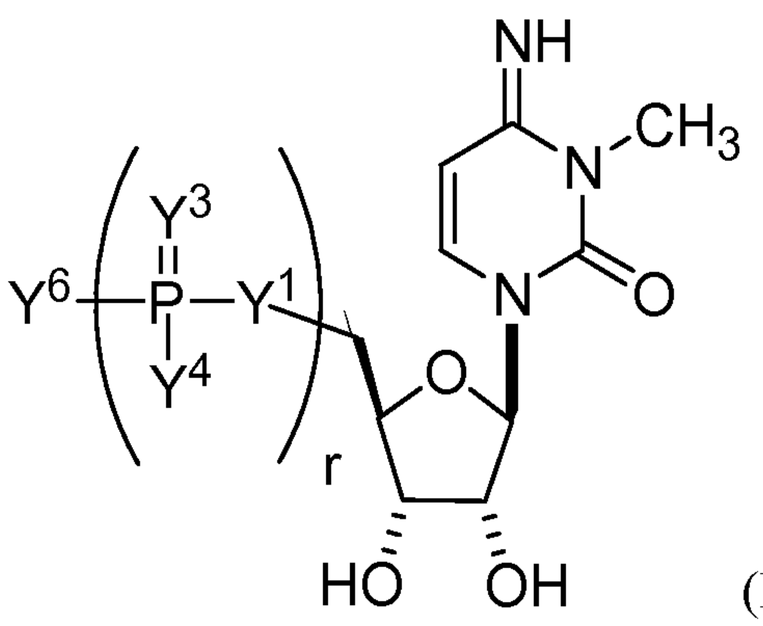
(BB- 129),



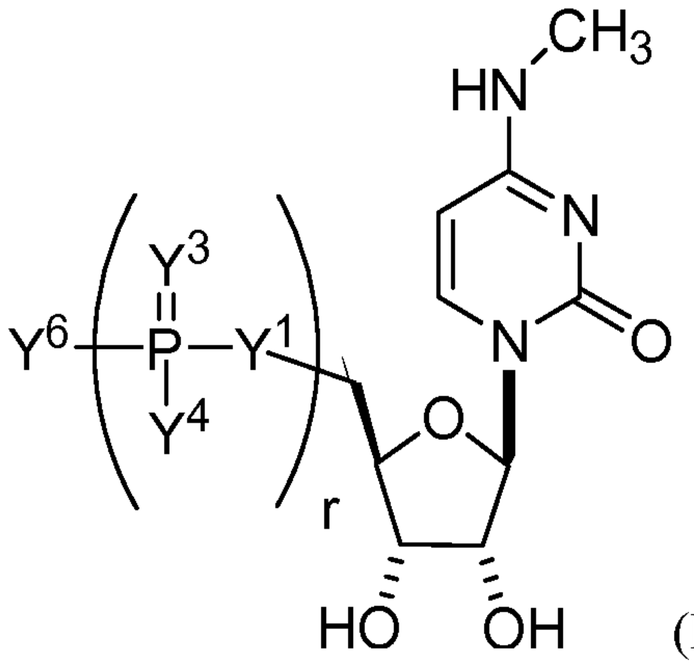
(BB- 130),



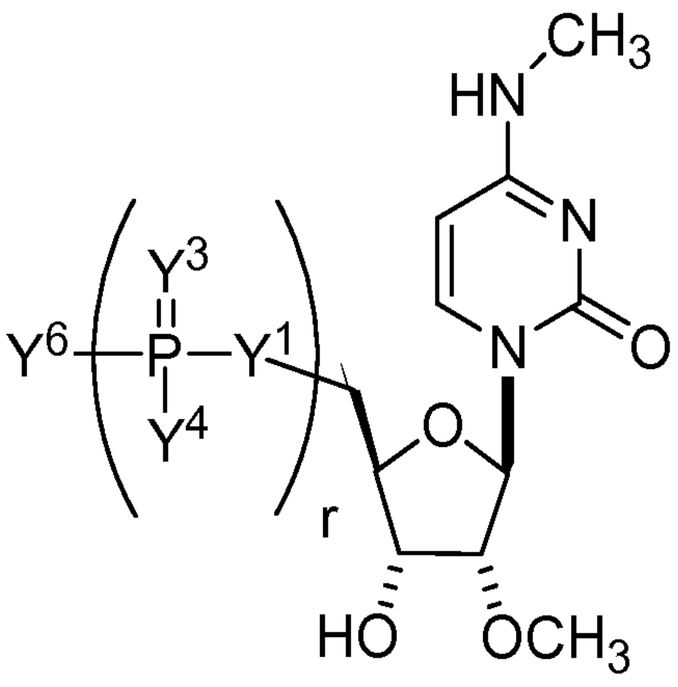
(BB- 131),



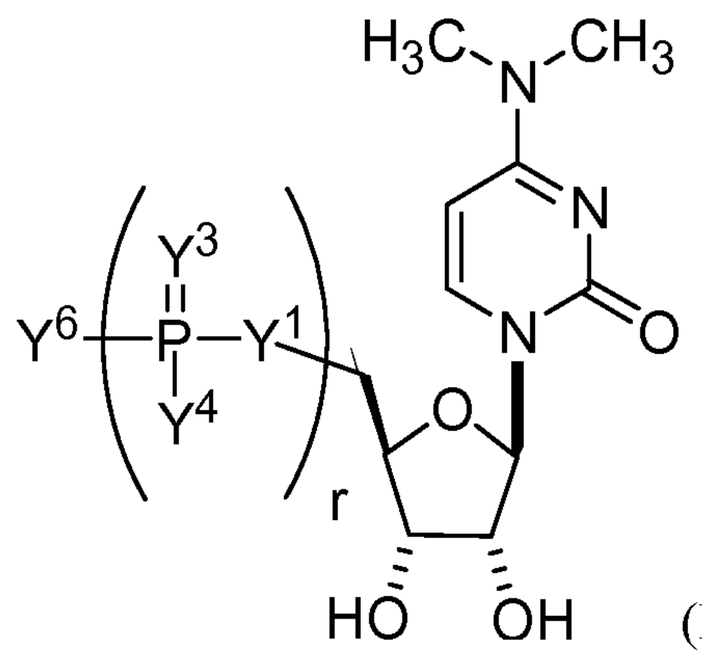
(BB- 132),



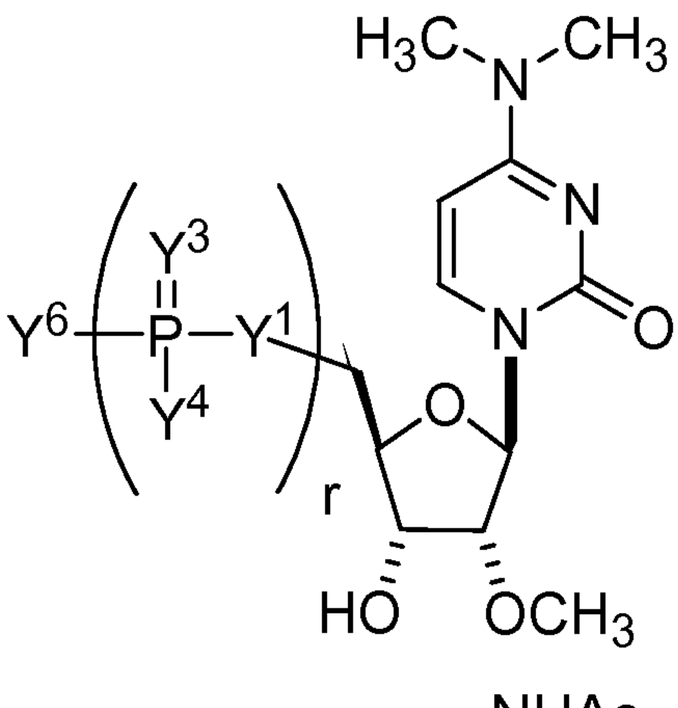
(BB- 133),



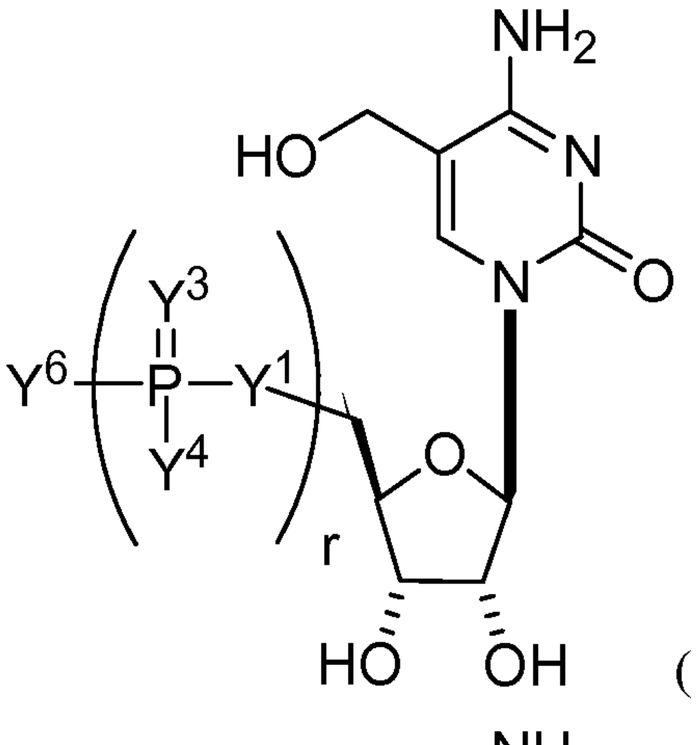
(BB- 134),



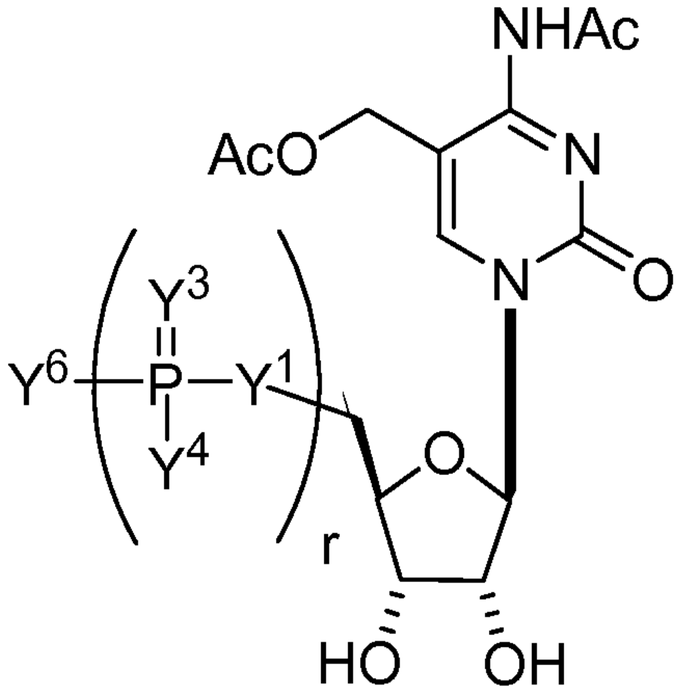
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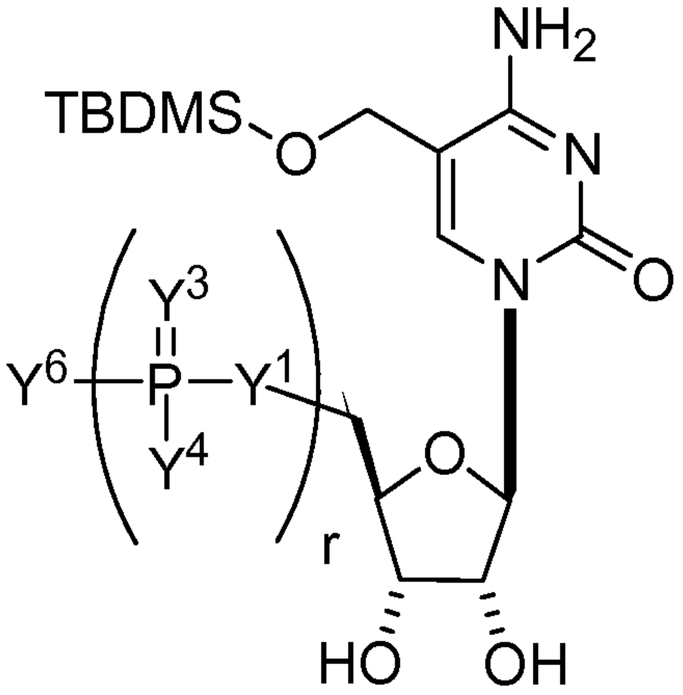
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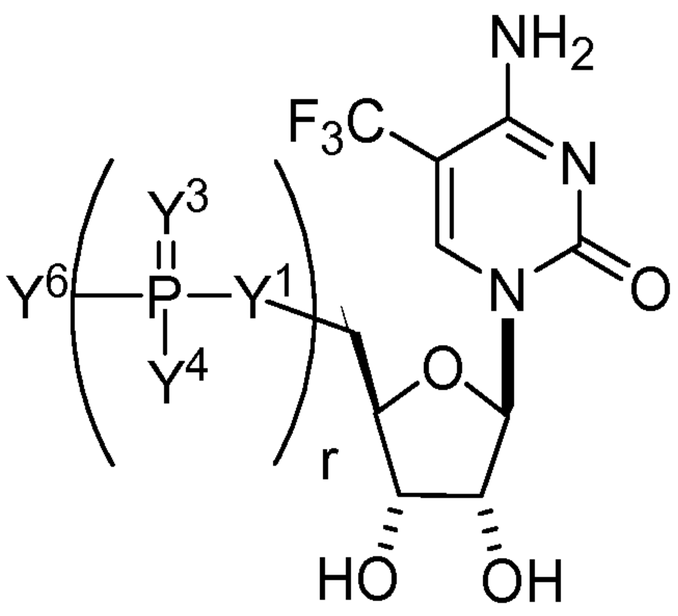
(BB- 137),



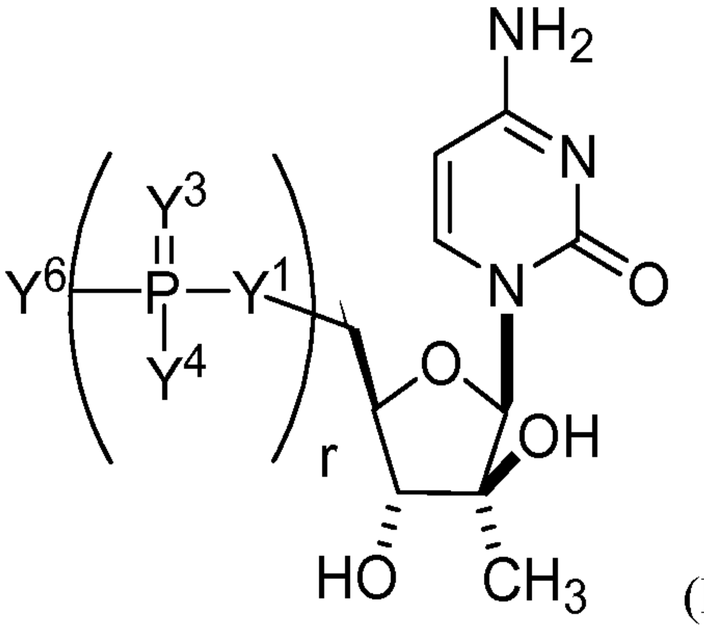
(BB- 138),



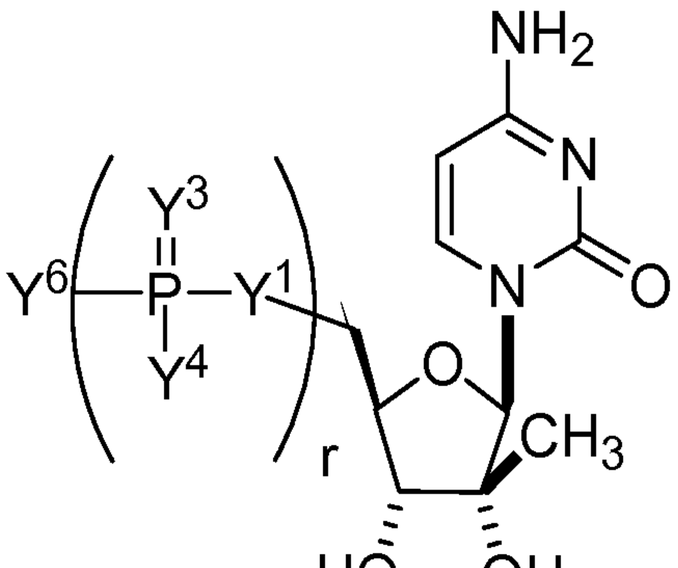
(BB- 139),



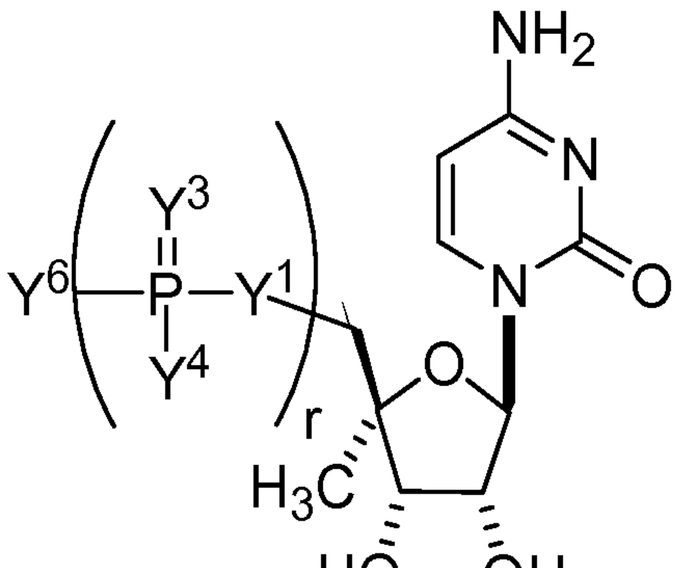
(BB- 140),



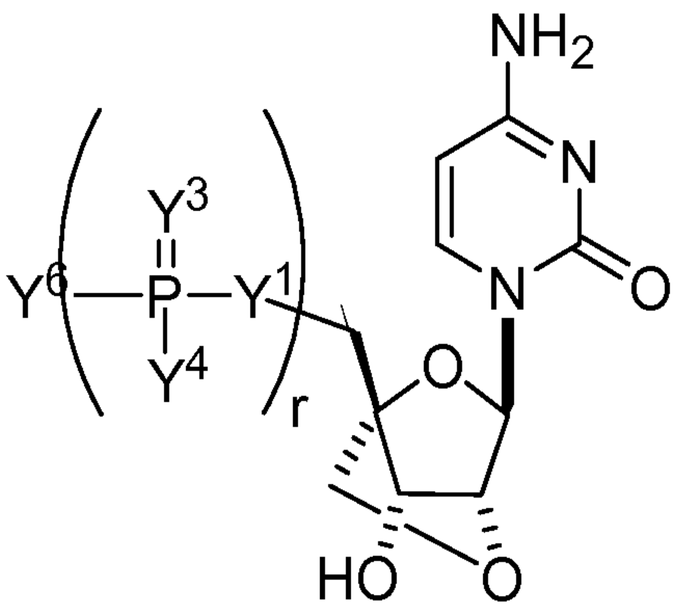
(BB- 141),



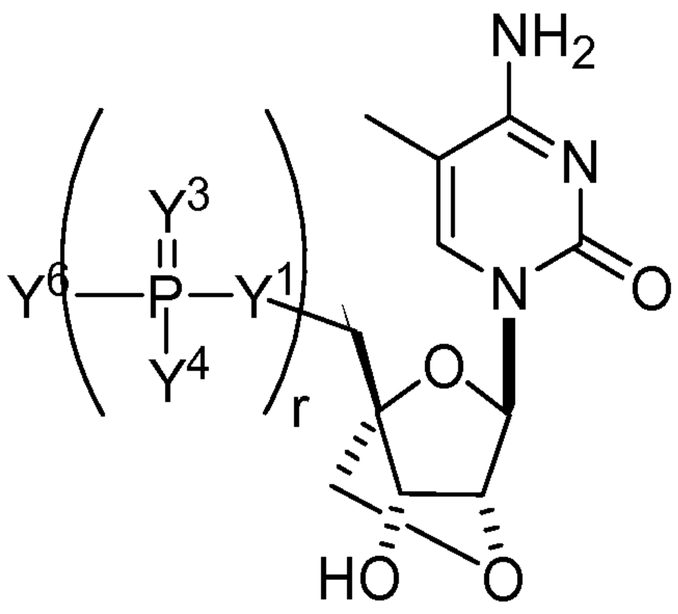
(BB- 142),



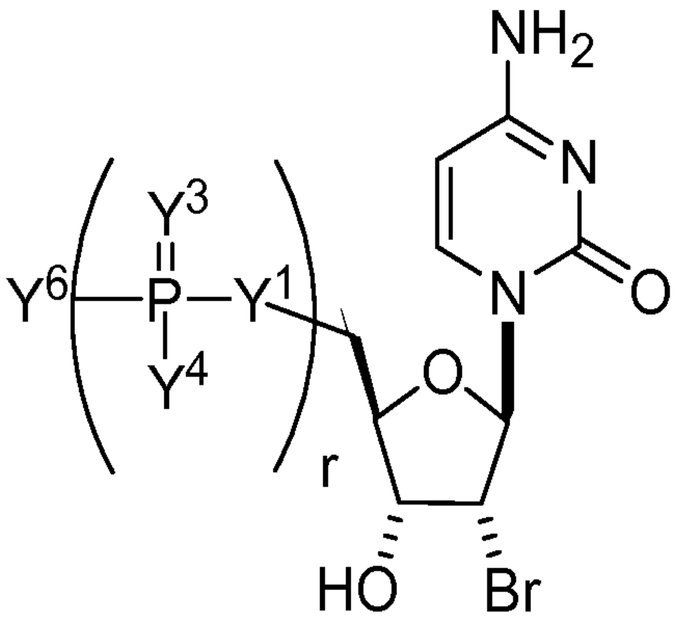
(BB- 143),



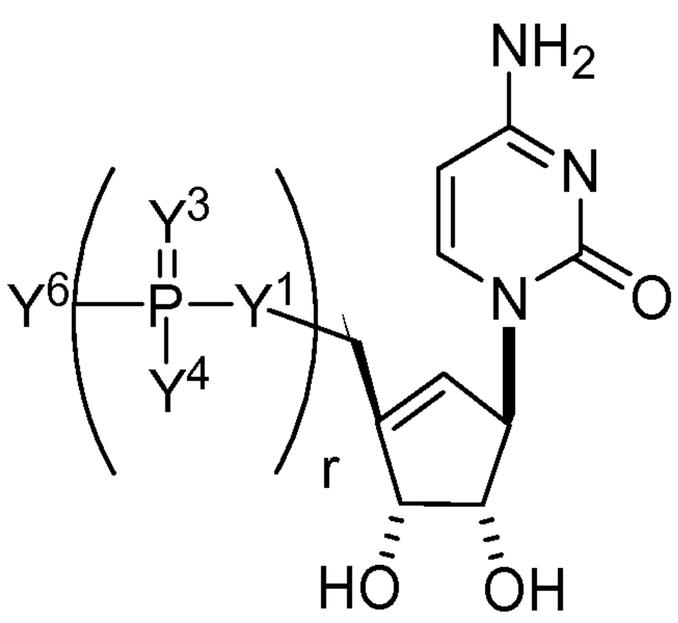
(BB- 144),



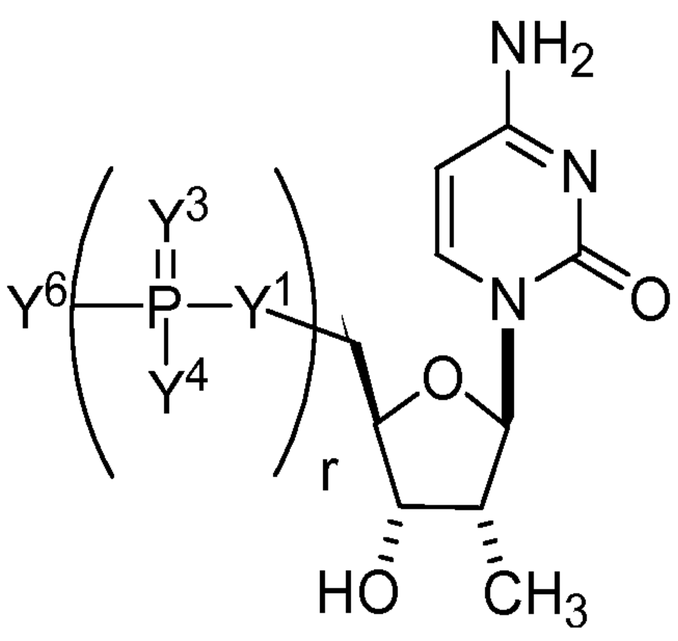
(BB- 145),



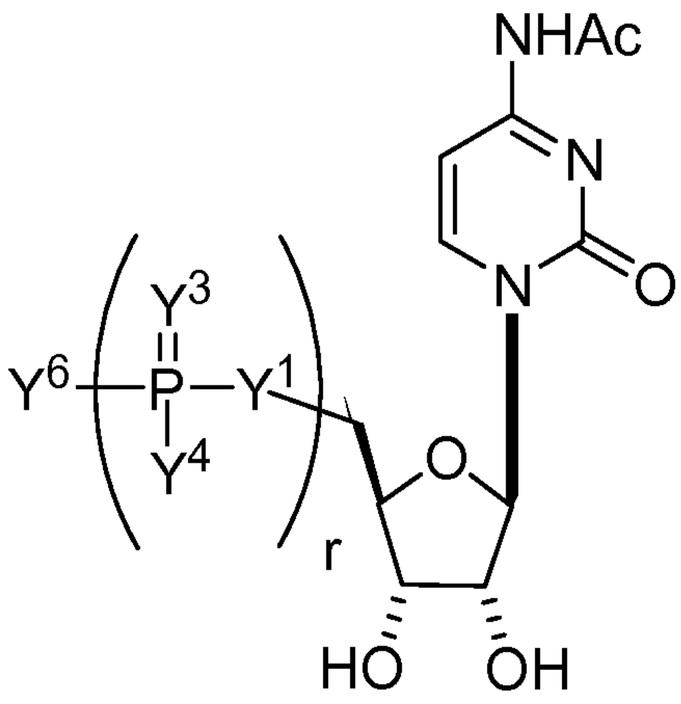
(BB- 146),



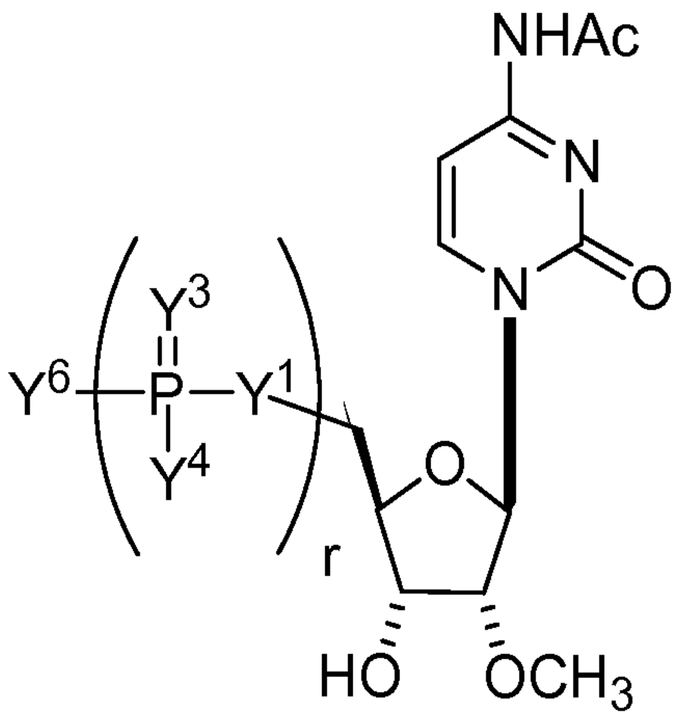
(BB- 147),



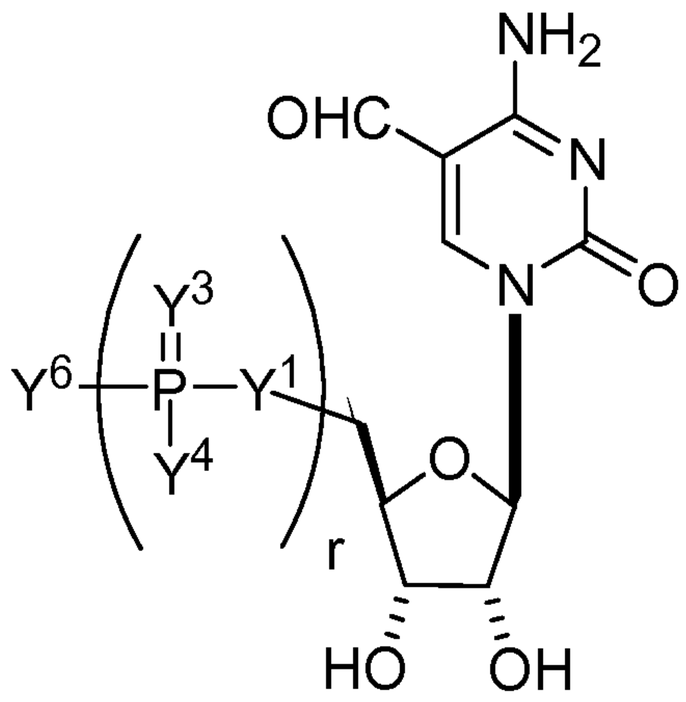
(BB- 148),



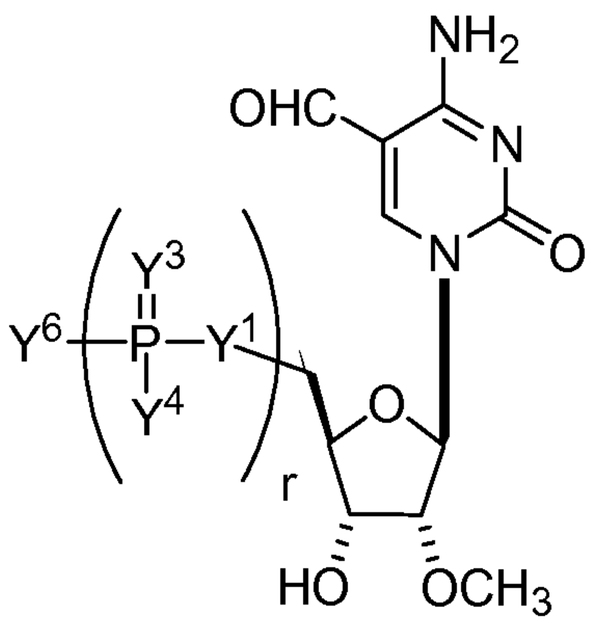
(BB- 149),



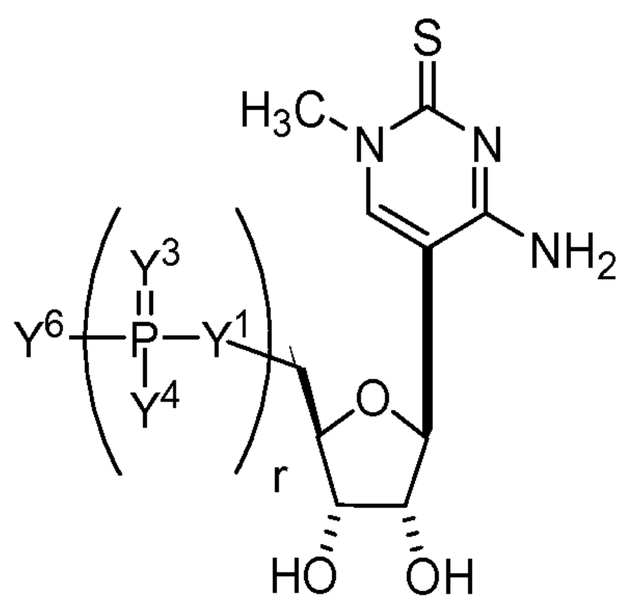
(BB- 150),



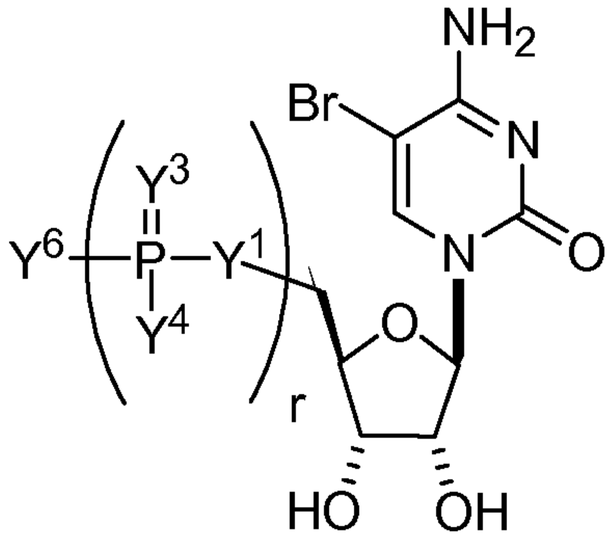
(BB- 151),



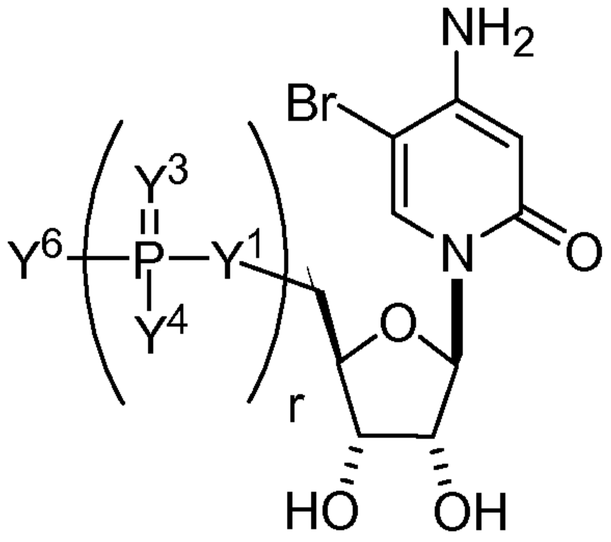
(BB- 152),



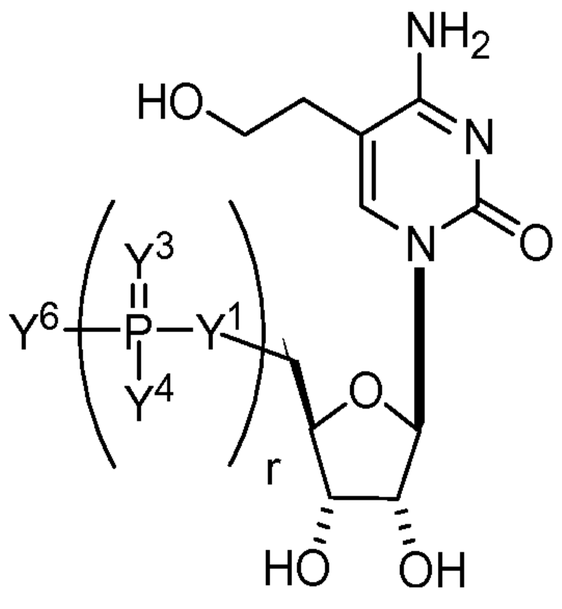
(BB- 153),



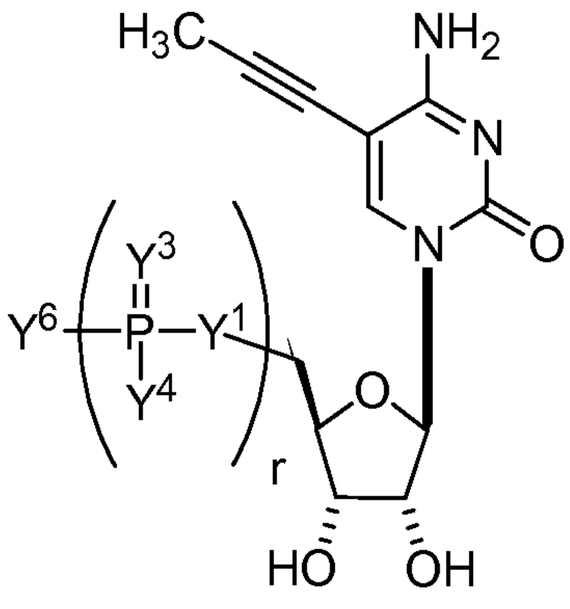
(BB- 154),



(BB- 155),

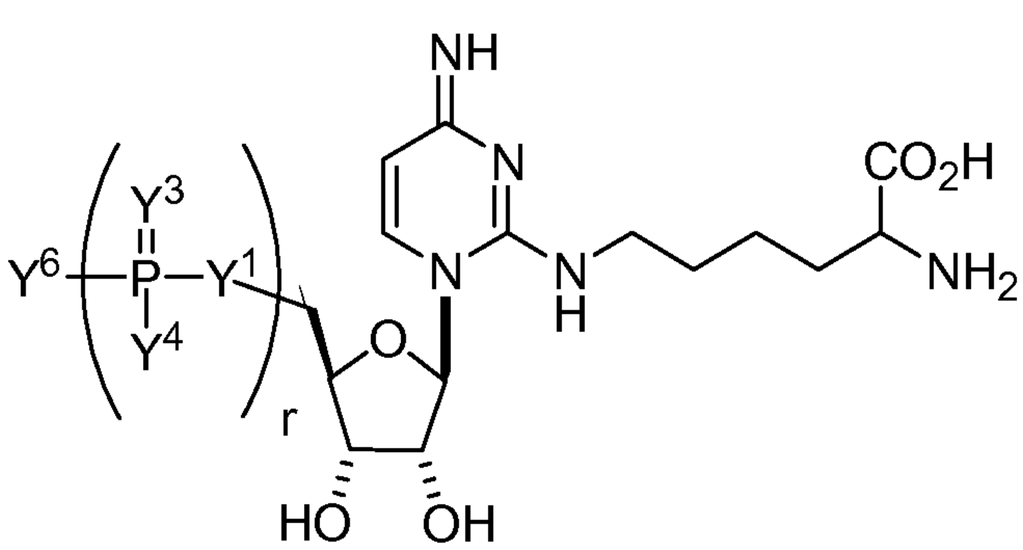


(BB- 156),

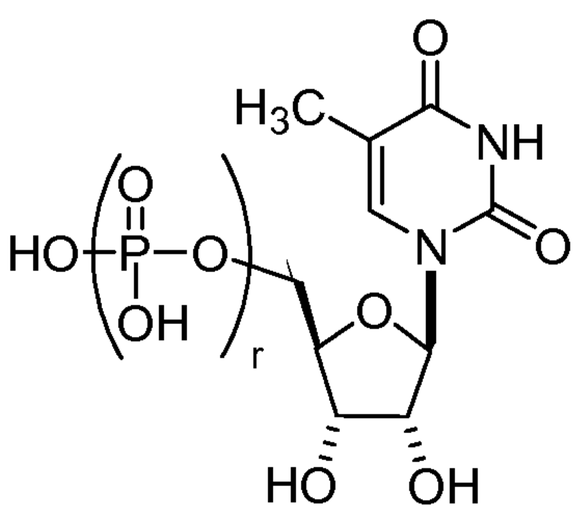


(BB- 157),

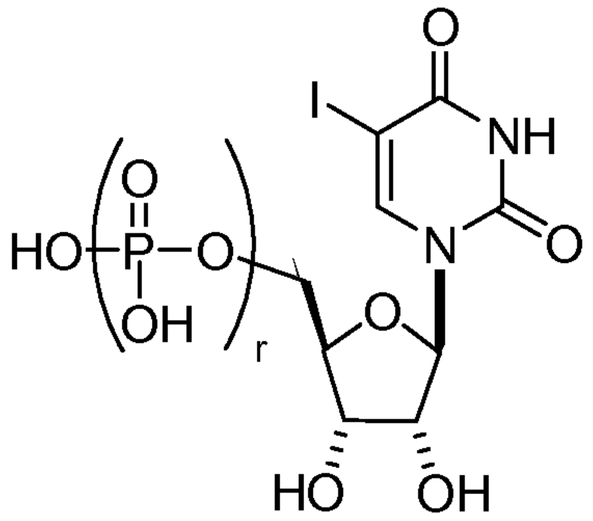
(BB- 158), and



(BB- 159), or a pharmaceutically acceptable salt or stereoisomer thereof, wherein Y^1, Y^3, Y^4, Y^6, and r are as described herein (e.g., each r is, independently, an integer from 0 to 5, such as from 0 to 3, from 1 to 3, or from 1 to 5)). For example, the building block molecule, which may be incorporated into a polynucleotide, primary construct, or mmRNA, can be:

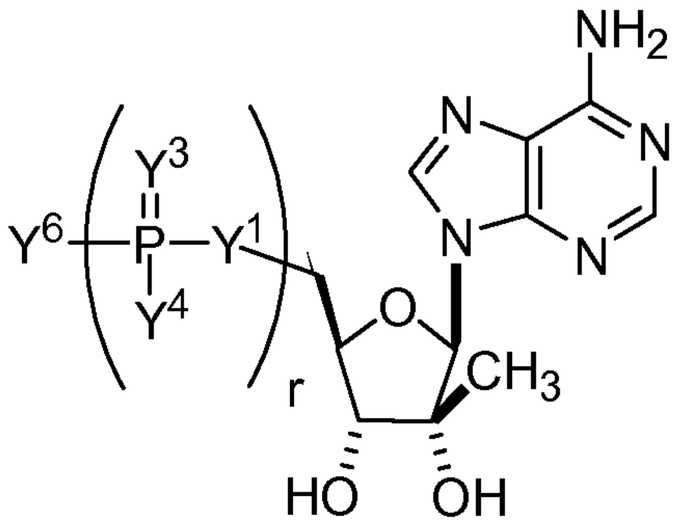


(BB- 160) or

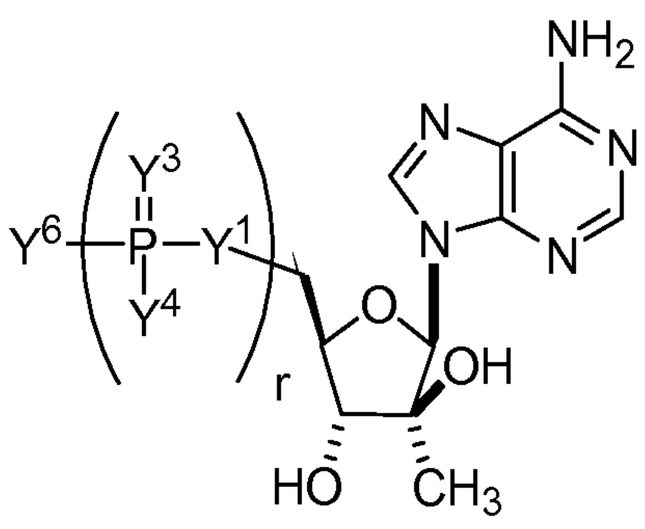


(BB- 161), or a pharmaceutically acceptable salt or stereoisomer thereof, wherein each r is, independently, an integer from 0 to 5 (e.g., from 0 to 3, from 1 to 3, or from 1 to 5).

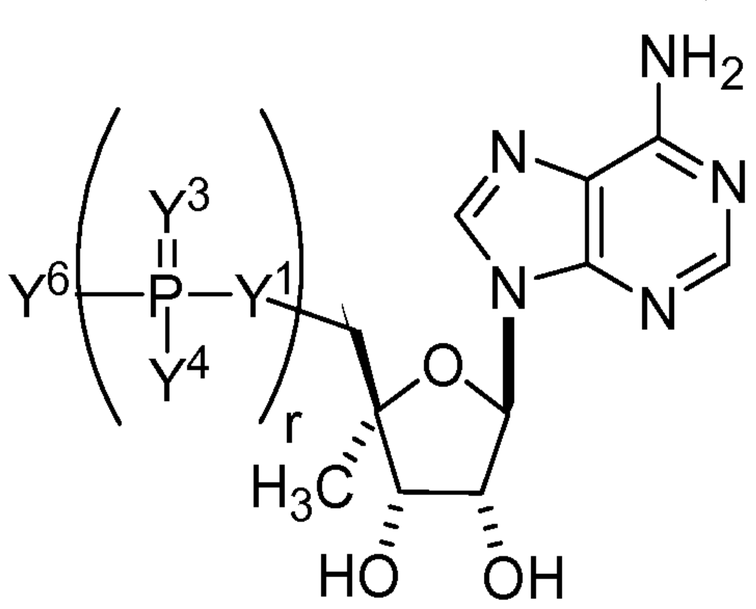
[000417] In some embodiments, the building block molecule, which may be incorporated into a polynucleotide, primary construct, or mmRNA, is a modified adenosine (e.g., selected from the group consisting of:



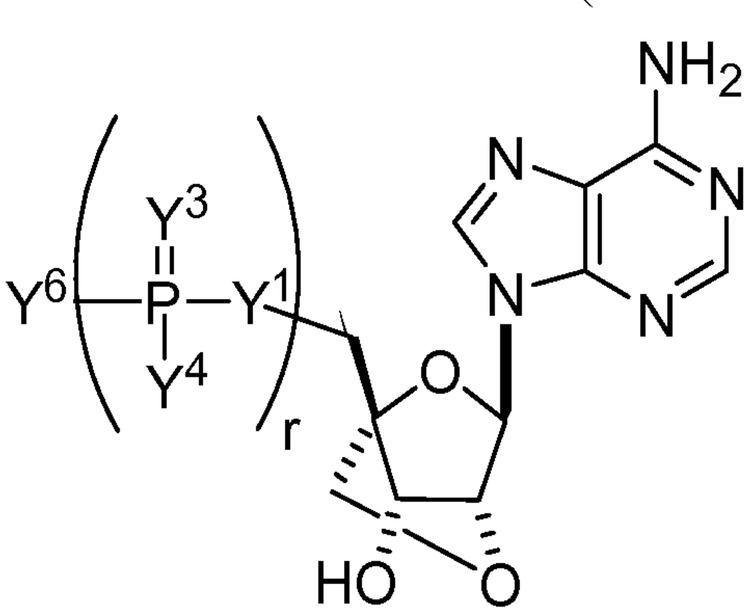
(BB- 162),



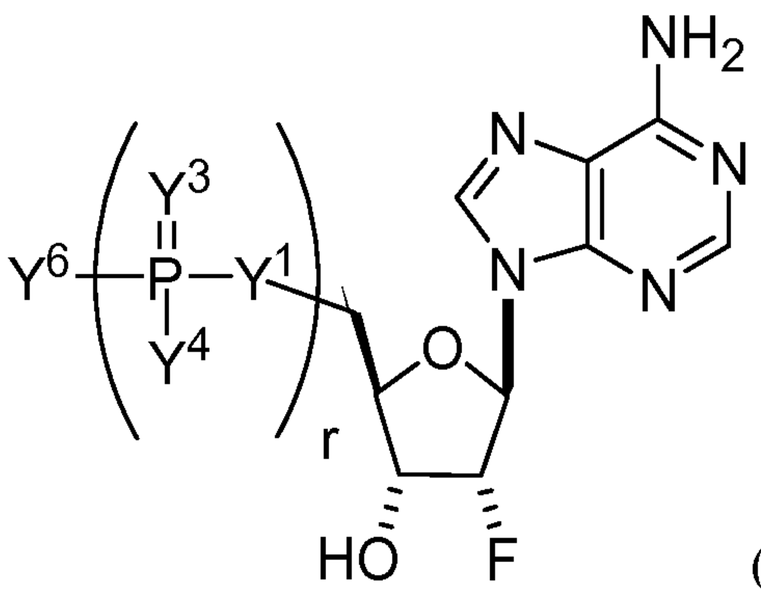
(BB- 163),



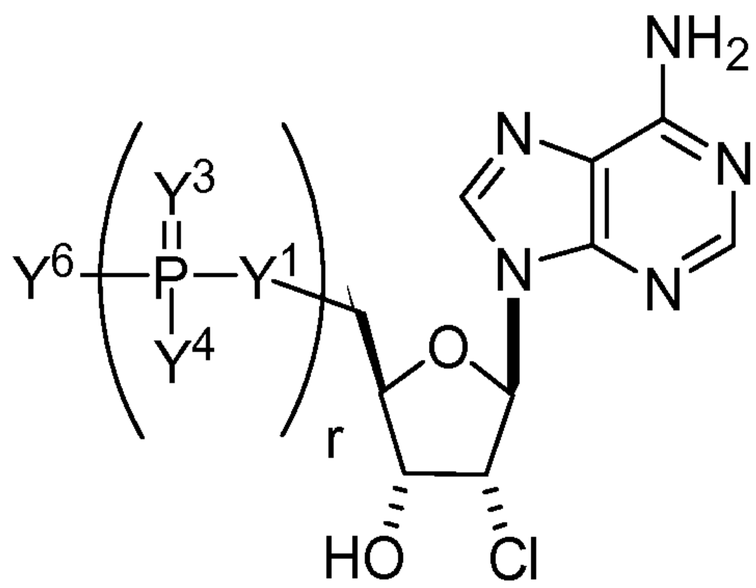
(BB- 164),



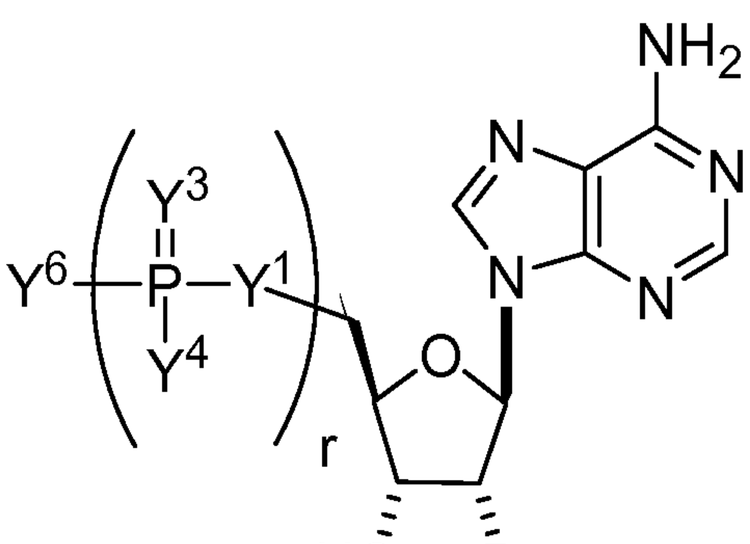
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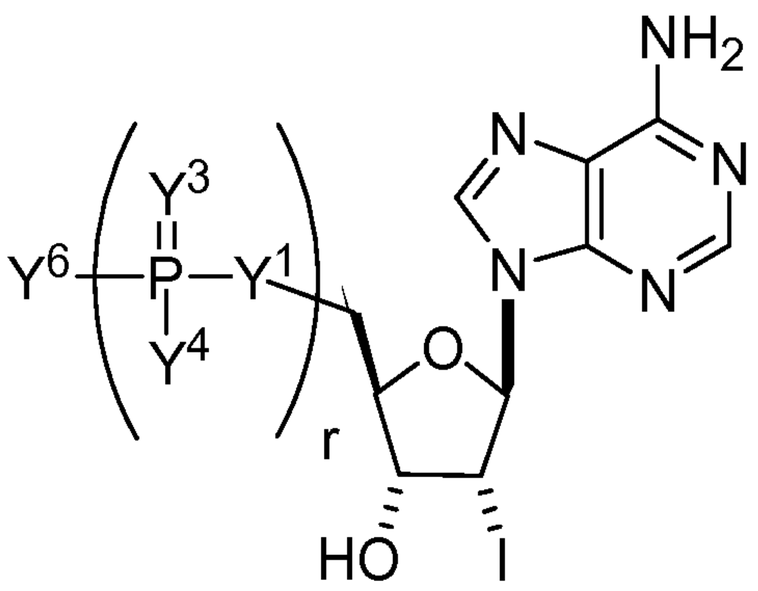


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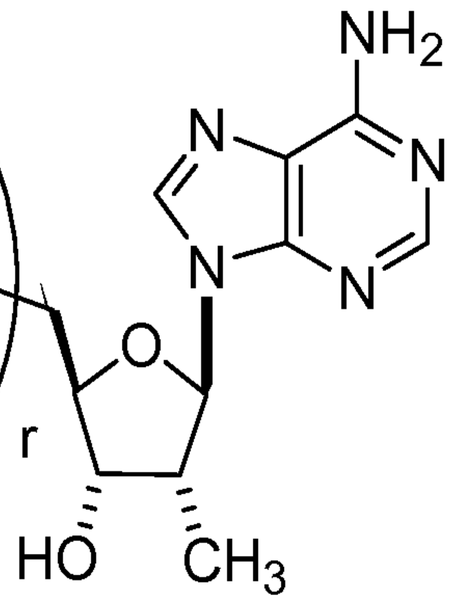


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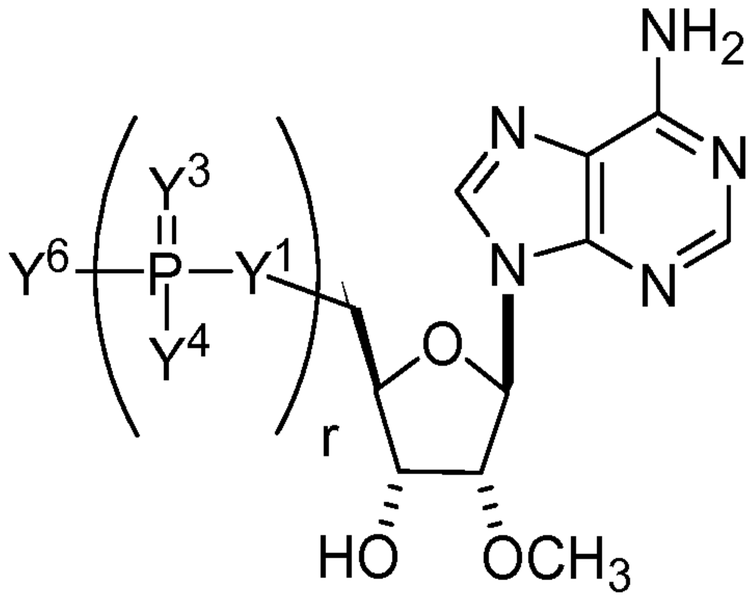
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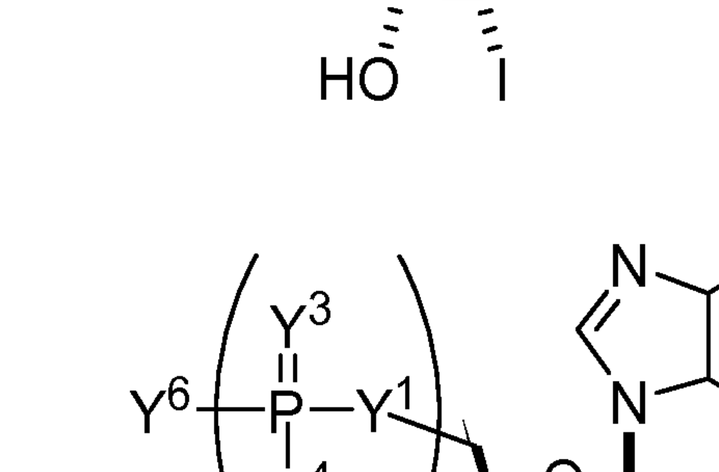
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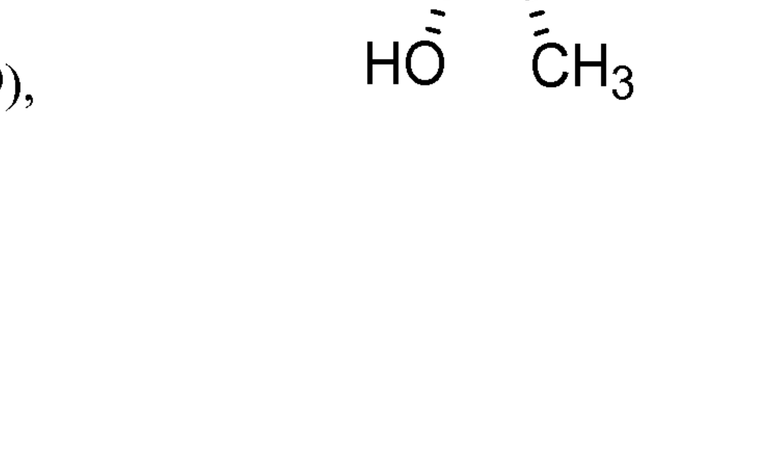
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(BB- 171),



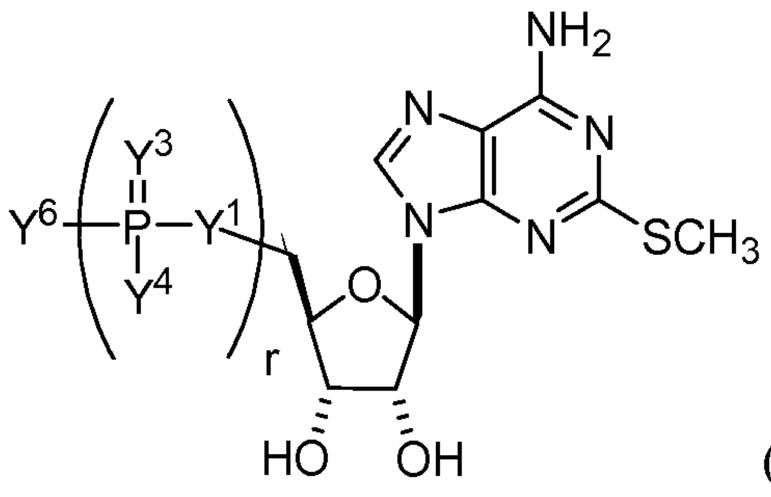
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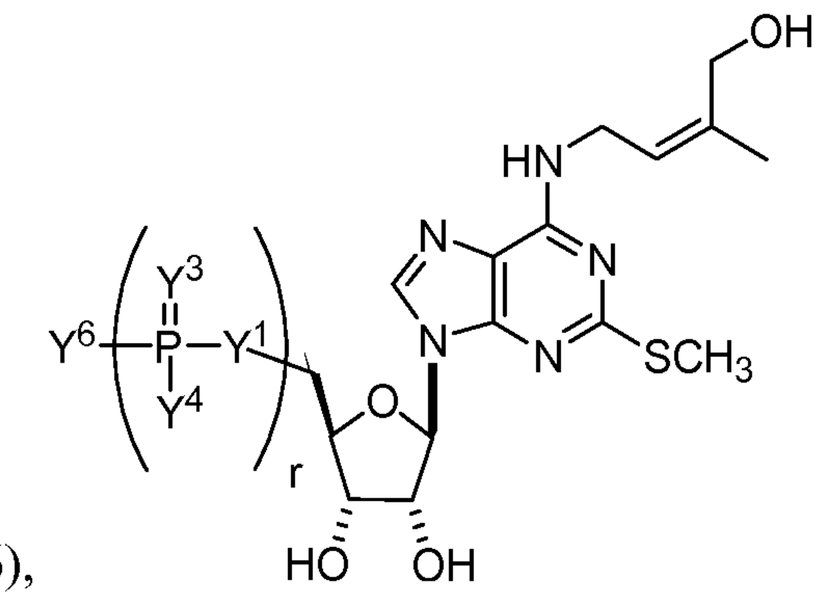
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(BB- 174),

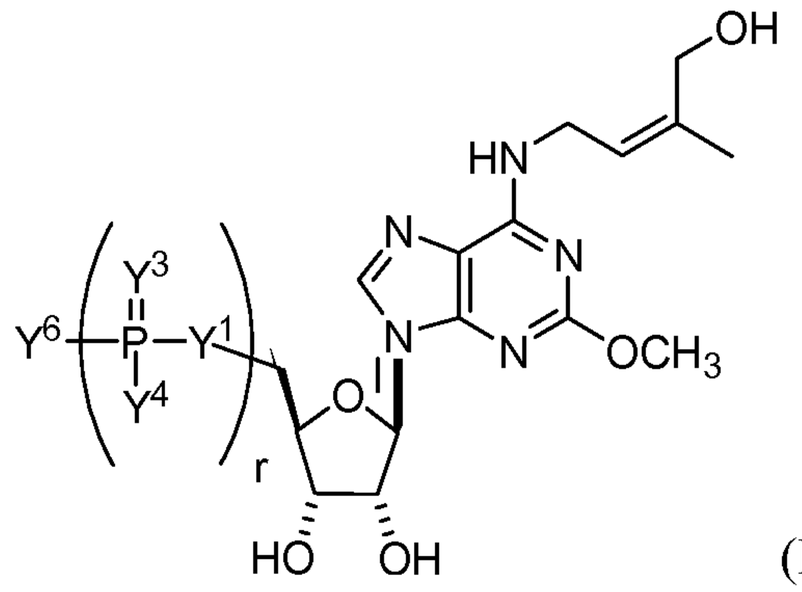
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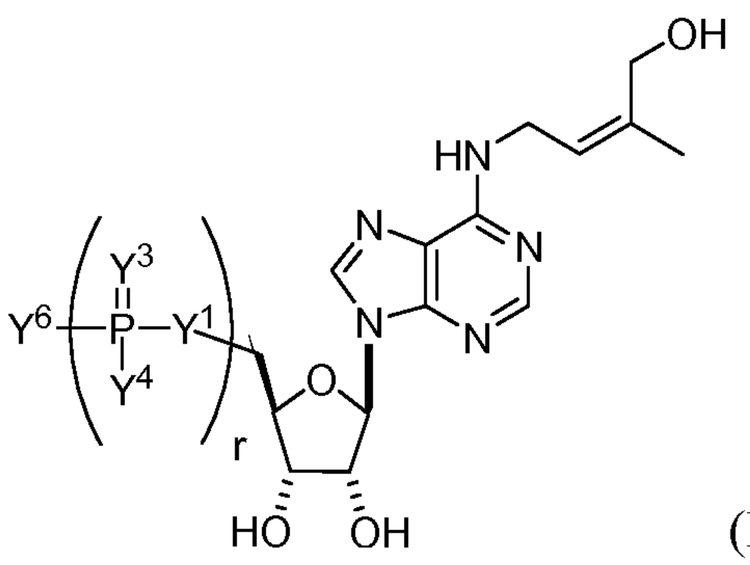
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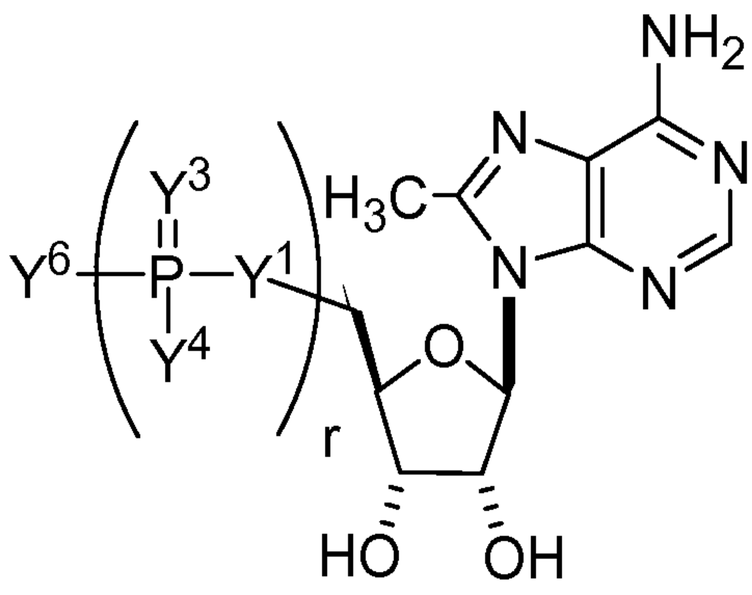
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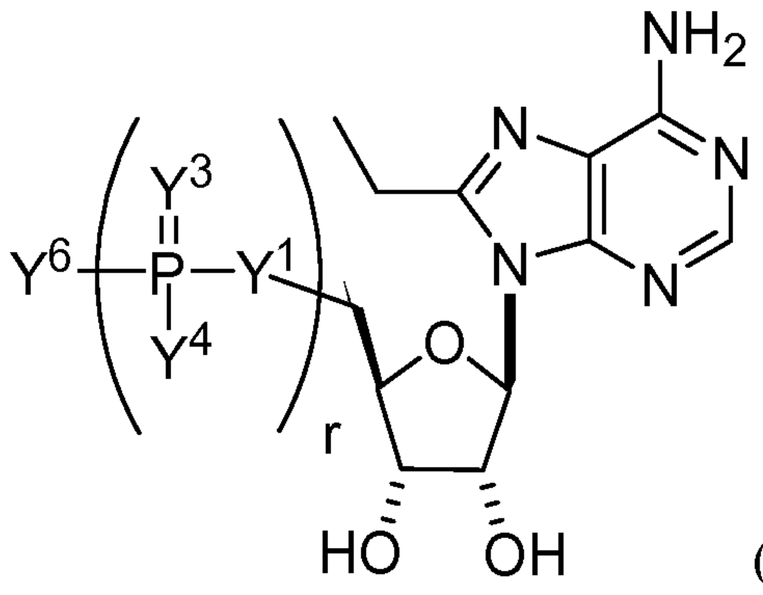
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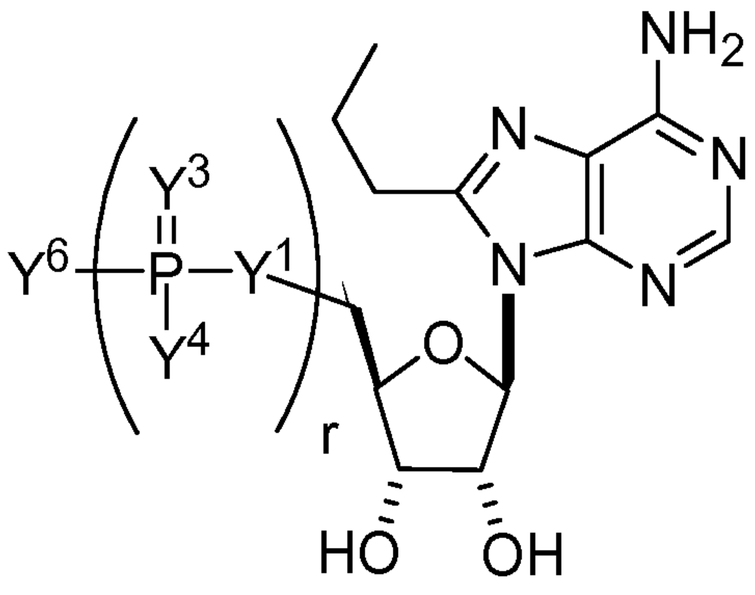
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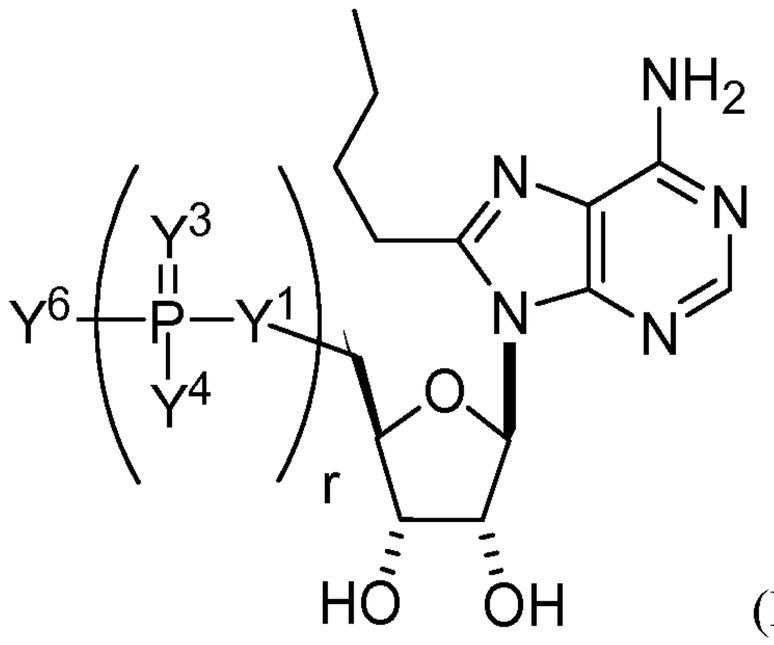


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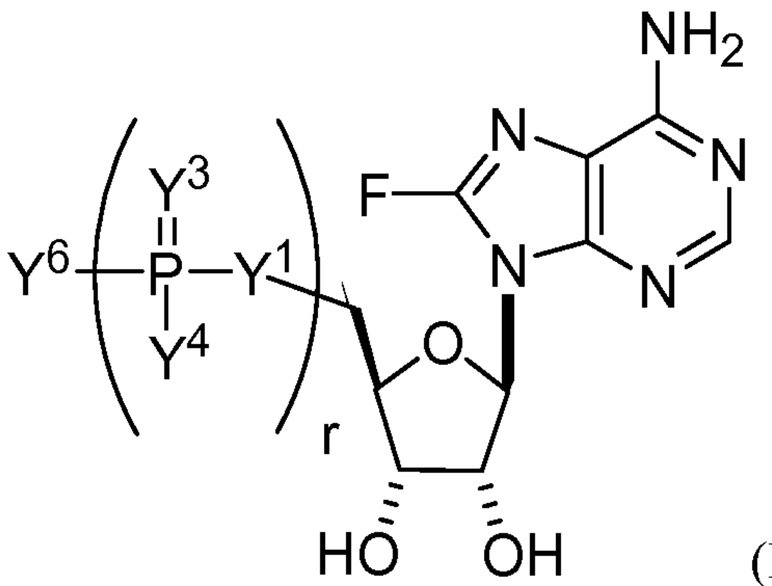


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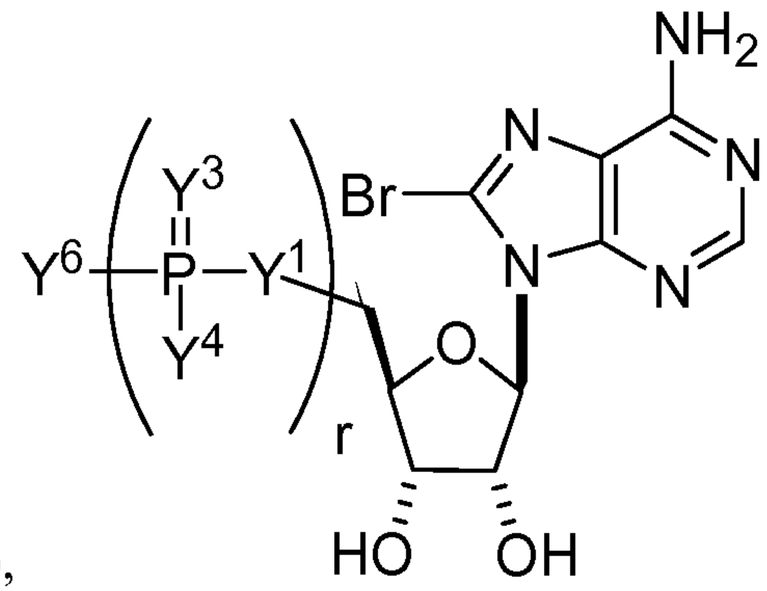




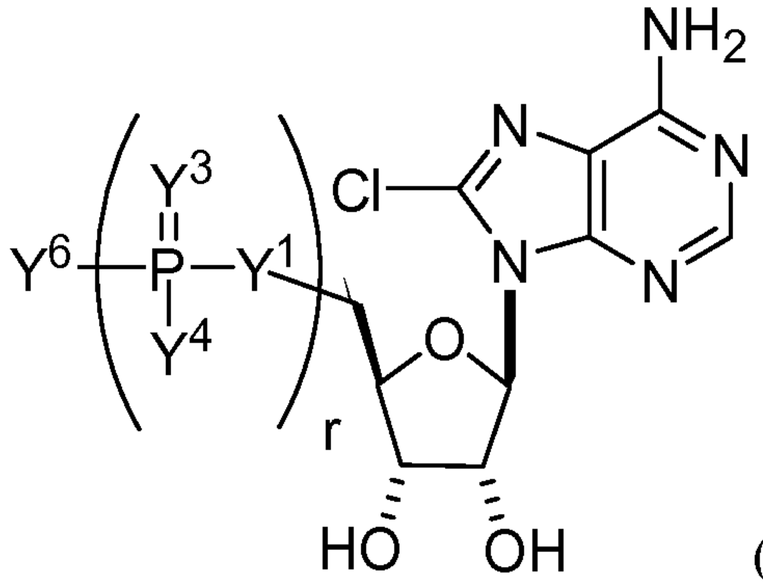
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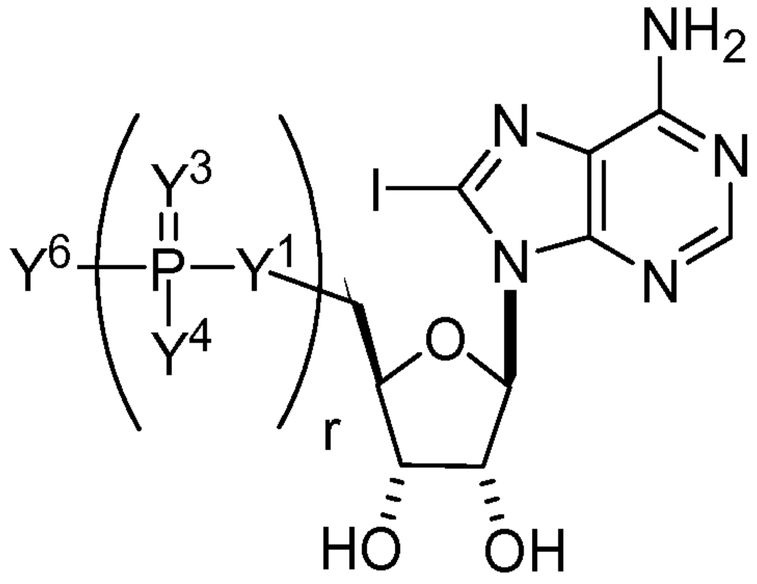
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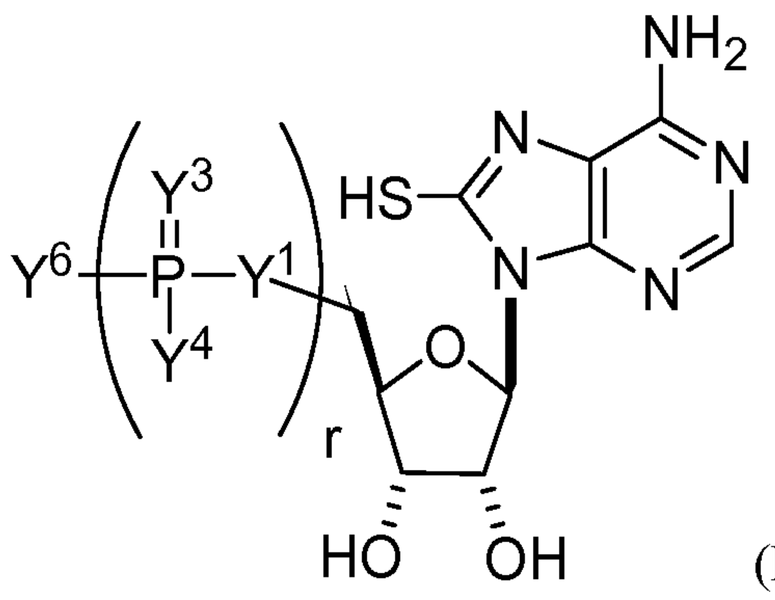
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(BB- 185),



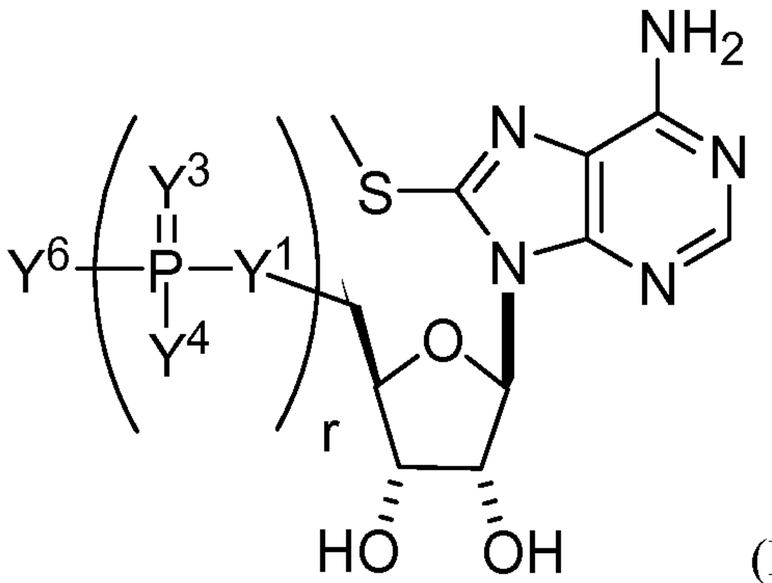
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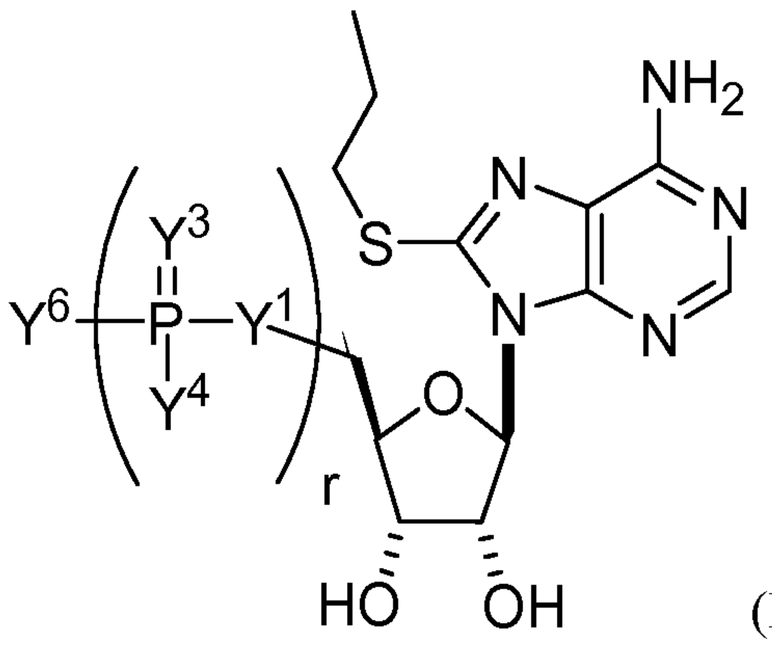
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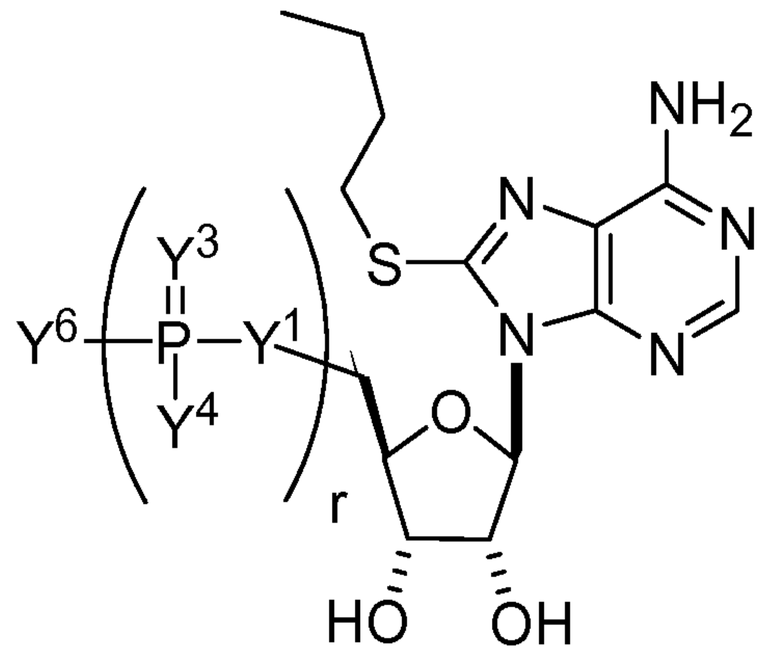
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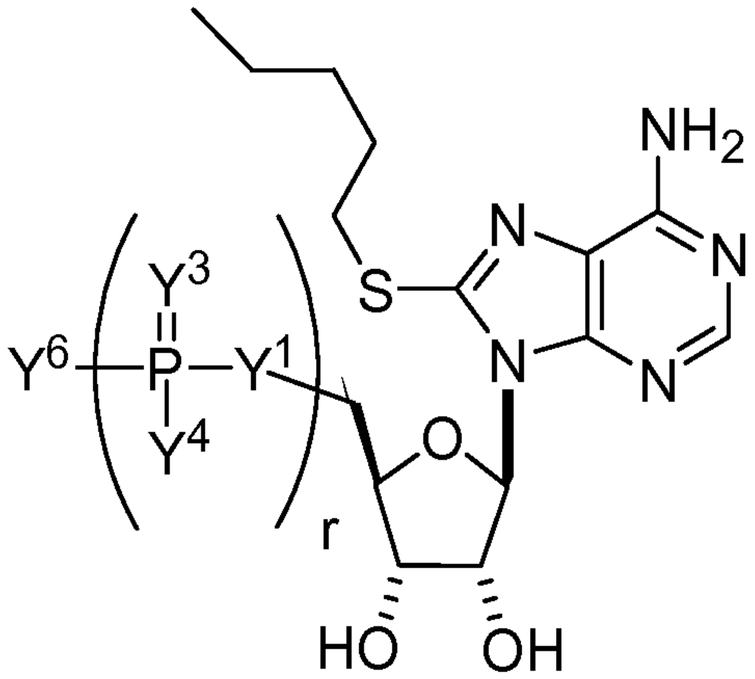
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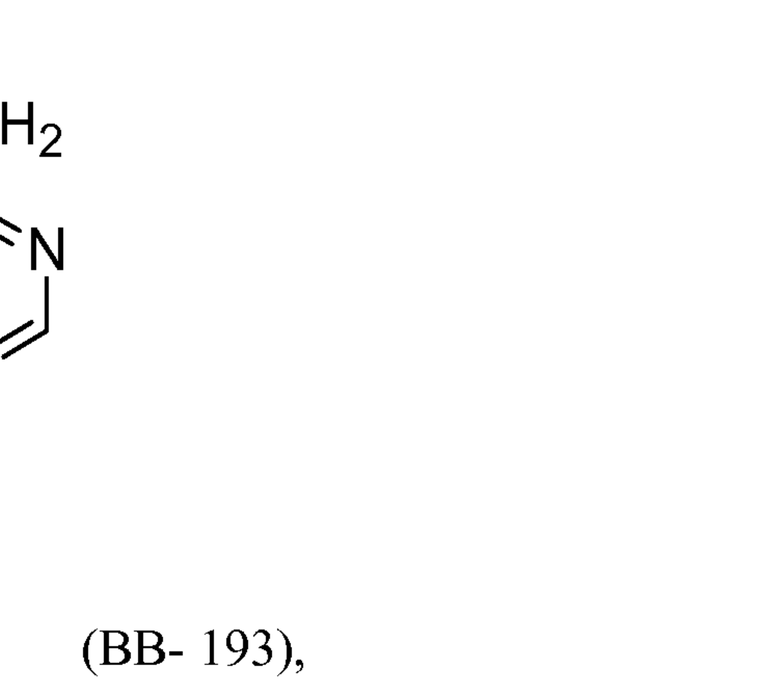
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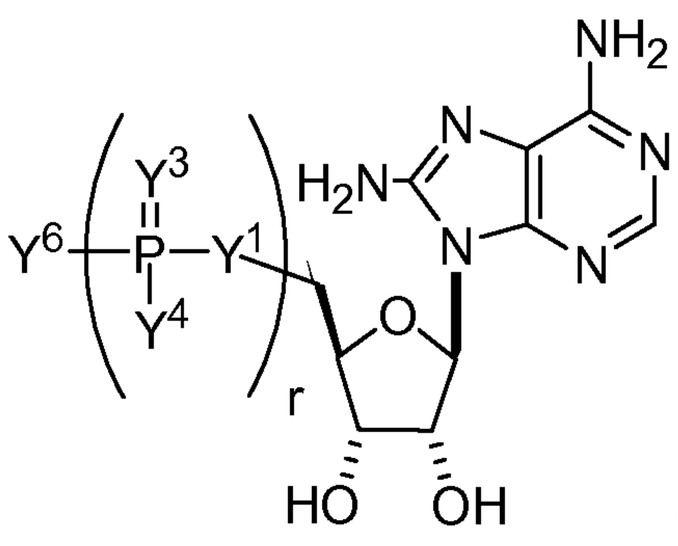
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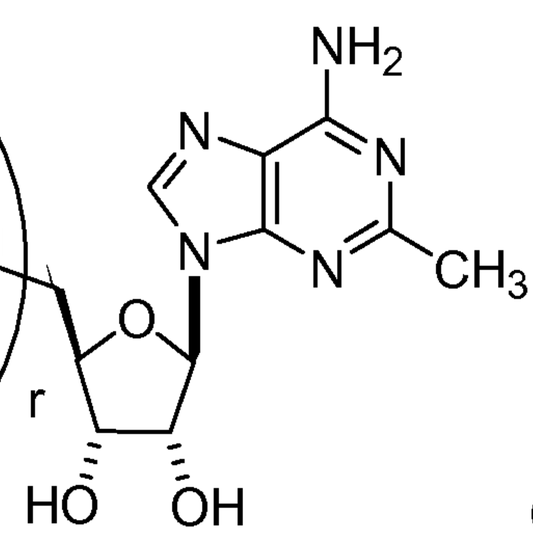
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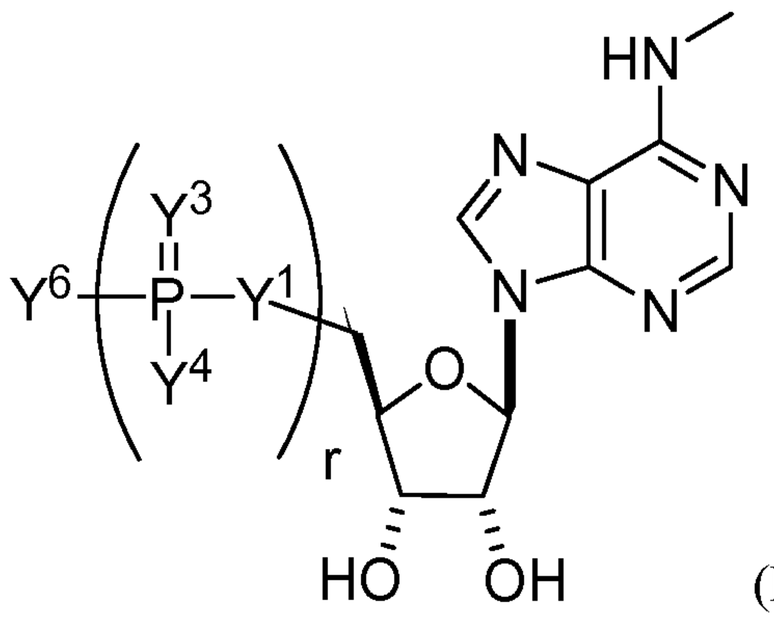
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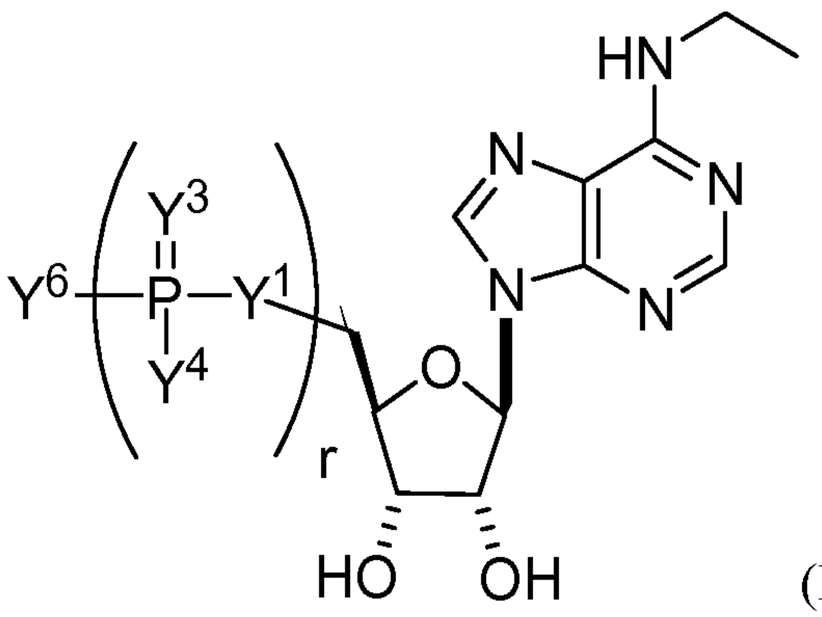
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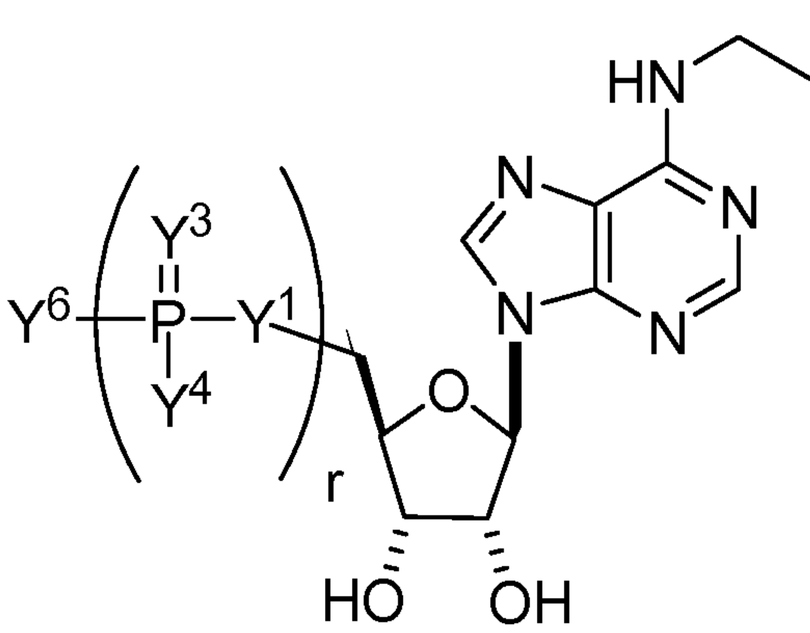
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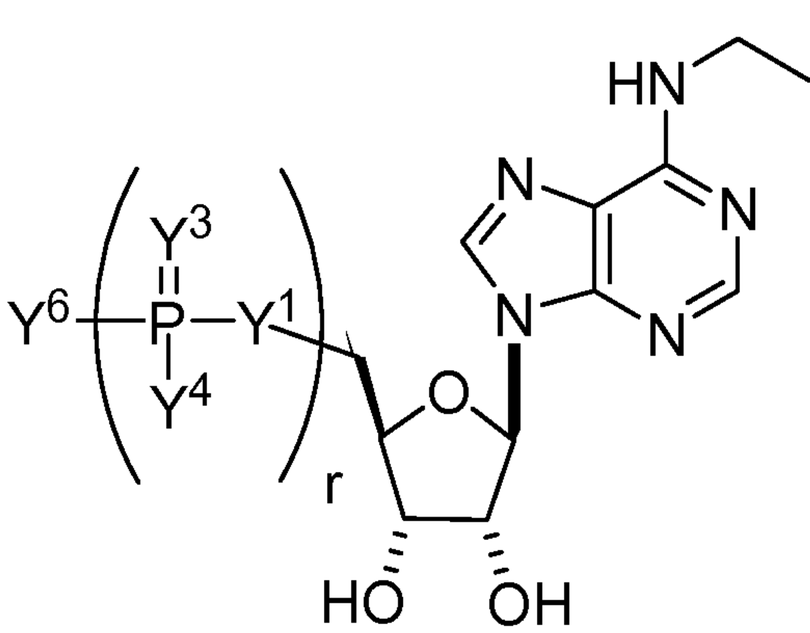
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(BB- 196),



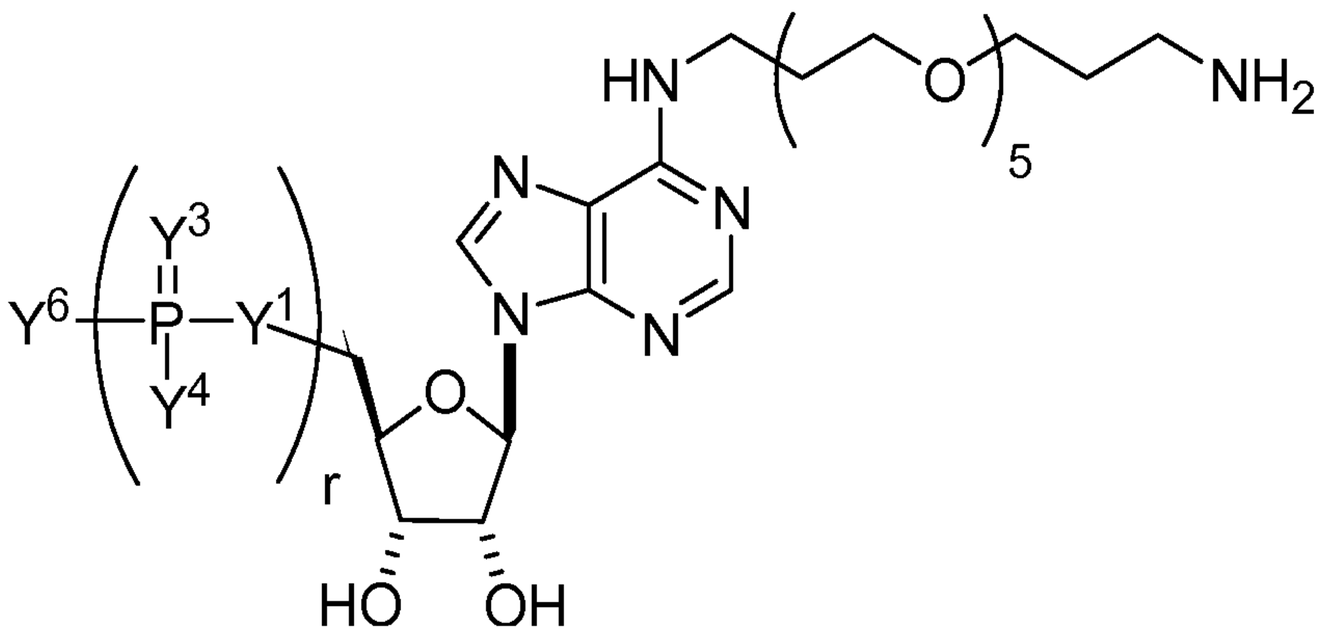
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(BB- 198),

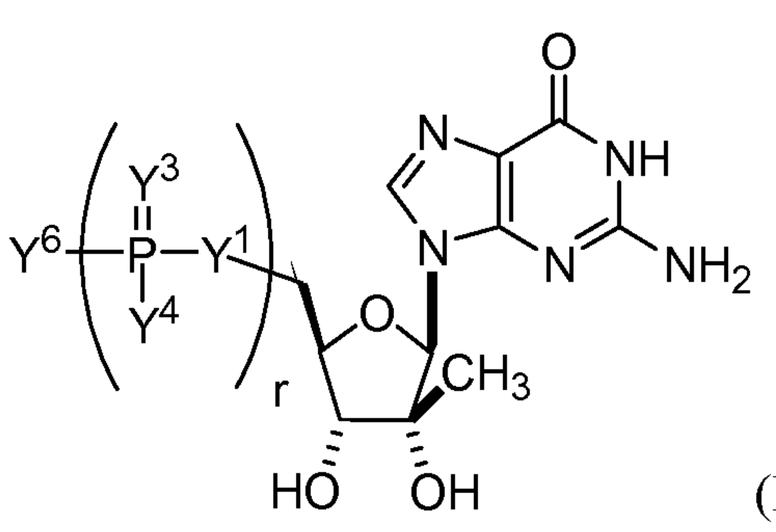


(BB- 199), and

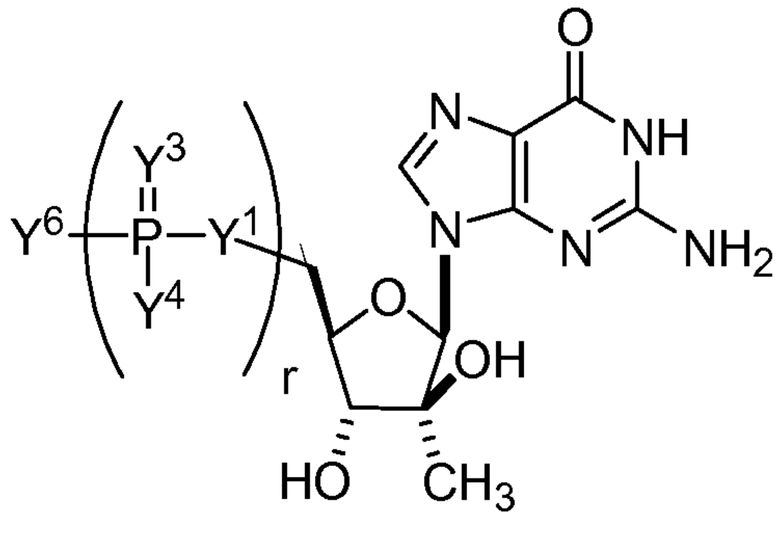


(BB- 200) or a pharmaceutically acceptable salt or stereoisomer thereof, wherein Y^1, Y^3, Y^4, Y^6, and r are as described herein (e.g., each r is, independently, an integer from 0 to 5, such as from 0 to 3, from 1 to 3, or from 1 to 5)).

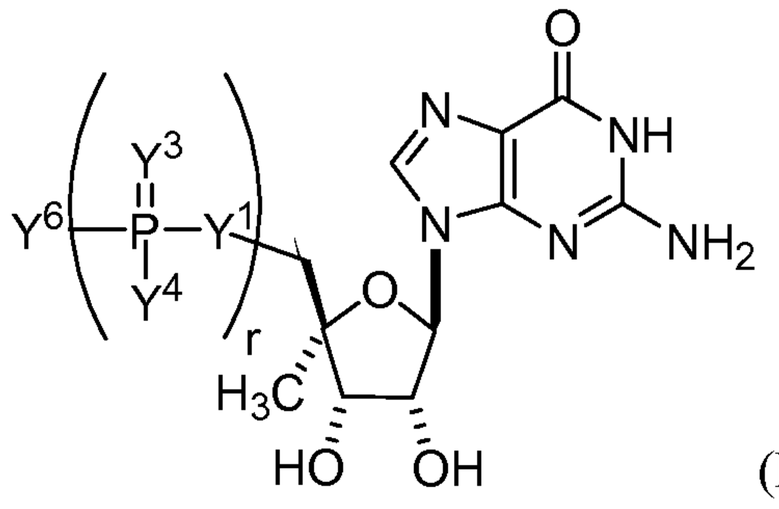
[000418] In some embodiments, the building block molecule, which may be incorporated into a polynucleotide, primary construct, or mmRNA, is a modified guanosine (e.g., selected from the group consisting of:



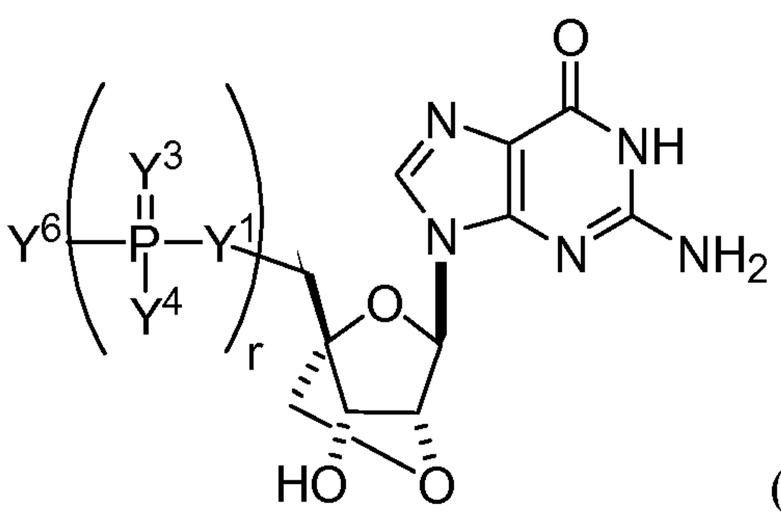
(BB- 201),



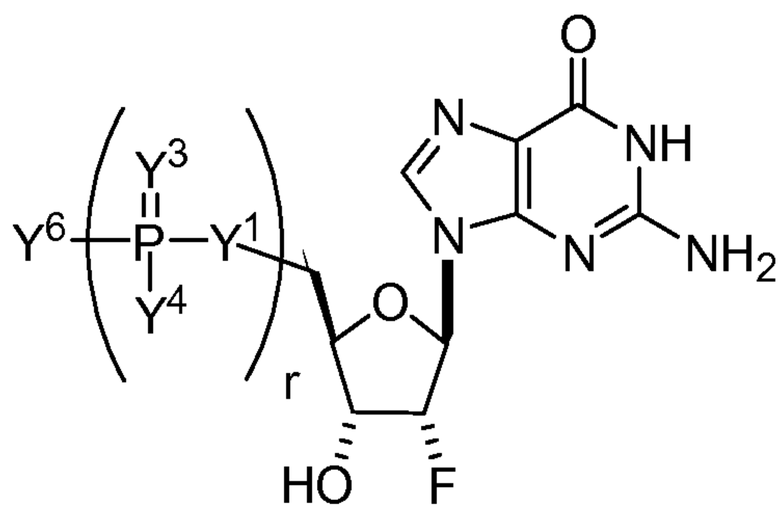
(BB- 202),

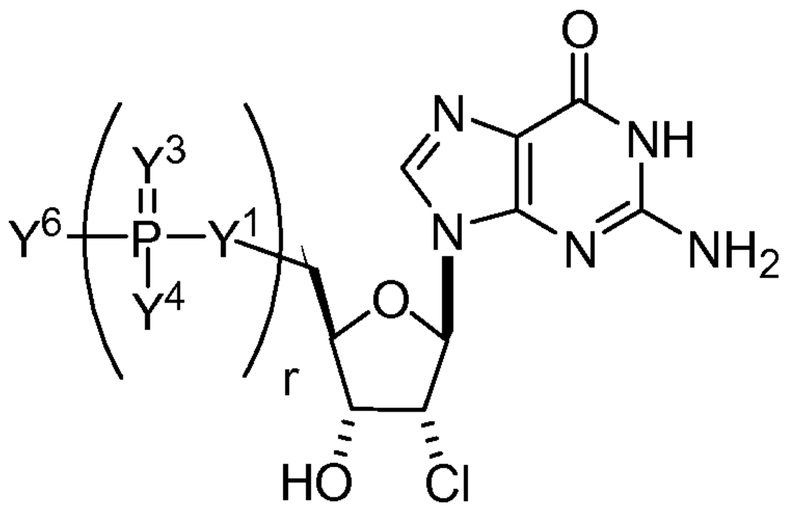


(BB- 203),

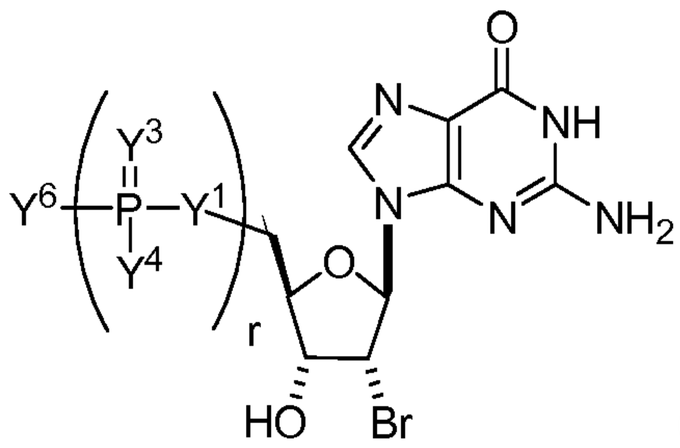


(BB- 204),

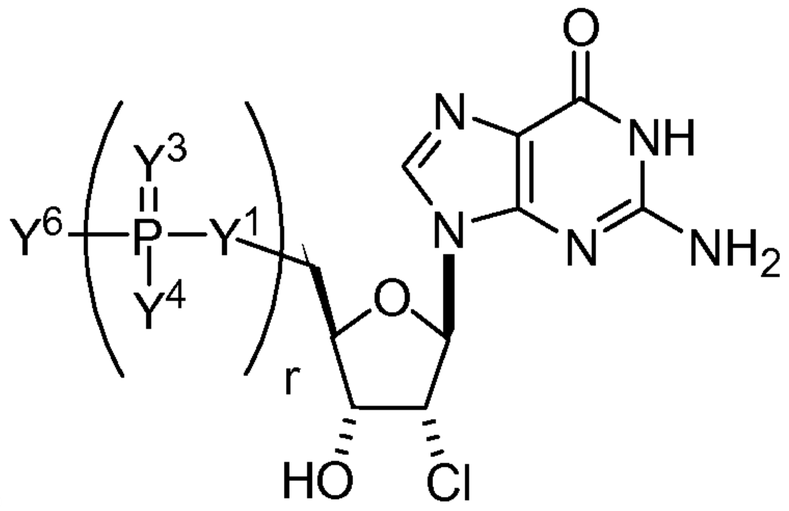




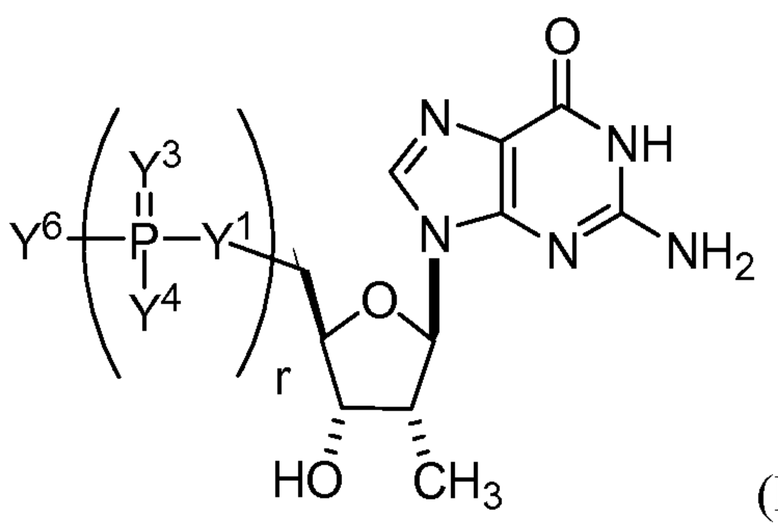
(BB- 205),



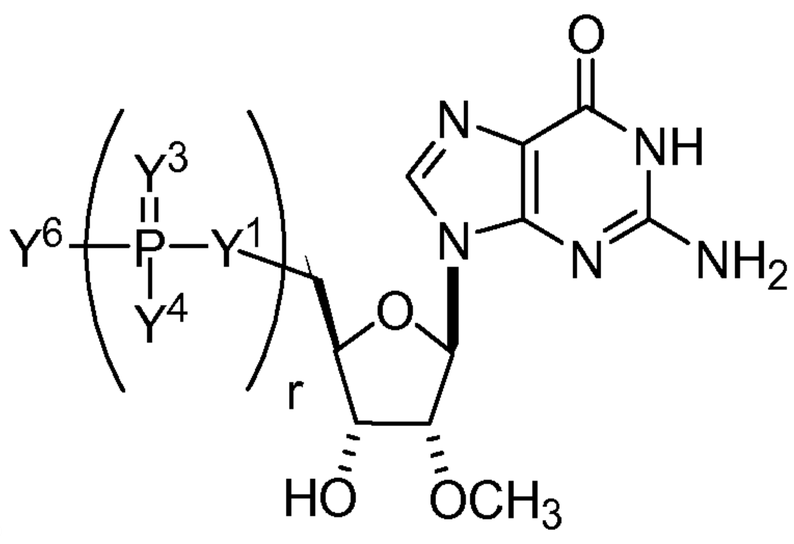
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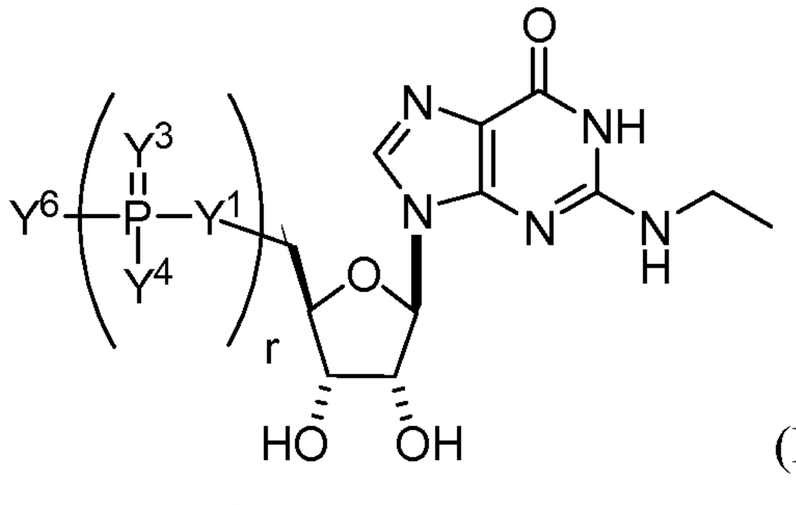


(BB- 208),

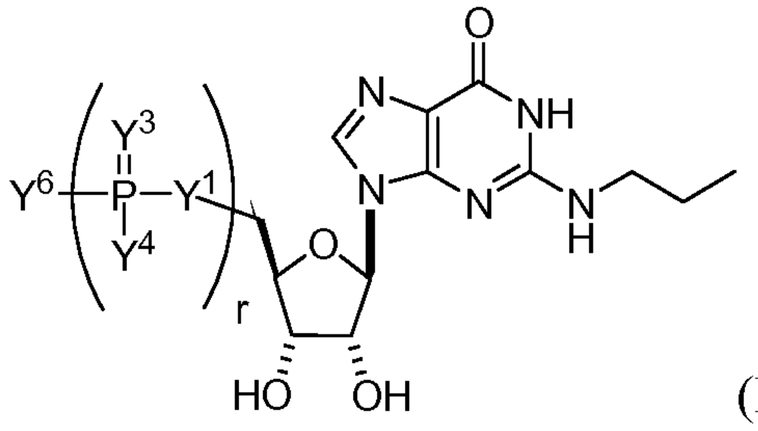


(BB- 209),

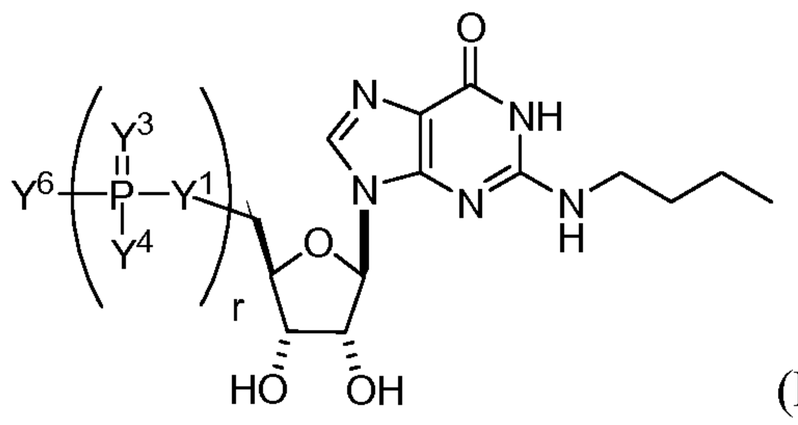




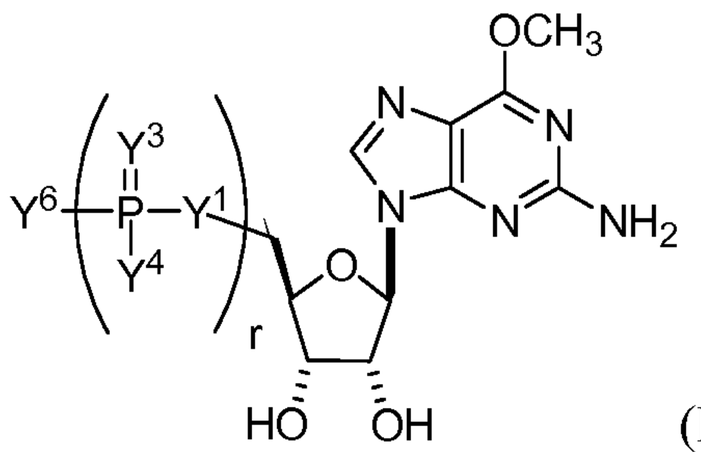
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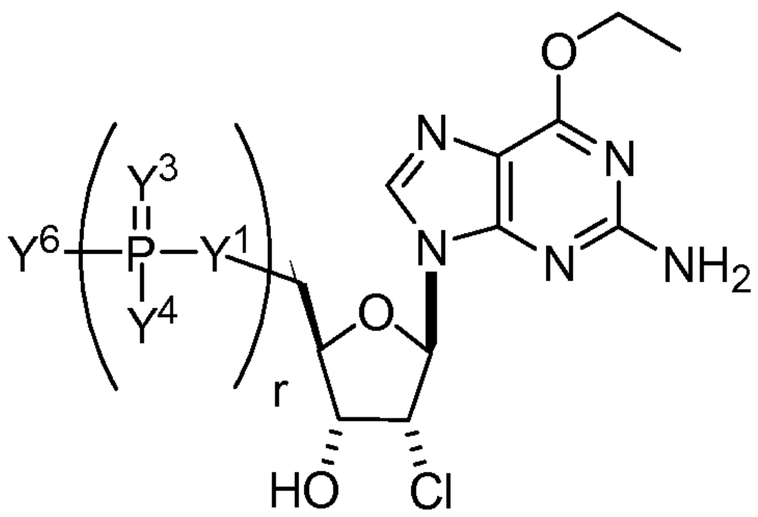
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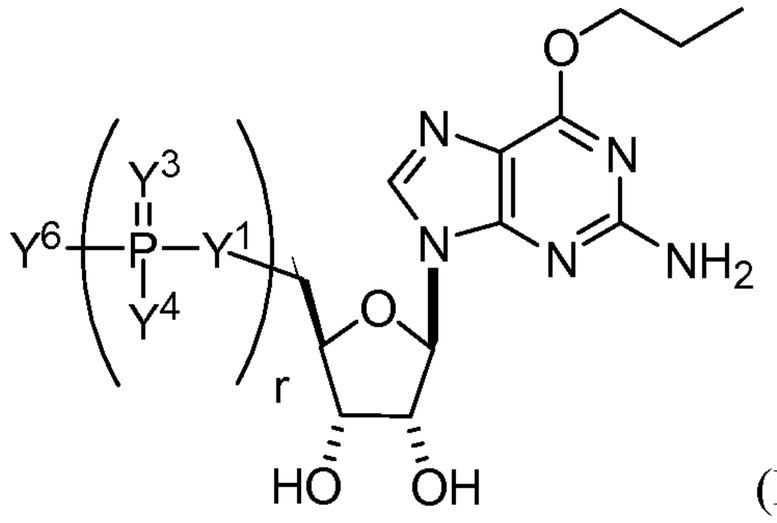
(BB-



213),

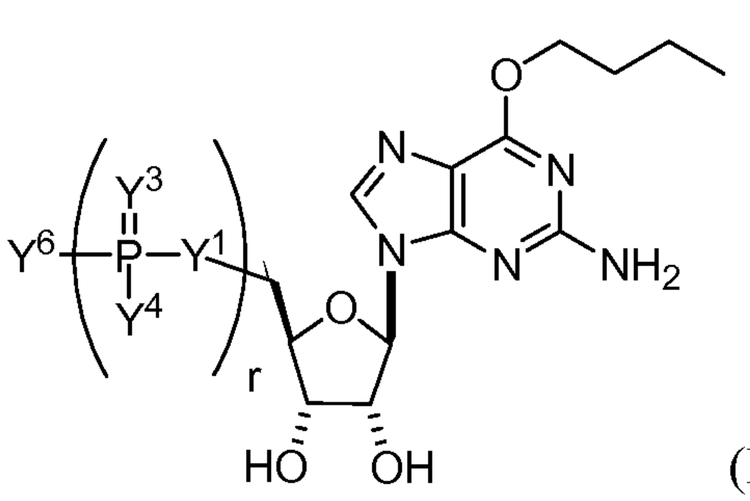


(BB- 214),

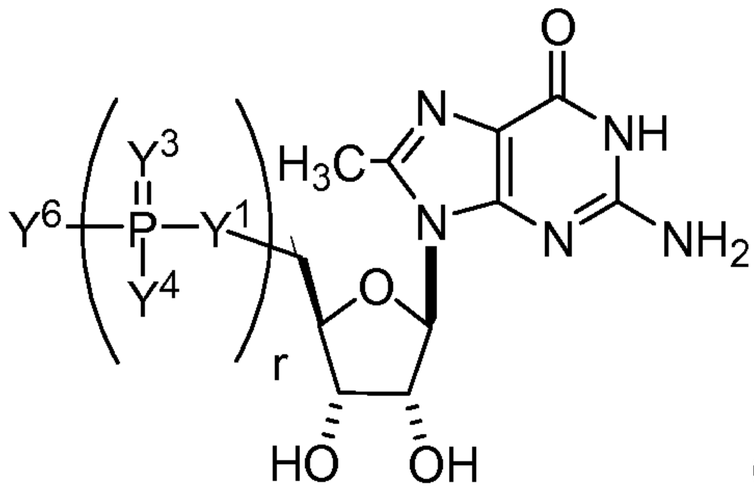


(BB- 215),

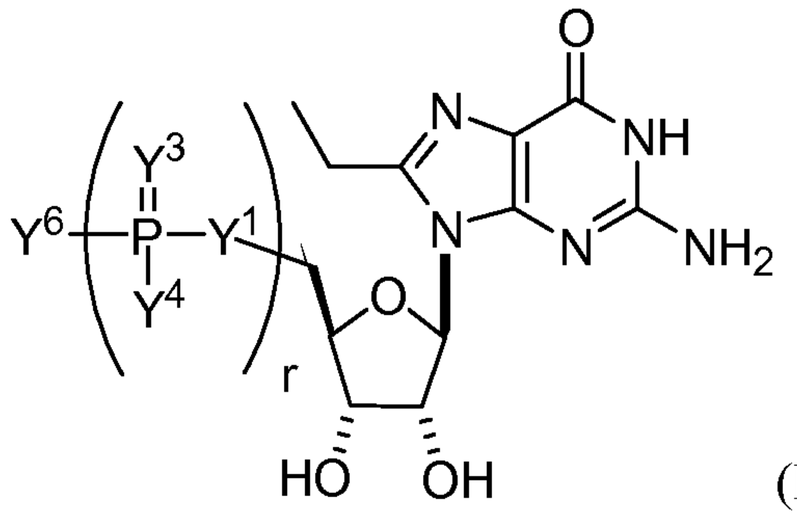
(BB- 216),



(BB- 217),

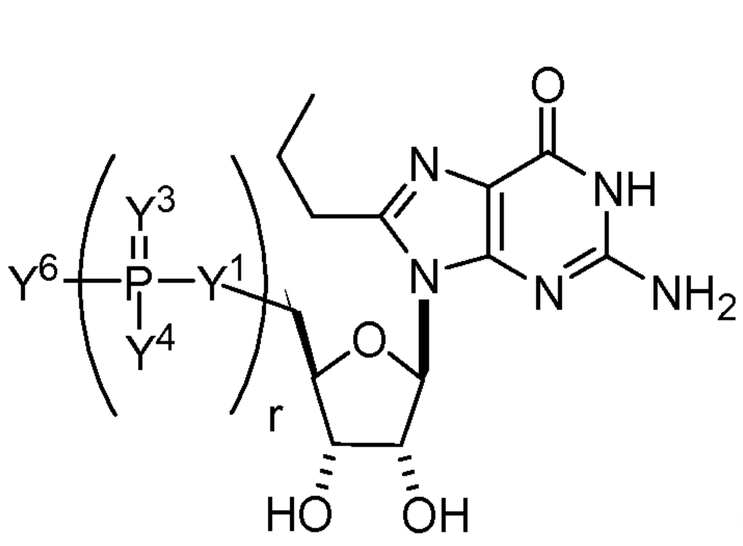


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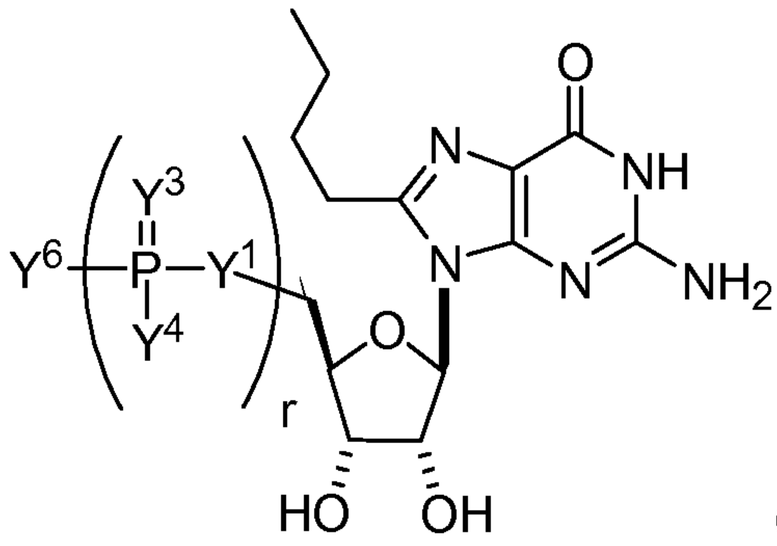


218),

(BB- 219),

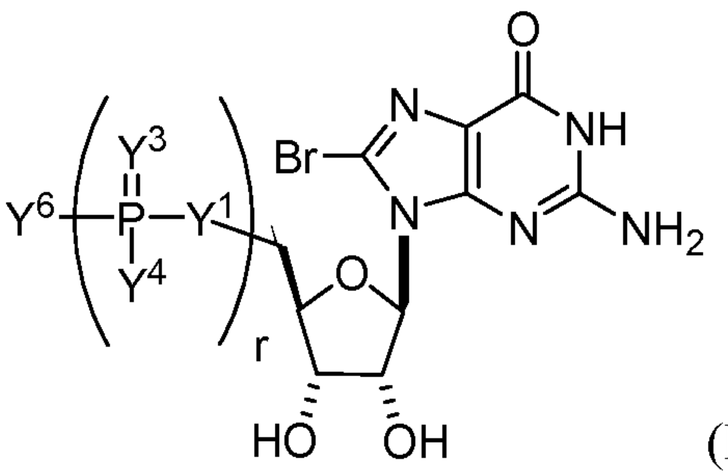


(BB- 220),



(BB-

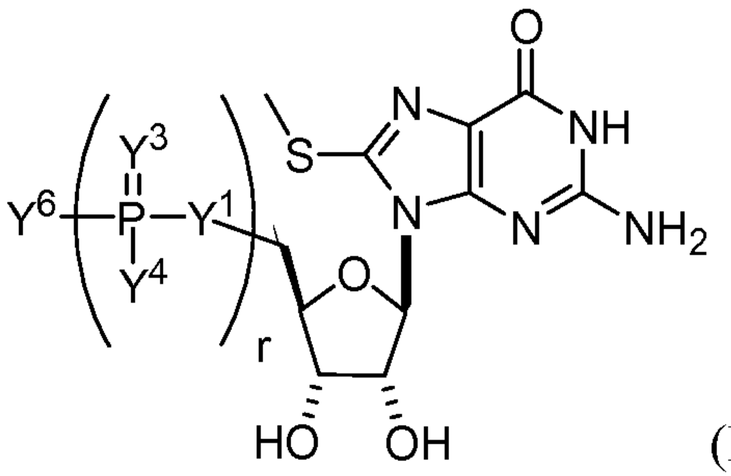
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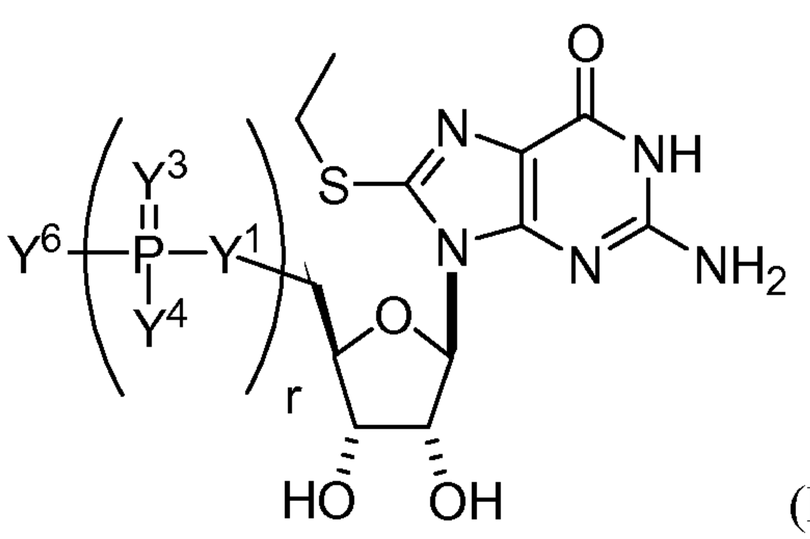
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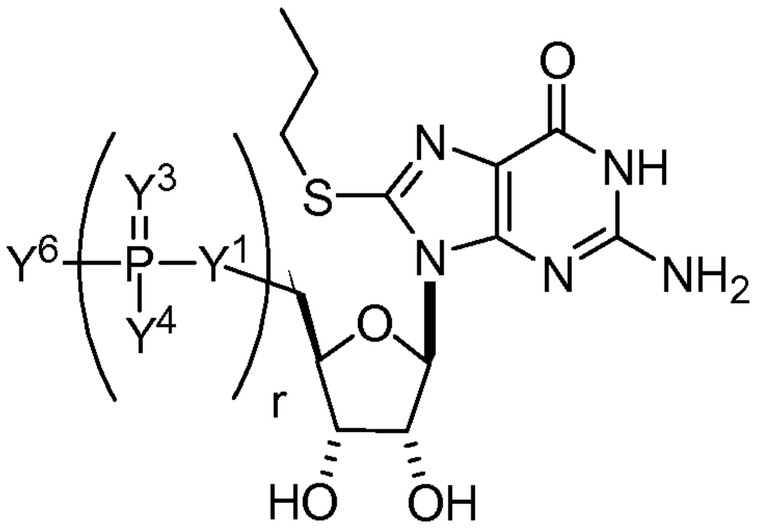
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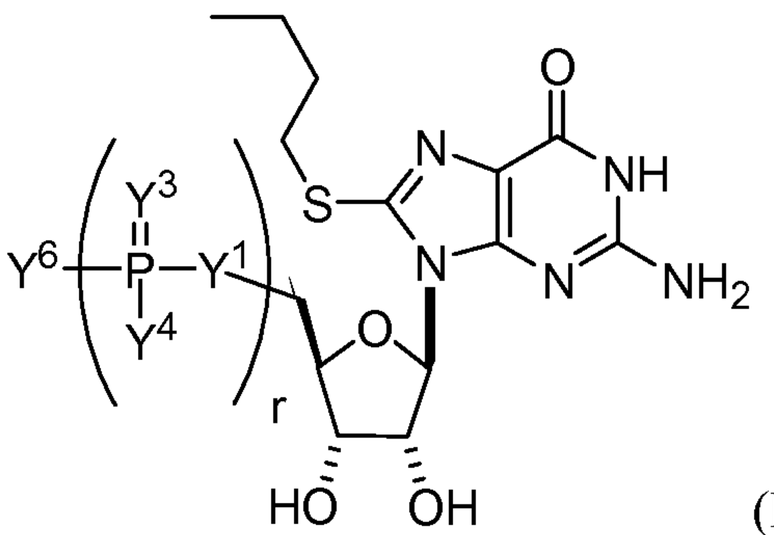
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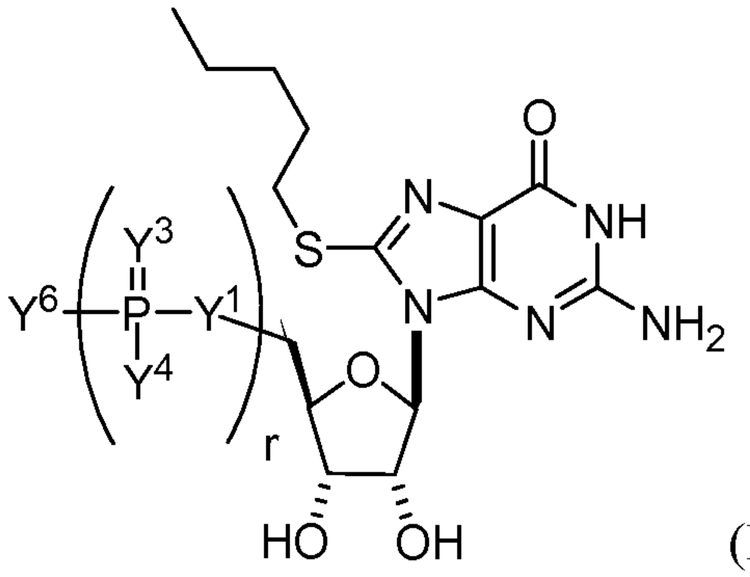
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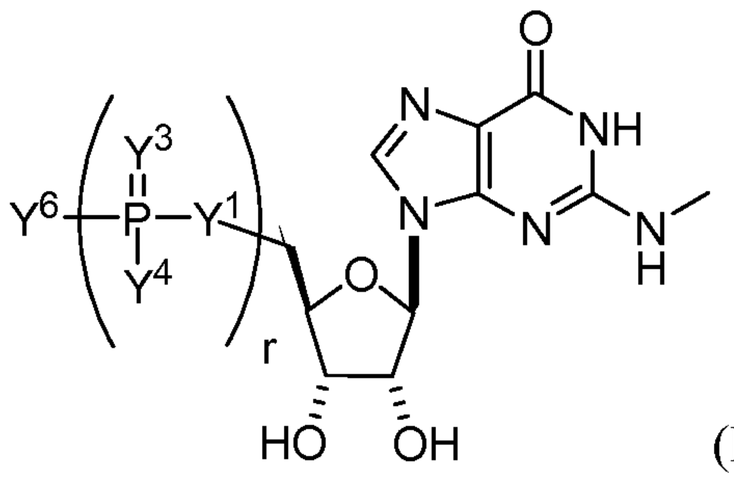
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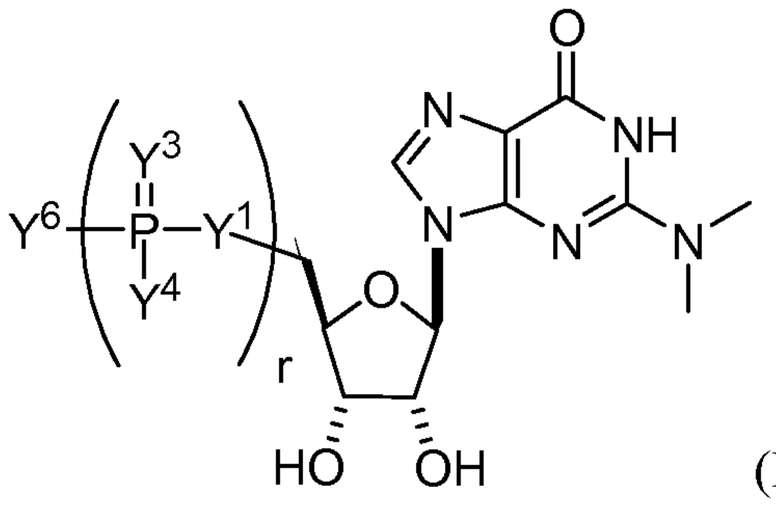
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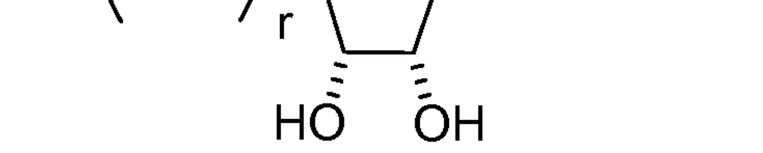


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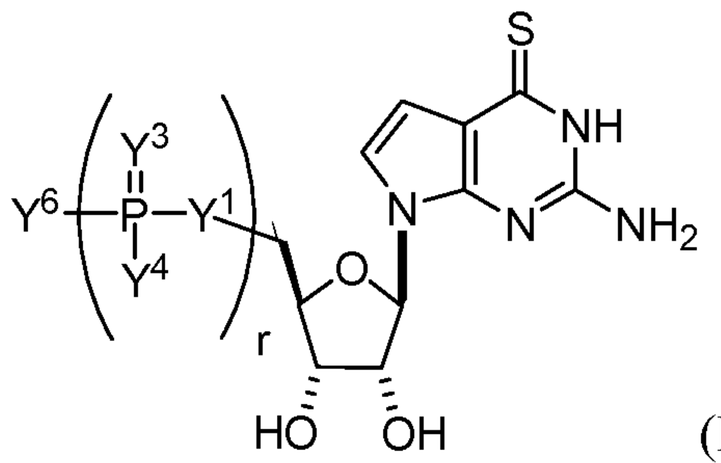


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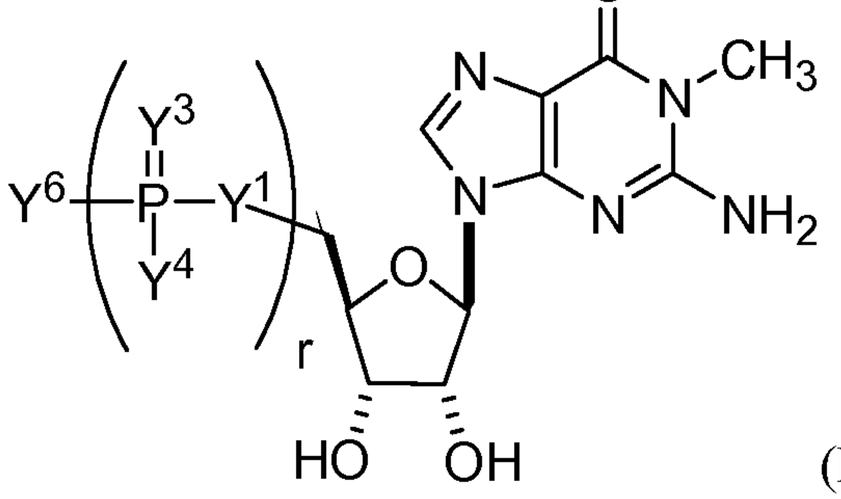
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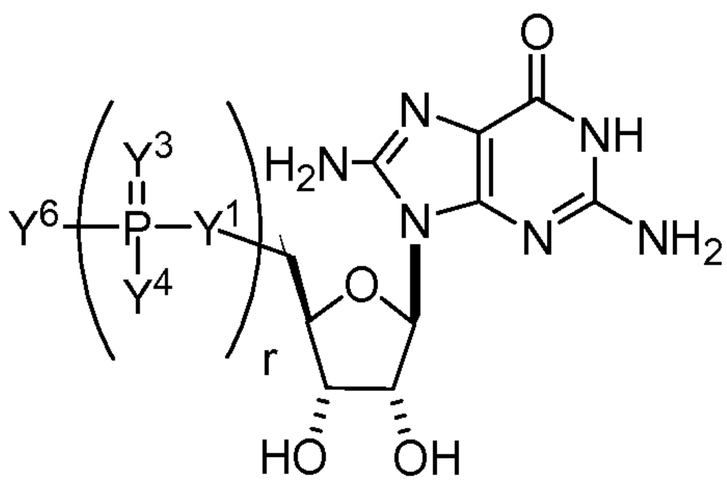
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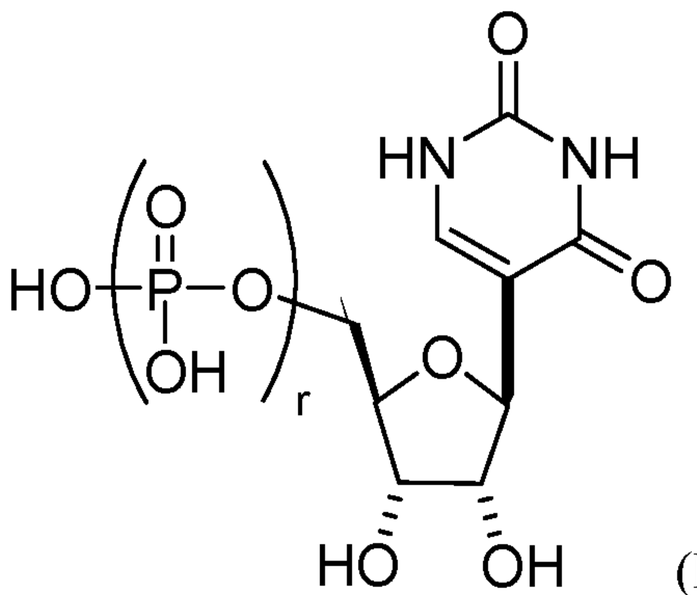
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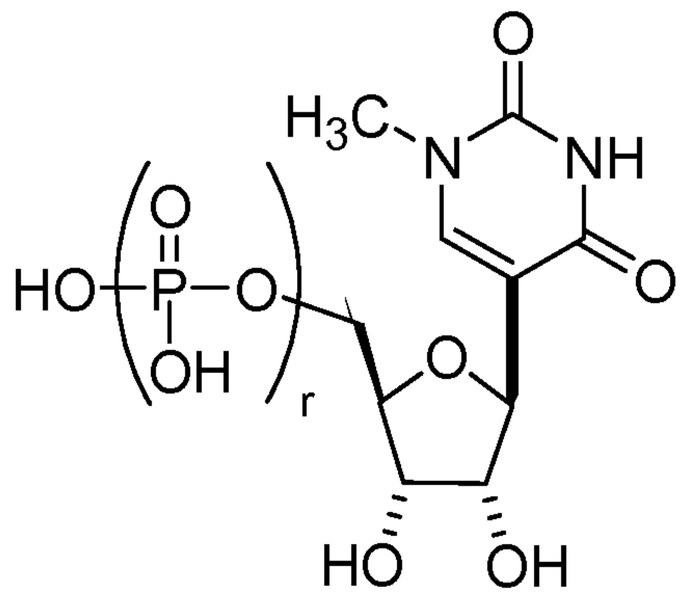
(BB- 237), or a pharmaceutically acceptable salt or stereoisomer thereof, wherein Y^1, Y^3,

Y^4, Y^6, and r are as described herein (e.g., each r is, independently, an integer from 0 to 5, such as from 0 to 3, from 1 to 3, or from 1 to 5)).

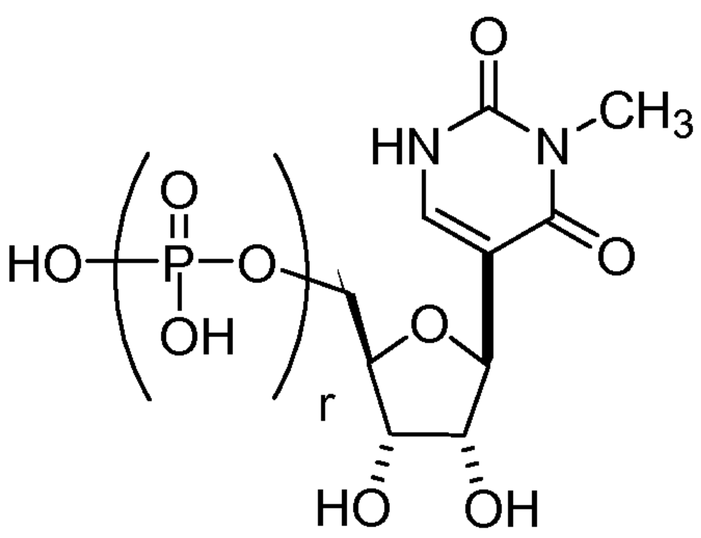
[000419] In some embodiments, the chemical modification can include replacement of C group at C-5 of the ring (e.g., for a pyrimidine nucleoside, such as cytosine or uracil) with N (e.g., replacement of the >CH group at C-5 with $>NR^{N1}$ group, wherein R^N1 is H or optionally substituted alkyl). For example, the building block molecule, which may be incorporated into a polynucleotide, primary construct, or mmRNA, can be:



(BB- 238) or

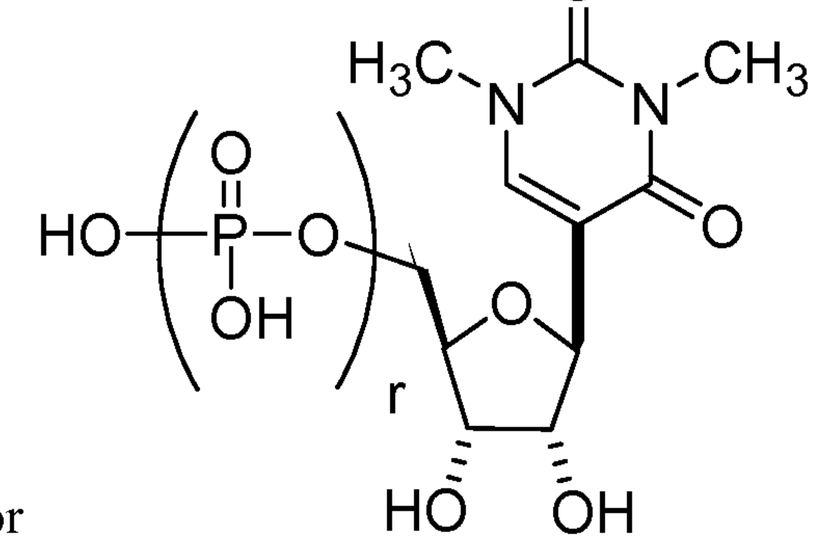


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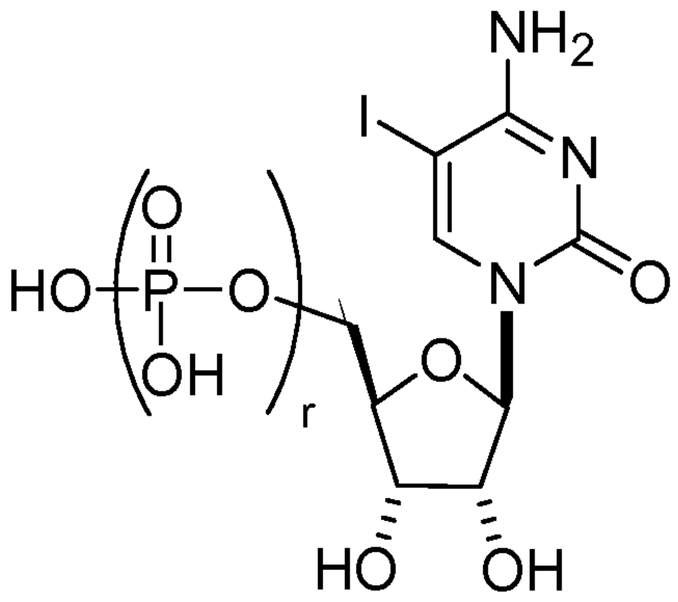
or

(BB- 240) or

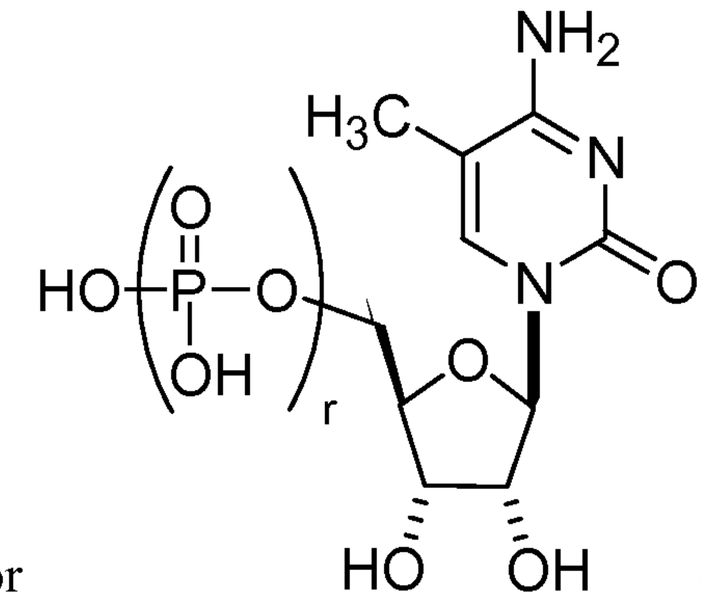


(BB- 241), or a pharmaceutically acceptable salt or stereoisomer thereof, wherein each r is, independently, an integer from 0 to 5 (e.g., from 0 to 3, from 1 to 3, or from 1 to 5).

[000420] In another embodiment, the chemical modification can include replacement of the hydrogen at C-5 of cytosine with halo (e.g., Br, Cl, F, or I) or optionally substituted alkyl (e.g., methyl). For example, the building block molecule, which may be incorporated into a polynucleotide, primary construct, or mmRNA, can be:

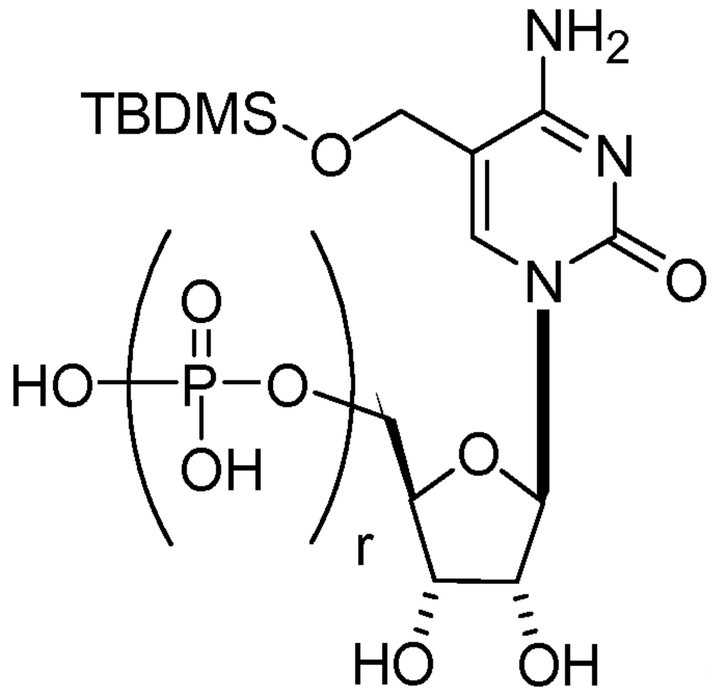


(BB- 242) or

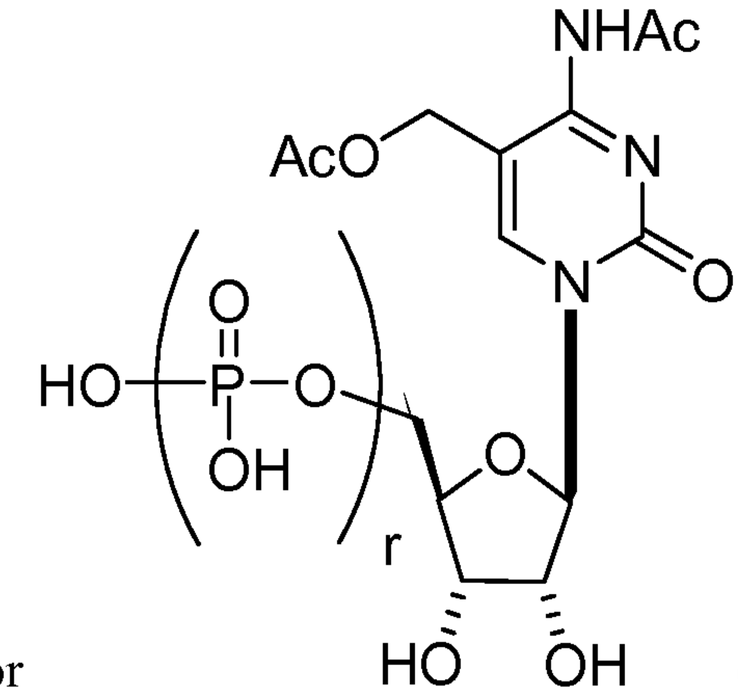


(BB- 243)

or

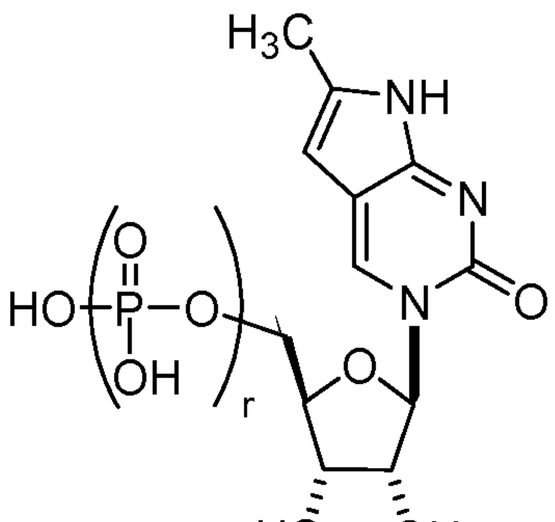


(BB- 244) or



(BB- 245), or a pharmaceutically acceptable salt or stereoisomer thereof, wherein each r is, independently, an integer from 0 to 5 (e.g., from 0 to 3, from 1 to 3, or from 1 to 5).

[000421] In yet a further embodiment, the chemical modification can include a fused ring that is formed by the NH_2 at the C-4 position and the carbon atom at the C-5 position. For example, the building block molecule, which may be incorporated into a polynucleotide, primary construct, or mmRNA, can be:



(BB- 246), or a pharmaceutically acceptable salt or stereoisomer thereof, wherein each r is, independently, an integer from 0 to 5 (e.g., from 0 to 3, from 1 to 3, or from 1 to 5).

Modifications on the Sugar

[000422] The modified nucleosides and nucleotides (e.g., building block molecules), which may be incorporated into a polynucleotide, primary construct, or mmRNA (e.g., RNA or mRNA, as described herein), can be modified on the sugar of the ribonucleic acid. For example, the 2′ hydroxyl group (OH) can be modified or replaced with a number of different substituents. Exemplary substitutions at the 2′-position include, but are not limited to, H, halo, optionally substituted C_{1-6} alkyl; optionally substituted C_{1-6} alkoxy; optionally substituted C_{6-10} aryloxy; optionally substituted C_{3-8} cycloalkyl; optionally substituted C_{3-8} cycloalkoxy; optionally substituted C_{6-10} aryloxy; optionally substituted C_{6-10} aryl-C_{1-6} alkoxy, optionally substituted C_{1-12} (heterocyclyl)oxy; a sugar (e.g., ribose, pentose, or any described herein); a polyethyleneglycol (PEG), -$O(CH_2CH_2O)_nCH_2CH_2OR$, where R is H or optionally substituted alkyl, and n is an integer from 0 to 20 (e.g., from 0 to 4, from 0 to 8, from 0 to 10, from 0 to 16, from 1 to 4, from 1 to 8, from 1 to 10, from 1 to 16, from 1 to 20, from 2 to 4, from 2 to 8, from 2 to 10, from 2 to 16, from 2 to 20, from 4 to 8, from 4 to 10, from 4 to 16, and from 4 to 20); “locked” nucleic acids (LNA) in which the 2′-hydroxyl is connected by a C_{1-6} alkylene or C_{1-6} heteroalkylene bridge to the 4’-carbon of the same ribose sugar, where exemplary bridges included methylene, propylene, ether, or amino bridges; aminoalkyl, as defined herein; aminoalkoxy, as defined herein; amino as defined herein; and amino acid, as defined herein

Generally, RNA includes the sugar group ribose, which is a 5-membered ring having an oxygen. Exemplary, non-limiting modified nucleotides include replacement of the oxygen in ribose (e.g., with S, Se, or alkylene, such as methylene or ethylene); addition of a double bond (e.g., to replace ribose with cyclopentenyl or cyclohexenyl); ring contraction of ribose (e.g., to form a 4-membered ring of cyclobutane or oxetane); ring expansion of ribose (e.g., to form a 6- or 7-membered ring having an additional carbon or heteroatom, such as for anhydrohexitol, altritol, mannitol, cyclohexanyl, cyclohexenyl, and morpholino that also has a phosphoramidate backbone); multicyclic forms (e.g., tricyclo; and “unlocked” forms, such as glycol nucleic acid (GNA) (e.g., R-GNA or S-GNA, where ribose is replaced by glycol units attached to phosphodiester bonds), threose nucleic acid (TNA, where ribose is replace with α-L-threofuranosyl-(3′→2′)) , and peptide nucleic acid (PNA, where 2-amino-ethyl-glycine linkages replace the ribose and

phosphodiester backbone). The sugar group can also contain one or more carbons that possess the opposite stereochemical configuration than that of the corresponding carbon in ribose. Thus, a polynucleotide, primary construct, or mmRNA molecule can include nucleotides containing, e.g., arabinose, as the sugar.

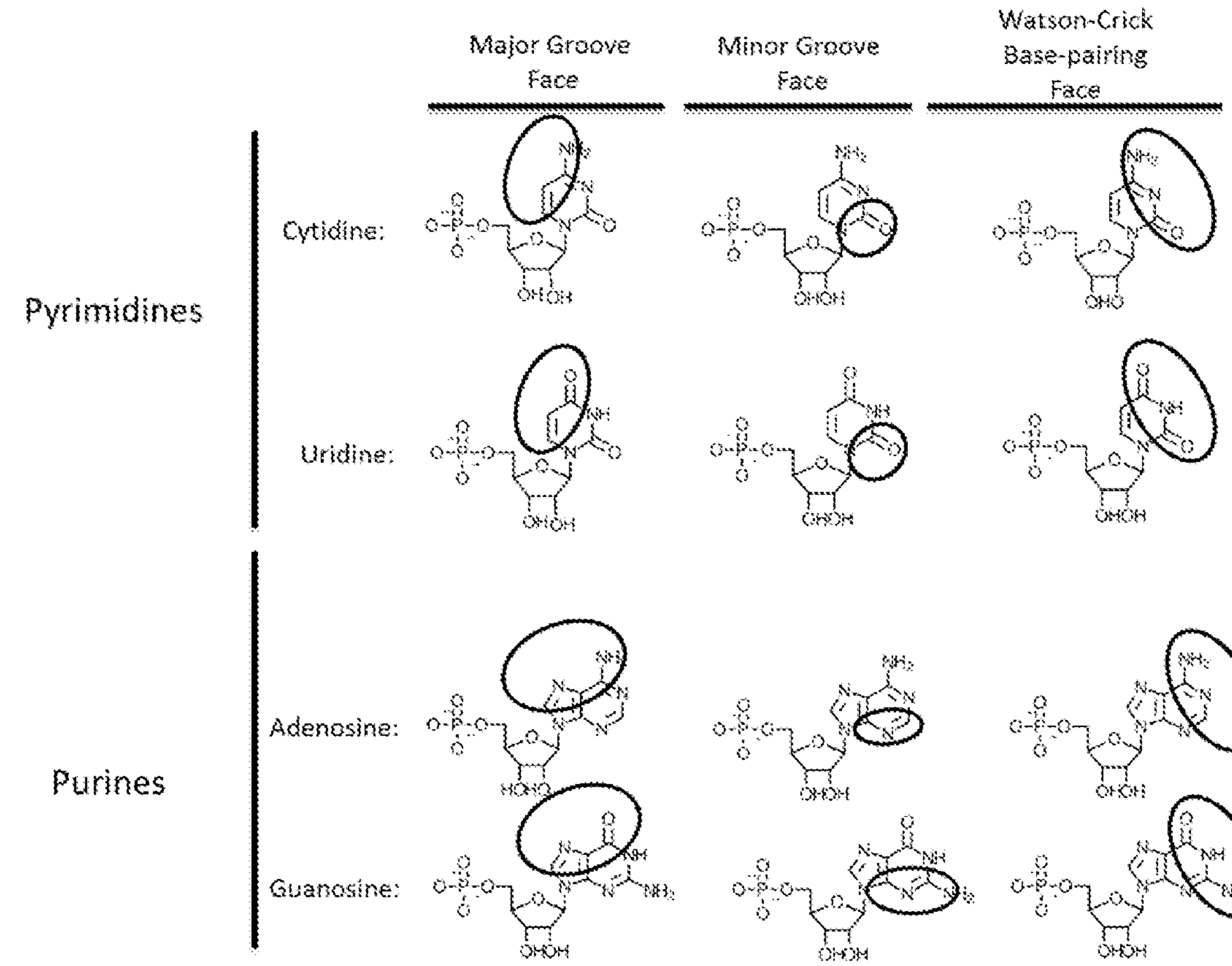
Modifications on the Nucleobase

[000423] The present disclosure provides for modified nucleosides and nucleotides. As described herein "nucleoside" is defined as a compound containing a sugar molecule (e.g., a pentose or ribose) or a derivative thereof in combination with an organic base (e.g., a purine or pyrimidine) or a derivative thereof (also referred to herein as "nucleobase"). As described herein, "nucleotide" is defined as a nucleoside including a phosphate group. The modified nucleotides may by synthesized by any useful method, as described herein (e.g., chemically, enzymatically, or recombinantly to include one or more modified or non-natural nucleosides).

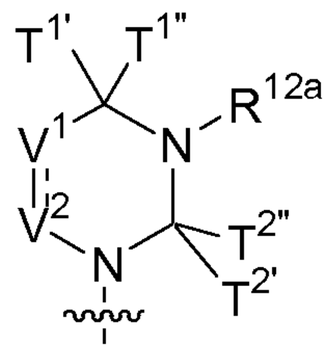
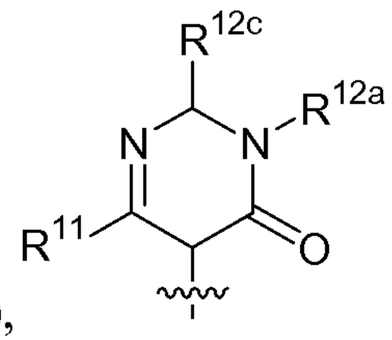
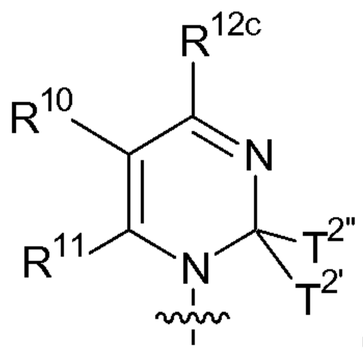
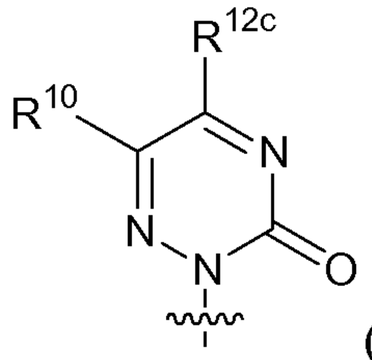
[000424] The modified nucleotide base pairing encompasses not only the standard adenosine-thymine, adenosine-uracil, or guanosine-cytosine base pairs, but also base pairs formed between nucleotides and/or modified nucleotides comprising non-standard or modified bases, wherein the arrangement of hydrogen bond donors and hydrogen bond acceptors permits hydrogen bonding between a non-standard base and a standard base or between two complementary non-standard base structures. One example of such non-standard base pairing is the base pairing between the modified nucleotide inosine and adenine, cytosine or uracil.

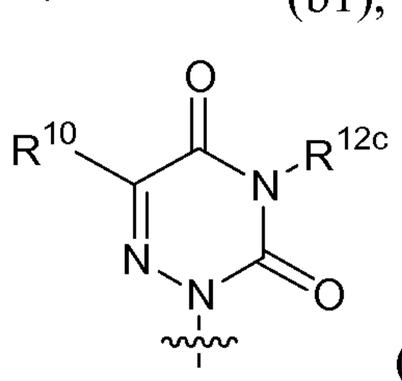
[000425] The modified nucleosides and nucleotides can include a modified nucleobase. Examples of nucleobases found in RNA include, but are not limited to, adenine, guanine, cytosine, and uracil. Examples of nucleobase found in DNA include, but are not limited to, adenine, guanine, cytosine, and thymine. These nucleobases can be modified or wholly replaced to provide polynucleotides, primary constructs, or mmRNA molecules having enhanced properties, e.g., resistance to nucleases through disruption of the binding of a major groove binding partner. Table 8 below identifies the chemical faces of each canonical nucleotide. Circles identify the atoms comprising the respective chemical regions.

Table 8



[000426] In some embodiments, B is a modified uracil. Exemplary modified uracils include those having Formula (b1)-(b5):

 (b1),  (b2),  (b3),  (b4),

 or (b5), or a pharmaceutically acceptable salt or stereoisomer thereof,

[000427] wherein

[000428] ═ is a single or double bond;

[000429] each of $T^{1'}$, $T^{1''}$, $T^{2'}$, and $T^{2''}$ is, independently, H, optionally substituted alkyl, optionally substituted alkoxy, or optionally substituted thioalkoxy, or the combination of $T^{1'}$ and $T^{1''}$ or the combination of $T^{2'}$ and $T^{2''}$ join together (e.g., as in T^2) to form O (oxo), S (thio), or Se (seleno);

[000430] each of V^1 and V^2 is, independently, O, S, $N(R^{Vb})_{nv}$, or $C(R^{Vb})_{nv}$, wherein nv is an integer from 0 to 2 and each R^{Vb} is, independently, H, halo, optionally substituted amino acid, optionally substituted alkyl, optionally substituted haloalkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted hydroxyalkyl, optionally substituted hydroxyalkenyl, optionally substituted hydroxyalkynyl, optionally substituted aminoalkyl (e.g., substituted with an N-protecting group, such as any described herein, e.g., trifluoroacetyl), optionally substituted aminoalkenyl, optionally substituted aminoalkynyl, optionally substituted acylaminoalkyl (e.g., substituted with an N-protecting group, such as any described herein, e.g., trifluoroacetyl), optionally substituted alkoxycarbonylalkyl, optionally substituted alkoxycarbonylalkenyl, optionally substituted alkoxycarbonylalkynyl, or optionally substituted alkynyloxy (e.g., optionally substituted with any substituent described herein, such as those selected from (1)-(21) for alkyl);

[000431] R^{10} is H, halo, optionally substituted amino acid, hydroxy, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted aminoalkyl, optionally substituted hydroxyalkyl, optionally substituted hydroxyalkenyl, optionally substituted hydroxyalkynyl, optionally substituted aminoalkenyl, optionally substituted aminoalkynyl, optionally substituted alkoxy, optionally substituted alkoxycarbonylalkyl, optionally substituted alkoxycarbonylalkenyl, optionally substituted alkoxycarbonylalkynyl, optionally substituted alkoxycarbonylalkoxy, optionally substituted carboxyalkoxy, optionally substituted carboxyalkyl, or optionally substituted carbamoylalkyl;

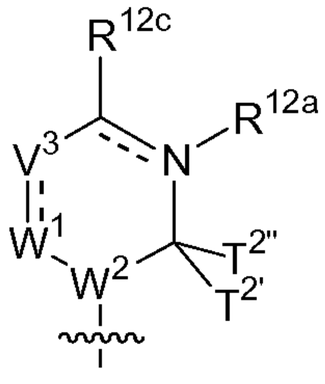
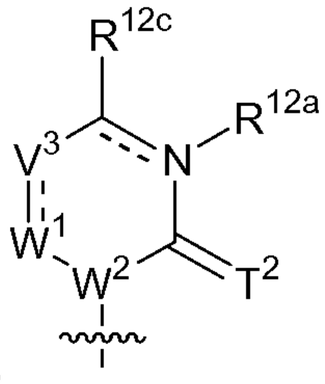
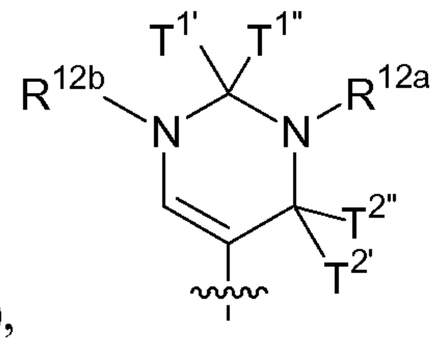
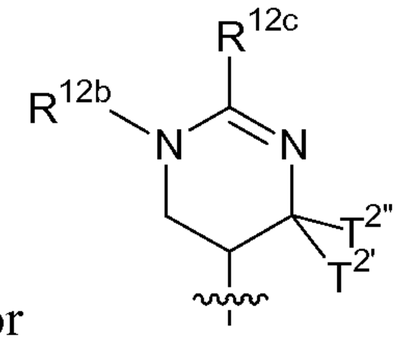
[000432] R^{11} is H or optionally substituted alkyl;

[000433] R^{12a} is H, optionally substituted alkyl, optionally substituted hydroxyalkyl, optionally substituted hydroxyalkenyl, optionally substituted hydroxyalkynyl, optionally substituted aminoalkyl, optionally substituted aminoalkenyl, or optionally substituted

aminoalkynyl, optionally substituted carboxyalkyl (e.g., optionally substituted with hydroxy), optionally substituted carboxyalkoxy, optionally substituted carboxyaminoalkyl, or optionally substituted carbamoylalkyl; and

[000434] R^{12c} is H, halo, optionally substituted alkyl, optionally substituted alkoxy, optionally substituted thioalkoxy, optionally substituted amino, optionally substituted hydroxyalkyl, optionally substituted hydroxyalkenyl, optionally substituted hydroxyalkynyl, optionally substituted aminoalkyl, optionally substituted aminoalkenyl, or optionally substituted aminoalkynyl.

[000435] Other exemplary modified uracils include those having Formula (b6)-(b9):


(b6),

(b7),

(b8), or


(b9), or a pharmaceutically acceptable salt or stereoisomer thereof,

[000436] wherein

[000437] $\overset{\cdots}{=}$ is a single or double bond;

[000438] each of $T^{1'}$, $T^{1''}$, $T^{2'}$, and $T^{2''}$ is, independently, H, optionally substituted alkyl, optionally substituted alkoxy, or optionally substituted thioalkoxy, or the combination of $T^{1'}$ and $T^{1''}$ join together (e.g., as in T^1) or the combination of $T^{2'}$ and $T^{2''}$ join together (e.g., as in T^2) to form O (oxo), S (thio), or Se (seleno), or each T^1 and T^2 is, independently, O (oxo), S (thio), or Se (seleno);

[000439] each of W^1 and W^2 is, independently, $N(R^{Wa})_{nw}$ or $C(R^{Wa})_{nw}$, wherein nw is an integer from 0 to 2 and each R^{Wa} is, independently, H, optionally substituted alkyl, or optionally substituted alkoxy;

[000440] each V^3 is, independently, O, S, $N(R^{Va})_{nv}$, or $C(R^{Va})_{nv}$, wherein nv is an integer from 0 to 2 and each R^{Va} is, independently, H, halo, optionally substituted amino acid, optionally substituted alkyl, optionally substituted hydroxyalkyl, optionally substituted hydroxyalkenyl, optionally substituted hydroxyalkynyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted heterocyclyl, optionally substituted alkheterocyclyl, optionally substituted alkoxy, optionally substituted

alkenyloxy, or optionally substituted alkynyloxy , optionally substituted aminoalkyl (e.g., substituted with an N-protecting group, such as any described herein, e.g., trifluoroacetyl, or sulfoalkyl), optionally substituted aminoalkenyl, optionally substituted aminoalkynyl, optionally substituted acylaminoalkyl (e.g., substituted with an N-protecting group, such as any described herein, e.g., trifluoroacetyl), optionally substituted alkoxycarbonylalkyl, optionally substituted alkoxycarbonylalkenyl, optionally substituted alkoxycarbonylalkynyl, optionally substituted alkoxycarbonylacyl, optionally substituted alkoxycarbonylalkoxy, optionally substituted carboxyalkyl (e.g., optionally substituted with hydroxy and/or an *O*-protecting group), optionally substituted carboxyalkoxy, optionally substituted carboxyaminoalkyl, or optionally substituted carbamoylalkyl (e.g., optionally substituted with any substituent described herein, such as those selected from (1)-(21) for alkyl), and wherein R^{Va} and R^{12c} taken together with the carbon atoms to which they are attached can form optionally substituted cycloalkyl, optionally substituted aryl, or optionally substituted heterocyclyl (e.g., a 5- or 6-membered ring);

[000441] R^{12a} is H, optionally substituted alkyl, optionally substituted hydroxyalkyl, optionally substituted hydroxyalkenyl, optionally substituted hydroxyalkynyl, optionally substituted aminoalkyl, optionally substituted aminoalkenyl, optionally substituted aminoalkynyl, optionally substituted carboxyalkyl (e.g., optionally substituted with hydroxy and/or an *O*-protecting group), optionally substituted carboxyalkoxy, optionally substituted carboxyaminoalkyl, optionally substituted carbamoylalkyl, or absent;

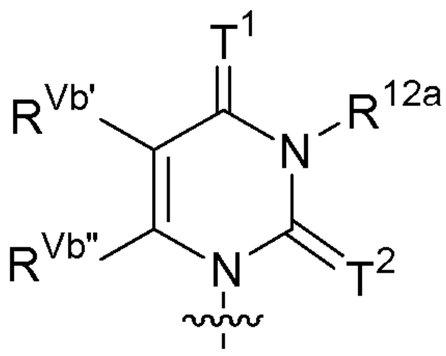
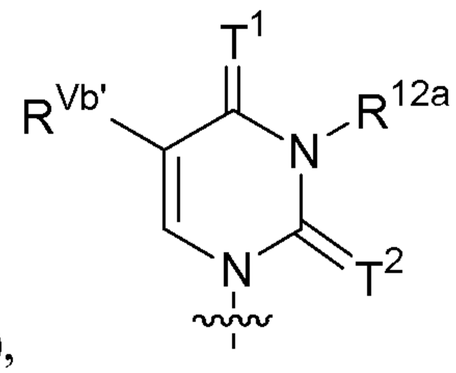
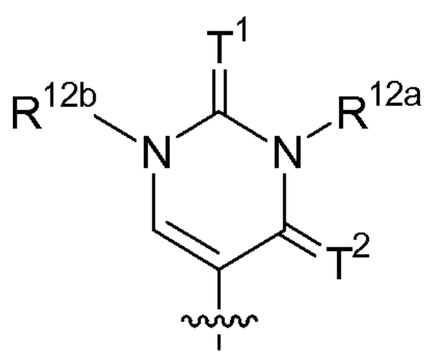
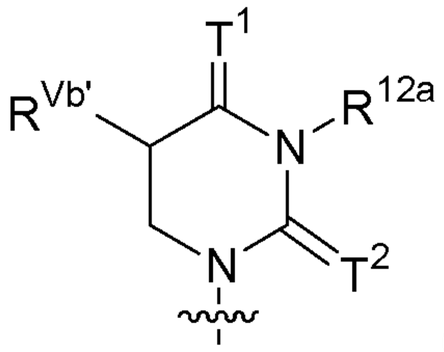
[000442] R^{12b} is H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted hydroxyalkyl, optionally substituted hydroxyalkenyl, optionally substituted hydroxyalkynyl, optionally substituted aminoalkyl, optionally substituted aminoalkenyl, optionally substituted aminoalkynyl, optionally substituted alkaryl, optionally substituted heterocyclyl, optionally substituted alkheterocyclyl, optionally substituted amino acid, optionally substituted alkoxycarbonylacyl, optionally substituted alkoxycarbonylalkoxy, optionally substituted alkoxycarbonylalkyl, optionally substituted alkoxycarbonylalkenyl, optionally substituted alkoxycarbonylalkynyl, optionally substituted alkoxycarbonylalkoxy, optionally substituted carboxyalkyl (e.g., optionally substituted with hydroxy and/or an *O*-protecting

group), optionally substituted carboxyalkoxy, optionally substituted carboxyaminoalkyl, or optionally substituted carbamoylalkyl,

[000443] wherein the combination of R^{12b} and $T^{1'}$ or the combination of R^{12b} and R^{12c} can join together to form optionally substituted heterocyclyl; and

[000444] R^{12c} is H, halo, optionally substituted alkyl, optionally substituted alkoxy, optionally substituted thioalkoxy, optionally substituted amino, optionally substituted aminoalkyl, optionally substituted aminoalkenyl, or optionally substituted aminoalkynyl.

[000445] Further exemplary modified uracils include those having Formula (b28)-(b31):


(b28),

(b29),

(b30), or

(b31), or a pharmaceutically acceptable salt or stereoisomer thereof,

[000446] wherein

[000447] each of T^1 and T^2 is, independently, O (oxo), S (thio), or Se (seleno);

[000448] each $R^{Vb'}$ and $R^{Vb''}$ is, independently, H, halo, optionally substituted amino acid, optionally substituted alkyl, optionally substituted haloalkyl, optionally substituted hydroxyalkyl, optionally substituted hydroxyalkenyl, optionally substituted hydroxyalkynyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted aminoalkyl (e.g., substituted with an N-protecting group, such as any described herein, e.g., trifluoroacetyl, or sulfoalkyl), optionally substituted aminoalkenyl, optionally substituted aminoalkynyl, optionally substituted acylaminoalkyl (e.g., substituted with an N-protecting group, such as any described herein, e.g., trifluoroacetyl), optionally substituted alkoxycarbonylalkyl, optionally substituted alkoxycarbonylalkenyl, optionally substituted alkoxycarbonylalkynyl, optionally

substituted alkoxycarbonylacyl, optionally substituted alkoxycarbonylalkoxy, optionally substituted carboxyalkyl (e.g., optionally substituted with hydroxy and/or an *O*-protecting group), optionally substituted carboxyalkoxy, optionally substituted carboxyaminoalkyl, or optionally substituted carbamoylalkyl (e.g., optionally substituted with any substituent described herein, such as those selected from (1)-(21) for alkyl) (e.g., $R^{Vb'}$ is optionally substituted alkyl, optionally substituted alkenyl, or optionally substituted aminoalkyl, e.g., substituted with an N-protecting group, such as any described herein, e.g., trifluoroacetyl, or sulfoalkyl);

[000449] R^{12a} is H, optionally substituted alkyl, optionally substituted carboxyaminoalkyl, optionally substituted aminoalkyl (e.g., e.g., substituted with an N-protecting group, such as any described herein, e.g., trifluoroacetyl, or sulfoalkyl), optionally substituted aminoalkenyl, or optionally substituted aminoalkynyl; and

[000450] R^{12b} is H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted hydroxyalkyl, optionally substituted hydroxyalkenyl, optionally substituted hydroxyalkynyl, optionally substituted aminoalkyl, optionally substituted aminoalkenyl, optionally substituted aminoalkynyl (e.g., e.g., substituted with an N-protecting group, such as any described herein, e.g., trifluoroacetyl, or sulfoalkyl),

[000451] optionally substituted alkoxycarbonylacyl, optionally substituted alkoxycarbonylalkoxy, optionally substituted alkoxycarbonylalkyl, optionally substituted alkoxycarbonylalkenyl, optionally substituted alkoxycarbonylalkynyl, optionally substituted alkoxycarbonylalkoxy, optionally substituted carboxyalkoxy, optionally substituted carboxyalkyl, or optionally substituted carbamoylalkyl.

[000452] In particular embodiments, T^1 is O (oxo), and T^2 is S (thio) or Se (seleno). In other embodiments, T^1 is S (thio), and T^2 is O (oxo) or Se (seleno). In some embodiments, $R^{Vb'}$ is H, optionally substituted alkyl, or optionally substituted alkoxy.

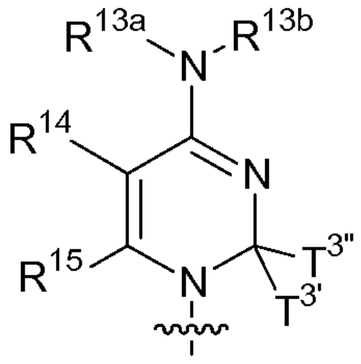
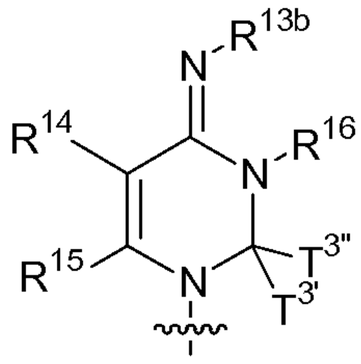
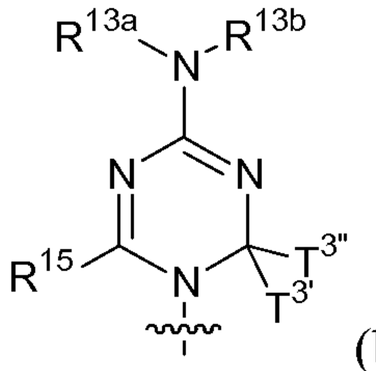
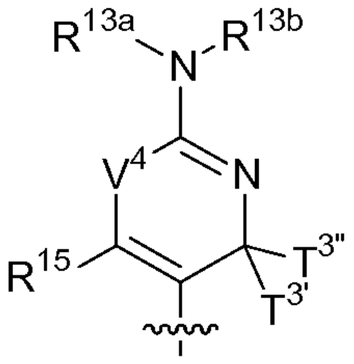
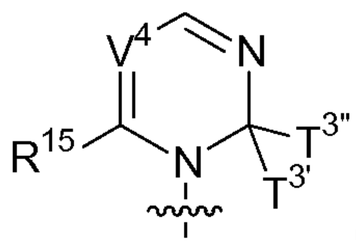
[000453] In other embodiments, each R^{12a} and R^{12b} is, independently, H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, or optionally substituted hydroxyalkyl. In particular embodiments, R^{12a} is H. In other embodiments, both R^{12a} and R^{12b} are H.

[000454] In some embodiments, each $R^{Vb'}$ of R^{12b} is, independently, optionally substituted aminoalkyl (e.g., substituted with an N-protecting group, such as any described herein, e.g., trifluoroacetyl, or sulfoalkyl), optionally substituted aminoalkenyl, optionally substituted aminoalkynyl, or optionally substituted acylaminoalkyl (e.g., substituted with an N-protecting group, such as any described herein, e.g., trifluoroacetyl). In some embodiments, the amino and/or alkyl of the optionally substituted aminoalkyl is substituted with one or more of optionally substituted alkyl, optionally substituted alkenyl, optionally substituted sulfoalkyl, optionally substituted carboxy (e.g., substituted with an *O*-protecting group), optionally substituted hydroxy (e.g., substituted with an *O*-protecting group), optionally substituted carboxyalkyl (e.g., substituted with an *O*-protecting group), optionally substituted alkoxycarbonylalkyl (e.g., substituted with an *O*-protecting group), or *N*-protecting group. In some embodiments, optionally substituted aminoalkyl is substituted with an optionally substituted sulfoalkyl or optionally substituted alkenyl. In particular embodiments, R^{12a} and $R^{Vb''}$ are both H. In particular embodiments, T^1 is O (oxo), and T^2 is S (thio) or Se (seleno).

[000455] In some embodiments, $R^{Vb'}$ is optionally substituted alkoxycarbonylalkyl or optionally substituted carbamoylalkyl.

[000456] In particular embodiments, the optional substituent for R^{12a}, R^{12b}, R^{12c}, or R^{Va} is a polyethylene glycol group (e.g., $-(CH_2)_{s2}(OCH_2CH_2)_{s1}(CH_2)_{s3}OR'$, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R' is H or C_{1-20} alkyl); or an amino-polyethylene glycol group (e.g., $-NR^{N1}(CH_2)_{s2}(CH_2CH_2O)_{s1}(CH_2)_{s3}NR^{N1}$, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and each R^{N1} is, independently, hydrogen or optionally substituted C_{1-6} alkyl).

[000457] In some embodiments, B is a modified cytosine. Exemplary modified cytosines include compounds of Formula (b10)-(b14):


(b10),

(b11),

(b12),

(b13), or

(b14), or a pharmaceutically acceptable salt or stereoisomer thereof,

wherein

[000458] each of $T^{3'}$ and $T^{3''}$ is, independently, H, optionally substituted alkyl, optionally substituted alkoxy, or optionally substituted thioalkoxy, or the combination of $T^{3'}$ and $T^{3''}$ join together (e.g., as in T^3) to form O (oxo), S (thio), or Se (seleno);

[000459] each V^4 is, independently, O, S, $N(R^{Vc})_{nv}$, or $C(R^{Vc})_{nv}$, wherein nv is an integer from 0 to 2 and each R^{Vc} is, independently, H, halo, optionally substituted amino acid, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted heterocyclyl, optionally substituted alkheterocyclyl, or optionally substituted alkynyloxy (e.g., optionally substituted with any substituent described herein, such as those selected from (1)-(21) for alkyl), wherein the combination of R^{13b} and R^{Vc} can be taken together to form optionally substituted heterocyclyl;

[000460] each V^5 is, independently, $N(R^{Vd})_{nv}$, or $C(R^{Vd})_{nv}$, wherein nv is an integer from 0 to 2 and each R^{Vd} is, independently, H, halo, optionally substituted amino acid, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted heterocyclyl, optionally substituted alkheterocyclyl, or optionally substituted alkynyloxy (e.g., optionally substituted with any substituent described herein, such as those selected from (1)-(21) for alkyl) (e.g., V^5 is –CH or N);

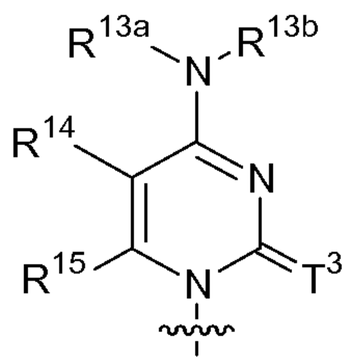
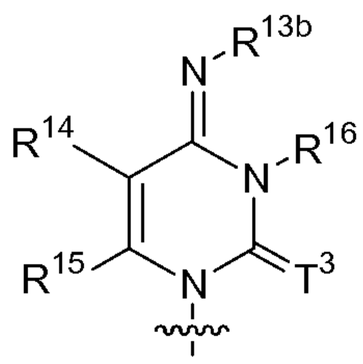
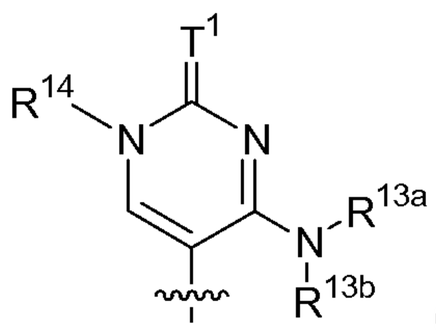
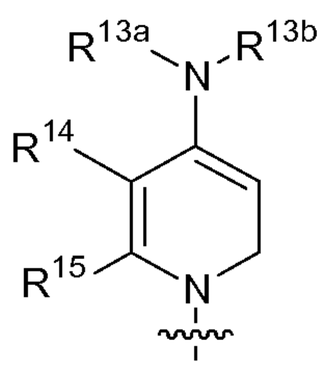
[000461] each of R^{13a} and R^{13b} is, independently, H, optionally substituted acyl, optionally substituted acyloxyalkyl, optionally substituted alkyl, or optionally substituted

alkoxy, wherein the combination of R^{13b} and R^{14} can be taken together to form optionally substituted heterocyclyl;

[000462] each R^{14} is, independently, H, halo, hydroxy, thiol, optionally substituted acyl, optionally substituted amino acid, optionally substituted alkyl, optionally substituted haloalkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted hydroxyalkyl (e.g., substituted with an *O*-protecting group), optionally substituted hydroxyalkenyl, optionally substituted hydroxyalkynyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted aminoalkoxy, optionally substituted alkoxyalkoxy, optionally substituted acyloxyalkyl, optionally substituted amino (e.g., -NHR, wherein R is H, alkyl, aryl, or phosphoryl), azido, optionally substituted aryl, optionally substituted heterocyclyl, optionally substituted alkheterocyclyl, optionally substituted aminoalkyl, optionally substituted aminoalkenyl, or optionally substituted aminoalkyl; and

[000463] each of R^{15} and R^{16} is, independently, H, optionally substituted alkyl, optionally substituted alkenyl, or optionally substituted alkynyl.

[000464] Further exemplary modified cytosines include those having Formula (b32)-(b35):


(b32),

(b33),

(b34), or

(b35), or a pharmaceutically acceptable salt or stereoisomer thereof,

[000465] wherein

[000466] each of T^1 and T^3 is, independently, O (oxo), S (thio), or Se (seleno);

[000467] each of R^{13a} and R^{13b} is, independently, H, optionally substituted acyl, optionally substituted acyloxyalkyl, optionally substituted alkyl, or optionally substituted

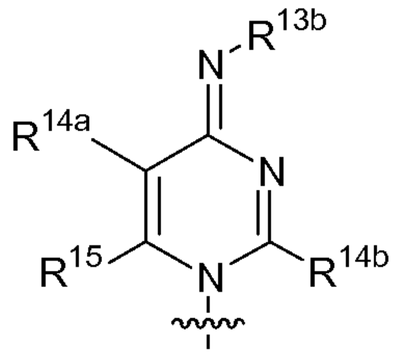
alkoxy, wherein the combination of R^{13b} and R^{14} can be taken together to form optionally substituted heterocyclyl;

[000468] each R^{14} is, independently, H, halo, hydroxy, thiol, optionally substituted acyl, optionally substituted amino acid, optionally substituted alkyl, optionally substituted haloalkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted hydroxyalkyl (e.g., substituted with an *O*-protecting group), optionally substituted hydroxyalkenyl, optionally substituted hydroxyalkynyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted aminoalkoxy, optionally substituted alkoxyalkoxy, optionally substituted acyloxyalkyl, optionally substituted amino (e.g., -NHR, wherein R is H, alkyl, aryl, or phosphoryl), azido, optionally substituted aryl, optionally substituted heterocyclyl, optionally substituted alkheterocyclyl, optionally substituted aminoalkyl (e.g., hydroxyalkyl, alkyl, alkenyl, or alkynyl), optionally substituted aminoalkenyl, or optionally substituted aminoalkynyl; and

[000469] each of R^{15} and R^{16} is, independently, H, optionally substituted alkyl, optionally substituted alkenyl, or optionally substituted alkynyl (e.g., R^{15} is H, and R^{16} is H or optionally substituted alkyl).

[000470] In some embodiments, R^{15} is H, and R^{16} is H or optionally substituted alkyl. In particular embodiments, R^{14} is H, acyl, or hydroxyalkyl. In some embodiments, R^{14} is halo. In some embodiments, both R^{14} and R^{15} are H. In some embodiments, both R^{15} and R^{16} are H. In some embodiments, each of R^{14} and R^{15} and R^{16} is H. In further embodiments, each of R^{13a} and R^{13b} is independently, H or optionally substituted alkyl.

[000471] Further non-limiting examples of modified cytosines include compounds of Formula (b36):



(b36) or a pharmaceutically acceptable salt or stereoisomer thereof,

[000472] wherein

[000473] each R^{13b} is, independently, H, optionally substituted acyl, optionally substituted acyloxyalkyl, optionally substituted alkyl, or optionally substituted alkoxy,

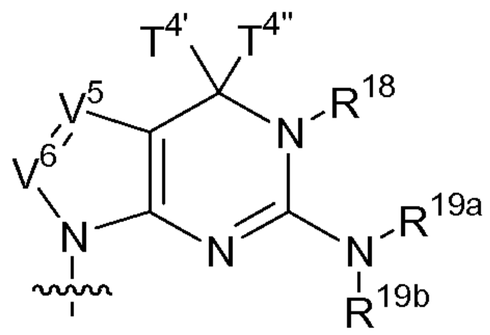
wherein the combination of R^{13b} and R^{14b} can be taken together to form optionally substituted heterocyclyl;

[000474] each R^{14a} and R^{14b} is, independently, H, halo, hydroxy, thiol, optionally substituted acyl, optionally substituted amino acid, optionally substituted alkyl, optionally substituted haloalkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted hydroxyalkyl (e.g., substituted with an *O*-protecting group), optionally substituted hydroxyalkenyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted aminoalkoxy, optionally substituted alkoxyalkoxy, optionally substituted acyloxyalkyl, optionally substituted amino (e.g., -NHR, wherein R is H, alkyl, aryl, phosphoryl, optionally substituted aminoalkyl, or optionally substituted carboxyaminoalkyl), azido, optionally substituted aryl, optionally substituted heterocyclyl, optionally substituted alkheterocyclyl, optionally substituted aminoalkyl, optionally substituted aminoalkenyl, or optionally substituted aminoalkynyl; and

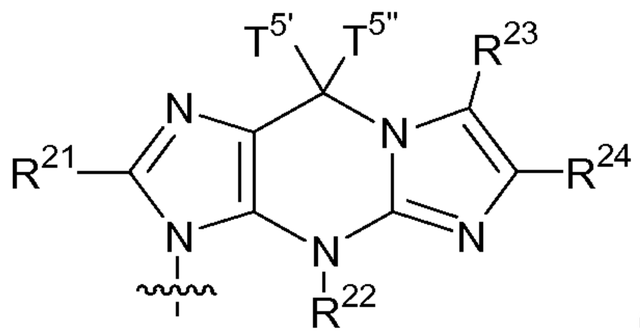
[000475] each of R^{15} is, independently, H, optionally substituted alkyl, optionally substituted alkenyl, or optionally substituted alkynyl.

[000476] In particular embodiments, R^{14b} is an optionally substituted amino acid (e.g., optionally substituted lysine). In some embodiments, R^{14a} is H.

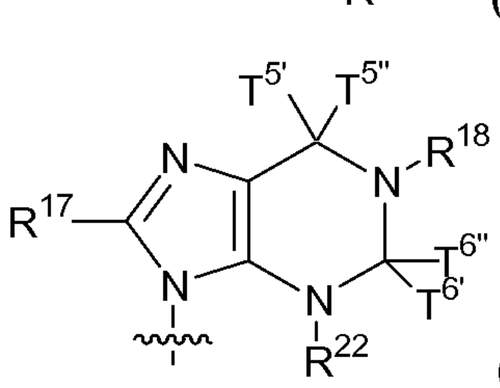
[000477] In some embodiments, B is a modified guanine. Exemplary modified guanines include compounds of Formula (b15)-(b17):



(b15),



(b16), or



(b17), or a pharmaceutically acceptable salt or stereoisomer thereof,

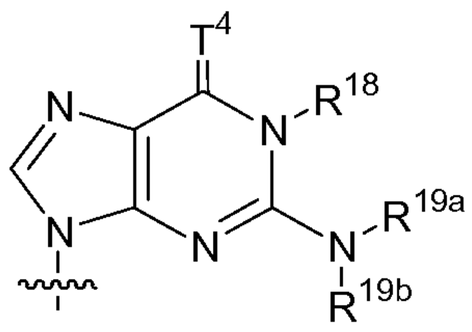
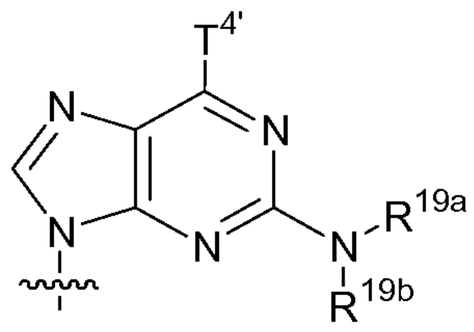
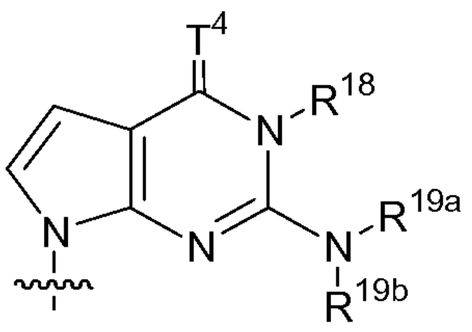
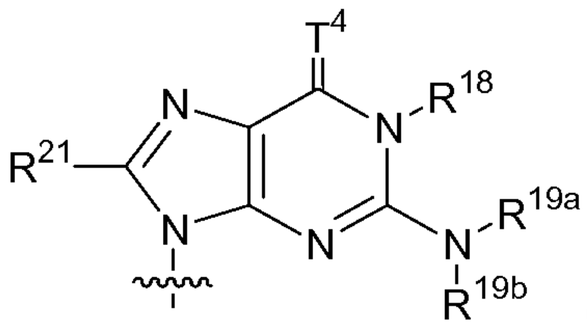
[000478] wherein

[000479] each of $T^{4'}$, $T^{4''}$, $T^{5'}$, $T^{5''}$, $T^{6'}$, and $T^{6''}$ is, independently, H, optionally substituted alkyl, or optionally substituted alkoxy, and wherein the combination of $T^{4'}$ and $T^{4''}$ (e.g., as in T^4) or the combination of $T^{5'}$ and $T^{5''}$ (e.g., as in T^5) or the combination of $T^{6'}$ and $T^{6''}$ (e.g., as in T^6) join together form O (oxo), S (thio), or Se (seleno);

[000480] each of V^5 and V^6 is, independently, O, S, $N(R^{Vd})_{nv}$, or $C(R^{Vd})_{nv}$, wherein nv is an integer from 0 to 2 and each R^{Vd} is, independently, H, halo, thiol, optionally substituted amino acid, cyano, amidine, optionally substituted aminoalkyl, optionally substituted aminoalkenyl, optionally substituted aminoalkynyl, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted alkoxy, optionally substituted alkenyloxy, or optionally substituted alkynyloxy (e.g., optionally substituted with any substituent described herein, such as those selected from (1)-(21) for alkyl) , optionally substituted thioalkoxy, or optionally substituted amino; and

[000481] each of R^{17}, R^{18}, R^{19a}, R^{19b}, R^{21}, R^{22}, R^{23}, and R^{24} is, independently, H, halo, thiol, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted thioalkoxy, optionally substituted amino, or optionally substituted amino acid.

[000482] Exemplary modified guanosines include compounds of Formula (b37)-(b40):


(b37),

(b38),

(b39),

or (b40), or a pharmaceutically acceptable salt or stereoisomer thereof,

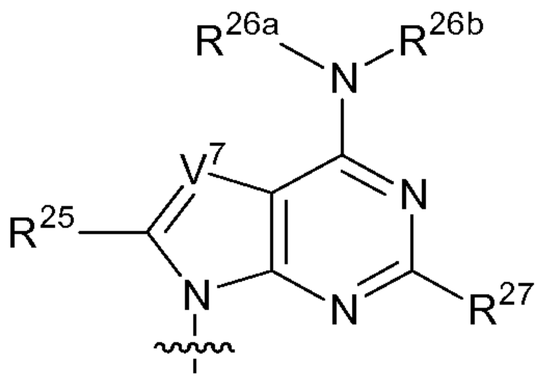
[000483] wherein

[000484] each of $T^{4'}$ is, independently, H, optionally substituted alkyl, or optionally substituted alkoxy, and each T^4 is, independently, O (oxo), S (thio), or Se (seleno);

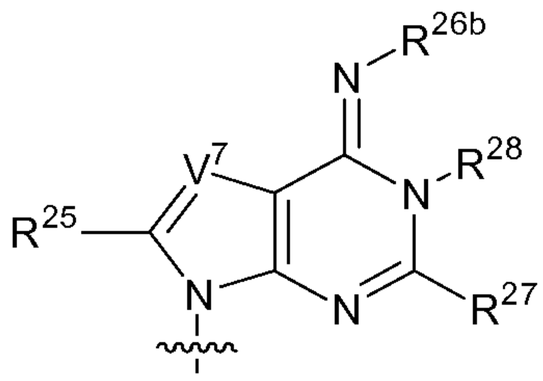
[000485] each of R^{18}, R^{19a}, R^{19b}, and R^{21} is, independently, H, halo, thiol, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted thioalkoxy, optionally substituted amino, or optionally substituted amino acid.

[000486] In some embodiments, R^{18} is H or optionally substituted alkyl. In further embodiments, T^4 is oxo. In some embodiments, each of R^{19a} and R^{19b} is, independently, H or optionally substituted alkyl.

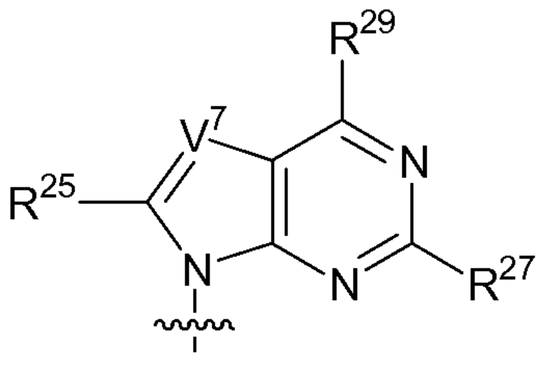
[000487] In some embodiments, B is a modified adenine. Exemplary modified adenines include compounds of Formula (b18)-(b20):



(b18),



(b19), or



(b20), or a pharmaceutically acceptable salt or stereoisomer thereof,

[000488] wherein

[000489] each V^7 is, independently, O, S, $N(R^{Ve})_{nv}$, or $C(R^{Ve})_{nv}$, wherein nv is an integer from 0 to 2 and each R^{Ve} is, independently, H, halo, optionally substituted amino acid, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted alkoxy, optionally substituted alkenyloxy, or optionally substituted alkynyloxy (e.g., optionally substituted with any substituent described herein, such as those selected from (1)-(21) for alkyl);

[000490] each R^{25} is, independently, H, halo, thiol, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted thioalkoxy, or optionally substituted amino;

[000491] each of R^{26a} and R^{26b} is, independently, H, optionally substituted acyl, optionally substituted amino acid, optionally substituted carbamoylalkyl, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally

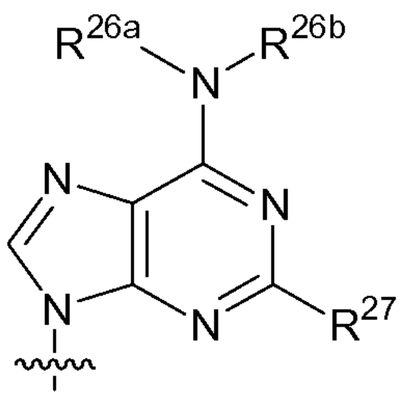
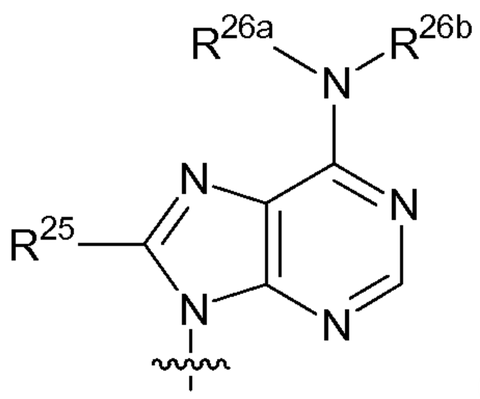
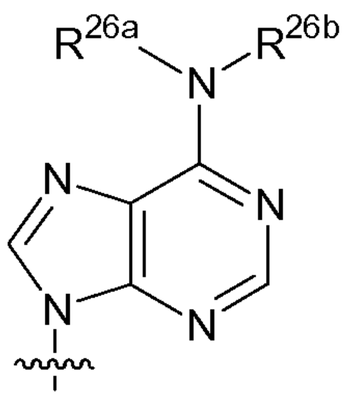
substituted hydroxyalkyl, optionally substituted hydroxyalkenyl, optionally substituted hydroxyalkynyl, optionally substituted alkoxy, or polyethylene glycol group (e.g., -$(CH_2)_{s2}(OCH_2CH_2)_{s1}(CH_2)_{s3}OR'$, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R' is H or C_{1-20} alkyl); or an amino-polyethylene glycol group (e.g., -$NR^{N1}(CH_2)_{s2}(CH_2CH_2O)_{s1}(CH_2)_{s3}NR^{N1}$, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and each R^{N1} is, independently, hydrogen or optionally substituted C_{1-6} alkyl);

[000492] each R^{27} is, independently, H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted alkoxy, optionally substituted thioalkoxy or optionally substituted amino;

[000493] each R^{28} is, independently, H, optionally substituted alkyl, optionally substituted alkenyl, or optionally substituted alkynyl; and

[000494] each R^{29} is, independently, H, optionally substituted acyl, optionally substituted amino acid, optionally substituted carbamoylalkyl, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted hydroxyalkyl, optionally substituted hydroxyalkenyl, optionally substituted alkoxy, or optionally substituted amino.

[000495] Exemplary modified adenines include compounds of Formula (b41)-(b43):

[000496]  (b41),  (b42), or  (b43), or a pharmaceutically acceptable salt or stereoisomer thereof,

[000497] wherein

[000498] each R^{25} is, independently, H, halo, thiol, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted thioalkoxy, or optionally substituted amino;

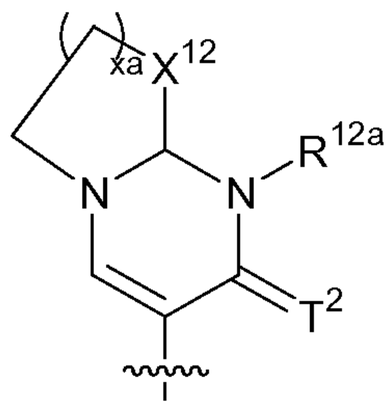
[000499] each of R^{26a} and R^{26b} is, independently, H, optionally substituted acyl, optionally substituted amino acid, optionally substituted carbamoylalkyl, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted hydroxyalkyl, optionally substituted hydroxyalkenyl, optionally substituted hydroxyalkynyl, optionally substituted alkoxy, or polyethylene glycol group (e.g., -$(CH_2)_{s2}(OCH_2CH_2)_{s1}(CH_2)_{s3}OR'$, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R' is H or C_{1-20} alkyl); or an amino-polyethylene glycol group (e.g., -$NR^{N1}(CH_2)_{s2}(CH_2CH_2O)_{s1}(CH_2)_{s3}NR^{N1}$, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and each R^{N1} is, independently, hydrogen or optionally substituted C_{1-6} alkyl); and

[000500] each R^{27} is, independently, H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted alkoxy, optionally substituted thioalkoxy, or optionally substituted amino.

[000501] In some embodiments, R^{26a} is H, and R^{26b} is optionally substituted alkyl. In some embodiments, each of R^{26a} and R^{26b} is, independently, optionally substituted alkyl. In particular embodiments, R^{27} is optionally substituted alkyl, optionally substituted alkoxy, or optionally substituted thioalkoxy. In other embodiments, R^{25} is optionally substituted alkyl, optionally substituted alkoxy, or optionally substituted thioalkoxy.

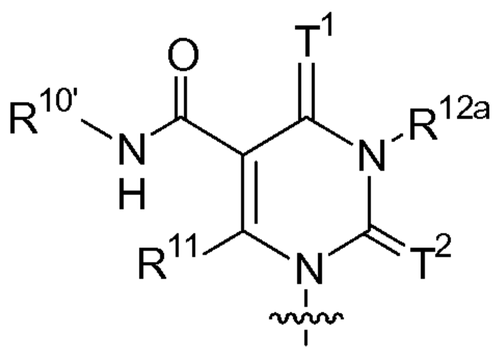
[000502] In particular embodiments, the optional substituent for R^{26a}, R^{26b}, or R^{29} is a polyethylene glycol group (e.g., -$(CH_2)_{s2}(OCH_2CH_2)_{s1}(CH_2)_{s3}OR'$, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and R' is H or C_{1-20} alkyl); or an amino-polyethylene glycol group (e.g., -$NR^{N1}(CH_2)_{s2}(CH_2CH_2O)_{s1}(CH_2)_{s3}NR^{N1}$, wherein s1 is an integer from 1 to 10 (e.g., from 1 to 6 or from 1 to 4), each of s2 and s3, independently, is an integer from 0 to 10 (e.g., from 0 to 4, from 0 to 6, from 1 to 4, from 1 to 6, or from 1 to 10), and each R^{N1} is, independently, hydrogen or optionally substituted C_{1-6} alkyl).

[000503] In some embodiments, B may have Formula (b21):



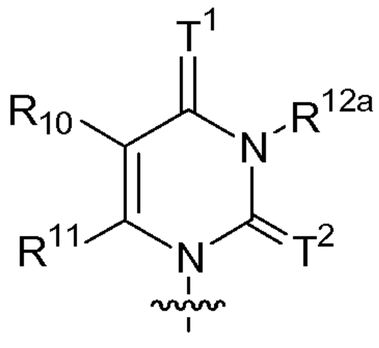
(b21), wherein X^{12} is, independently, O, S, optionally substituted alkylene (e.g., methylene), or optionally substituted heteroalkylene, xa is an integer from 0 to 3, and R^{12a} and T^2 are as described herein.

[000504] In some embodiments, B may have Formula (b22):



(b22), wherein $R^{10'}$ is, independently, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted aryl, optionally substituted heterocyclyl, optionally substituted aminoalkyl, optionally substituted aminoalkenyl, optionally substituted aminoalkynyl, optionally substituted alkoxy, optionally substituted alkoxycarbonylalkyl, optionally substituted alkoxycarbonylalkenyl, optionally substituted alkoxycarbonylalkynyl, optionally substituted alkoxycarbonylalkoxy, optionally substituted carboxyalkoxy, optionally substituted carboxyalkyl, or optionally substituted carbamoylalkyl, and R^{11}, R^{12a}, T^1, and T^2 are as described herein.

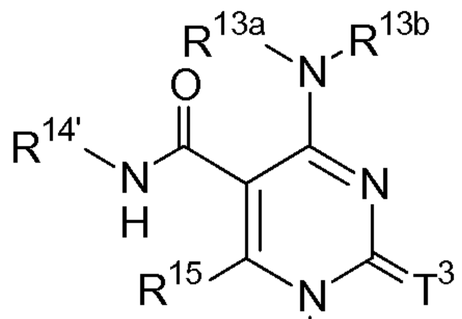
[000505] In some embodiments, B may have Formula (b23):



(b23), wherein R^{10} is optionally substituted heterocyclyl (e.g., optionally substituted furyl, optionally substitued thienyl, or optionally substitued pyrrolyl), optionally substituted aryl (e.g., optionally substituted phenyl or optionally substituted naphthyl), or any substituent described herein (e.g., for R^{10}) ;and wherein R^{11} (e.g., H or any substituent described herein), R^{12a} (e.g., H or any substituent described

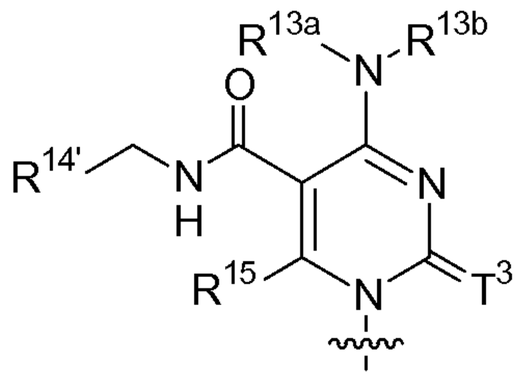
herein), T^1 (e.g., oxo or any substituent described herein), and T^2 (e.g., oxo or any substituent described herein) are as described herein.

In some embodiments, B may have Formula (b24):



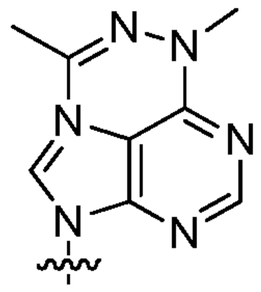
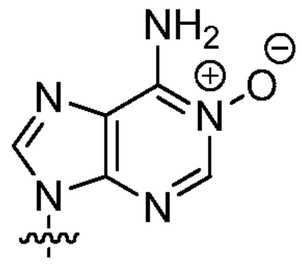
(b24), wherein $R^{14'}$ is, independently, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted aryl, optionally substituted heterocyclyl, optionally substituted alkaryl, optionally substituted alkheterocyclyl, optionally substituted aminoalkyl, optionally substituted aminoalkenyl, optionally substituted aminoalkynyl, optionally substituted alkoxy, optionally substituted alkoxycarbonylalkenyl, optionally substituted alkoxycarbonylalkynyl, optionally substituted alkoxycarbonylalkyl, optionally substituted alkoxycarbonylalkoxy, optionally substituted carboxyalkoxy, optionally substituted carboxyalkyl, or optionally substituted carbamoylalkyl, and R^{13a}, R^{13b}, R^{15}, and T^3 are as described herein.

[000506] In some embodiments, B may have Formula (b25):



(b25), wherein $R^{14'}$ is optionally substituted heterocyclyl (e.g., optionally substituted furyl, optionally substitued thienyl, or optionally substitued pyrrolyl), optionally substituted aryl (e.g., optionally substituted phenyl or optionally substituted naphthyl), or any substituent described herein (e.g., for R^{14} or $R^{14'}$); and wherein R^{13a} (e.g., H or any substituent described herein), R^{13b} (e.g., H or any substituent described herein), R^{15} (e.g., H or any substituent described herein), and T^3 (e.g., oxo or any substituent described herein) are as described herein.

[000507] In some embodiments, B is a nucleobase selected from the group consisting of cytosine, guanine, adenine, and uracil. In some embodiments, B may be:


(b26) or 
(b27).

[000508] In some embodiments, the modified nucleobase is a modified uracil. Exemplary nucleobases and nucleosides having a modified uracil include pseudouridine (ψ), pyridin-4-one ribonucleoside, 5-aza-uridine, 6-aza-uridine, 2-thio-5-aza-uridine, 2-thio-uridine (s^2U), 4-thio-uridine (s^4U), 4-thio-pseudouridine, 2-thio-pseudouridine, 5-hydroxy-uridine (ho^5U), 5-aminoallyl-uridine, 5-halo-uridine (e.g., 5-iodo-uridine or 5-bromo-uridine), 3-methyl-uridine (m^3U), 5-methoxy-uridine (mo^5U), uridine 5-oxyacetic acid (cmo^5U), uridine 5-oxyacetic acid methyl ester ($mcmo^5U$), 5-carboxymethyl-uridine (cm^5U), 1-carboxymethyl-pseudouridine, 5-carboxyhydroxymethyl-uridine (chm^5U), 5-carboxyhydroxymethyl-uridine methyl ester ($mchm^5U$), 5-methoxycarbonylmethyl-uridine (mcm^5U), 5-methoxycarbonylmethyl-2-thio-uridine (mcm^5s^2U), 5-aminomethyl-2-thio-uridine (nm^5s^2U), 5-methylaminomethyl-uridine (mnm^5U), 5-methylaminomethyl-2-thio-uridine (mnm^5s^2U), 5-methylaminomethyl-2-seleno-uridine (mnm^5se^2U), 5-carbamoylmethyl-uridine (ncm^5U), 5-carboxymethylaminomethyl-uridine ($cmnm^5U$), 5-carboxymethylaminomethyl-2-thio-uridine ($cmnm^5s^2U$), 5-propynyl-uridine, 1-propynyl-pseudouridine, 5-taurinomethyl-uridine (τm^5U), 1-taurinomethyl-pseudouridine, 5-taurinomethyl-2-thio-uridine(τm^5s^2U), 1-taurinomethyl-4-thio-pseudouridine, 5-methyl-uridine (m^5U, i.e., having the nucleobase deoxythymine), 1-methylpseudouridine ($m^1\psi$), 5-methyl-2-thio-uridine (m^5s^2U), 1-methyl-4-thio-pseudouridine ($m^1s^4\psi$), 4-thio-1-methyl-pseudouridine, 3-methyl-pseudouridine ($m^3\psi$), 2-thio-1-methyl-pseudouridine, 1-methyl-1-deaza-pseudouridine, 2-thio-1-methyl-1-deaza-pseudouridine, dihydrouridine (D), dihydropseudouridine, 5,6-dihydrouridine, 5-methyl-dihydrouridine (m^5D), 2-thio-dihydrouridine, 2-thio-dihydropseudouridine, 2-methoxy-uridine, 2-methoxy-4-thio-uridine, 4-methoxy-pseudouridine, 4-methoxy-2-thio-pseudouridine, N1-methyl-pseudouridine (also known as 1-methylpseudouridine ($m^1\psi$)), 3-(3-amino-3-carboxypropyl)uridine (acp^3U), 1-methyl-3-(3-amino-3-carboxypropyl)pseudouridine ($acp^3\psi$), 5-(isopentenylaminomethyl)uridine (inm^5U), 5-(isopentenylaminomethyl)-2-thio-uridine (inm^5s^2U), α-thio-uridine, 2′-O-methyl-uridine (Um), 5,2′-O-dimethyl-

uridine (m^5Um), 2′-O-methyl-pseudouridine (ψm), 2-thio-2′-O-methyl-uridine (s^2Um), 5-methoxycarbonylmethyl-2′-O-methyl-uridine (mcm^5Um), 5-carbamoylmethyl-2′-O-methyl-uridine (ncm^5Um), 5-carboxymethylaminomethyl-2′-O-methyl-uridine ($cmnm^5Um$), 3,2′-O-dimethyl-uridine (m^3Um), 5-(isopentenylaminomethyl)-2′-O-methyl-uridine (inm^5Um), 1-thio-uridine, deoxythymidine, 2'-F-ara-uridine, 2'-F-uridine, 2'-OH-ara-uridine, 5-(2-carbomethoxyvinyl) uridine, and 5-[3-(1-E-propenylamino)uridine.

[000509] In some embodiments, the modified nucleobase is a modified cytosine. Exemplary nucleobases and nucleosides having a modified cytosine include 5-aza-cytidine, 6-aza-cytidine, pseudoisocytidine, 3-methyl-cytidine (m^3C), N4-acetyl-cytidine (ac^4C), 5-formyl-cytidine (f^5C), N4-methyl-cytidine (m^4C), 5-methyl-cytidine (m^5C), 5-halo-cytidine (e.g., 5-iodo-cytidine), 5-hydroxymethyl-cytidine (hm^5C), 1-methyl-pseudoisocytidine, pyrrolo-cytidine, pyrrolo-pseudoisocytidine, 2-thio-cytidine (s^2C), 2-thio-5-methyl-cytidine, 4-thio-pseudoisocytidine, 4-thio-1-methyl-pseudoisocytidine, 4-thio-1-methyl-1-deaza-pseudoisocytidine, 1-methyl-1-deaza-pseudoisocytidine, zebularine, 5-aza-zebularine, 5-methyl-zebularine, 5-aza-2-thio-zebularine, 2-thio-zebularine, 2-methoxy-cytidine, 2-methoxy-5-methyl-cytidine, 4-methoxy-pseudoisocytidine, 4-methoxy-1-methyl-pseudoisocytidine, lysidine (k_2C), α-thio-cytidine, 2′-O-methyl-cytidine (Cm), 5,2′-O-dimethyl-cytidine (m^5Cm), N4-acetyl-2′-O-methyl-cytidine (ac^4Cm), N4,2′-O-dimethyl-cytidine (m^4Cm), 5-formyl-2′-O-methyl-cytidine (f^5Cm), N4,N4,2′-O-trimethyl-cytidine ($m^4{}_2Cm$), 1-thio-cytidine, 2'-F-ara-cytidine, 2'-F-cytidine, and 2'-OH-ara-cytidine.

[000510] In some embodiments, the modified nucleobase is a modified adenine. Exemplary nucleobases and nucleosides having a modified adenine include 2-amino-purine, 2, 6-diaminopurine, 2-amino-6-halo-purine (e.g., 2-amino-6-chloro-purine), 6-halo-purine (e.g., 6-chloro-purine), 2-amino-6-methyl-purine, 8-azido-adenosine, 7-deaza-adenine, 7-deaza-8-aza-adenine, 7-deaza-2-amino-purine, 7-deaza-8-aza-2-amino-purine, 7-deaza-2,6-diaminopurine, 7-deaza-8-aza-2,6-diaminopurine, 1-methyl-adenosine (m^1A), 2-methyl-adenine (m^2A), N6-methyl-adenosine (m^6A), 2-methylthio-N6-methyl-adenosine (ms^2m^6A), N6-isopentenyl-adenosine (i^6A), 2-methylthio-N6-isopentenyl-adenosine (ms^2i^6A), N6-(cis-hydroxyisopentenyl)adenosine (io^6A), 2-

methylthio-N6-(cis-hydroxyisopentenyl)adenosine (ms^2io^6A), N6-glycinylcarbamoyl-adenosine (g^6A), N6-threonylcarbamoyl-adenosine (t^6A), N6-methyl-N6-threonylcarbamoyl-adenosine (m^6t^6A), 2-methylthio-N6-threonylcarbamoyl-adenosine (ms^2g^6A), N6,N6-dimethyl-adenosine ($m^6{}_2A$), N6-hydroxynorvalylcarbamoyl-adenosine (hn^6A), 2-methylthio-N6-hydroxynorvalylcarbamoyl-adenosine (ms^2hn^6A), N6-acetyl-adenosine (ac^6A), 7-methyl-adenine, 2-methylthio-adenine, 2-methoxy-adenine, α-thio-adenosine, 2′-O-methyl-adenosine (Am), N6,2′-O-dimethyl-adenosine (m^6Am), N6,N6,2′-O-trimethyl-adenosine ($m^6{}_2Am$), 1,2′-O-dimethyl-adenosine (m^1Am), 2′-O-ribosyladenosine (phosphate) (Ar(p)), 2-amino-N6-methyl-purine, 1-thio-adenosine, 8-azido-adenosine, 2'-F-ara-adenosine, 2'-F-adenosine, 2'-OH-ara-adenosine, and N6-(19-amino-pentaoxanonadecyl)-adenosine.

[000511] In some embodiments, the modified nucleobase is a modified guanine. Exemplary nucleobases and nucleosides having a modified guanine include inosine (I), 1-methyl-inosine (m^1I), wyosine (imG), methylwyosine (mimG), 4-demethyl-wyosine (imG-14), isowyosine (imG2), wybutosine (yW), peroxywybutosine (o_2yW), hydroxywybutosine (OHyW), undermodified hydroxywybutosine (OHyW*), 7-deaza-guanosine, queuosine (Q), epoxyqueuosine (oQ), galactosyl-queuosine (galQ), mannosyl-queuosine (manQ), 7-cyano-7-deaza-guanosine ($preQ_0$), 7-aminomethyl-7-deaza-guanosine ($preQ_1$), archaeosine (G^+), 7-deaza-8-aza-guanosine, 6-thio-guanosine, 6-thio-7-deaza-guanosine, 6-thio-7-deaza-8-aza-guanosine, 7-methyl-guanosine (m^7G), 6-thio-7-methyl-guanosine, 7-methyl-inosine, 6-methoxy-guanosine, 1-methyl-guanosine (m^1G), N2-methyl-guanosine (m^2G), N2,N2-dimethyl-guanosine ($m^2{}_2G$), N2,7-dimethyl-guanosine ($m^{2,7}G$), N2, N2,7-dimethyl-guanosine ($m^{2,2,7}G$), 8-oxo-guanosine, 7-methyl-8-oxo-guanosine, 1-methyl-6-thio-guanosine, N2-methyl-6-thio-guanosine, N2,N2-dimethyl-6-thio-guanosine, α-thio-guanosine, 2′-O-methyl-guanosine (Gm), N2-methyl-2′-O-methyl-guanosine (m^2Gm), N2,N2-dimethyl-2′-O-methyl-guanosine ($m^2{}_2Gm$), 1-methyl-2′-O-methyl-guanosine (m^1Gm), N2,7-dimethyl-2′-O-methyl-guanosine ($m^{2,7}Gm$), 2′-O-methyl-inosine (Im), 1,2′-O-dimethyl-inosine (m^1Im), and 2′-O-ribosylguanosine (phosphate) (Gr(p)).

[000512] The nucleobase of the nucleotide can be independently selected from a purine, a pyrimidine, a purine or pyrimidine analog. For example, the nucleobase can each be

independently selected from adenine, cytosine, guanine, uracil, or hypoxanthine. In another embodiment, the nucleobase can also include, for example, naturally-occurring and synthetic derivatives of a base, including pyrazolo[3,4-d]pyrimidines, 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo (*e.g.,* 8-bromo), 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, deazaguanine, 7-deazaguanine, 3-deazaguanine, deazaadenine, 7-deazaadenine, 3-deazaadenine, pyrazolo[3,4-d]pyrimidine, imidazo[1,5-a]1,3,5 triazinones, 9-deazapurines, imidazo[4,5-d]pyrazines, thiazolo[4,5-d]pyrimidines, pyrazin-2-ones, 1,2,4-triazine, pyridazine; and 1,3,5 triazine. When the nucleotides are depicted using the shorthand A, G, C, T or U, each letter refers to the representative base and/or derivatives thereof, *e.g.*, A includes adenine or adenine analogs, *e.g.,* 7-deaza adenine).

Modifications on the Internucleoside Linkage

[000513] The modified nucleotides, which may be incorporated into a polynucleotide, primary construct, or mmRNA molecule, can be modified on the internucleoside linkage (e.g., phosphate backbone). Herein, in the context of the polynucleotide backbone, the phrases "phosphate" and "phosphodiester" are used interchangeably. Backbone phosphate groups can be modified by replacing one or more of the oxygen atoms with a different substituent. Further, the modified nucleosides and nucleotides can include the wholesale replacement of an unmodified phosphate moiety with another internucleoside linkage as described herein. Examples of modified phosphate groups include, but are not limited to, phosphorothioate, phosphoroselenates, boranophosphates,boranophosphate esters, hydrogen phosphonates, phosphoramidates, phosphorodiamidates, alkyl or aryl phosphonates, and phosphotriesters. Phosphorodithioates have both non-linking oxygens replaced by sulfur. The phosphate linker can also be modified by the replacement of a

linking oxygen with nitrogen (bridged phosphoramidates), sulfur (bridged phosphorothioates), and carbon (bridged methylene-phosphonates).

[000514] The α-thio substituted phosphate moiety is provided to confer stability to RNA and DNA polymers through the unnatural phosphorothioate backbone linkages. Phosphorothioate DNA and RNA have increased nuclease resistance and subsequently a longer half-life in a cellular environment. Phosphorothioate linked polynucleotides, primary constructs, or mmRNA molecules are expected to also reduce the innate immune response through weaker binding/activation of cellular innate immune molecules.

[000515] In specific embodiments, a modified nucleoside includes an alpha-thio-nucleoside (e.g., 5′-O-(1-thiophosphate)-adenosine, 5′-O-(1-thiophosphate)-cytidine (α-thio-cytidine), 5′-O-(1-thiophosphate)-guanosine, 5′-O-(1-thiophosphate)-uridine, or 5′-O-(1-thiophosphate)-pseudouridine).

[000516] Other internucleoside linkages that may be employed according to the present invention, including internucleoside linkages which do not contain a phosphorous atom, are described herein below.

Combinations of Modified Sugars, Nucleobases, and Internucleoside Linkages

[000517] The polynucleotides, primary constructs, and mmRNA of the invention can include a combination of modifications to the sugar, the nucleobase, and/or the internucleoside linkage. These combinations can include any one or more modifications described herein. For examples, any of the nucleotides described herein in Formulas (Ia), (Ia-1)-(Ia-3), (Ib)-(If), (IIa)-(IIp), (IIb-1), (IIb-2), (IIc-1)-(IIc-2), (IIn-1), (IIn-2), (IVa)-(IVl), and (IXa)-(IXr) can be combined with any of the nucleobases described herein (e.g., in Formulas (b1)-(b43) or any other described herein).

Synthesis of Polypeptides, Primary Constructs, and mmRNA Molecules

[000518] The polypeptides, primary constructs, and mmRNA molecules for use in accordance with the invention may be prepared according to any useful technique, as described herein. The modified nucleosides and nucleotides used in the synthesis of polynucleotides, primary constructs, and mmRNA molecules disclosed herein can be prepared from readily available starting materials using the following general methods and procedures. Where typical or preferred process conditions (e.g., reaction temperatures, times, mole ratios of reactants, solvents, pressures, etc.) are provided, a

skilled artisan would be able to optimize and develop additional process conditions. Optimum reaction conditions may vary with the particular reactants or solvent used, but such conditions can be determined by one skilled in the art by routine optimization procedures.

[000519] The processes described herein can be monitored according to any suitable method known in the art. For example, product formation can be monitored by spectroscopic means, such as nuclear magnetic resonance spectroscopy (e.g., ^{1}H or ^{13}C) infrared spectroscopy, spectrophotometry (e.g., UV-visible), or mass spectrometry, or by chromatography such as high performance liquid chromatography (HPLC) or thin layer chromatography.

[000520] Preparation of polypeptides, primary constructs, and mmRNA molecules of the present invention can involve the protection and deprotection of various chemical groups. The need for protection and deprotection, and the selection of appropriate protecting groups can be readily determined by one skilled in the art. The chemistry of protecting groups can be found, for example, in Greene, et al., *Protective Groups in Organic Synthesis*, 2d. Ed., Wiley & Sons, 1991, which is incorporated herein by reference in its entirety.

[000521] The reactions of the processes described herein can be carried out in suitable solvents, which can be readily selected by one of skill in the art of organic synthesis. Suitable solvents can be substantially nonreactive with the starting materials (reactants), the intermediates, or products at the temperatures at which the reactions are carried out, i.e., temperatures which can range from the solvent's freezing temperature to the solvent's boiling temperature. A given reaction can be carried out in one solvent or a mixture of more than one solvent. Depending on the particular reaction step, suitable solvents for a particular reaction step can be selected.

[000522] Resolution of racemic mixtures of modified nucleosides and nucleotides can be carried out by any of numerous methods known in the art. An example method includes fractional recrystallization using a "chiral resolving acid" which is an optically active, salt-forming organic acid. Suitable resolving agents for fractional recrystallization methods are, for example, optically active acids, such as the D and L forms of tartaric acid, diacetyltartaric acid, dibenzoyltartaric acid, mandelic acid, malic acid, lactic acid or

the various optically active camphorsulfonic acids. Resolution of racemic mixtures can also be carried out by elution on a column packed with an optically active resolving agent (*e.g.*, dinitrobenzoylphenylglycine). Suitable elution solvent composition can be determined by one skilled in the art.

Modified nucleosides and nucleotides (e.g., building block molecules) can be prepared according to the synthetic methods described in Ogata et al., J. Org. Chem. 74:2585-2588 (2009); Purmal et al., Nucl. Acids Res. 22(1): 72-78, (1994); Fukuhara et al., Biochemistry, 1(4): 563-568 (1962); and Xu et al., Tetrahedron, 48(9): 1729-1740 (1992), each of which are incorporated by reference in their entirety.

[000523] The polypeptides, primary constructs, and mmRNA of the invention may or may not be uniformly modified along the entire length of the molecule. For example, one or more or all types of nucleotide (*e.g.*, purine or pyrimidine, or any one or more or all of A, G, U, C) may or may not be uniformly modified in a polynucleotide of the invention, or in a given predetermined sequence region thereof (*e.g.* one or more of the sequence regions represented in Figure 1). In some embodiments, all nucleotides X in a polynucleotide of the invention (or in a given sequence region thereof) are modified, wherein X may any one of nucleotides A, G, U, C, or any one of the combinations A+G, A+U, A+C, G+U, G+C, U+C, A+G+U, A+G+C, G+U+C or A+G+C.

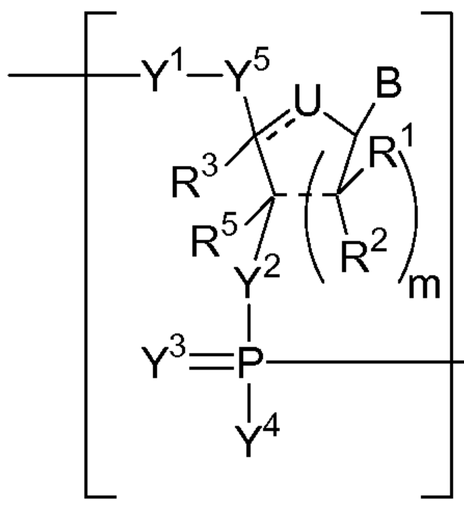
[000524] Different sugar modifications, nucleotide modifications, and/or internucleoside linkages (e.g., backbone structures) may exist at various positions in the polynucleotide, primary construct, or mmRNA. One of ordinary skill in the art will appreciate that the nucleotide analogs or other modification(s) may be located at any position(s) of a polynucleotide, primary construct, or mmRNA such that the function of the polynucleotide, primary construct, or mmRNA is not substantially decreased. A modification may also be a 5′ or 3′ terminal modification. The polynucleotide, primary construct, or mmRNA may contain from about 1% to about 100% modified nucleotides (either in relation to overall nucleotide content, or in relation to one or more types of nucleotide, i.e. any one or more of A, G, U or C) or any intervening percentage (e.g., from 1% to 20%, from 1% to 25%, from 1% to 50%, from 1% to 60%, from 1% to 70%, from 1% to 80%, from 1% to 90%, from 1% to 95%, from 10% to 20%, from 10% to 25%, from 10% to 50%, from 10% to 60%, from 10% to 70%, from 10% to 80%, from

10% to 90%, from 10% to 95%, from 10% to 100%, from 20% to 25%, from 20% to 50%, from 20% to 60%, from 20% to 70%, from 20% to 80%, from 20% to 90%, from 20% to 95%, from 20% to 100%, from 50% to 60%, from 50% to 70%, from 50% to 80%, from 50% to 90%, from 50% to 95%, from 50% to 100%, from 70% to 80%, from 70% to 90%, from 70% to 95%, from 70% to 100%, from 80% to 90%, from 80% to 95%, from 80% to 100%, from 90% to 95%, from 90% to 100%, and from 95% to 100%).

[000525] In some embodiments, the polynucleotide, primary construct, or mmRNA includes a modified pyrimidine (e.g., a modified uracil/uridine/U or modified cytosine/cytidine/C). In some embodiments, the uracil or uridine (generally: U) in the polynucleotide, primary construct, or mmRNA molecule may be replaced with from about 1% to about 100% of a modified uracil or modified uridine (e.g., from 1% to 20%, from 1% to 25%, from 1% to 50%, from 1% to 60%, from 1% to 70%, from 1% to 80%, from 1% to 90%, from 1% to 95%, from 10% to 20%, from 10% to 25%, from 10% to 50%, from 10% to 60%, from 10% to 70%, from 10% to 80%, from 10% to 90%, from 10% to 95%, from 10% to 100%, from 20% to 25%, from 20% to 50%, from 20% to 60%, from 20% to 70%, from 20% to 80%, from 20% to 90%, from 20% to 95%, from 20% to 100%, from 50% to 60%, from 50% to 70%, from 50% to 80%, from 50% to 90%, from 50% to 95%, from 50% to 100%, from 70% to 80%, from 70% to 90%, from 70% to 95%, from 70% to 100%, from 80% to 90%, from 80% to 95%, from 80% to 100%, from 90% to 95%, from 90% to 100%, and from 95% to 100% of a modified uracil or modified uridine). The modified uracil or uridine can be replaced by a compound having a single unique structure or by a plurality of compounds having different structures (e.g., 2, 3, 4 or more unique structures, as described herein). In some embodiments, the cytosine or cytidine (generally: C) in the polynucleotide, primary construct, or mmRNA molecule may be replaced with from about 1% to about 100% of a modified cytosine or modified cytidine (e.g., from 1% to 20%, from 1% to 25%, from 1% to 50%, from 1% to 60%, from 1% to 70%, from 1% to 80%, from 1% to 90%, from 1% to 95%, from 10% to 20%, from 10% to 25%, from 10% to 50%, from 10% to 60%, from 10% to 70%, from 10% to 80%, from 10% to 90%, from 10% to 95%, from 10% to 100%, from 20% to 25%, from 20% to 50%, from 20% to 60%, from 20% to 70%, from

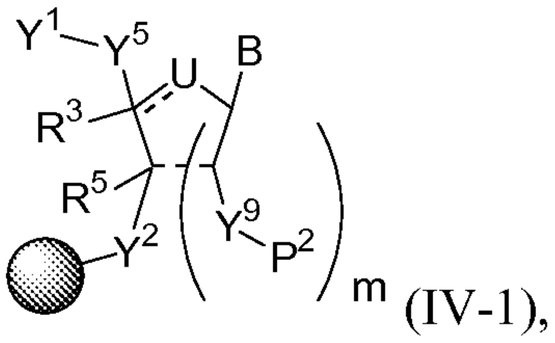
20% to 80%, from 20% to 90%, from 20% to 95%, from 20% to 100%, from 50% to 60%, from 50% to 70%, from 50% to 80%, from 50% to 90%, from 50% to 95%, from 50% to 100%, from 70% to 80%, from 70% to 90%, from 70% to 95%, from 70% to 100%, from 80% to 90%, from 80% to 95%, from 80% to 100%, from 90% to 95%, from 90% to 100%, and from 95% to 100% of a modified cytosine or modified cytidine). The modified cytosine or cytidine can be replaced by a compound having a single unique structure or by a plurality of compounds having different structures (e.g., 2, 3, 4 or more unique structures, as described herein).

In some embodiments, the present disclosure provides methods of synthesizing a polynucleotide, primary construct, or mmRNA (e.g., the first region, first flanking region, or second flanking region) including n number of linked nucleosides having Formula (Ia-1):



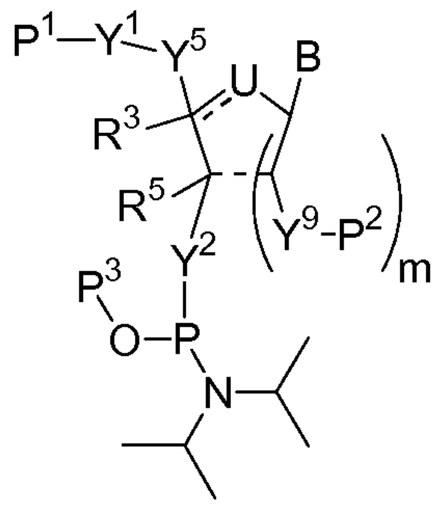
(Ia-1), comprising:

a) reacting a nucleotide of Formula (IV-1):



(IV-1),

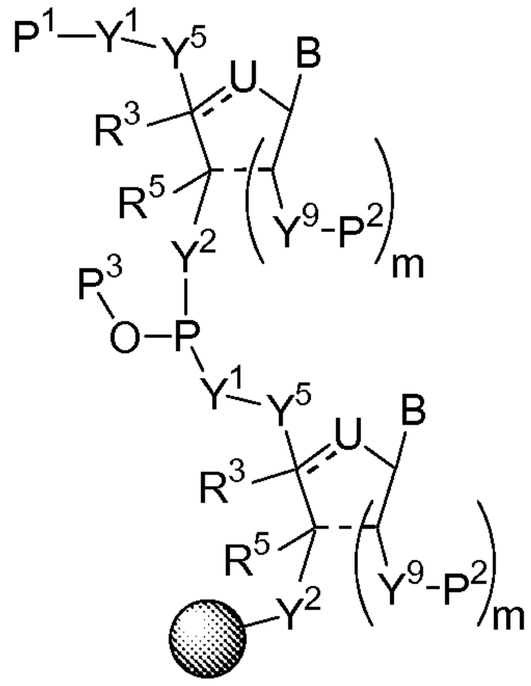
with a phosphoramidite compound of Formula (V-1):



(V-1),

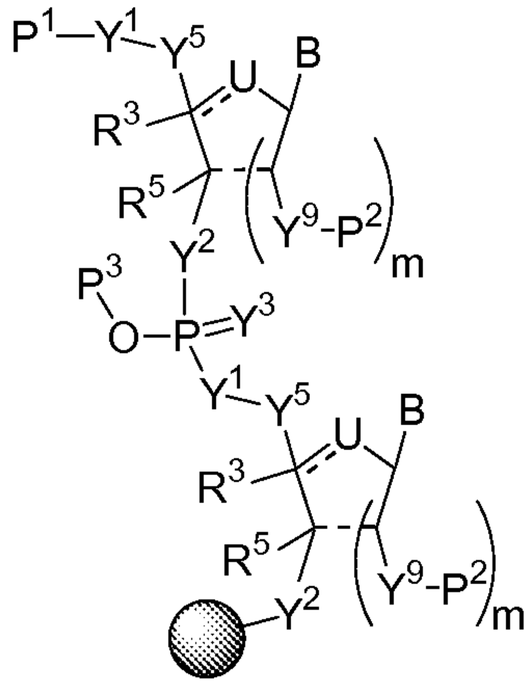
wherein Y^9 is H, hydroxy, phosphoryl, pyrophosphate, sulfate, amino, thiol, optionally substituted amino acid, or a peptide (e.g., including from 2 to 12 amino acids); and each P^1, P^2, and P^3 is, independently, a suitable protecting group; and  denotes a solid support;

to provide a polynucleotide, primary construct, or mmRNA of Formula (VI-1):



(VI-1), and

b) oxidizing or sulfurizing the polynucleotide, primary construct, or mmRNA of Formula (V) to yield a polynucleotide, primary construct, or mmRNA of Formula (VII-1):



(VII-1), and

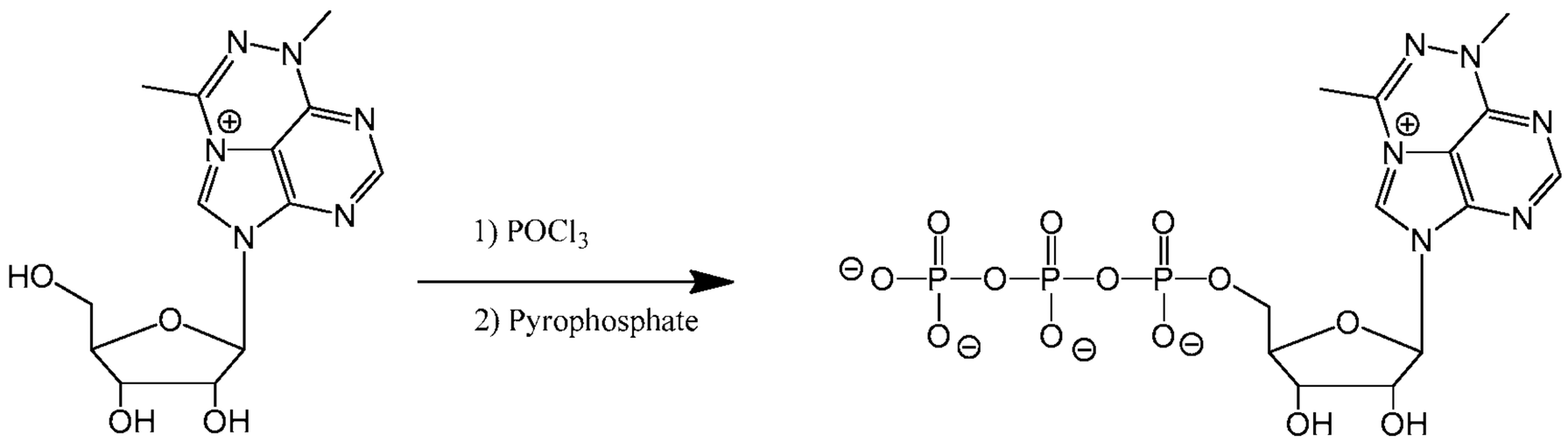
c) removing the protecting groups to yield the polynucleotide, primary construct, or mmRNA of Formula (Ia).

[000526] In some embodiments, steps a) and b) are repeated from 1 to about 10,000 times. In some embodiments, the methods further comprise a nucleotide (e.g., mmRNA molecule) selected from the group consisting of A, C, G and U adenosine, cytosine, guanosine, and uracil. In some embodiments, the nucleobase may be a pyrimidine or derivative thereof. In some embodiments, the polynucleotide, primary construct, or mmRNA is translatable.

[000527] Other components of polynucleotides, primary constructs, and mmRNA are optional, and are beneficial in some embodiments. For example, a 5′ untranslated region (UTR) and/or a 3′UTR are provided, wherein either or both may independently contain one or more different nucleotide modifications. In such embodiments, nucleotide modifications may also be present in the translatable region. Also provided are polynucleotides, primary constructs, and mmRNA containing a Kozak sequence.

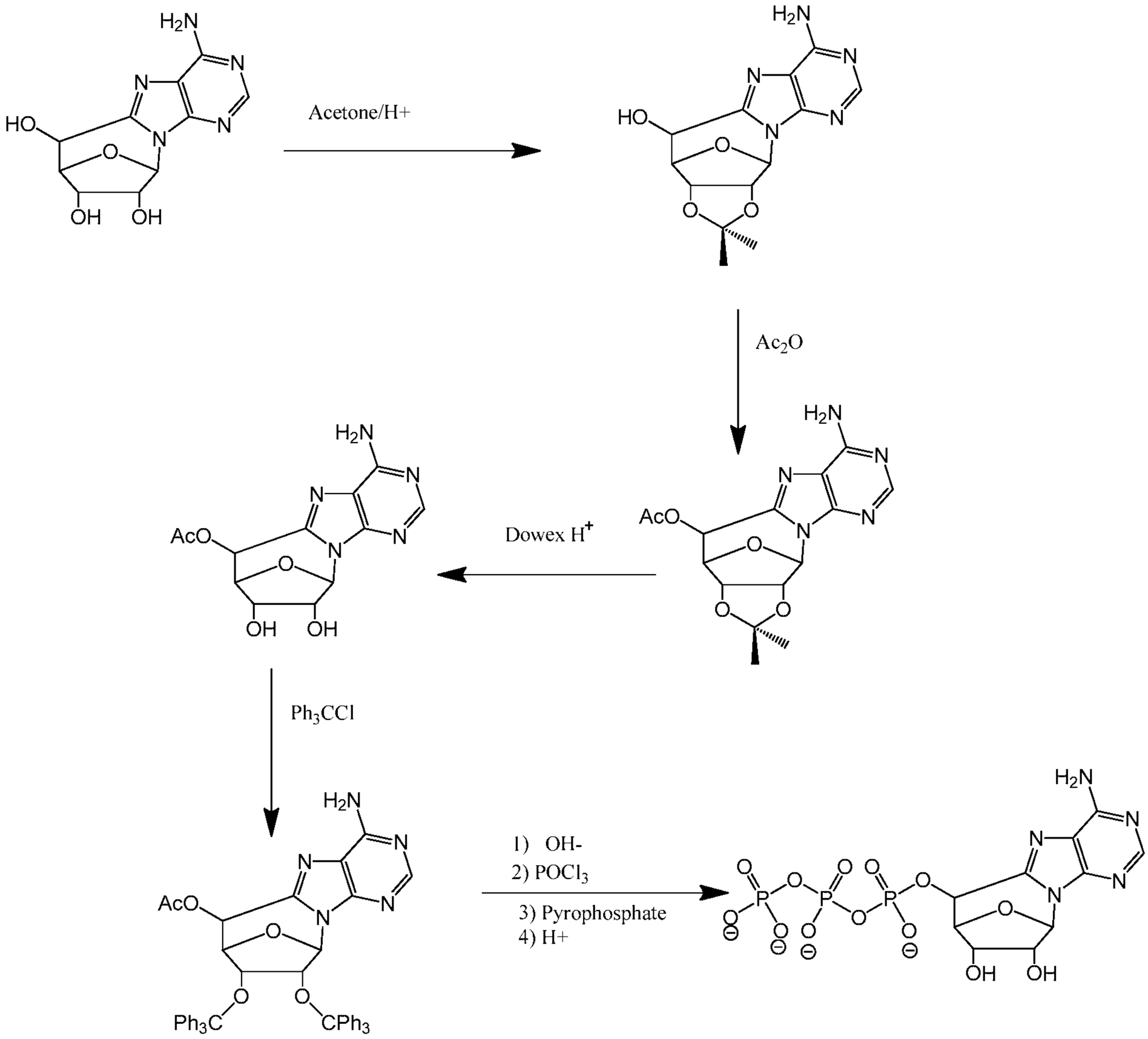
[000528] Exemplary syntheses of modified nucleotides, which are incorporated into a modified nucleic acid or mmRNA, e.g., RNA or mRNA, are provided below in Scheme 1 through Scheme 11. Scheme 1 provides a general method for phosphorylation of nucleosides, including modified nucleosides.

Scheme 1



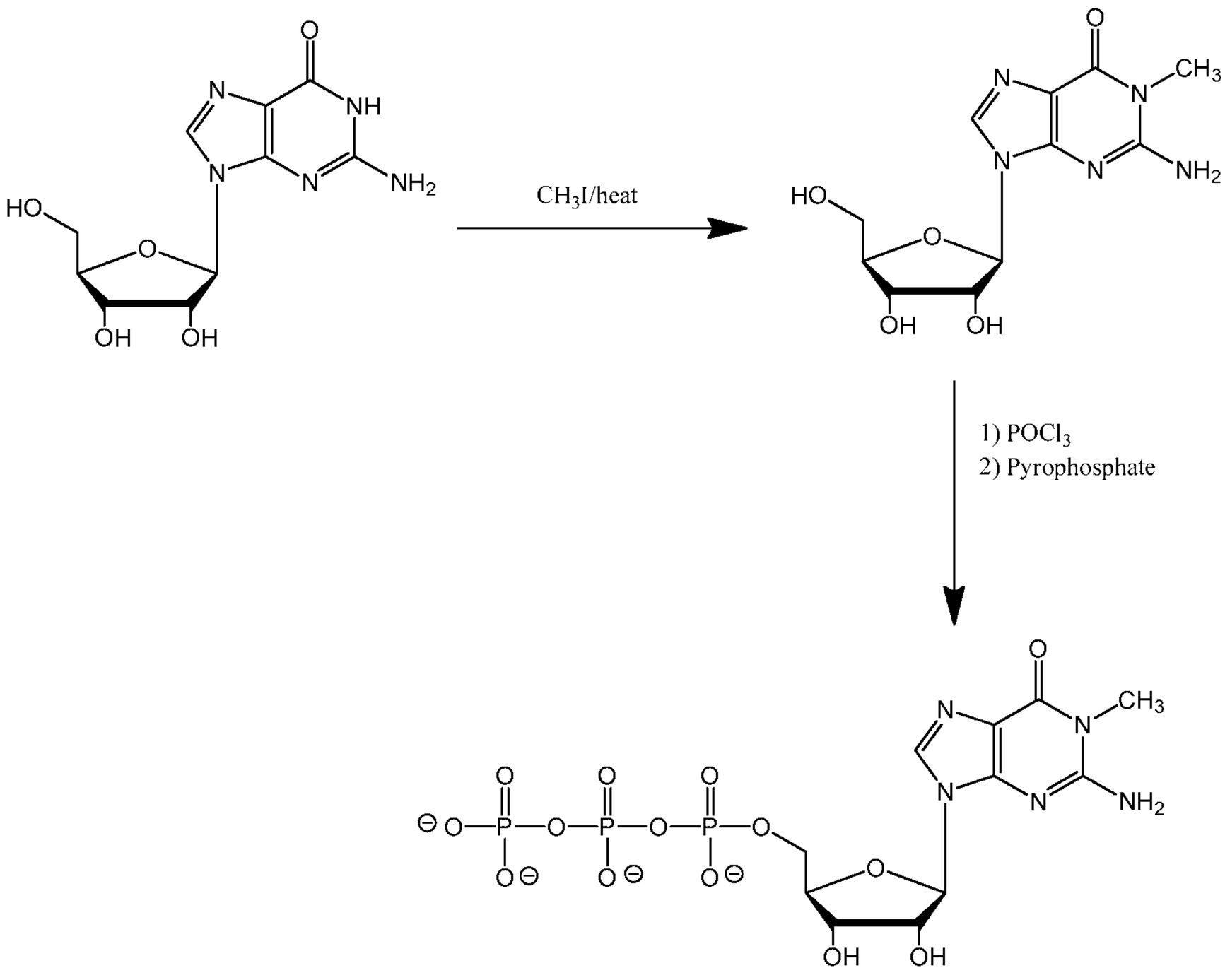
[000529] Various protecting groups may be used to control the reaction. For example, Scheme 2 provides the use of multiple protecting and deprotecting steps to promote phosphorylation at the 5′ position of the sugar, rather than the 2′ and 3′ hydroxyl groups.

Scheme 2

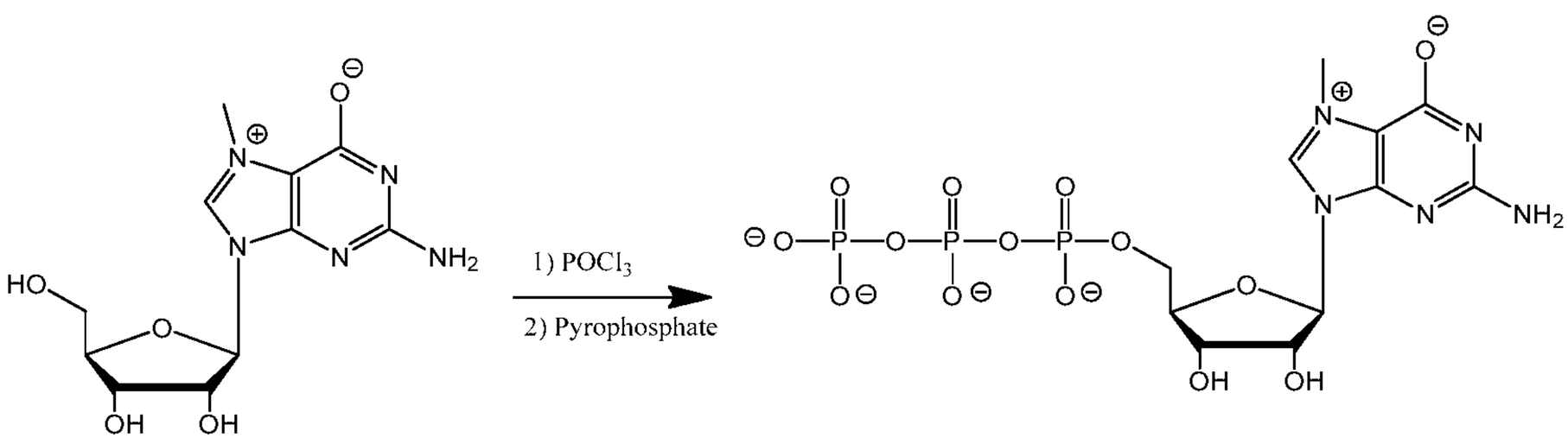


[000530] Modified nucleotides can be synthesized in any useful manner. Schemes 3, 4, and 7 provide exemplary methods for synthesizing modified nucleotides having a modified purine nucleobase; and Schemes 5 and 6 provide exemplary methods for synthesizing modified nucleotides having a modified pseudouridine or pseudoisocytidine, respectively.

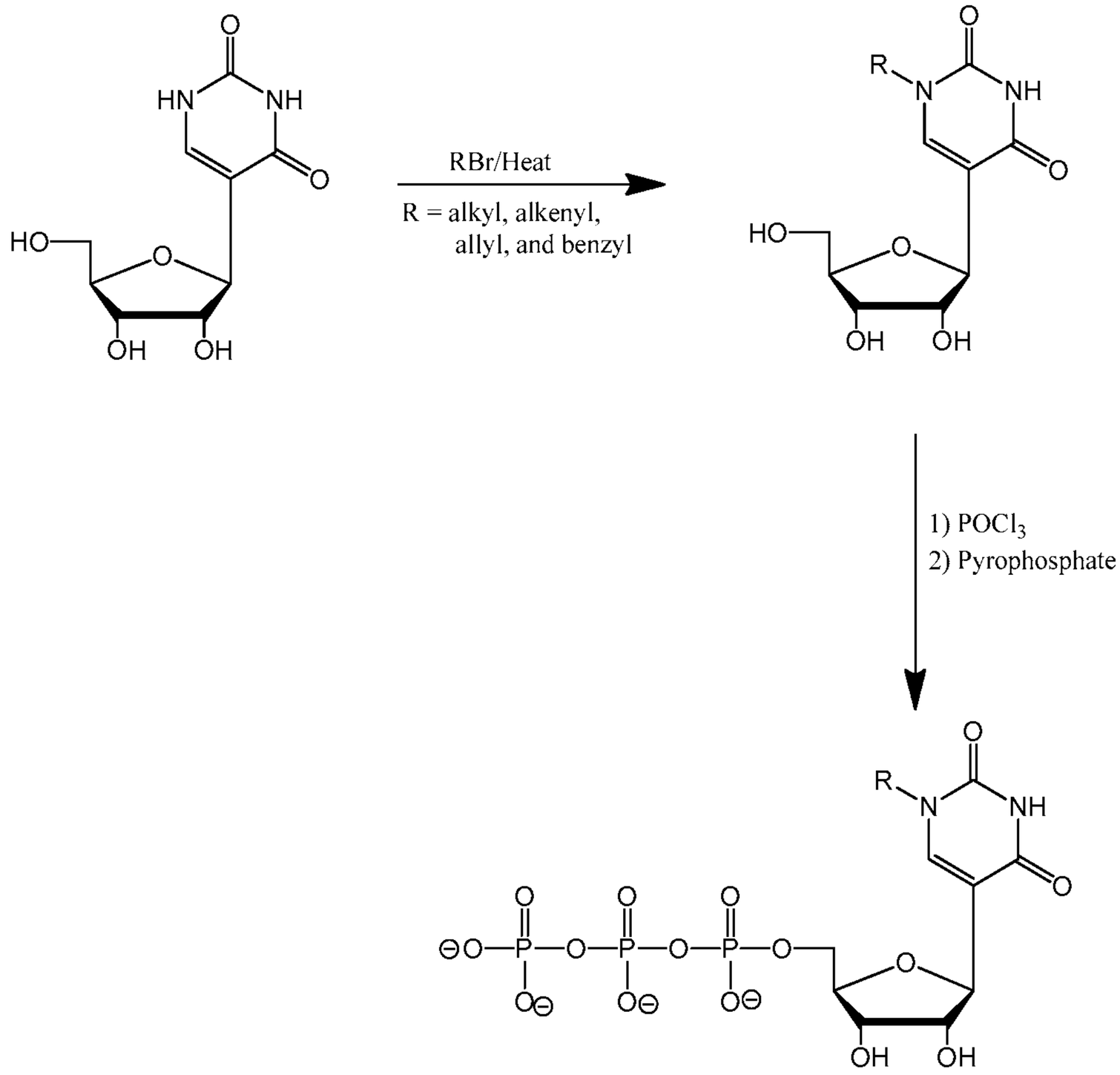
Scheme 3



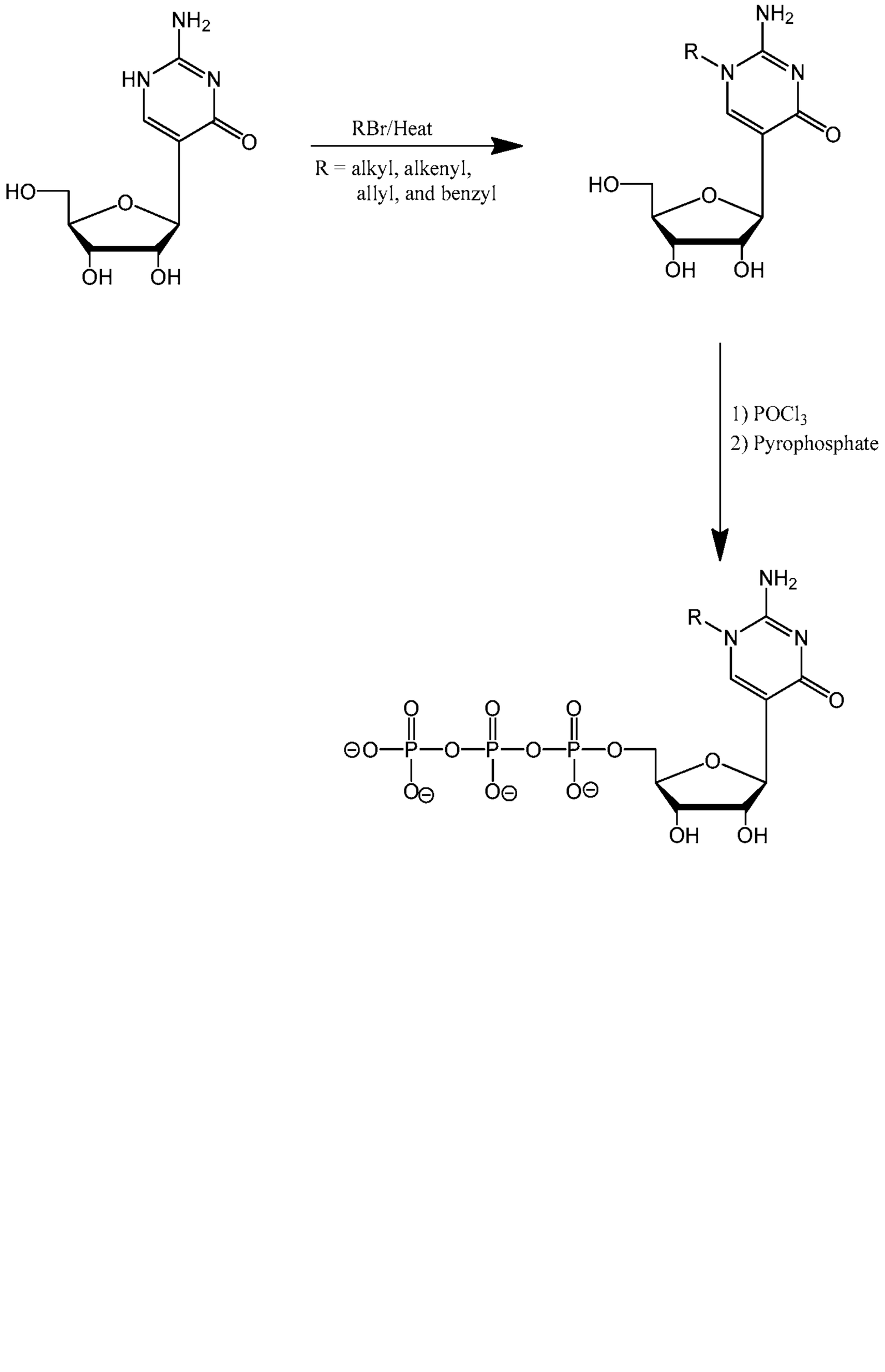
Scheme 4



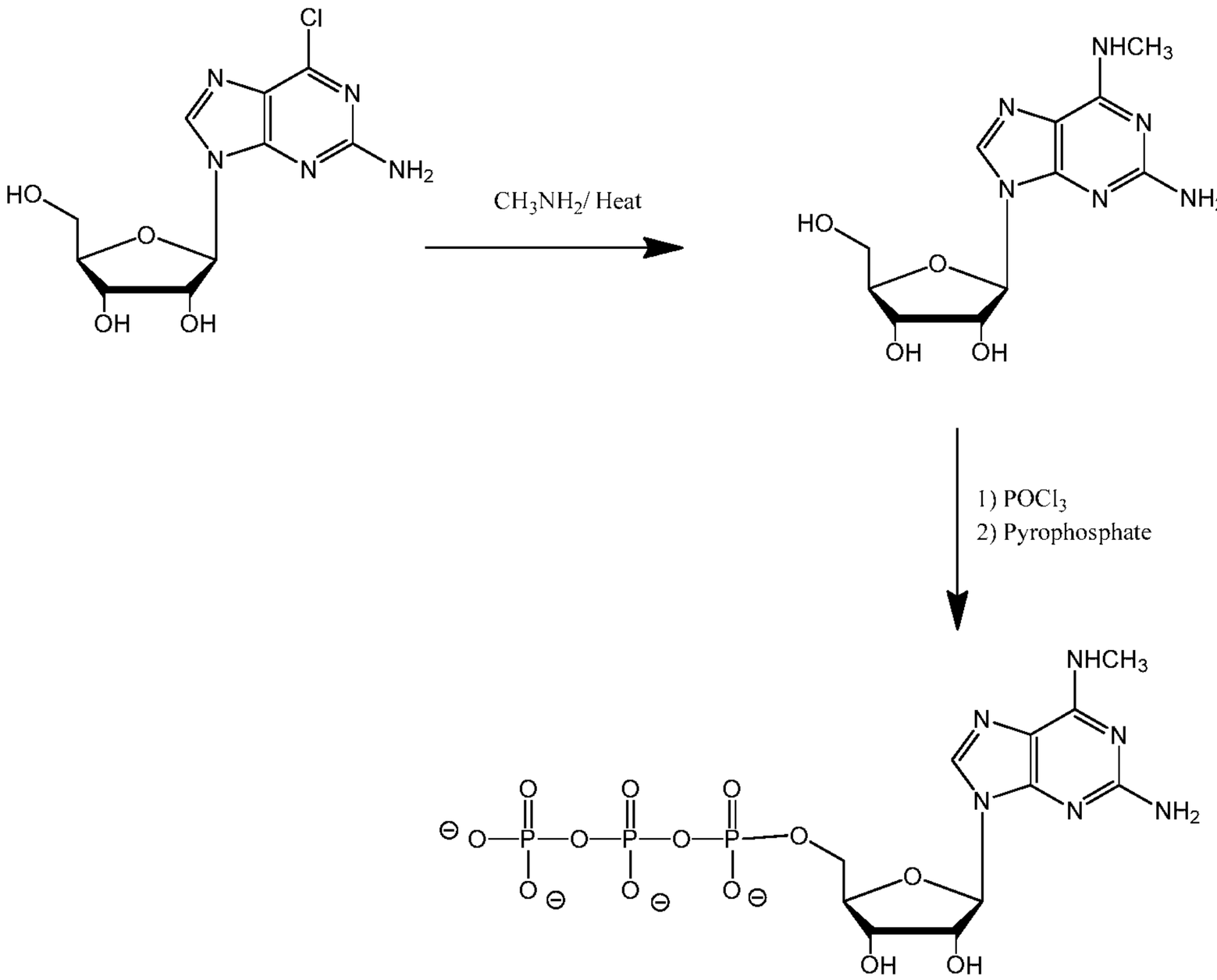
Scheme 5


HN
NH
O
HO
O
OH
OH
RBr/Heat
R = alkyl, alkenyl,
allyl, and benzyl
R
N
NH
O
HO
O
OH
OH
1) POCl3
2) Pyrophosphate
R
N
NH
O
O
O

Scheme 6

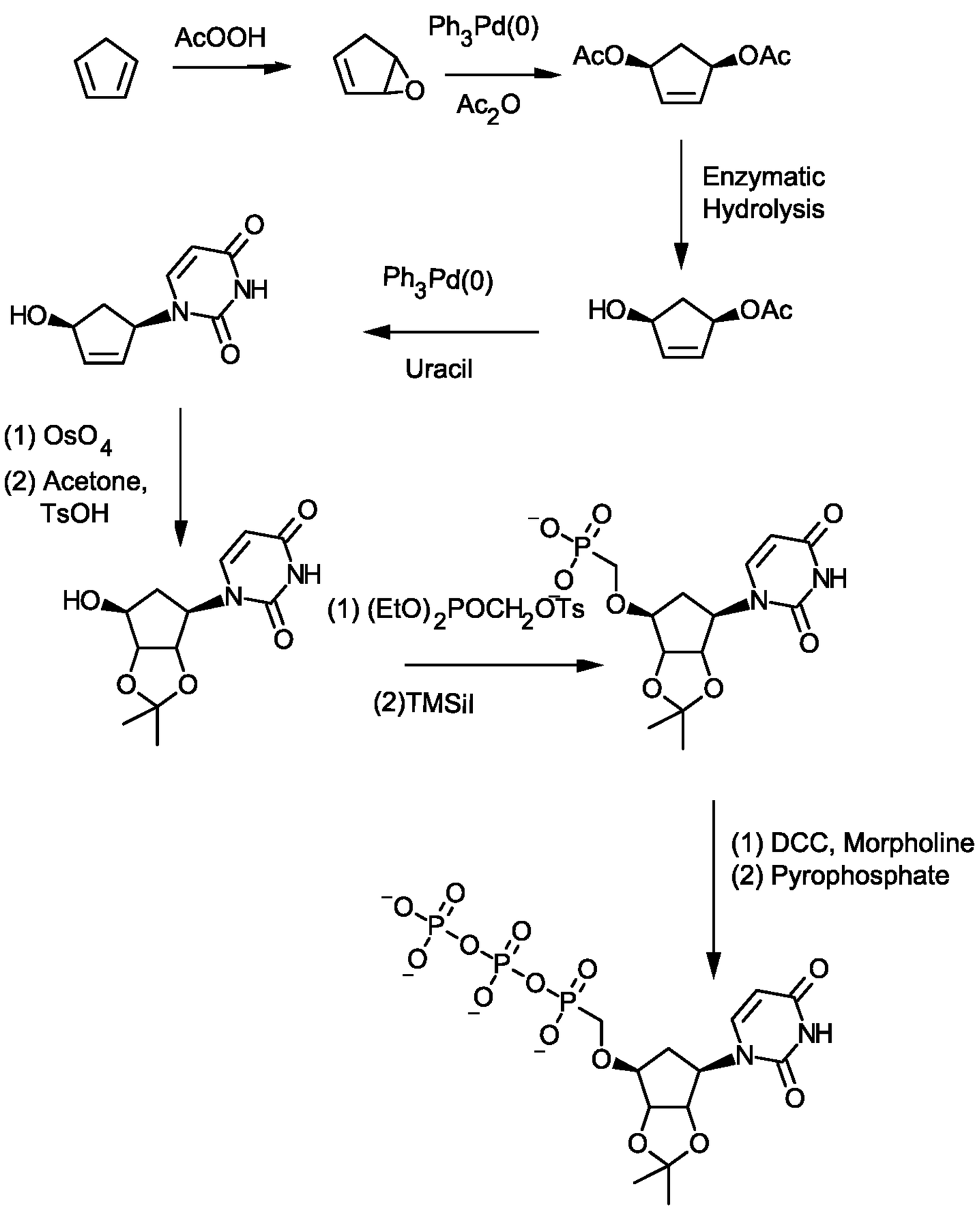

NH2
HN
N
O
HO
O
OH
OH
RBr/Heat
R = alkyl, alkenyl,
allyl, and benzyl
R
1) POCl3
2) Pyrophosphate

Scheme 7

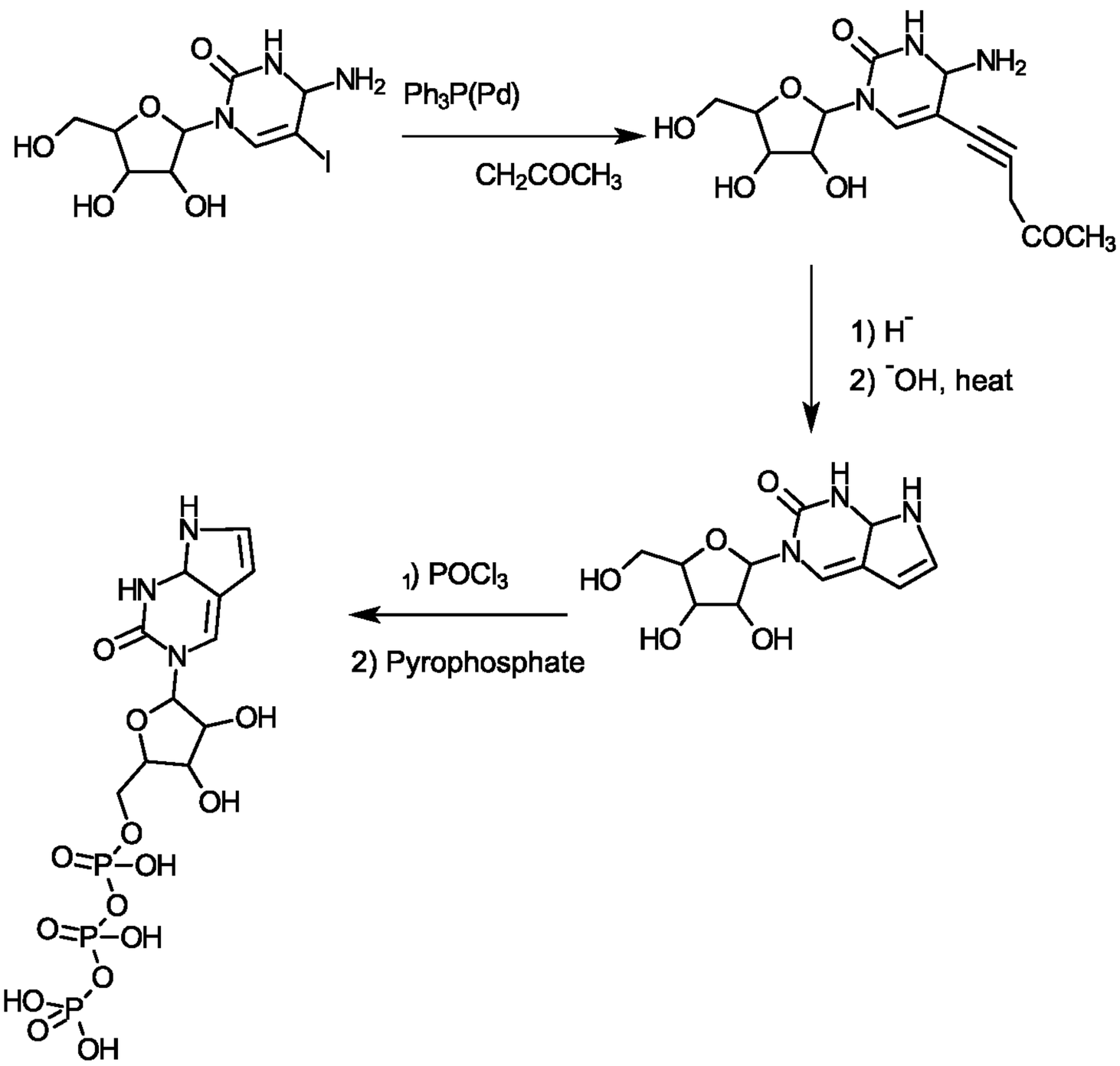


[000531] Schemes 8 and 9 provide exemplary syntheses of modified nucleotides. Scheme 10 provides a non-limiting biocatalytic method for producing nucleotides.

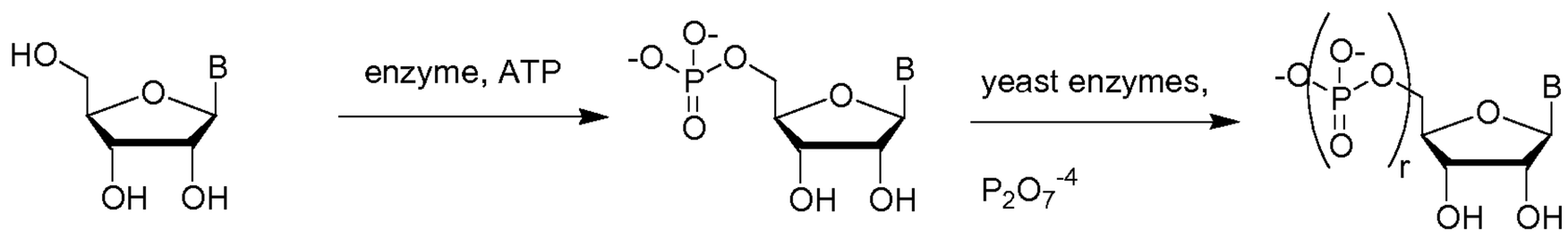
Scheme 8


AcOOH
Ph3Pd(0)
Ac2O
AcO
OAc
Enzymatic Hydrolysis
HO
OAc
Ph3Pd(0)
Uracil
HO
NH
N
O
(1) OsO4
(2) Acetone, TsOH
(1) (EtO)2POCH2OTs
(2)TMSiI
(1) DCC, Morpholine
(2) Pyrophosphate

Scheme 9



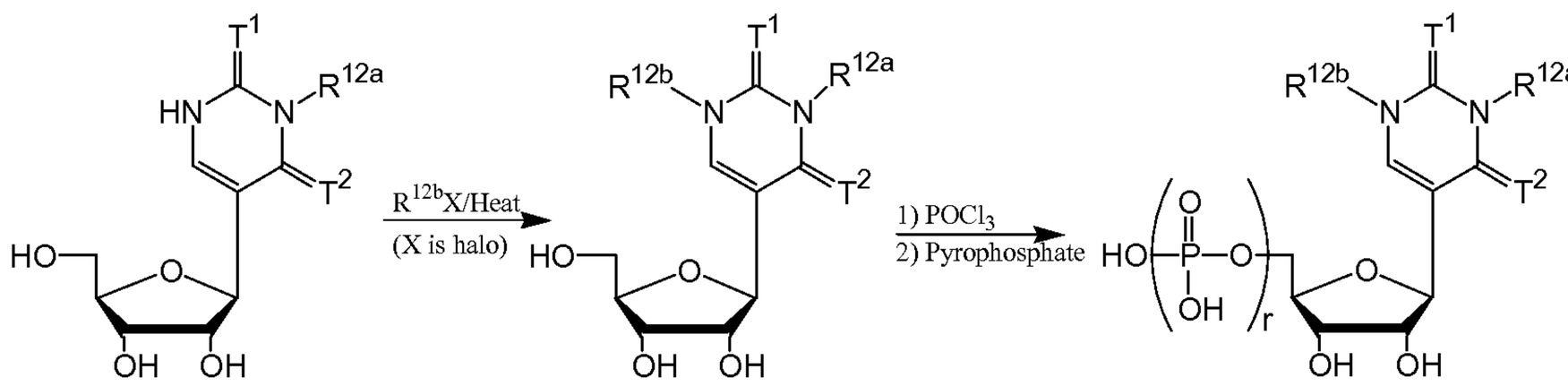
Scheme 10



[000532] Scheme 11 provides an exemplary synthesis of a modified uracil, where the N1 position is modified with R^{12b}, as provided elsewhere, and the 5′-position of ribose is phosphorylated. T^1, T^2, R^{12a}, R^{12b}, and r are as provided herein. This synthesis, as well as optimized versions thereof, can be used to modify other pyrimidine nucleobases and purine nucleobases (see e.g., Formulas (b1)-(b43)) and/or to install one or more phosphate groups (e.g., at the 5′ position of the sugar). This alkylating reaction can also be used to include one or more optionally substituted alkyl group at any reactive group

(e.g., amino group) in any nucleobase described herein (e.g., the amino groups in the Watson-Crick base-pairing face for cytosine, uracil, adenine, and guanine).

Scheme 11



Combinations of Nucleotides in mmRNA

[000533] Further examples of modified nucleotides and modified nucleotide combinations are provided below in Table 9. These combinations of modified nucleotides can be used to form the polypeptides, primary constructs, or mmRNA of the invention. Unless otherwise noted, the modified nucleotides may be completely substituted for the natural nucleotides of the modified nucleic acids or mmRNA of the invention. As a non-limiting example, the natural nucleotide uridine may be substituted with a modified nucleoside described herein. In another non-limiting example, the natural nucleotide uridine may be partially substituted (e.g., about 0.1%, 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 99.9%) with at least one of the modified nucleoside disclosed herein.

Table 9

Modified Nucleotide	**Modified Nucleotide Combination**
α-thio-cytidine	α-thio-cytidine/5-iodo-uridine
	α-thio-cytidine/N1-methyl-pseudouridine
	α-thio-cytidine/α-thio-uridine
	α-thio-cytidine/5-methyl-uridine
	α-thio-cytidine/pseudo-uridine
	about 50% of the cytosines are α-thio-cytidine
pseudoisocytidine	pseudoisocytidine/5-iodo-uridine
	pseudoisocytidine/N1-methyl-pseudouridine
	pseudoisocytidine/α-thio-uridine
	pseudoisocytidine/5-methyl-uridine
	pseudoisocytidine/pseudouridine
	about 25% of cytosines are pseudoisocytidine
	pseudoisocytidine/about 50% of uridines are N1-methyl-pseudouridine and about 50% of uridines are pseudouridine

	pseudoisocytidine/about 25% of uridines are N1-methyl-pseudouridine and about 25% of uridines are pseudouridine
pyrrolo-cytidine	pyrrolo-cytidine/5-iodo-uridine
	pyrrolo-cytidine/N1-methyl-pseudouridine
	pyrrolo-cytidine/α-thio-uridine
	pyrrolo-cytidine/5-methyl-uridine
	pyrrolo-cytidine/pseudouridine
	about 50% of the cytosines are pyrrolo-cytidine
5-methyl-cytidine	5-methyl-cytidine/5-iodo-uridine
	5-methyl-cytidine/N1-methyl-pseudouridine
	5-methyl-cytidine/α-thio-uridine
	5-methyl-cytidine/5-methyl-uridine
	5-methyl-cytidine/pseudouridine
	about 25% of cytosines are 5-methyl-cytidine
	about 50% of cytosines are 5-methyl-cytidine
	5-methyl-cytidine/5-methoxy-uridine
	5-methyl-cytidine/5-bromo-uridine
	5-methyl-cytidine/2-thio-uridine
	5-methyl-cytidine/about 50% of uridines are 2-thio-uridine
	about 50% of uridines are 5-methyl-cytidine/ about 50% of uridines are 2-thio-uridine
N4-acetyl-cytidine	N4-acetyl-cytidine /5-iodo-uridine
	N4-acetyl-cytidine /N1-methyl-pseudouridine
	N4-acetyl-cytidine /α-thio-uridine
	N4-acetyl-cytidine /5-methyl-uridine
	N4-acetyl-cytidine /pseudouridine
	about 50% of cytosines are N4-acetyl-cytidine
	about 25% of cytosines are N4-acetyl-cytidine
	N4-acetyl-cytidine /5-methoxy-uridine
	N4-acetyl-cytidine /5-bromo-uridine
	N4-acetyl-cytidine /2-thio-uridine
	about 50% of cytosines are N4-acetyl-cytidine/ about 50% of uridines are 2-thio-uridine

[000534] Further examples of modified nucleotide combinations are provided below in Table 10. These combinations of modified nucleotides can be used to form the polypeptides, primary constructs, or mmRNA of the invention.

Table 10

Modified Nucleotide	**Modified Nucleotide Combination**
modified cytidine having one or more nucleobases of Formula (b10)	modified cytidine with (b10)/pseudouridine
	modified cytidine with (b10)/N1-methyl-pseudouridine
	modified cytidine with (b10)/5-methoxy-uridine
	modified cytidine with (b10)/5-methyl-uridine

	modified cytidine with (b10)/5-bromo-uridine
	modified cytidine with (b10)/2-thio-uridine
	about 50% of cytidine substituted with modified cytidine (b10)/ about 50% of uridines are 2-thio-uridine
modified cytidine having one or more nucleobases of Formula (b32)	modified cytidine with (b32)/pseudouridine
	modified cytidine with (b32)/N1-methyl-pseudouridine
	modified cytidine with (b32)/5-methoxy-uridine
	modified cytidine with (b32)/5-methyl-uridine
	modified cytidine with (b32)/5-bromo-uridine
	modified cytidine with (b32)/2-thio-uridine
	about 50% of cytidine substituted with modified cytidine (b32)/ about 50% of uridines are 2-thio-uridine
modified uridine having one or more nucleobases of Formula (b1)	modified uridine with (b1)/ N4-acetyl-cytidine
	modified uridine with (b1)/ 5-methyl-cytidine
modified uridine having one or more nucleobases of Formula (b8)	modified uridine with (b8)/ N4-acetyl-cytidine
	modified uridine with (b8)/ 5-methyl-cytidine
modified uridine having one or more nucleobases of Formula (b28)	modified uridine with (b28)/ N4-acetyl-cytidine
	modified uridine with (b28)/ 5-methyl-cytidine
modified uridine having one or more nucleobases of Formula (b29)	modified uridine with (b29)/ N4-acetyl-cytidine
	modified uridine with (b29)/ 5-methyl-cytidine
modified uridine having one or more nucleobases of Formula (b30)	modified uridine with (b30)/ N4-acetyl-cytidine
	modified uridine with (b30)/ 5-methyl-cytidine

[000535] In some embodiments, at least 25% of the cytosines are replaced by a compound of Formula (b10)-(b14) (e.g., at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or about 100%).

[000536] In some embodiments, at least 25% of the uracils are replaced by a compound of Formula (b1)-(b9) (e.g., at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or about 100%).

[000537] In some embodiments, at least 25% of the cytosines are replaced by a compound of Formula (b10)-(b14), and at least 25% of the uracils are replaced by a compound of Formula (b1)-(b9) (e.g., at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or about 100%).

IV. Pharmaceutical Compositions

Formulation, Administration, Delivery and Dosing

[000538] The present invention provides polynucleotides, primary constructs and mmRNA compositions and complexes in combination with one or more pharmaceutically acceptable excipients. Pharmaceutical compositions may optionally comprise one or more additional active substances, e.g. therapeutically and/or prophylactically active substances. General considerations in the formulation and/or manufacture of pharmaceutical agents may be found, for example, in *Remington: The Science and Practice of Pharmacy* 21st ed., Lippincott Williams & Wilkins, 2005 (incorporated herein by reference).

[000539] In some embodiments, compositions are administered to humans, human patients or subjects. For the purposes of the present disclosure, the phrase "active ingredient" generally refers to polynucleotides, primary constructs and mmRNA to be delivered as described herein.

[000540] Although the descriptions of pharmaceutical compositions provided herein are principally directed to pharmaceutical compositions which are suitable for administration to humans, it will be understood by the skilled artisan that such compositions are generally suitable for administration to any other animal, *e.g.,* to non-human animals, e.g. non-human mammals. Modification of pharmaceutical compositions suitable for administration to humans in order to render the compositions suitable for administration to various animals is well understood, and the ordinarily skilled veterinary pharmacologist can design and/or perform such modification with merely ordinary, if any, experimentation. Subjects to which administration of the pharmaceutical compositions is contemplated include, but are not limited to, humans and/or other primates; mammals, including commercially relevant mammals such as cattle, pigs,

horses, sheep, cats, dogs, mice, and/or rats; and/or birds, including commercially relevant birds such as poultry, chickens, ducks, geese, and/or turkeys.

[000541] Formulations of the pharmaceutical compositions described herein may be prepared by any method known or hereafter developed in the art of pharmacology. In general, such preparatory methods include the step of bringing the active ingredient into association with an excipient and/or one or more other accessory ingredients, and then, if necessary and/or desirable, dividing, shaping and/or packaging the product into a desired single- or multi-dose unit.

[000542] A pharmaceutical composition in accordance with the invention may be prepared, packaged, and/or sold in bulk, as a single unit dose, and/or as a plurality of single unit doses. As used herein, a "unit dose" is discrete amount of the pharmaceutical composition comprising a predetermined amount of the active ingredient. The amount of the active ingredient is generally equal to the dosage of the active ingredient which would be administered to a subject and/or a convenient fraction of such a dosage such as, for example, one-half or one-third of such a dosage.

[000543] Relative amounts of the active ingredient, the pharmaceutically acceptable excipient, and/or any additional ingredients in a pharmaceutical composition in accordance with the invention will vary, depending upon the identity, size, and/or condition of the subject treated and further depending upon the route by which the composition is to be administered. By way of example, the composition may comprise between 0.1% and 100%, e.g., between .5 and 50%, between 1-30%, between 5-80%, at least 80% (w/w) active ingredient.

Formulations

[000544] The polynucleotide, primary construct, and mmRNA of the invention can be formulated using one or more excipients to: (1) increase stability; (2) increase cell transfection; (3) permit the sustained or delayed release (e.g., from a depot formulation of the polynucleotide, primary construct, or mmRNA); (4) alter the biodistribution (e.g., target the polynucleotide, primary construct, or mmRNA to specific tissues or cell types); (5) increase the translation of encoded protein *in vivo*; and/or (6) alter the release profile of encoded protein in vivo. In addition to traditional excipients such as any and all solvents, dispersion media, diluents, or other liquid vehicles, dispersion or suspension

aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, excipients of the present invention can include, without limitation, lipidoids, liposomes, lipid nanoparticles, polymers, lipoplexes, core-shell nanoparticles, peptides, proteins, cells transfected with polynucleotide, primary construct, or mmRNA (e.g., for transplantation into a subject), hyaluronidase, nanoparticle mimics and combinations thereof. Accordingly, the formulations of the invention can include one or more excipients, each in an amount that together increases the stability of the polynucleotide, primary construct, or mmRNA, increases cell transfection by the polynucleotide, primary construct, or mmRNA, increases the expression of polynucleotide, primary construct, or mmRNA encoded protein, and/or alters the release profile of polynucleotide, primary construct, or mmRNA encoded proteins. Further, the primary construct and mmRNA of the present invention may be formulated using self-assembled nucleic acid nanoparticles.

[000545] Formulations of the pharmaceutical compositions described herein may be prepared by any method known or hereafter developed in the art of pharmacology. In general, such preparatory methods include the step of associating the active ingredient with an excipient and/or one or more other accessory ingredients.

[000546] A pharmaceutical composition in accordance with the present disclosure may be prepared, packaged, and/or sold in bulk, as a single unit dose, and/or as a plurality of single unit doses. As used herein, a "unit dose" refers to a discrete amount of the pharmaceutical composition comprising a predetermined amount of the active ingredient. The amount of the active ingredient may generally be equal to the dosage of the active ingredient which would be administered to a subject and/or a convenient fraction of such a dosage including, but not limited to, one-half or one-third of such a dosage.

[000547] Relative amounts of the active ingredient, the pharmaceutically acceptable excipient, and/or any additional ingredients in a pharmaceutical composition in accordance with the present disclosure may vary, depending upon the identity, size, and/or condition of the subject being treated and further depending upon the route by which the composition is to be administered. For example, the composition may comprise between 0.1% and 99% (w/w) of the active ingredient.

[000548] In some embodiments, the formulations described herein may contain at least one mmRNA. As a non-limiting example, the formulations may contain 1, 2, 3, 4 or 5 mmRNA. In one embodiment the formulation may contain modified mRNA encoding proteins selected from categories such as, but not limited to, human proteins, veterinary proteins, bacterial proteins, biological proteins, antibodies, immunogenic proteins, therapeutic peptides and proteins, secreted proteins, plasma membrane proteins, cytoplasmic and cytoskeletal proteins, intrancellular membrane bound proteins, nuclear proteins, proteins associated with human disease and/or proteins associated with non-human diseases. In one embodiment, the formulation contains at least three modified mRNA encoding proteins. In one embodiment, the formulation contains at least five modified mRNA encoding proteins.

[000549] Pharmaceutical formulations may additionally comprise a pharmaceutically acceptable excipient, which, as used herein, includes, but is not limited to, any and all solvents, dispersion media, diluents, or other liquid vehicles, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, and the like, as suited to the particular dosage form desired. Various excipients for formulating pharmaceutical compositions and techniques for preparing the composition are known in the art (see Remington: The Science and Practice of Pharmacy, 21st Edition, A. R. Gennaro, Lippincott, Williams & Wilkins, Baltimore, MD, 2006; incorporated herein by reference in its entirety). The use of a conventional excipient medium may be contemplated within the scope of the present disclosure, except insofar as any conventional excipient medium may be incompatible with a substance or its derivatives, such as by producing any undesirable biological effect or otherwise interacting in a deleterious manner with any other component(s) of the pharmaceutical composition.

[000550] In some embodiments, the particle size of the lipid nanoparticle may be increased and/or decreased. The change in particle size may be able to help counter biological reaction such as, but not limited to, inflammation or may increase the biological effect of the modified mRNA delivered to mammals.

[000551] Pharmaceutically acceptable excipients used in the manufacture of pharmaceutical compositions include, but are not limited to, inert diluents, surface active

agents and/or emulsifiers, preservatives, buffering agents, lubricating agents, and/or oils. Such excipients may optionally be included in the pharmaceutical formulations of the invention.

Lipidoids

[000552] The synthesis of lipidoids has been extensively described and formulations containing these compounds are particularly suited for delivery of polynucleotides, primary constructs or mmRNA (see Mahon et al., Bioconjug Chem. 2010 21:1448-1454; Schroeder et al., J Intern Med. 2010 267:9-21; Akinc et al., Nat Biotechnol. 2008 26:561-569; Love et al., Proc Natl Acad Sci U S A. 2010 107:1864-1869; Siegwart et al., Proc Natl Acad Sci U S A. 2011 108:12996-3001; all of which are incorporated herein in their entireties).

[000553] While these lipidoids have been used to effectively deliver double stranded small interfering RNA molecules in rodents and non-human primates (see Akinc et al., Nat Biotechnol. 2008 26:561-569; Frank-Kamenetsky et al., Proc Natl Acad Sci U S A. 2008 105:11915-11920; Akinc et al., Mol Ther. 2009 17:872-879; Love et al., Proc Natl Acad Sci U S A. 2010 107:1864-1869; Leuschner et al., Nat Biotechnol. 2011 29:1005-1010; all of which is incorporated herein in their entirety), the present disclosure describes their formulation and use in delivering single stranded polynucleotides, primary constructs, or mmRNA. Complexes, micelles, liposomes or particles can be prepared containing these lipidoids and therefore, can result in an effective delivery of the polynucleotide, primary construct, or mmRNA, as judged by the production of an encoded protein, following the injection of a lipidoid formulation via localized and/or systemic routes of administration. Lipidoid complexes of polynucleotides, primary constructs, or mmRNA can be administered by various means including, but not limited to, intravenous, intramuscular, or subcutaneous routes.

[000554] *In vivo* delivery of nucleic acids may be affected by many parameters, including, but not limited to, the formulation composition, nature of particle PEGylation, degree of loading, oligonucleotide to lipid ratio, and biophysical parameters such as, but not limited to, particle size (Akinc et al., Mol Ther. 2009 17:872-879; herein incorporated by reference in its entirety). As an example, small changes in the anchor chain length of poly(ethylene glycol) (PEG) lipids may result in significant effects on in vivo efficacy.

Formulations with the different lipidoids, including, but not limited to penta[3-(1-laurylaminopropionyl)]-triethylenetetramine hydrochloride (TETA–5LAP; aka 98N12-5, see Murugaiah et al., Analytical Biochemistry, 401:61 (2010); herein incorporated by reference in its entirety), C12-200 (including derivatives and variants), and MD1, can be tested for *in vivo* activity.

[000555] The lipidoid referred to herein as "98N12-5" is disclosed by Akinc et al., Mol Ther. 2009 17:872-879 and is incorporated by reference in its entirety. (See Figure 2)

[000556] The lipidoid referred to herein as "C12-200" is disclosed by Love et al., Proc Natl Acad Sci U S A. 2010 107:1864-1869 (see Figure 2) and Liu and Huang, Molecular Therapy. 2010 669-670 (see Figure 2); both of which are herein incorporated by reference in their entirety. The lipidoid formulations can include particles comprising either 3 or 4 or more components in addition to polynucleotide, primary construct, or mmRNA. As an example, formulations with certain lipidoids, include, but are not limited to, 98N12-5 and may contain 42% lipidoid, 48% cholesterol and 10% PEG (C14 alkyl chain length). As another example, formulations with certain lipidoids, include, but are not limited to, C12-200 and may contain 50% lipidoid, 10% disteroylphosphatidyl choline, 38.5% cholesterol, and 1.5% PEG-DMG.

[000557] In one embodiment, a polynucleotide, primary construct, or mmRNA formulated with a lipidoid for systemic intravenous administration can target the liver. For example, a final optimized intravenous formulation using polynucleotide, primary construct, or mmRNA, and comprising a lipid molar composition of 42% 98N12-5, 48% cholesterol, and 10% PEG-lipid with a final weight ratio of about 7.5 to 1 total lipid to polynucleotide, primary construct, or mmRNA, and a C14 alkyl chain length on the PEG lipid, with a mean particle size of roughly 50–60 nm, can result in the distribution of the formulation to be greater than 90% to the liver.(see, Akinc et al., Mol Ther. 2009 17:872-879; herein incorporated by reference in its entirety). In another example, an intravenous formulation using a C12-200 (see US provisional application 61/175,770 and published international application WO2010129709, each of which is herein incorporated by reference in their entirety) lipidoid may have a molar ratio of 50/10/38.5/1.5 of C12-200/disteroylphosphatidyl choline/cholesterol/PEG-DMG, with a weight ratio of 7 to 1 total lipid to polynucleotide, primary construct, or mmRNA, and a mean particle size of

80 nm may be effective to deliver polynucleotide, primary construct, or mmRNA to hepatocytes (see, Love et al., Proc Natl Acad Sci U S A. 2010 107:1864-1869 herein incorporated by reference in its entirety). In another embodiment, an MD1 lipidoid-containing formulation may be used to effectively deliver polynucleotide, primary construct, or mmRNA to hepatocytes *in vivo*. The characteristics of optimized lipidoid formulations for intramuscular or subcutaneous routes may vary significantly depending on the target cell type and the ability of formulations to diffuse through the extracellular matrix into the blood stream. While a particle size of less than 150 nm may be desired for effective hepatocyte delivery due to the size of the endothelial fenestrae (see, Akinc et al., Mol Ther. 2009 17:872-879 herein incorporated by reference in its entirety), use of a lipidoid-formulated polynucleotide, primary construct, or mmRNA to deliver the formulation to other cells types including, but not limited to, endothelial cells, myeloid cells, and muscle cells may not be similarly size-limited. Use of lipidoid formulations to deliver siRNA in vivo to other non-hepatocyte cells such as myeloid cells and endothelium has been reported (see Akinc et al., Nat Biotechnol. 2008 26:561-569; Leuschner et al., Nat Biotechnol. 2011 29:1005-1010; Cho et al. Adv. Funct. Mater. 2009 19:3112-3118; 8th International Judah Folkman Conference, Cambridge, MA October 8-9, 2010; each of which is herein incorporated by reference in its entirety). Effective delivery to myeloid cells, such as monocytes, lipidoid formulations may have a similar component molar ratio. Different ratios of lipidoids and other components including, but not limited to, disteroylphosphatidyl choline, cholesterol and PEG-DMG, may be used to optimize the formulation of the polynucleotide, primary construct, or mmRNA for delivery to different cell types including, but not limited to, hepatocytes, myeloid cells, muscle cells, etc. For example, the component molar ratio may include, but is not limited to, 50% C12-200, 10% disteroylphosphatidyl choline, 38.5% cholesterol, and %1.5 PEG-DMG (see Leuschner et al., Nat Biotechnol 2011 29:1005-1010; herein incorporated by reference in its entirety). The use of lipidoid formulations for the localized delivery of nucleic acids to cells (such as, but not limited to, adipose cells and muscle cells) via either subcutaneous or intramuscular delivery, may not require all of the formulation components desired for systemic delivery, and as such may comprise only the lipidoid and the polynucleotide, primary construct, or mmRNA.

[000558] Combinations of different lipidoids may be used to improve the efficacy of polynucleotide, primary construct, or mmRNA directed protein production as the lipidoids may be able to increase cell transfection by the polynucleotide, primary construct, or mmRNA; and/or increase the translation of encoded protein (see Whitehead et al., Mol. Ther. 2011, 19:1688-1694, herein incorporated by reference in its entirety).

Liposomes, Lipoplexes, and Lipid Nanoparticles

[000559] The polynucleotide, primary construct, and mmRNA of the invention can be formulated using one or more liposomes, lipoplexes, or lipid nanoparticles. In one embodiment, pharmaceutical compositions of polynucleotide, primary construct, or mmRNA include liposomes. Liposomes are artificially-prepared vesicles which may primarily be composed of a lipid bilayer and may be used as a delivery vehicle for the administration of nutrients and pharmaceutical formulations. Liposomes can be of different sizes such as, but not limited to, a multilamellar vesicle (MLV) which may be hundreds of nanometers in diameter and may contain a series of concentric bilayers separated by narrow aqueous compartments, a small unicellular vesicle (SUV) which may be smaller than 50 nm in diameter, and a large unilamellar vesicle (LUV) which may be between 50 and 500 nm in diameter. Liposome design may include, but is not limited to, opsonins or ligands in order to improve the attachment of liposomes to unhealthy tissue or to activate events such as, but not limited to, endocytosis. Liposomes may contain a low or a high pH in order to improve the delivery of the pharmaceutical formulations.

[000560] The formation of liposomes may depend on the physicochemical characteristics such as, but not limited to, the pharmaceutical formulation entrapped and the liposomal ingredients , the nature of the medium in which the lipid vesicles are dispersed, the effective concentration of the entrapped substance and its potential toxicity, any additional processes involved during the application and/or delivery of the vesicles, the optimization size, polydispersity and the shelf-life of the vesicles for the intended application, and the batch-to-batch reproducibility and possibility of large-scale production of safe and efficient liposomal products.

[000561] In one embodiment, pharmaceutical compositions described herein may include, without limitation, liposomes such as those formed from 1,2-dioleyloxy-*N,N*-

dimethylaminopropane (DODMA) liposomes, DiLa2 liposomes from Marina Biotech (Bothell, WA), 1,2-dilinoleyloxy-3-dimethylaminopropane (DLin-DMA), 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-KC2-DMA), and MC3 (US20100324120; herein incorporated by reference in its entirety) and liposomes which may deliver small molecule drugs such as, but not limited to, DOXIL® from Janssen Biotech, Inc. (Horsham, PA).

[000562] In one embodiment, pharmaceutical compositions described herein may include, without limitation, liposomes such as those formed from the synthesis of stabilized plasmid-lipid particles (SPLP) or stabilized nucleic acid lipid particle (SNALP) that have been previously described and shown to be suitable for oligonucleotide delivery in vitro and in vivo (see Wheeler et al. Gene Therapy. 1999 6:271-281; Zhang et al. Gene Therapy. 1999 6:1438-1447; Jeffs et al. Pharm Res. 2005 22:362-372; Morrissey et al., Nat Biotechnol. 2005 2:1002-1007; Zimmermann et al., Nature. 2006 441:111-114; Heyes et al. J Contr Rel. 2005 107:276-287; Semple et al. Nature Biotech. 2010 28:172-176; Judge et al. J Clin Invest. 2009 119:661-673; deFougerolles *Hum Gene Ther.* 2008 19:125-132; all of which are incorporated herein in their entireties). The original manufacture method by Wheeler et al. was a detergent dialysis method, which was later improved by Jeffs et al. and is referred to as the spontaneous vesicle formation method. The liposome formulations are composed of 3 to 4 lipid components in addition to the polynucleotide, primary construct, or mmRNA. As an example a liposome can contain, but is not limited to, 55% cholesterol, 20% disteroylphosphatidyl choline (DSPC), 10% PEG-S-DSG, and 15% 1,2-dioleyloxy-*N,N*-dimethylaminopropane (DODMA), as described by Jeffs et al. As another example, certain liposome formulations may contain, but are not limited to, 48% cholesterol, 20% DSPC, 2% PEG-c-DMA, and 30% cationic lipid, where the cationic lipid can be 1,2-distearloxy-*N,N*-dimethylaminopropane (DSDMA), DODMA, DLin-DMA, or 1,2-dilinolenyloxy-3-dimethylaminopropane (DLenDMA), as described by Heyes et al.

[000563] In one embodiment, pharmaceutical compositions may include liposomes which may be formed to deliver mmRNA which may encode at least one immunogen. The mmRNA may be encapsulated by the liposome and/or it may be contained in an aqueous core which may then be encapsulated by the liposome (see International Pub.

Nos. WO2012031046, WO2012031043, WO2012030901 and WO2012006378; each of which is herein incorporated by reference in their entirety). In another embodiment, the mmRNA which may encode an immunogen may be formulated in a cationic oil-in-water emulsion where the emulsion particle comprises an oil core and a cationic lipid which can interact with the mmRNA anchoring the molecule to the emulsion particle (see International Pub. No. WO2012006380; herein incorporated by reference in its entirety).

In yet another embodiment, the lipid formulation may include at least cationic lipid, a lipid which may enhance transfection and a least one lipid which contains a hydrophilic head group linked to a lipid moiety (International Pub. No. WO2011076807 and U.S. Pub. No. 20110200582; each of which is herein incorporated by reference in their entirety). In another embodiment, the polynucleotides, primary constructs and/or mmRNA encoding an immunogen may be formulated in a lipid vesicle which may have crosslinks between functionalized lipid bilayers (see U.S. Pub. No. 20120177724, herein incorporated by reference in its entirety).

[000564] In one embodiment, the polynucleotides, primary constructs and/or mmRNA may be formulated in a lipid vesicle which may have crosslinks between functionalized lipid bilayers.

[000565] In one embodiment, the polynucleotides, primary constructs and/or mmRNA may be formulated in a liposome comprising a cationic lipid. The liposome may have a molar ratio of nitrogen atoms in the cationic lipid to the phophates in the RNA (N:P ratio) of between 1:1 and 20:1 as described in International Publication No. WO2013006825, herein incorporated by reference in its entirety. In another embodiment, the liposome may have a N:P ratio of greater than 20:1 or less than 1:1.

[000566] In one embodiment, the polynucleotides, primary constructs and/or mmRNA may be formulated in a lipid-polycation complex. The formation of the lipid-polycation complex may be accomplished by methods known in the art and/or as described in U.S. Pub. No. 20120178702, herein incorporated by reference in its entirety. As a non-limiting example, the polycation may include a cationic peptide or a polypeptide such as, but not limited to, polylysine, polyornithine and/or polyarginine and the cationic peptides described in International Pub. No. WO2012013326; herein incorporated by reference in its entirety. In another embodiment, the polynucleotides, primary constructs and/or

mmRNA may be formulated in a lipid-polycation complex which may further include a neutral lipid such as, but not limited to, cholesterol or dioleoyl phosphatidylethanolamine (DOPE).

[000567] The liposome formulation may be influenced by, but not limited to, the selection of the cationic lipid component, the degree of cationic lipid saturation, the nature of the PEGylation, ratio of all components and biophysical parameters such as size. In one example by Semple et al. (Semple et al. Nature Biotech. 2010 28:172-176; herein incorporated by reference in its entirety), the liposome formulation was composed of 57.1 % cationic lipid, 7.1% dipalmitoylphosphatidylcholine, 34.3 % cholesterol, and 1.4% PEG-c-DMA. As another example, changing the composition of the cationic lipid could more effectively deliver siRNA to various antigen presenting cells (Basha et al. Mol Ther. 2011 19:2186-2200; herein incorporated by reference in its entirety).

[000568] In some embodiments, the ratio of PEG in the lipid nanoparticle (LNP) formulations may be increased or decreased and/or the carbon chain length of the PEG lipid may be modified from C14 to C18 to alter the pharmacokinetics and/or biodistribution of the LNP formulations. As a non-limiting example, LNP formulations may contain 1-5% of the lipid molar ratio of PEG-c-DOMG as compared to the cationic lipid, DSPC and cholesterol. In another embodiment the PEG-c-DOMG may be replaced with a PEG lipid such as, but not limited to, PEG- DSG (1,2-Distearoyl-sn-glycerol, methoxypolyethylene glycol) or PEG-DPG (1,2-Dipalmitoyl-sn-glycerol, methoxypolyethylene glycol). The cationic lipid may be selected from any lipid known in the art such as, but not limited to, DLin-MC3-DMA, DLin-DMA, C12-200 and DLin-KC2-DMA.

[000569] In one embodiment, the polynucleotides, primary constructs or mmRNA may be formulated in a lipid nanoparticle such as those described in International Publication No. WO2012170930, herein incorporated by reference in its entirety.

[000570] In one embodiment, the cationic lipid may be selected from, but not limited to, a cationic lipid described in International Publication Nos. WO2012040184, WO2011153120, WO2011149733, WO2011090965, WO2011043913, WO2011022460, WO2012061259, WO2012054365, WO2012044638, WO2010080724, WO201021865 and WO2008103276, US Patent Nos. 7,893,302, 7,404,969 and 8,283,333 and US Patent

Publication No. US20100036115 and US20120202871; each of which is herein incorporated by reference in their entirety. In another embodiment, the cationic lipid may be selected from, but not limited to, formula A described in International Publication Nos. WO2012040184, WO2011153120, WO2011149733, WO2011090965, WO2011043913, WO2011022460, WO2012061259, WO2012054365 and WO2012044638; each of which is herein incorporated by reference in their entirety. In yet another embodiment, the cationic lipid may be selected from, but not limited to, formula CLI-CLXXIX of International Publication No. WO2008103276, formula CLI-CLXXIX of US Patent No. 7,893,302, formula CLI-CLXXXXII of US Patent No. 7,404,969 and formula I-VI of US Patent Publication No. US20100036115; each of which is herein incorporated by reference in their entirety. As a non-limiting example, the cationic lipid may be selected from (20Z,23Z)-N,N-dimethylnonacosa-20,23-dien-10-amine, (17Z,20Z)-N,N-dimemylhexacosa-17,20-dien-9-amine, (1Z,19Z)-N5N-dimethylpentacosa-l 6, 19-dien-8-amine, (13Z,16Z)-N,N-dimethyldocosa-13,16-dien-5-amine, (12Z,15Z)-N,N-dimethylhenicosa-12,15-dien-4-amine, (14Z,17Z)-N,N-dimethyltricosa-14,17-dien-6-amine, (15Z,18Z)-N,N-dimethyltetracosa-15,18-dien-7-amine, (18Z,21Z)-N,N-dimethylheptacosa-18,21-dien-10-amine, (15Z,18Z)-N,N-dimethyltetracosa-15,18-dien-5-amine, (14Z,17Z)-N,N-dimethyltricosa-14,17-dien-4-amine, (19Z,22Z)-N,N-dimeihyloctacosa-19,22-dien-9-amine, (18Z,21 Z)-N,N-dimethylheptacosa- 18 ,21 -dien-8 –amine, (17Z,20Z)-N,N-dimethylhexacosa- 17,20-dien-7-amine, (16Z,19Z)-N,N-dimethylpentacosa-16,19-dien-6-amine, (22Z,25Z)-N,N-dimethylhentriaconta-22,25-dien-10-amine, (21 Z ,24Z)-N,N-dimethyltriaconta-21,24-dien-9-amine, (18Z)-N,N-dimetylheptacos-18-en-10-amine, (17Z)-N,N-dimethylhexacos-17-en-9-amine, (19Z,22Z)-N,N-dimethyloctacosa-19,22-dien-7-amine, N,N-dimethylheptacosan-10-amine, (20Z,23Z)-N-ethyl-N-methylnonacosa-20,23-dien-l0-amine, 1-[(11Z,14Z)-l-nonylicosa-11,14-dien-l-yl] pyrrolidine, (20Z)-N,N-dimethylheptacos-20-en-l 0-amine, (15Z)-N,N-dimethyl eptacos-15-en-l 0-amine, (14Z)-N,N-dimethylnonacos-14-en-l0-amine, (17Z)-N,N-dimethylnonacos-17-en-l0-amine, (24Z)-N,N-dimethyltritriacont-24-en-l0-amine, (20Z)-N,N-dimethylnonacos-20-en-l 0-amine, (22Z)-N,N-dimethylhentriacont-22-en-l0-amine, (16Z)-N,N-dimethylpentacos-16-en-8-amine, (12Z,15Z)-N,N-dimethyl-2-nonylhenicosa-12,15-dien-1–amine, (13Z,16Z)-

N,N-dimethyl-3-nonyldocosa-l3,16-dien-l–amine, N,N-dimethyl-l-[(lS,2R)-2-octylcyclopropyl] eptadecan-8-amine, 1-[(1S,2R)-2-hexylcyclopropyl]-N,N-dimethylnonadecan-10-amine, N,N-dimethyl-1-[(1S ,2R)-2-octylcyclopropyl]nonadecan-10-amine, N,N-dimethyl-21-[(lS,2R)-2-octylcyclopropyl]henicosan-l0-amine,N,N-dimethyl-1-[(1S,2S)-2-{[(lR,2R)-2-pentylcyclopropyl]methyl}cyclopropyl]nonadecan-10-amine,N,N-dimethyl-1-[(1S,2R)-2-octylcyclopropyl]hexadecan-8-amine, N,N-dimethyl-[(lR,2S)-2-undecylcyclopropyl]tetradecan-5-amine, N,N-dimethyl-3-{7-[(1S,2R)-2-octylcyclopropyl]heptyl} dodecan-1–amine, 1-[(1R,2S)-2-hepty lcyclopropyl]-N,N-dimethyloctadecan-9–amine, 1-[(1S,2R)-2-decylcyclopropyl]-N,N-dimethylpentadecan-6-amine, N,N-dimethyl-l-[(lS,2R)-2-octylcyclopropyl]pentadecan-8-amine, R-N,N-dimethyl-1-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]-3-(octyloxy)propan-2-amine, S-N,N-dimethyl-1-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]-3-(octyloxy)propan-2-amine, 1-{2-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]-1-[(octyloxy)methyl]ethyl}pyrrolidine, (2S)-N,N-dimethyl-1-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]-3-[(5Z)-oct-5-en-1-yloxy]propan-2-amine, 1-{2-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]-1-[(octyloxy)methyl]ethyl}azetidine, (2S)-1-(hexyloxy)-N,N-dimethyl-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-2-amine, (2S)-1-(heptyloxy)-N,N-dimethyl-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-2-amine, N,N-dimethyl-1-(nonyloxy)-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-2-amine, N,N-dimethyl-1-[(9Z)-octadec-9-en-1-yloxy]-3-(octyloxy)propan-2-amine; (2S)-N,N-dimethyl-1-[(6Z,9Z,12Z)-octadeca-6,9,12-trien-1-yloxy]-3-(octyloxy)propan-2-amine, (2S)-1-[(11Z,14Z)-icosa-11,14-dien-1-yloxy]-N,N-dimethyl-3-(pentyloxy)propan-2-amine, (2S)-1-(hexyloxy)-3-[(11Z,14Z)-icosa-11,14-dien-1-yloxy]-N,N-dimethylpropan-2-amine, 1-[(11Z,14Z)-icosa-11,14-dien-1-yloxy]-N,N-dimethy1-3-(octyloxy)propan-2-amine, 1-[(13Z,16Z)-docosa-l3,16-dien-l-yloxy]-N,N-dimethyl-3-(octyloxy)propan-2-amine, (2S)-1-[(13Z,16Z)-docosa-13,16-dien-1-yloxy]-3-(hexyloxy)-N,N-dimethylpropan-2-amine, (2S)-1-[(13Z)-docos-13-en-1-yloxy]-3-(hexyloxy)-N,N-dimethylpropan-2-amine, 1-[(13Z)-docos-13-en-1-yloxy]-N,N-dimethyl-3-(octyloxy)propan-2-amine, 1-[(9Z)-hexadec-9-en-1-yloxy]-N,N-dimethyl-3-(octyloxy)propan-2-amine, (2R)-N,N-dimethyl-H(1-metoylo ctyl)oxy]-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-2-amine, (2R)-1-[(3,7-dimethyloctyl)oxy]-N,N-dimethyl-3-[(9Z,12Z)-octadeca-9,12-dien-1-yloxy]propan-

2-amine, N,N-dimethyl-1-(octyloxy)-3-({8-[(1S,2S)-2-{[(1R,2R)-2-pentylcyclopropyl]methyl}cyclopropyl]octyl}oxy)propan-2-amine, N,N-dimethyl-1-{[8-(2-oc1ylcyclopropyl)octyl]oxy}-3-(octyloxy)propan-2-amine and (11E,20Z,23Z)-N,N-dimethylnonacosa-11,20,2-trien-10-amine or a pharmaceutically acceptable salt or stereoisomer thereof.

[000571] In one embodiment, the lipid may be a cleavable lipid such as those described in International Publication No. WO2012170889, herein incorporated by reference in its entirety.

[000572] In one embodiment, the cationic lipid may be synthesized by methods known in the art and/or as described in International Publication Nos. WO2012040184, WO2011153120, WO2011149733, WO2011090965, WO2011043913, WO2011022460, WO2012061259, WO2012054365, WO2012044638, WO2010080724 and WO201021865; each of which is herein incorporated by reference in their entirety.

[000573] In one embodiment, the LNP formulations of the polynucleotides, primary constructs and/or mmRNA may contain PEG-c-DOMG at 3% lipid molar ratio. In another embodiment, the LNP formulations polynucleotides, primary constructs and/or mmRNA may contain PEG-c-DOMG at 1.5% lipid molar ratio.

[000574] In one embodiment, the pharmaceutical compositions of the polynucleotides, primary constructs and/or mmRNA may include at least one of the PEGylated lipids described in International Publication No. 2012099755, herein incorporated by reference.

[000575] In one embodiment, the LNP formulation may contain PEG-DMG 2000 (1,2-dimyristoyl-sn-glycero-3-phophoethanolamine-N-[methoxy(polyethylene glycol)-2000). In one embodiment, the LNP formulation may contain PEG-DMG 2000, a cationic lipid known in the art and at least one other component. In another embodiment, the LNP formulation may contain PEG-DMG 2000, a cationic lipid known in the art, DSPC and cholesterol. As a non-limiting example, the LNP formulation may contain PEG-DMG 2000, DLin-DMA, DSPC and cholesterol. As another non-limiting example the LNP formulation may contain PEG-DMG 2000, DLin-DMA, DSPC and cholesterol in a molar ratio of 2:40:10:48 (see e.g., Geall et al., Nonviral delivery of self-amplifying RNA vaccines, PNAS 2012; PMID: 22908294; herein incorporated by reference in its entirety). . As another non-limiting example, modified RNA described herein may be formulated

in a nanoparticle to be delivered by a parenteral route as described in U.S. Pub. No. 20120207845; herein incorporated by reference in its entirety.

In one embodiment, the LNP formulation may be formulated by the methods described in International Publication Nos. WO2011127255 or WO2008103276, each of which is herein incorporated by reference in their entirety. As a non-limiting example, modified RNA described herein may be encapsulated in LNP formulations as described in WO2011127255 and/or WO2008103276; each of which is herein incorporated by reference in their entirety.

In one embodiment, LNP formulations described herein may comprise a polycationic composition. As a non-limiting example, the polycationic composition may be selected from formula 1-60 of US Patent Publication No. US20050222064; herein incorporated by reference in its entirety. In another embodiment, the LNP formulations comprising a polycationic composition may be used for the delivery of the modified RNA described herein *in vivo* and/or *in vitro*.

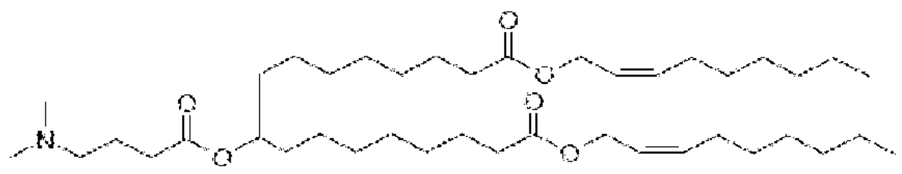
[000576] In one embodiment, the LNP formulations described herein may additionally comprise a permeability enhancer molecule. Non-limiting permeability enhancer molecules are described in US Patent Publication No. US20050222064; herein incorporated by reference in its entirety.

[000577] In one embodiment, the pharmaceutical compositions may be formulated in liposomes such as, but not limited to, DiLa2 liposomes (Marina Biotech, Bothell, WA), SMARTICLES® (Marina Biotech, Bothell, WA), neutral DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) based liposomes (e.g., siRNA delivery for ovarian cancer (Landen et al. Cancer Biology & Therapy 2006 5(12)1708-1713); herein incorporated by reference in its entirety) and hyaluronan-coated liposomes (Quiet Therapeutics, Israel).

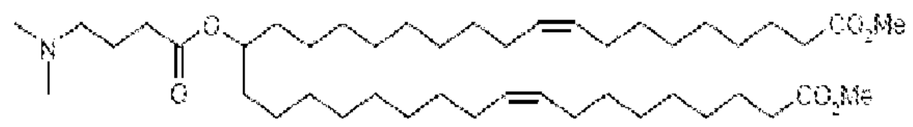
[000578] The nanoparticle formulations may be a carbohydrate nanoparticle comprising a carbohydrate carrier and a modified nucleic acid molecule (e.g., mmRNA). As a non-limiting example, the carbohydrate carrier may include, but is not limited to, an anhydride-modified phytoglycogen or glycogen-type material, phtoglycogen octenyl succinate, phytoglycogen beta-dextrin, anhydride-modified phytoglycogen beta-dextrin. (See e.g., International Publication No. WO2012109121; herein incorporated by reference in its entirety).

[000579] Lipid nanoparticle formulations may be improved by replacing the cationic lipid with a biodegradable cationic lipid which is known as a rapidly eliminated lipid nanoparticle (reLNP). Ionizable cationic lipids, such as, but not limited to, DLinDMA, DLin-KC2-DMA, and DLin-MC3-DMA, have been shown to accumulate in plasma and tissues over time and may be a potential source of toxicity. The rapid metabolism of the rapidly eliminated lipids can improve the tolerability and therapeutic index of the lipid nanoparticles by an order of magnitude from a 1 mg/kg dose to a 10 mg/kg dose in rat. Inclusion of an enzymatically degraded ester linkage can improve the degradation and metabolism profile of the cationic component, while still maintaining the activity of the reLNP formulation. The ester linkage can be internally located within the lipid chain or it may be terminally located at the terminal end of the lipid chain. The internal ester linkage may replace any carbon in the lipid chain.

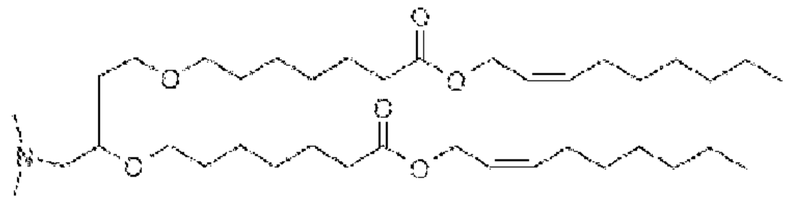
[000580] In one embodiment, the internal ester linkage may be located on either side of the saturated carbon. Non-limiting examples of reLNPs include,



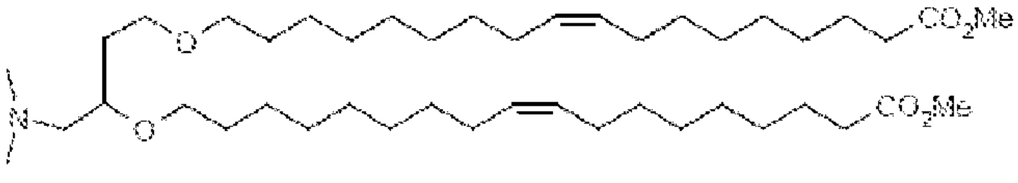
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and



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[000581] In one embodiment, an immune response may be elicited by delivering a lipid nanoparticle which may include a nanospecies, a polymer and an immunogen. (U.S. Publication No. 20120189700 and International Publication No. WO2012099805; each of which is herein incorporated by reference in their entirety). The polymer may encapsulate the nanospecies or partially encapsulate the nanospecies. The immunogen may be a recombinant protein, a modified RNA and/or a primary construct described

herein. In one embodiment, the lipid nanoparticle may be formulated for use in a vaccine such as, but not limited to, against a pathogen.

[000582] Lipid nanoparticles may be engineered to alter the surface properties of particles so the lipid nanoparticles may penetrate the mucosal barrier. Mucus is located on mucosal tissue such as, but not limted to, oral (e.g., the buccal and esophageal membranes and tonsil tissue), ophthalmic, gastrointestinal (e.g., stomach, small intestine, large intestine, colon, rectum), nasal, respiratory (e.g., nasal, pharyngeal, tracheal and bronchial membranes), genital (e.g., vaginal, cervical and urethral membranes). Nanoparticles larger than 10-200 nm which are preferred for higher drug encapsulation efficiency and the ability to provide the sustained delivery of a wide array of drugs have been thought to be too large to rapidly diffuse through mucosal barriers. Mucus is continuously secreted, shed, discarded or digested and recycled so most of the trapped particles may be removed from the mucosla tissue within seconds or within a few hours. Large polymeric nanoparticles (200nm -500nm in diameter) which have been coated densely with a low molecular weight polyethylene glycol (PEG) diffused through mucus only 4 to 6-fold lower than the same particles diffusing in water (Lai et al. PNAS 2007 104(5):1482-487; Lai et al. Adv Drug Deliv Rev. 2009 61(2): 158-171; each of which is herein incorporated by reference in their entirety). The transport of nanoparticles may be determined using rates of permeation and/or fluorescent microscopy techniques including, but not limited to, fluorescence recovery after photobleaching (FRAP) and high resolution multiple particle tracking (MPT). As a non-limiting example, compositions which can penetrate a mucosal barrier may be made as described in U.S. Pat. No. 8,241,670, herein incorporated by reference in its entirety.

[000583] The lipid nanoparticle engineered to penetrate mucus may comprise a polymeric material (i.e. a polymeric core) and/or a polymer-vitamin conjugate and/or a tri-block co-polymer. The polymeric material may include, but is not limited to, polyamines, polyethers, polyamides, polyesters, polycarbamates, polyureas, polycarbonates, poly(styrenes), polyimides, polysulfones, polyurethanes, polyacetylenes, polyethylenes, polyethyeneimines, polyisocyanates, polyacrylates, polymethacrylates, polyacrylonitriles, and polyarylates. The polymeric material may be biodegradable and/or biocompatible. The polymeric material may additionally be irradiated. As a non-limiting

example, the polymeric material may be gamma irradiated (See e.g., International App. No. WO201282165, herein incorporated by reference in its entirety). Non-limiting examples of specific polymers include poly(caprolactone) (PCL), ethylene vinyl acetate polymer (EVA), poly(lactic acid) (PLA), poly(L-lactic acid) (PLLA), poly(glycolic acid) (PGA), poly(lactic acid-co-glycolic acid) (PLGA), poly(L-lactic acid-co-glycolic acid) (PLLGA), poly(D,L-lactide) (PDLA), poly(L-lactide) (PLLA), poly(D,L-lactide-co-caprolactone), poly(D,L-lactide-co-caprolactone-co-glycolide), poly(D,L-lactide-co-PEO-co-D,L-lactide), poly(D,L-lactide-co-PPO-co-D,L-lactide), polyalkyl cyanoacralate, polyurethane, poly-L-lysine (PLL), hydroxypropyl methacrylate (HPMA), polyethyleneglycol, poly-L-glutamic acid, poly(hydroxy acids), polyanhydrides, polyorthoesters, poly(ester amides), polyamides, poly(ester ethers), polycarbonates, polyalkylenes such as polyethylene and polypropylene, polyalkylene glycols such as poly(ethylene glycol) (PEG), polyalkylene oxides (PEO), polyalkylene terephthalates such as poly(ethylene terephthalate), polyvinyl alcohols (PVA), polyvinyl ethers, polyvinyl esters such as poly(vinyl acetate), polyvinyl halides such as poly(vinyl chloride) (PVC), polyvinylpyrrolidone, polysiloxanes, polystyrene (PS), polyurethanes, derivatized celluloses such as alkyl celluloses, hydroxyalkyl celluloses, cellulose ethers, cellulose esters, nitro celluloses, hydroxypropylcellulose, carboxymethylcellulose, polymers of acrylic acids, such as poly(methyl(meth)acrylate) (PMMA), poly(ethyl(meth)acrylate), poly(butyl(meth)acrylate), poly(isobutyl(meth)acrylate), poly(hexyl(meth)acrylate), poly(isodecyl(meth)acrylate), poly(lauryl(meth)acrylate), poly(phenyl(meth)acrylate), poly(methyl acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate), poly(octadecyl acrylate) and copolymers and mixtures thereof, polydioxanone and its copolymers, polyhydroxyalkanoates, polypropylene fumarate, polyoxymethylene, poloxamers, poly(ortho)esters, poly(butyric acid), poly(valeric acid), poly(lactide-co-caprolactone), and trimethylene carbonate, polyvinylpyrrolidone.The lipid nanoparticle may be coated or associated with a co-polymer such as, but not limited to, a block co-polymer (such as a branched polyether-polyamide block copolymer described in International Publication No. WO2013012476, herein incorporated by reference in its entirety), and (poly(ethylene glycol))-(poly(propylene oxide))-(poly(ethylene glycol)) triblock copolymer (see e.g., US Publication 20120121718 and

US Publication 20100003337 and U.S. Pat. No. 8,263,665; each of which is herein incorporated by reference in their entirety). The co-polymer may be a polymer that is generally regarded as safe (GRAS) and the formation of the lipid nanoparticle may be in such a way that no new chemical entities are created. For example, the lipid nanoparticle may comprise poloxamers coating PLGA nanoparticles without forming new chemical entities which are still able to rapidly penetrate human mucus (Yang et al. Angew. Chem. Int. Ed. 2011 50:2597-2600; herein incorporated by reference in its entirety).

[000584] The vitamin of the polymer-vitamin conjugate may be vitamin E. The vitamin portion of the conjugate may be substituted with other suitable components such as, but not limited to, vitamin A, vitamin E, other vitamins, cholesterol, a hydrophobic moiety, or a hydrophobic component of other surfactants (e.g., sterol chains, fatty acids, hydrocarbon chains and alkylene oxide chains).

[000585] The lipid nanoparticle engineered to penetrate mucus may include surface altering agents such as, but not limited to, mmRNA, anionic proteins (e.g., bovine serum albumin), surfactants (e.g., cationic surfactants such as for example dimethyldioctadecyl-ammonium bromide), sugars or sugar derivatives (e.g., cyclodextrin), nucleic acids, polymers (e.g., heparin, polyethylene glycol and poloxamer), mucolytic agents (e.g., N-acetylcysteine, mugwort, bromelain, papain, clerodendrum, acetylcysteine, bromhexine, carbocisteine, eprazinone, mesna, ambroxol, sobrerol, domiodol, letosteine, stepronin, tiopronin, gelsolin, thymosin β4 dornase alfa, neltenexine, erdosteine) and various DNases including rhDNase.. The surface altering agent may be embedded or enmeshed in the particle's surface or disposed (e.g., by coating, adsorption, covalent linkage, or other process) on the surface of the lipid nanoparticle. (see e.g., US Publication 20100215580 and US Publication 20080166414; each of which is herein incorporated by reference in their entirety).

[000586] The mucus penetrating lipid nanoparticles may comprise at least one mmRNA described herein. The mmRNA may be encapsulated in the lipid nanoparticle and/or disposed on the surface of the paricle. The mmRNA may be covalently coupled to the lipid nanoparticle. Formulations of mucus penetrating lipid nanoparticles may comprise a plurality of nanoparticles. Further, the formulations may contain particles which may interact with the mucus and alter the structural and/or adhesive properties of the

surrounding mucus to decrease mucoadhesion which may increase the delivery of the mucus penetrating lipid nanoparticles to the mucosal tissue.

[000587] In one embodiment, the polynucleotide, primary construct, or mmRNA is formulated as a lipoplex, such as, without limitation, the ATUPLEX™ system, the DACC system, the DBTC system and other siRNA-lipoplex technology from Silence Therapeutics (London, United Kingdom), STEMFECT™ from STEMGENT® (Cambridge, MA), and polyethylenimine (PEI) or protamine-based targeted and non-targeted delivery of nucleic acids acids (Aleku et al. Cancer Res. 2008 68:9788-9798; Strumberg et al. Int J Clin Pharmacol Ther 2012 50:76-78; Santel et al., Gene Ther 2006 13:1222-1234; Santel et al., Gene Ther 2006 13:1360-1370; Gutbier et al., Pulm Pharmacol. Ther. 2010 23:334-344; Kaufmann et al. Microvasc Res 2010 80:286-293Weide et al. J Immunother. 2009 32:498-507; Weide et al. J Immunother. 2008 31:180-188; Pascolo Expert Opin. Biol. Ther. 4:1285-1294; Fotin-Mleczek et al., 2011 J. Immunother. 34:1-15; Song et al., Nature Biotechnol. 2005, 23:709-717; Peer et al., Proc Natl Acad Sci U S A. 2007 6;104:4095-4100; deFougerolles *Hum Gene Ther.* 2008 19:125-132; all of which are incorporated herein by reference in its entirety).

[000588] In one embodiment such formulations may also be constructed or compositions altered such that they passively or actively are directed to different cell types *in vivo*, including but not limited to hepatocytes, immune cells, tumor cells, endothelial cells, antigen presenting cells, and leukocytes (Akinc et al. Mol Ther. 2010 18:1357-1364; Song et al., Nat Biotechnol. 2005 23:709-717; Judge et al., J Clin Invest. 2009 119:661-673; Kaufmann et al., Microvasc Res 2010 80:286-293; Santel et al., Gene Ther 2006 13:1222-1234; Santel et al., Gene Ther 2006 13:1360-1370; Gutbier et al., Pulm Pharmacol. Ther. 2010 23:334-344; Basha et al., Mol. Ther. 2011 19:2186-2200; Fenske and Cullis, Expert Opin Drug Deliv. 2008 5:25-44; Peer et al., Science. 2008 319:627-630; Peer and Lieberman, Gene Ther. 2011 18:1127-1133; all of which are incorporated herein by reference in its entirety). One example of passive targeting of formulations to liver cells includes the DLin-DMA, DLin-KC2-DMA and DLin-MC3-DMA-based lipid nanoparticle formulations which have been shown to bind to apolipoprotein E and promote binding and uptake of these formulations into hepatocytes in vivo (Akinc et al. Mol Ther. 2010 18:1357-1364; herein incorporated by reference in

its entirety). Formulations can also be selectively targeted through expression of different ligands on their surface as exemplified by, but not limited by, folate, transferrin, N-acetylgalactosamine (GalNAc), and antibody targeted approaches (Kolhatkar et al., Curr Drug Discov Technol. 2011 8:197-206; Musacchio and Torchilin, *Front Biosci.* 2011 16:1388-1412; Yu et al., Mol Membr Biol. 2010 27:286-298; Patil et al., Crit Rev Ther Drug Carrier Syst. 2008 25:1-61; Benoit et al., Biomacromolecules. 2011 12:2708-2714; Zhao et al., Expert Opin Drug Deliv. 2008 5:309-319; Akinc et al., Mol Ther. 2010 18:1357-1364; Srinivasan et al., Methods Mol Biol. 2012 820:105-116; Ben-Arie et al., Methods Mol Biol. 2012 757:497-507; Peer 2010 J Control Release. 20:63-68; Peer et al., Proc Natl Acad Sci U S A. 2007 104:4095-4100; Kim et al., Methods Mol Biol. 2011 721:339-353; Subramanya et al., Mol Ther. 2010 18:2028-2037; Song et al., Nat Biotechnol. 2005 23:709-717; Peer et al., Science. 2008 319:627-630; Peer and Lieberman, Gene Ther. 2011 18:1127-1133; all of which are incorporated herein by reference in its entirety)..

[000589] In one embodiment, the polynucleotide, primary construct, or mmRNA is formulated as a solid lipid nanoparticle. A solid lipid nanoparticle (SLN) may be spherical with an average diameter between 10 to 1000 nm. SLN possess a solid lipid core matrix that can solubilize lipophilic molecules and may be stabilized with surfactants and/or emulsifiers. In a further embodiment, the lipid nanoparticle may be a self-assembly lipid-polymer nanoparticle (see Zhang et al., ACS Nano, 2008, 2 (8), pp 1696–1702; herein incorporated by reference in its entirety).

[000590] Liposomes, lipoplexes, or lipid nanoparticles may be used to improve the efficacy of polynucleotide, primary construct, or mmRNA directed protein production as these formulations may be able to increase cell transfection by the polynucleotide, primary construct, or mmRNA; and/or increase the translation of encoded protein. One such example involves the use of lipid encapsulation to enable the effective systemic delivery of polyplex plasmid DNA (Heyes et al., Mol Ther. 2007 15:713-720; herein incorporated by reference in its entirety). The liposomes, lipoplexes, or lipid nanoparticles may also be used to increase the stability of the polynucleotide, primary construct, or mmRNA.

[000591] In one embodiment, the the polynucleotides, primary constructs, and/or the mmRNA of the present invention can be formulated for controlled release and/or targeted delivery. As used herein, "controlled release" refers to a pharmaceutical composition or compound release profile that conforms to a particular pattern of release to effect a therapeutic outcome. In one embodiment, the polynucleotides, primary constructs or the mmRNA may be encapsulated into a delivery agent described herein and/or known in the art for controlled release and/or targeted delivery. As used herein, the term "encapsulate" means to enclose, surround or encase. As it relates to the formulation of the compounds of the invention, encapsulation may be substantial, complete or partial. The term "substitantially encapsulated" means that at least greater than 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, 99, 99.9, 99.9 or greater than 99.999% of the pharmaceutical composition or compound of the invention may be enclosed, surrounded or encased within the delivery agent. "Partially encapsulation" means that less than 10, 10, 20, 30, 40 50 or less of the pharmaceutical composition or compound of the invention may be enclosed, surrounded or encased within the delivery agent. Advantageously, encapsulation may be determined by measuring the escape or the activity of the pharmaceutical composition or compound of the invention using fluorescence and/or electron micrograph. For example, at least 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, 99, 99.9, 99.99 or greater than 99.99% of the pharmaceutical composition or compound of the invention are encapsulated in the delivery agent.

[000592] In one embodiment, the controlled release formulation may include, but is not limited to, tri-block co-polymers. As a non-limiting example, the formulation may include two different types of tri-block co-polymers (International Pub. No. WO2012131104 and WO2012131106; each of which is herein incorporated by reference in its entirety).

[000593] In another embodiment, the the polynucleotides, primary constructs, or the mmRNA may be encapsulated into a lipid nanoparticle or a rapidly eliminated lipid nanoparticle and the lipid nanoparticles or a rapidly eliminated lipid nanoparticle may then be encapsulated into a polymer, hydrogel and/or surgical sealant described herein and/or known in the art. As a non-limiting example, the polymer, hydrogel or surgical sealant may be PLGA, ethylene vinyl acetate (EVAc), poloxamer, GELSITE®

(Nanotherapeutics, Inc. Alachua, FL), HYLENEX® (Halozyme Therapeutics, San Diego CA), surgical sealants such as fibrinogen polymers (Ethicon Inc. Cornelia, GA), TISSELL® (Baxter International, Inc Deerfield, IL), PEG-based sealants, and COSEAL® (Baxter International, Inc Deerfield, IL).

[000594] In another embodiment, the lipid nanoparticle may be encapsulated into any polymer known in the art which may form a gel when injected into a subject. As another non-limiting example, the lipid nanoparticle may be encapsulated into a polymer matrix which may be biodegradable.

[000595] In one embodiment, the the polynucleotide, primary construct, or mmRNA formulation for controlled release and/or targeted delivery may also include at least one controlled release coating. Controlled release coatings include, but are not limited to, OPADRY®, polyvinylpyrrolidone/vinyl acetate copolymer, polyvinylpyrrolidone, hydroxypropyl methylcellulose, hydroxypropyl cellulose, hydroxyethyl cellulose, EUDRAGIT RL®, EUDRAGIT RS® and cellulose derivatives such as ethylcellulose aqueous dispersions (AQUACOAT® and SURELEASE®).

[000596] In one embodiment, the controlled release and/or targeted delivery formulation may comprise at least one degradable polyester which may contain polycationic side chains. Degradeable polyesters include, but are not limited to, poly(serine ester), poly(L-lactide-co-L-lysine), poly(4-hydroxy-L-proline ester), and combinations thereof. In another embodiment, the degradable polyesters may include a PEG conjugation to form a PEGylated polymer.

[000597] In one embodiment, the the polynucleotides, primary constructs, and/or the mmRNA of the present invention may be encapsulated in a therapeutic nanoparticle. Therapeutic nanoparticles may be formulated by methods described herein and known in the art such as, but not limited to, International Pub Nos. WO2010005740, WO2010030763, WO2010005721, WO2010005723, WO2012054923, US Pub. Nos. US20110262491, US20100104645, US20100087337, US20100068285, US20110274759, US20100068286 and US20120288541 and US Pat No. 8,206,747, 8,293,276, 8,318,208 and 8,318,211 each of which is herein incorporated by reference in their entirety. In another embodiment, therapeutic polymer nanoparticles may be

identified by the methods described in US Pub No. US20120140790, herein incorporated by reference in its entirety.

[000598] In one embodiment, the therapeutic nanoparticle may be formulated for sustained release. As used herein, "sustained release" refers to a pharmaceutical composition or compound that conforms to a release rate over a specific period of time. The period of time may include, but is not limited to, hours, days, weeks, months and years. As a non-limiting example, the sustained release nanoparticle may comprise a polymer and a therapeutic agent such as, but not limited to, the the polynucleotides, primary constructs, and mmRNA of the present invention (see International Pub No. 2010075072 and US Pub No. US20100216804, US20110217377 and US20120201859, each of which is herein incorporated by reference in their entirety).

[000599] In one embodiment, the therapeutic nanoparticles may be formulated to be target specific. As a non-limiting example, the thereapeutic nanoparticles may include a corticosteroid (see International Pub. No. WO2011084518; herein incorporated by reference in its entirety). In one embodiment, the therapeutic nanoparticles may be formulated to be cancer specific. As a non-limiting example, the therapeutic nanoparticles may be formulated in nanoparticles described in International Pub No. WO2008121949, WO2010005726, WO2010005725, WO2011084521 and US Pub No. US20100069426, US20120004293 and US20100104655, each of which is herein incorporated by reference in their entirety.

[000600] In one embodiment, the nanoparticles of the present invention may comprise a polymeric matrix. As a non-limiting example, the nanoparticle may comprise two or more polymers such as, but not limited to, polyethylenes, polycarbonates, polyanhydrides, polyhydroxyacids, polypropylfumerates, polycaprolactones, polyamides, polyacetals, polyethers, polyesters, poly(orthoesters), polycyanoacrylates, polyvinyl alcohols, polyurethanes, polyphosphazenes, polyacrylates, polymethacrylates, polycyanoacrylates, polyureas, polystyrenes, polyamines, polylysine, poly(ethylene imine), poly(serine ester), poly(L-lactide-co-L-lysine), poly(4-hydroxy-L-proline ester) or combinations thereof.

[000601] In one embodiment, the therapeutic nanoparticle comprises a diblock copolymer. In one embodiment, the diblock copolymer may include PEG in combination

with a polymer such as, but not limited to, polyethylenes, polycarbonates, polyanhydrides, polyhydroxyacids, polypropylfumerates, polycaprolactones, polyamides, polyacetals, polyethers, polyesters, poly(orthoesters), polycyanoacrylates, polyvinyl alcohols, polyurethanes, polyphosphazenes, polyacrylates, polymethacrylates, polycyanoacrylates, polyureas, polystyrenes, polyamines, polylysine, poly(ethylene imine), poly(serine ester), poly(L-lactide-co-L-lysine), poly(4-hydroxy-L-proline ester) or combinations thereof.

[000602] As a non-limiting example the therapeutic nanoparticle comprises a PLGA-PEG block copolymer (see US Pub. No. US20120004293 and US Pat No. 8,236,330, each of which is herein incorporated by reference in their entirety). In another non-limiting example, the therapeutic nanoparticle is a stealth nanoparticle comprising a diblock copolymer of PEG and PLA or PEG and PLGA (see US Pat No 8,246,968 and International Publication No. WO2012166923, each of which is herein incorporated by reference in its entirety).

[000603] In one embodiment, the therapeutic nanoparticle may comprise a multiblock copolymer (See e.g., U.S. Pat. No. 8,263,665 and 8,287,910; each of which is herein incorporated by reference in its entirety).

[000604] In one embodiment, the block copolymers described herein may be included in a polyion complex comprising a non-polymeric micelle and the block copolymer. (See e.g., U.S. Pub. No. 20120076836; herein incorporated by reference in its entirety).

[000605] In one embodiment, the therapeutic nanoparticle may comprise at least one acrylic polymer. Acrylic polymers include but are not limited to, acrylic acid, methacrylic acid, acrylic acid and methacrylic acid copolymers, methyl methacrylate copolymers, ethoxyethyl methacrylates, cyanoethyl methacrylate, amino alkyl methacrylate copolymer, poly(acrylic acid), poly(methacrylic acid), polycyanoacrylates and combinations thereof.

[000606] In one embodiment, the therapeutic nanoparticles may comprise at least one cationic polymer described herein and/or known in the art.

[000607] In one embodiment, the therapeutic nanoparticles may comprise at least one amine-containing polymer such as, but not limited to polylysine, polyethylene imine,

poly(amidoamine) dendrimers, poly(beta-amino esters) (See e.g., U.S. Pat. No. 8,287,849; herein incorporated by reference in its entirety) and combinations thereof.

[000608] In one embodiment, the therapeutic nanoparticles may comprise at least one degradable polyester which may contain polycationic side chains. Degradeable polyesters include, but are not limited to, poly(serine ester), poly(L-lactide-co-L-lysine), poly(4-hydroxy-L-proline ester), and combinations thereof. In another embodiment, the degradable polyesters may include a PEG conjugation to form a PEGylated polymer.

[000609] In another embodiment, the therapeutic nanoparticle may include a conjugation of at least one targeting ligand. The targeting ligand may be any ligand known in the art such as, but not limited to, a monoclonal antibody. (Kirpotin et al, Cancer Res. 2006 66:6732-6740; herein incorporated by reference in its entirety).

[000610] In one embodiment, the therapeutic nanoparticle may be formulated in an aqueous solution which may be used to target cancer (see International Pub No. WO2011084513 and US Pub No. US20110294717, each of which is herein incorporated by reference in their entirety).

[000611] In one embodiment, the polynucleotides, primary constructs, or mmRNA may be encapsulated in, linked to and/or associated with synthetic nanocarriers. . Synthetic nanocarriers include, but are not limited to, those described in International Pub. Nos. WO2010005740, WO2010030763, WO201213501, WO2012149252, WO2012149255, WO2012149259, WO2012149265, WO2012149268, WO2012149282, WO2012149301, WO2012149393, WO2012149405, WO2012149411, WO2012149454 and WO2013019669, and US Pub. Nos. US20110262491, US20100104645, US20100087337 and US20120244222, each of which is herein incorporated by reference in their entirety. The synthetic nanocarriers may be formulated using methods known in the art and/or described herein. As a non-limiting example, the synthetic nanocarriers may be formulated by the methods described in International Pub Nos. WO2010005740, WO2010030763 and WO201213501and US Pub. Nos. US20110262491, US20100104645, US20100087337 and US2012024422, each of which is herein incorporated by reference in their entirety. In another embodiment, the synthetic nanocarrier formulations may be lyophilized by methods described in International Pub.

No. WO2011072218 and US Pat No. 8,211,473; each of which is herein incorporated by reference in their entirety.

[000612] In one embodiment, the synthetic nanocarriers may contain reactive groups to release the polynucleotides, primary constructs and/or mmRNA described herein (see International Pub. No. WO20120952552 and US Pub No. US20120171229, each of which is herein incorporated by reference in their entirety).

[000613] In one embodiment, the synthetic nanocarriers may contain an immunostimulatory agent to enhance the immune response from delivery of the synthetic nanocarrier. As a non-limiting example, the synthetic nanocarrier may comprise a Th1 immunostimulatory agent which may enhance a Th1-based response of the immune system (see International Pub No. WO2010123569 and US Pub. No. US20110223201, each of which is herein incorporated by reference in its entirety).

[000614] In one embodiment, the synthetic nanocarriers may be formulated for targeted release. In one embodiment, the synthetic nanocarrier is formulated to release the polynucleotides, primary constructs and/or mmRNA at a specified pH and/or after a desired time interval. As a non-limiting example, the synthetic nanoparticle may be formulated to release the polynucleotides, primary constructs and/or mmRNA after 24 hours and/or at a pH of 4.5 (see International Pub. Nos. WO2010138193 and WO2010138194 and US Pub Nos. US20110020388 and US20110027217, each of which is herein incorporated by reference in their entireties).

[000615] In one embodiment, the synthetic nanocarriers may be formulated for controlled and/or sustained release of the polynucleotides, primary constructs and/or mmRNA described herein. As a non-limiting example, the synthetic nanocarriers for sustained release may be formulated by methods known in the art, described herein and/or as described in International Pub No. WO2010138192 and US Pub No. 20100303850, each of which is herein incorporated by reference in their entirety.

[000616] In one embodiment, the synthetic nanocarrier may be formulated for use as a vaccine. In one embodiment, the synthetic nanocarrier may encapsulate at least one polynucleotide, primary construct and/or mmRNA which encode at least one antigen. As a non-limiting example, the synthetic nanocarrier may include at least one antigen and an excipient for a vaccine dosage form (see International Pub No. WO2011150264 and US

Pub No. US20110293723, each of which is herein incorporated by reference in their entirety). As another non-limiting example, a vaccine dosage form may include at least two synthetic nanocarriers with the same or different antigens and an excipient (see International Pub No. WO2011150249 and US Pub No. US20110293701, each of which is herein incorporated by reference in their entirety). The vaccine dosage form may be selected by methods described herein, known in the art and/or described in International Pub No. WO2011150258 and US Pub No. US20120027806, each of which is herein incorporated by reference in their entirety).

[000617] In one embodiment, the synthetic nanocarrier may comprise at least one polynucleotide, primary construct and/or mmRNA which encodes at least one adjuvant. As non-limiting example, the adjuvant may comprise dimethyldioctadecylammonium-bromide, dimethyldioctadecylammonium-chloride, dimethyldioctadecylammonium-phosphate or dimethyldioctadecylammonium-acetate (DDA) and an apolar fraction or part of said apolar fraction of a total lipid extract of a mycobacterium (See e.g, U.S. Pat. No. 8,241,610; herein incorporated by reference in its entirety). In another embodiment, the synthetic nanocarrier may comprise at least one polynucleotide, primary construct and/or mmRNA and an adjuvant. As a non-limiting example, the synthetic nanocarrier comprising and adjuvant may be formulated by the methods described in International Pub No. WO2011150240 and US Pub No. US20110293700, each of which is herein incorporated by reference in its entirety.

[000618] In one embodiment, the synthetic nanocarrier may encapsulate at least one polynucleotide, primary construct and/or mmRNA which encodes a peptide, fragment or region from a virus. As a non-limiting example, the synthetic nanocarrier may include, but is not limited to, the nanocarriers described in International Pub No. WO2012024621, WO201202629, WO2012024632 and US Pub No. US20120064110, US20120058153 and US20120058154, each of which is herein incorporated by reference in their entirety.

[000619] In one embodiment, the synthetic nanocarrier may be coupled to a polynucleotide, primary construct or mmRNA which may be able to trigger a humoral and/or cytotoxic T lymphocyte (CTL) response (See e.g., International Publication No. WO2013019669, herein incorporated by reference in its entirety).

[000620] In one embodiment, the nanoparticle may be optimized for oral administration. The nanoparticle may comprise at least one cationic biopolymer such as, but not limited to, chitosan or a derivative thereof. As a non-limiting example, the nanoparticle may be formulated by the methods described in U.S. Pub. No. 20120282343; herein incorporated by reference in its entirety.

Polymers, Biodegradable Nanoparticles, and Core-Shell Nanoparticles

[000621] The polynucleotide, primary construct, and mmRNA of the invention can be formulated using natural and/or synthetic polymers. Non-limiting examples of polymers which may be used for delivery include, but are not limited to, DYNAMIC POLYCONJUGATE® (Arrowhead Reasearch Corp., Pasadena, CA) formulations from MIRUS® Bio (Madison, WI) and Roche Madison (Madison, WI), PHASERX™ polymer formulations such as, without limitation, SMARTT POLYMER TECHNOLOGY™ (PHASERX®, Seattle, WA), DMRI/DOPE, poloxamer, VAXFECTIN® adjuvant from Vical (San Diego, CA), chitosan, cyclodextrin from Calando Pharmaceuticals (Pasadena, CA), dendrimers and poly(lactic-co-glycolic acid) (PLGA) polymers. RONDEL™ (RNAi/Oligonucleotide Nanoparticle Delivery) polymers (Arrowhead Research Corporation, Pasadena, CA) and pH responsive co-block polymers such as, but not limited to, PHASERX® (Seattle, WA).

[000622] A non-limiting example of chitosan formulation includes a core of positively charged chitosan and an outer portion of negatively charged substrate (U.S. Pub. No. 20120258176; herein incorporated by reference in its entirety). Chitosan includes, but is not limited to N-trimethyl chitosan, mono-N-carboxymethyl chitosan (MCC), N-palmitoyl chitosan (NPCS), EDTA-chitosan, low molecular weight chitosan, chitosan derivatives, or combinations thereof.

[000623] In one embodiment, the polymers used in the present invention have undergone processing to reduce and/or inhibit the attachement of unwanted substances such as, but not limited to, bacteria, to the surface of the polymer. The polymer may be processed by methods known and/or described in the art and/or described in International Pub. No. WO2012150467, herein incorporated by reference in its entirety.

[000624] A non-limiting example of PLGA formulations include, but are not limited to, PLGA injectable depots (e.g., ELIGARD® which is formed by dissolving PLGA in 66%

N-methyl-2-pyrrolidone (NMP) and the remainder being aqueous solvent and leuprolide. Once injected, the PLGA and leuprolide peptide precipitates into the subcutaneous space).

[000625] Many of these polymer approaches have demonstrated efficacy in delivering oligonucleotides *in vivo* into the cell cytoplasm (reviewed in deFougerolles *Hum Gene Ther.* 2008 19:125-132; herein incorporated by reference in its entirety). Two polymer approaches that have yielded robust *in vivo* delivery of nucleic acids, in this case with small interfering RNA (siRNA), are dynamic polyconjugates and cyclodextrin-based nanoparticles. The first of these delivery approaches uses dynamic polyconjugates and has been shown *in vivo* in mice to effectively deliver siRNA and silence endogenous target mRNA in hepatocytes (Rozema et al., Proc Natl Acad Sci U S A. 2007 104:12982-12887; herein incorporated by reference in its entirety). This particular approach is a multicomponent polymer system whose key features include a membrane-active polymer to which nucleic acid, in this case siRNA, is covalently coupled via a disulfide bond and where both PEG (for charge masking) and *N*-acetylgalactosamine (for hepatocyte targeting) groups are linked via pH-sensitive bonds (Rozema et al., Proc Natl Acad Sci U S A. 2007 104:12982-12887; herein incorporated by reference in its entirety). On binding to the hepatocyte and entry into the endosome, the polymer complex disassembles in the low-pH environment, with the polymer exposing its positive charge, leading to endosomal escape and cytoplasmic release of the siRNA from the polymer. Through replacement of the *N*-acetylgalactosamine group with a mannose group, it was shown one could alter targeting from asialoglycoprotein receptor-expressing hepatocytes to sinusoidal endothelium and Kupffer cells. Another polymer approach involves using transferrin-targeted cyclodextrin-containing polycation nanoparticles. These nanoparticles have demonstrated targeted silencing of the *EWS-FLI1* gene product in transferrin receptor-expressing Ewing's sarcoma tumor cells (Hu-Lieskovan *et al*., Cancer Res.2005 65: 8984-8982; herein incorporated by reference in its entirety) and siRNA formulated in these nanoparticles was well tolerated in non-human primates (Heidel *et al*., Proc Natl Acad Sci USA 2007 104:5715-21; herein incorporated by reference in its entirety). Both of these delivery strategies incorporate rational approaches using both targeted delivery and endosomal escape mechanisms.

[000626] The polymer formulation can permit the sustained or delayed release of polynucleotide, primary construct, or mmRNA (e.g., following intramuscular or subcutaneous injection). The altered release profile for the polynucleotide, primary construct, or mmRNA can result in, for example, translation of an encoded protein over an extended period of time. The polymer formulation may also be used to increase the stability of the polynucleotide, primary construct, or mmRNA. Biodegradable polymers have been previously used to protect nucleic acids other than mmRNA from degradation and been shown to result in sustained release of payloads in vivo (Rozema et al., Proc Natl Acad Sci U S A. 2007 104:12982-12887; Sullivan et al., Expert Opin Drug Deliv. 2010 7:1433-1446; Convertine et al., Biomacromolecules. 2010 Oct 1; Chu et al., Acc Chem Res. 2012 Jan 13; Manganiello et al., Biomaterials. 2012 33:2301-2309; Benoit et al., Biomacromolecules. 2011 12:2708-2714; Singha et al., Nucleic Acid Ther. 2011 2:133-147; deFougerolles Hum Gene Ther. 2008 19:125-132; Schaffert and Wagner, Gene Ther. 2008 16:1131-1138; Chaturvedi et al., Expert Opin Drug Deliv. 2011 8:1455-1468; Davis, Mol Pharm. 2009 6:659-668; Davis, Nature 2010 464:1067-1070; each of which is herein incorporated by reference in its entirety).

[000627] In one embodiment, the pharmaceutical compositions may be sustained release formulations. In a further embodiment, the sustained release formulations may be for subcutaneous delivery. Sustained release formulations may include, but are not limited to, PLGA microspheres, ethylene vinyl acetate (EVAc), poloxamer, GELSITE® (Nanotherapeutics, Inc. Alachua, FL), HYLENEX® (Halozyme Therapeutics, San Diego CA), surgical sealants such as fibrinogen polymers (Ethicon Inc. Cornelia, GA), TISSELL® (Baxter International, Inc Deerfield, IL), PEG-based sealants, and COSEAL® (Baxter International, Inc Deerfield, IL).

[000628] As a non-limiting example modified mRNA may be formulated in PLGA microspheres by preparing the PLGA microspheres with tunable release rates (e.g., days and weeks) and encapsulating the modified mRNA in the PLGA microspheres while maintaining the integrity of the modified mRNA during the encapsulation process. EVAc are non-biodegradeable, biocompatible polymers which are used extensively in pre-clinical sustained release implant applications (e.g., extended release products Ocusert a pilocarpine ophthalmic insert for glaucoma or progestasert a sustained release

progesterone intrauterine deivce; transdermal delivery systems Testoderm, Duragesic and Selegiline; catheters). Poloxamer F-407 NF is a hydrophilic, non-ionic surfactant triblock copolymer of polyoxyethylene-polyoxypropylene-polyoxyethylene having a low viscosity at temperatures less than 5°C and forms a solid gel at temperatures greater than 15°C. PEG-based surgical sealants comprise two synthetic PEG components mixed in a delivery device which can be prepared in one minute, seals in 3 minutes and is reabsorbed within 30 days. GELSITE® and natural polymers are capable of in-situ gelation at the site of administration. They have been shown to interact with protein and peptide therapeutic candidates through ionic ineraction to provide a stabilizing effect.

[000629] Polymer formulations can also be selectively targeted through expression of different ligands as exemplified by, but not limited by, folate, transferrin, and N-acetylgalactosamine (GalNAc) (Benoit et al., Biomacromolecules. 2011 12:2708-2714; Rozema et al., Proc Natl Acad Sci U S A. 2007 104:12982-12887; Davis, Mol Pharm. 2009 6:659-668; Davis, Nature 2010 464:1067-1070; each of which is herein incorporated by reference in its entirety).

[000630] The modified nucleic acid, and mmRNA of the invention may be formulated with or in a polymeric compound. The polymer may include at least one polymer such as, but not limited to, polyethenes, polyethylene glycol (PEG), poly(l-lysine)(PLL), PEG grafted to PLL, cationic lipopolymer, biodegradable cationic lipopolymer, polyethyleneimine (PEI), cross-linked branched poly(alkylene imines), a polyamine derivative, a modified poloxamer, a biodegradable polymer, elastic biodegradable polymer, biodegradable block copolymer, biodegradable random copolymer, biodegradable polyester copolymer, biodegradable polyester block copolymer, biodegradable polyester block random copolymer, multiblock copolymers, linear biodegradable copolymer, poly[α-(4-aminobutyl)-L-glycolic acid) (PAGA), biodegradable cross-linked cationic multi-block copolymers, polycarbonates, polyanhydrides, polyhydroxyacids, polypropylfumerates, polycaprolactones, polyamides, polyacetals, polyethers, polyesters, poly(orthoesters), polycyanoacrylates, polyvinyl alcohols, polyurethanes, polyphosphazenes, polyacrylates, polymethacrylates, polycyanoacrylates, polyureas, polystyrenes, polyamines, polylysine, poly(ethylene imine), poly(serine ester), poly(L-lactide-co-L-lysine), poly(4-hydroxy-L-proline ester),

acrylic polymers, amine-containing polymers, dextran polymers, dextran polymer derivatives or or combinations thereof .

[000631] As a non-limiting example, the modified nucleic acid or mmRNA of the invention may be formulated with the polymeric compound of PEG grafted with PLL as described in U.S. Pat. No. 6,177,274; herein incorporated by reference in its entirety. The formulation may be used for transfecting cells *in vitro* or for *in vivo* delivery of the modified nucleic acid and mmRNA. In another example, the modified nucleic acid and mmRNA may be suspended in a solution or medium with a cationic polymer, in a dry pharmaceutical composition or in a solution that is capable of being dried as described in U.S. Pub. Nos. 20090042829 and 20090042825; each of which are herein incorporated by reference in their entireties.

[000632] As another non-limiting example the polynucleotides, primary constructs or mmRNA of the invention may be formulated with a PLGA-PEG block copolymer (see US Pub. No. US20120004293 and US Pat No. 8,236,330, herein incorporated by reference in their entireties) or PLGA-PEG-PLGA block copolymers (See U.S. Pat. No. 6,004,573, herein incorporated by reference in its entirety). As a non-limiting example, the polynucleotides, primary constructs or mmRNA of the invention may be formulated with a diblock copolymer of PEG and PLA or PEG and PLGA (see US Pat No 8,246,968, herein incorporated by reference in its entirety).

[000633] A polyamine derivative may be used to deliver nucleic acids or to treat and/or prevent a disease or to be included in an implantable or injectable device (U.S. Pub. No. 20100260817 herein incorporated by reference in its entirety). As a non-limiting example, a pharmaceutical composition may include the modified nucleic acids and mmRNA and the polyamine derivative described in U.S. Pub. No. 20100260817 (the contents of which are incorporated herein by reference in its entirety. As a non-limiting example the polynucleotides, primary constructs and mmRNA of the present invention may be delivered using a polyaminde polymer such as, but not limited to, a polymer comprising a 1,3-dipolar addition polymer prepared by combining a carbohydrate diazide monomer with a dilkyne unite comprising oligoamines (U.S. Pat. No. 8,236,280; herein incorporated by reference in its entirety).

[000634] In one embodiment, the polynucleotides, primary constructs or mmRNA of the present invention may be formulated with at least one polymer and/or derivatives thereof described in International Publication Nos. WO2011115862, WO2012082574 and WO2012068187 and U.S. Pub. No. 20120283427, each of which are herein incorporated by reference in their entireties. In another embodiment, the modified nucleic acid or mmRNA of the present invention may be formulated with a polymer of formula Z as described in WO2011115862, herein incorporated by reference in its entirety. In yet another embodiment, the modified nucleic acid or mmRNA may be formulated with a polymer of formula Z, Z' or Z'' as described in International Pub. Nos. WO2012082574 or WO2012068187 and U.S. Pub. No. 2012028342, each of which are herein incorporated by reference in their entireties. The polymers formulated with the modified RNA of the present invention may be synthesized by the methods described in International Pub. Nos. WO2012082574 or WO2012068187, each of which are herein incorporated by reference in their entireties.

[000635] The polynucleotides, primary constructs or mmRNA of the invention may be formulated with at least one acrylic polymer. Acrylic polymers include but are not limited to, acrylic acid, methacrylic acid, acrylic acid and methacrylic acid copolymers, methyl methacrylate copolymers, ethoxyethyl methacrylates, cyanoethyl methacrylate, amino alkyl methacrylate copolymer, poly(acrylic acid), poly(methacrylic acid), polycyanoacrylates and combinations thereof.

[000636] Formulations of polynucleotides, primary constructs or mmRNA of the invention may include at least one amine-containing polymer such as, but not limited to polylysine, polyethylene imine, poly(amidoamine) dendrimers or combinations thereof.

[000637] For example, the modified nucleic acid or mmRNA of the invention may be formulated in a pharmaceutical compound including a poly(alkylene imine), a biodegradable cationic lipopolymer, a biodegradable block copolymer, a biodegradable polymer, or a biodegradable random copolymer, a biodegradable polyester block copolymer, a biodegradable polyester polymer, a biodegradable polyester random copolymer, a linear biodegradable copolymer, PAGA, a biodegradable cross-linked cationic multi-block copolymer or combinations thereof. The biodegradable cationic lipopolymer may be made by methods known in the art and/or described in U.S. Pat. No.

6,696,038, U.S. App. Nos. 20030073619 and 20040142474 each of which is herein incorporated by reference in their entireties. The poly(alkylene imine) may be made using methods known in the art and/or as described in U.S. Pub. No. 20100004315, herein incorporated by reference in its entirety. The biodegradabale polymer, biodegradable block copolymer, the biodegradable random copolymer, biodegradable polyester block copolymer, biodegradable polyester polymer, or biodegradable polyester random copolymer may be made using methods known in the art and/or as described in U.S. Pat. Nos. 6,517,869 and 6,267,987, the contents of which are each incorporated herein by reference in their entirety. The linear biodegradable copolymer may be made using methods known in the art and/or as described in U.S. Pat. No. 6,652,886. The PAGA polymer may be made using methods known in the art and/or as described in U.S. Pat. No. 6,217,912 herein incorporated by reference in its entirety. The PAGA polymer may be copolymerized to form a copolymer or block copolymer with polymers such as but not limited to, poly-L-lysine, polyargine, polyornithine, histones, avidin, protamines, polylactides and poly(lactide-co-glycolides). The biodegradable cross-linked cationic multi-block copolymers may be made my methods known in the art and/or as described in U.S. Pat. No. 8,057,821 or U.S. Pub. No. 2012009145 each of which are herein incorporated by reference in their entireties. For example, the multi-block copolymers may be synthesized using linear polyethyleneimine (LPEI) blocks which have distinct patterns as compared to branched polyethyleneimines. Further, the composition or pharmaceutical composition may be made by the methods known in the art, described herein, or as described in U.S. Pub. No. 20100004315 or U.S. Pat. Nos. 6,267,987 and 6,217,912 each of which are herein incorporated by reference in their entireties.

[000638] The polynucleotides, primary constructs, and mmRNA of the invention may be formulated with at least one degradable polyester which may contain polycationic side chains. Degradeable polyesters include, but are not limited to, poly(serine ester), poly(L-lactide-co-L-lysine), poly(4-hydroxy-L-proline ester), and combinations thereof. In another embodiment, the degradable polyesters may include a PEG conjugation to form a PEGylated polymer.

[000639] The polynucleotides, primary construct, mmRNA of the invention may be formulated with at least one crosslinkable polyester. Crosslinkable polyesters include

those known in the art and described in US Pub. No. 20120269761, herein incorporated by reference in its entirety.

[000640] In one embodiment, the polymers described herein may be conjugated to a lipid-terminating PEG. As a non-limiting example, PLGA may be conjugated to a lipid-terminating PEG forming PLGA-DSPE-PEG. As another non-limiting example, PEG conjugates for use with the present invention are described in International Publication No. WO2008103276, herein incorporated by reference in its entirety. The polymers may be conjugated using a ligand conjugate such as, but not limited to, the conjugates described in U.S. Pat. No. 8,273,363, herein incorporated by reference in its entirety.

[000641] In one embodiment, the modified RNA described herein may be conjugated with another compound. Non-limiting examples of conjugates are described in US Patent Nos. 7,964,578 and 7,833,992, each of which are herein incorporated by reference in their entireties. In another embodiment, modified RNA of the present invention may be conjugated with conjugates of formula 1-122 as described in US Patent Nos. 7,964,578 and 7,833,992, each of which are herein incorporated by reference in their entireties. The polynucleotides, primary constructs and/or mmRNA described herein may be conjugated with a metal such as, but not limited to, gold. (See e.g., Giljohann et al. Journ. Amer. Chem. Soc. 2009 131(6): 2072-2073; herein incorporated by reference in its entirety). In another embodiment, the polynucleotides, primary constructs and/or mmRNA described herein may be conjugated and/or encapsulated in gold-nanoparticles. (Interantional Pub. No. WO201216269 and U.S. Pub. No. 20120302940; each of which is herein incorporated by reference in its entirety).

[000642] As described in U.S. Pub. No. 20100004313, herein incorporated by reference in its entirety, a gene delivery composition may include a nucleotide sequence and a poloxamer. For example, the modified nucleic acid and mmRNA of the present inveition may be used in a gene delivery composition with the poloxamer described in U.S. Pub. No. 20100004313.

[000643] In one embodiment, the polymer formulation of the present invention may be stabilized by contacting the polymer formulation, which may include a cationic carrier, with a cationic lipopolymer which may be covalently linked to cholesterol and polyethylene glycol groups. The polymer formulation may be contacted with a cationic

lipopolymer using the methods described in U.S. Pub. No. 20090042829 herein incorporated by reference in its entirety. The cationic carrier may include, but is not limited to, polyethylenimine, poly(trimethylenimine), poly(tetramethylenimine), polypropylenimine, aminoglycoside-polyamine, dideoxy-diamino-b-cyclodextrin, spermine, spermidine, poly(2-dimethylamino)ethyl methacrylate, poly(lysine), poly(histidine), poly(arginine), cationized gelatin, dendrimers, chitosan, 1,2-Dioleoyl-3-Trimethylammonium-Propane(DOTAP), N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA), 1-[2-(oleoyloxy)ethyl]-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride (DOTIM), 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA), 3B-[N—(N′,N′-Dimethylaminoethane)-carbamoyl]Cholesterol Hydrochloride (DC-Cholesterol HCl) diheptadecylamidoglycyl spermidine (DOGS), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide (DMRIE), N,N-dioleyl-N,N-dimethylammonium chloride DODAC) and combinations thereof.

[000644] The polynucleotides, primary constructs and/or mmRNA of the invention may be formulated in a polyplex of one or more polymers (U.S. Pub. No. 20120237565 and 20120270927; each of which is herein incorporated by reference in its entirety). In one embodiment, the polyplex comprises two or more cationic polymers. The catioinic polymer may comprise a poly(ethylene imine) (PEI) such as linear PEI.

[000645] The polynucleotide, primary construct, and mmRNA of the invention can also be formulated as a nanoparticle using a combination of polymers, lipids, and/or other biodegradable agents, such as, but not limited to, calcium phosphate. Components may be combined in a core-shell, hybrid, and/or layer-by-layer architecture, to allow for fine-tuning of the nanoparticle so to delivery of the polynucleotide, primary construct and mmRNA may be enhanced (Wang et al., Nat Mater. 2006 5:791-796; Fuller et al., Biomaterials. 2008 29:1526-1532; DeKoker et al., Adv Drug Deliv Rev. 2011 63:748-761; Endres et al., Biomaterials. 2011 32:7721-7731; Su et al., Mol Pharm. 2011 Jun 6;8(3):774-87; herein incorporated by reference in its entirety). As a non-limiting example, the nanoparticle may comprise a plurality of polymers such as, but not limited to hydrophilic-hydrophobic polymers (e.g., PEG-PLGA), hydrophobic polymers (e.g.,

PEG) and/or hydrophilic polymers (International Pub. No. WO20120225129; herein incorporated by reference in its entirety).

[000646] Biodegradable calcium phosphate nanoparticles in combination with lipids and/or polymers have been shown to deliver polynucleotides, primary constructs and mmRNA *in vivo*. In one embodiment, a lipid coated calcium phosphate nanoparticle, which may also contain a targeting ligand such as anisamide, may be used to deliver the polynucleotide, primary construct and mmRNA of the present invention. For example, to effectively deliver siRNA in a mouse metastatic lung model a lipid coated calcium phosphate nanoparticle was used (Li et al., J Contr Rel. 2010 142: 416-421; Li et al., J Contr Rel. 2012 158:108-114; Yang et al., Mol Ther. 2012 20:609-615; herein incorporated by reference in its entirety). This delivery system combines both a targeted nanoparticle and a component to enhance the endosomal escape, calcium phosphate, in order to improve delivery of the siRNA.

[000647] In one embodiment, calcium phosphate with a PEG-polyanion block copolymer may be used to delivery polynucleotides, primary constructs and mmRNA (Kazikawa et al., J Contr Rel. 2004 97:345-356; Kazikawa et al., J Contr Rel. 2006 111:368-370; herein incorporated by reference in its entirety).

[000648] In one embodiment, a PEG-charge-conversional polymer (Pitella et al., Biomaterials. 2011 32:3106-3114) may be used to form a nanoparticle to deliver the polynucleotides, primary constructs and mmRNA of the present invention. The PEG-charge-conversional polymer may improve upon the PEG-polyanion block copolymers by being cleaved into a polycation at acidic pH, thus enhancing endosomal escape.

[000649] The use of core-shell nanoparticles has additionally focused on a high-throughput approach to synthesize cationic cross-linked nanogel cores and various shells (Siegwart et al., Proc Natl Acad Sci U S A. 2011 108:12996-13001). The complexation, delivery, and internalization of the polymeric nanoparticles can be precisely controlled by altering the chemical composition in both the core and shell components of the nanoparticle. For example, the core-shell nanoparticles may efficiently deliver siRNA to mouse hepatocytes after they covalently attach cholesterol to the nanoparticle.

[000650] In one embodiment, a hollow lipid core comprising a middle PLGA layer and an outer neutral lipid layer containg PEG may be used to delivery of the polynucleotide,

primary construct and mmRNA of the present invention. As a non-limiting example, in mice bearing a luciferease-expressing tumor, it was determined that the lipid-polymer-lipid hybrid nanoparticle significantly suppressed luciferase expression, as compared to a conventional lipoplex (Shi et al, Angew Chem Int Ed. 2011 50:7027-7031; herein incorporated by reference in its entirety).

[000651] In one embodiment, the lipid nanoparticles may comprise a core of the modified nucleic acid molecules disclosed herein and a polymer shell. The polymer shell may be any of the polymers described herein and are known in the art. In an additional embodiment, the polymer shell may be used to protect the modified nucleic acids in the core.

[000652] Core–shell nanoparticles for use with the modified nucleic acid molecules of the present invention are described and may be formed by the methods described in U.S. Pat. No. 8,313,777 herein incorporated by reference in its entirety.

[000653] In one embodiment, the core-shell nanoparticles may comprise a core of the modified nucleic acid molecules disclosed herein and a polymer shell. The polymer shell may be any of the polymers described herein and are known in the art. In an additional embodiment, the polymer shell may be used to protect the modified nucleic acid molecules in the core. As a non-limiting example, the core-shell nanoparticle may be used to treat an eye disease or disorder (See e.g. US Publication No. 20120321719, herein incorporated by reference in its entirety).

[000654] In one embodiment, the polymer used with the formulations described herein may be a modified polymer (such as, but not limited to, a modified polyacetal) as described in International Publication No. WO2011120053, herein incorporated by reference in its entirety.

Peptides and Proteins

[000655] The polynucleotide, primary construct, and mmRNA of the invention can be formulated with peptides and/or proteins in order to increase transfection of cells by the polynucleotide, primary construct, or mmRNA. In one embodiment, peptides such as, but not limited to, cell penetrating peptides and proteins and peptides that enable intracellular delivery may be used to deliver pharmaceutical formulations. A non-limiting example of a cell penetrating peptide which may be used with the pharmaceutical formulations of the

present invention includes a cell-penetrating peptide sequence attached to polycations that facilitates delivery to the intracellular space, e.g., HIV-derived TAT peptide, penetratins, transportans, or hCT derived cell-penetrating peptides (see, e.g., Caron et al., Mol. Ther. 3(3):310-8 (2001); Langel, Cell-Penetrating Peptides: Processes and Applications (CRC Press, Boca Raton FL, 2002); El-Andaloussi et al., Curr. Pharm. Des. 11(28):3597-611 (2003); and Deshayes et al., Cell. Mol. Life Sci. 62(16):1839-49 (2005), all of which are incorporated herein by reference in their entirety). The compositions can also be formulated to include a cell penetrating agent, e.g., liposomes, which enhance delivery of the compositions to the intracellular space. Polynucleotides, primary constructs, and mmRNA of the invention may be complexed to peptides and/or proteins such as, but not limited to, peptides and/or proteins from Aileron Therapeutics (Cambridge, MA) and Permeon Biologics (Cambridge, MA) in order to enable intracellular delivery (Cronican et al., ACS Chem. Biol. 2010 5:747-752; McNaughton et al., Proc. Natl. Acad. Sci. USA 2009 106:6111-6116; Sawyer, Chem Biol Drug Des. 2009 73:3-6; Verdine and Hilinski, Methods Enzymol. 2012;503:3-33; all of which are herein incorporated by reference in its entirety).

[000656] In one embodiment, the cell-penetrating polypeptide may comprise a first domain and a second domain. The first domain may comprise a supercharged polypeptide. The second domain may comprise a protein-binding partner. As used herein, "protein-binding partner" includes, but are not limited to, antibodies and functional fragments thereof, scaffold proteins, or peptides. The cell-penetrating polypeptide may further comprise an intracellular binding partner for the protein-binding partner. The cell-penetrating polypeptide may be capable of being secreted from a cell where the polynucleotide, primary construct, or mmRNA may be introduced.

[000657] Formulations of the including peptides or proteins may be used to increase cell transfection by the polynucleotide, primary construct, or mmRNA, alter the biodistribution of the polynucleotide, primary construct, or mmRNA (e.g., by targeting specific tissues or cell types), and/or increase the translation of encoded protein. (See e.g., International Pub. No. WO2012110636; herein incorporated by reference in its entirety).

Cells

[000658] The polynucleotide, primary construct, and mmRNA of the invention can be transfected *ex vivo* into cells, which are subsequently transplanted into a subject. As non-limiting examples, the pharmaceutical compositions may include red blood cells to deliver modified RNA to liver and myeloid cells, virosomes to deliver modified RNA in virus-like particles (VLPs), and electroporated cells such as, but not limited to, from MAXCYTE® (Gaithersburg, MD) and from ERYTECH® (Lyon, France) to deliver modified RNA. Examples of use of red blood cells, viral particles and electroporated cells to deliver payloads other than mmRNA have been documented (Godfrin et al., Expert Opin Biol Ther. 2012 12:127-133; Fang et al., Expert Opin Biol Ther. 2012 12:385-389; Hu et al., Proc Natl Acad Sci U S A. 2011 108:10980-10985; Lund et al., Pharm Res. 2010 27:400-420; Huckriede et al., J Liposome Res. 2007;17:39-47; Cusi, Hum Vaccin. 2006 2:1-7; de Jonge et al., Gene Ther. 2006 13:400-411; all of which are herein incorporated by reference in its entirety).

[000659] The polynucleotides, primary constructs and mmRNA may be delivered in synthetic VLPs synthesized by the methods described in International Pub No. WO2011085231 and US Pub No. 20110171248, each of which are herein incorporated by reference in their entireties.

[000660] Cell-based formulations of the polynucleotide, primary construct, and mmRNA of the invention may be used to ensure cell transfection (e.g., in the cellular carrier), alter the biodistribution of the polynucleotide, primary construct, or mmRNA (e.g., by targeting the cell carrier to specific tissues or cell types), and/or increase the translation of encoded protein.

[000661] A variety of methods are known in the art and suitable for introduction of nucleic acid into a cell, including viral and non-viral mediated techniques. Examples of typical non-viral mediated techniques include, but are not limited to, electroporation, calcium phosphate mediated transfer, nucleofection, sonoporation, heat shock, magnetofection, liposome mediated transfer, microinjection, microprojectile mediated transfer (nanoparticles), cationic polymer mediated transfer (DEAE-dextran, polyethylenimine, polyethylene glycol (PEG) and the like) or cell fusion.

[000662] The technique of sonoporation, or cellular sonication, is the use of sound (e.g., ultrasonic frequencies) for modifying the permeability of the cell plasma membrane. Sonoporation methods are known to those in the art and are used to deliver nucleic acids *in vivo* (Yoon and Park, Expert Opin Drug Deliv. 2010 7:321-330; Postema and Gilja, Curr Pharm Biotechnol. 2007 8:355-361; Newman and Bettinger, Gene Ther. 2007 14:465-475; all herein incorporated by reference in their entirety). Sonoporation methods are known in the art and are also taught for example as it relates to bacteria in US Patent Publication 20100196983 and as it relates to other cell types in, for example, US Patent Publication 20100009424, each of which are incorporated herein by reference in their entirety.

[000663] Electroporation techniques are also well known in the art and are used to deliver nucleic acids *in vivo* and clinically (Andre et al., Curr Gene Ther. 2010 10:267-280; Chiarella et al., Curr Gene Ther. 2010 10:281-286; Hojman, Curr Gene Ther. 2010 10:128-138; all herein incorporated by reference in their entirety). In one embodiment, polynucleotides, primary constructs or mmRNA may be delivered by electroporation as described in Example 8.

Hyaluronidase

[000664] The intramuscular or subcutaneous localized injection of polynucleotide, primary construct, or mmRNA of the invention can include hyaluronidase, which catalyzes the hydrolysis of hyaluronan. By catalyzing the hydrolysis of hyaluronan, a constituent of the interstitial barrier, hyaluronidase lowers the viscosity of hyaluronan, thereby increasing tissue permeability (Frost, Expert Opin. Drug Deliv. (2007) 4:427-440; herein incorporated by reference in its entirety). It is useful to speed their dispersion and systemic distribution of encoded proteins produced by transfected cells. Alternatively, the hyaluronidase can be used to increase the number of cells exposed to a polynucleotide, primary construct, or mmRNA of the invention administered intramuscularly or subcutaneously.

Nanoparticle Mimics

[000665] The polynucleotide, primary construct or mmRNA of the invention may be encapsulated within and/or absorbed to a nanoparticle mimic. A nanoparticle mimic can mimic the delivery function organisms or particles such as, but not limited to, pathogens,

viruses, bacteria, fungus, parasites, prions and cells. As a non-limiting example the polynucleotide, primary construct or mmRNA of the invention may be encapsulated in a non-viron particle which can mimic the delivery function of a virus (see International Pub. No. WO2012006376 herein incorporated by reference in its entirety).

Nanotubes

[000666] The polynucleotides, primary constructs or mmRNA of the invention can be attached or otherwise bound to at least one nanotube such as, but not limited to, rosette nanotubes, rosette nanotubes having twin bases with a linker, carbon nanotubes and/or single-walled carbon nanotubes, The polynucleotides, primary constructs or mmRNA may be bound to the nanotubes through forces such as, but not limited to, steric, ionic, covalent and/or other forces.

[000667] In one embodiment, the nanotube can release one or more polynucleotides, primary constructs or mmRNA into cells. The size and/or the surface structure of at least one nanotube may be altered so as to govern the interaction of the nanotubes within the body and/or to attach or bind to the polynucleotides, primary constructs or mmRNA disclosed herein. In one embodiment, the building block and/or the functional groups attached to the building block of the at least one nanotube may be altered to adjust the dimensions and/or properties of the nanotube. As a non-limiting example, the length of the nanotubes may be altered to hinder the nanotubes from passing through the holes in the walls of normal blood vessels but still small enough to pass through the larger holes in the blood vessels of tumor tissue.

[000668] In one embodiment, at least one nanotube may also be coated with delivery enhancing compounds including polymers, such as, but not limited to, polyethylene glycol. In another embodiment, at least one nanotube and/or the polynucleotides, primary constructs or mmRNA may be mixed with pharmaceutically acceptable excipients and/or delivery vehicles.

[000669] In one embodiment, the polynucleotides, primary constructs or mmRNA are attached and/or otherwise bound to at least one rosette nanotube. The rosette nanotubes may be formed by a process known in the art and/or by the process described in International Publication No. WO2012094304, herein incorporated by reference in its entirety. At least one polynucleotide, primary construct and/or mmRNA may be attached

and/or otherwise bound to at least one rosette nanotube by a process as described in International Publication No. WO2012094304, herein incorporated by reference in its entirety, where rosette nanotubes or modules forming rosette nanotubes are mixed in aqueous media with at least one polynucleotide, primary construct and/or mmRNA under conditions which may cause at least one polynucleotide, primary construct or mmRNA to attach or otherwise bind to the rosette nanotubes.

[000670] In one embodiment, the polynucleotides, primary constructs or mmRNA may be attached to and/or otherwise bound to at least one carbon nanotube. As a non-limiting example, the polynucleotides, primary constructs or mmRNA may be bound to a linking agent and the linked agent may be bound to the carbon nanotube (See e.g., U.S. Pat No. 8,246,995; herein incorporated by reference in its entirety). The carbon nanotube may be a single-walled nanotube (See e.g., U.S. Pat No. 8,246,995; herein incorporated by reference in its entirety).

Conjugates

[000671] The polynucleotides, primary constructs, and mmRNA of the invention include conjugates, such as a polynucleotide, primary construct, or mmRNA covalently linked to a carrier or targeting group, or including two encoding regions that together produce a fusion protein (e.g., bearing a targeting group and therapeutic protein or peptide).

[000672] The conjugates of the invention include a naturally occurring substance, such as a protein (e.g., human serum albumin (HSA), low-density lipoprotein (LDL), high-density lipoprotein (HDL), or globulin); an carbohydrate (e.g., a dextran, pullulan, chitin, chitosan, inulin, cyclodextrin or hyaluronic acid); or a lipid. The ligand may also be a recombinant or synthetic molecule, such as a synthetic polymer, e.g., a synthetic polyamino acid, an oligonucleotide (e.g. an aptamer). Examples of polyamino acids include polyamino acid is a polylysine (PLL), poly L-aspartic acid, poly L-glutamic acid, styrene-maleic acid anhydride copolymer, poly(L-lactide-co-glycolied) copolymer, divinyl ether-maleic anhydride copolymer, N-(2-hydroxypropyl)methacrylamide copolymer (HMPA), polyethylene glycol (PEG), polyvinyl alcohol (PVA), polyurethane, poly(2-ethylacryllic acid), N-isopropylacrylamide polymers, or polyphosphazine. Example of polyamines include: polyethylenimine, polylysine (PLL), spermine,

spermidine, polyamine, pseudopeptide-polyamine, peptidomimetic polyamine, dendrimer polyamine, arginine, amidine, protamine, cationic lipid, cationic porphyrin, quaternary salt of a polyamine, or an alpha helical peptide.

[000673] Representative U.S. patents that teach the preparation of polynucleotide conjugates, particularly to RNA, include, but are not limited to, U.S. Pat. Nos. 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941; 6,294,664; 6,320,017; 6,576,752; 6,783,931; 6,900,297; 7,037,646; each of which is herein incorporated by reference in their entireties.

[000674] In one embodiment, the conjugate of the present invention may function as a carrier for the modified nucleic acids and mmRNA of the present invention. The conjugate may comprise a cationic polymer such as, but not limited to, polyamine, polylysine, polyalkylenimine, and polyethylenimine which may be grafted to with poly(ethylene glycol). As a non-limiting example, the conjugate may be similar to the polymeric conjugate and the method of synthesizing the polymeric conjugate described in U.S. Pat. No. 6,586,524 herein incorporated by reference in its entirety.

[000675] The conjugates can also include targeting groups, e.g., a cell or tissue targeting agent, e.g., a lectin, glycoprotein, lipid or protein, e.g., an antibody, that binds to a specified cell type such as a kidney cell. A targeting group can be a thyrotropin, melanotropin, lectin, glycoprotein, surfactant protein A, Mucin carbohydrate, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-gulucosamine multivalent mannose, multivalent fucose, glycosylated polyaminoacids, multivalent galactose, transferrin, bisphosphonate, polyglutamate, polyaspartate, a lipid, cholesterol, a steroid, bile acid, folate, vitamin B12, biotin, an RGD peptide, an RGD peptide mimetic or an aptamer.

[000676] Targeting groups can be proteins, e.g., glycoproteins, or peptides, e.g., molecules having a specific affinity for a co-ligand, or antibodies e.g., an antibody, that binds to a specified cell type such as a cancer cell, endothelial cell, or bone cell. Targeting groups may also include hormones and hormone receptors. They can also include non-peptidic species, such as lipids, lectins, carbohydrates, vitamins, cofactors, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-gulucosamine multivalent mannose, multivalent fucose, or aptamers. The ligand can be, for example, a lipopolysaccharide, or an activator of p38 MAP kinase.

[000677] The targeting group can be any ligand that is capable of targeting a specific receptor. Examples include, without limitation, folate, GalNAc, galactose, mannose, mannose-6P, apatamers, integrin receptor ligands, chemokine receptor ligands, transferrin, biotin, serotonin receptor ligands, PSMA, endothelin, GCPII, somatostatin, LDL, and HDL ligands. In particular embodiments, the targeting group is an aptamer. The aptamer can be unmodified or have any combination of modifications disclosed herein.

[000678] In one embodiment, pharmaceutical compositions of the present invention may include chemical modifications such as, but not limited to, modifications similar to locked nucleic acids.

[000679] Representative U.S. Patents that teach the preparation of locked nucleic acid (LNA) such as those from Santaris, include, but are not limited to, the following: U.S. Pat. Nos. 6,268,490; 6,670,461; 6,794,499; 6,998,484; 7,053,207; 7,084,125; and 7,399,845, each of which is herein incorporated by reference in its entirety.

[000680] Representative U.S. patents that teach the preparation of PNA compounds include, but are not limited to, U.S. Pat. Nos. 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found, for example, in Nielsen *et al.*, Science, 1991, 254, 1497-1500.

[000681] Some embodiments featured in the invention include polynucleotides, primary constructs or mmRNA with phosphorothioate backbones and oligonucleosides with other modified backbones, and in particular --CH_2--NH--CH_2--, --CH_2--N(CH_3)--O--CH_2-- [known as a methylene (methylimino) or MMI backbone], --CH_2--O--N(CH_3)--CH_2--, --CH_2--N(CH_3)--N(CH_3)--CH_2-- and --N(CH_3)--CH_2--CH_2--[wherein the native

phosphodiester backbone is represented as --O—$P(O)_2$--O--CH_2--] of the above-referenced U.S. Pat. No. 5,489,677, and the amide backbones of the above-referenced U.S. Pat. No. 5,602,240. In some embodiments, the polynucletotides featured herein have morpholino backbone structures of the above-referenced U.S. Pat. No. 5,034,506.

[000682] Modifications at the 2′ position may also aid in delivery. Preferably, modifications at the 2′ position are not located in a polypeptide-coding sequence, i.e., not in a translatable region. Modifications at the 2′ position may be located in a 5′UTR, a 3′UTR and/or a tailing region. Modifications at the 2′ position can include one of the following at the 2' position: H (i.e., 2′-deoxy); F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Exemplary suitable modifications include $O[(CH_2)_nO]_mCH_3$, $O(CH_2)_nOCH_3$, $O(CH_2)_nNH_2$, $O(CH_2)_nCH_3$, $O(CH_2)_nONH_2$, and $O(CH_2)_nON[(CH_2)_nCH_3)]_2$, where n and m are from 1 to about 10. In other embodiments, the polynucleotides, primary constructs or mmRNA include one of the following at the 2' position: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2CH_3, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties, or a group for improving the pharmacodynamic properties, and other substituents having similar properties. In some embodiments, the modification includes a 2'-methoxyethoxy (2'-O--$CH_2CH_2OCH_3$, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin *et al*., *Helv. Chim. Acta*, 1995, 78:486-504) *i.e*., an alkoxy-alkoxy group. Another exemplary modification is 2'-dimethylaminooxyethoxy, *i.e*., a $O(CH_2)_2ON(CH_3)_2$ group, also known as 2'-DMAOE, as described in examples herein below, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethylaminoethoxyethyl or 2'-DMAEOE), *i.e*., 2'-O--CH_2--O--CH_2--$N(CH_2)_2$, also described in examples herein below. Other modifications include 2'-methoxy (2'-OCH_3), 2'-aminopropoxy (2'-$OCH_2CH_2CH_2NH_2$) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked dsRNAs and the 5' position of 5' terminal nucleotide. Polynucleotides of the

invention may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative U.S. patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S. Pat. Nos. 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920 and each of which is herein incorporated by reference.

[000683] In still other embodiments, the polynucleotide, primary construct, or mmRNA is covalently conjugated to a cell penetrating polypeptide. The cell-penetrating peptide may also include a signal sequence. The conjugates of the invention can be designed to have increased stability; increased cell transfection; and/or altered the biodistribution (e.g., targeted to specific tissues or cell types).

[000684] In one embodiment, the polynucleotides, primary constructs or mmRNA may be conjugated to an agent to enhance delivery. As a non-limiting example, the agent may be a monomer or polymer such as a targeting monomer or a polymer having targeting blocks as described in International Publication No. WO2011062965, herein incorporated by reference in its entirety. In another non-limiting example, the agent may be a transport agent covalently coupled to the polynucleotides, primary constructs or mmRNA of the present invention (See e.g., U.S. Pat. Nos. 6,835.393 and 7,374,778, each of which is herein incorporated by reference in its entirety). In yet another non-limiting example, the agent may be a membrane barrier transport enhancing agent such as those described in U.S. Pat. Nos. 7,737,108 and 8,003,129, each of which is herein incorporated by reference in its entirety.

[000685] In another embodiment, polynucleotides, primary constructs or mmRNA may be conjugated to SMARTT POLYMER TECHNOLOGY® (PHASERX®, Inc. Seattle, WA).

Self-Assembled Nanoparticles

Nucleic Acid Self-Assembled Nanoparticles

[000686] Self-assembled nanoparticles have a well-defined size which may be precisely controlled as the nucleic acid strands may be easily reprogrammable. For example, the optimal particle size for a cancer-targeting nanodelivery carrier is 20-100 nm as a

diameter greater than 20 nm avoids renal clearance and enhances delivery to certain tumors through enhanced permeability and retention effect. Using self-assembled nucleic acid nanoparticles a single uniform population in size and shape having a precisely controlled spatial orientation and density of cancer-targeting ligands for enhanced delivery. As a non-limiting example, oligonucleotide nanoparticles were prepared using programmable self-assembly of short DNA fragments and therapeutic siRNAs. These nanoparticles are molecularly identical with controllable particle size and target ligand location and density. The DNA fragments and siRNAs self-assembled into a one-step reaction to generate DNA/siRNA tetrahedral nanoparticles for targeted *in vivo* delivery. (Lee et al., Nature Nanotechnology 2012 7:389-393; herein incorporated by reference in its entirety).

[000687] In one embodiment, the polynucleotides, primary constructs and/or mmRNA disclosed herein may be formulated as self-assembled nanoparticles. As a non-limiting example, nucleic acids may be used to make nanoparticles which may be used in a delivery system for the polynucleotides, primary constructs and/or mmRNA of the present invention (See e.g., International Pub. No. WO2012125987; herein incorporated by reference in its entirety).

[000688] In one embodiment, the nucleic acid self-assembled nanoparticles may comprise a core of the polynucleotides, primary constructs or mmRNA disclosed herein and a polymer shell. The polymer shell may be any of the polymers described herein and are known in the art. In an additional embodiment, the polymer shell may be used to protect the polynucleotides, primary contructs and mmRNA in the core.

Polymer-Based Self-Assembled Nanoparticles

[000689] Polymers may be used to form sheets which self-assembled into nanoparticles. These nanoparticles may be used to deliver the polynucleotides, primary constructs and mmRNA of the present invention. In one embodiment, these self-assembled nanoparticles may be microsponges formed of long polymers of RNA hairpins which form into crystalline 'pleated' sheets before self-assembling into microsponges. These microsponges are densely-packed sponge like microparticles which may function as an efficient carrier and may be able to deliver cargo to a cell. The microsponges may be from 1um to 300 nm in diameter. The microsponges may be complexed with other

agents known in the art to form larger microsponges. As a non-limiting example, the microsponge may be complexed with an agent to form an outer layer to promote cellular uptake such as polycation polyethyleneime (PEI). This complex can form a 250-nm diameter particle that can remain stable at high temperatures (150°C) (Grabow and Jaegar, Nature Materials 2012, 11:269-269; herein incorporated by reference in its entirety). Additionally these microsponges may be able to exhibit an extraordinary degree of protection from degradation by ribonucleases.

[000690] In another embodiment, the polymer-based self-assembled nanoparticles such as, but not limited to, microsponges, may be fully programmable nanoparticles. The geometry, size and stoichiometry of the nanoparticle may be precisely controlled to create the optimal nanoparticle for delivery of cargo such as, but not limited to, polynucleotides, primary constructs and/or mmRNA.

[000691] In one embodiment, the polymer based nanoparticles may comprise a core of the polynucleotides, primary constructs and/or mmRNA disclosed herein and a polymer shell. The polymer shell may be any of the polymers described herein and are known in the art. In an additional embodiment, the polymer shell may be used to protect the polynucleotides, primary construct and/or mmRNA in the core.

[000692] In yet another embodiment, the polymer based nanoparticle may comprise a non-nucleic acid polymer comprising a plurality of heterogenous monomers such as those described in Interantional Publication No. WO2013009736, herein incorporated by reference in its entirety.

Inorganic Nanoparticles

[000693] The polynucleotides, primary constructs and/or mmRNAs of the present invention may be formulated in inorganic nanoparticles (U.S. Pat. No. 8,257,745, herein incorporated by reference in its entirety). The inorganic nanoparticles may include, but are not limited to, clay substances that are water swellable. As a non-limiting example, the inorganic nanoparticle may include synthetic smectite clays which are made from simple silicates (See e.g., U.S. Pat. No. 5,585,108 and 8,257,745 each of which are herein incorporated by reference in their entirety).

[000694] In one embodiment, the inorganic nanoparticles may comprise a core of the modified nucleic acids disclosed herein and a polymer shell. The polymer shell may be

any of the polymers described herein and are known in the art. In an additional embodiment, the polymer shell may be used to protect the modified nucleic acids in the core.

Semi-conductive and Metallic Nanoparticles

[000695] The polynucleotides, primary constructs and/or mmRNAs of the present invention may be formulated in water-dispersible nanoparticle comprising a semiconductive or metallic material (U.S. Pub. No. 20120228565; herein incorporated by reference in its entirety) or formed in a magnetic nanoparticle (U.S. Pub. No. 20120265001 and 20120283503; each of which is herein incorporated by reference in its entirety). The water-dispersible nanoparticles may be hydrophobic nanoparticles or hydrophilic nanoparticles.

[000696] In one embodiment, the semi-conductive and/or metallic nanoparticles may comprise a core of the polynucleotides, primary constructs and/or mmRNA disclosed herein and a polymer shell. The polymer shell may be any of the polymers described herein and are known in the art. In an additional embodiment, the polymer shell may be used to protect the polynucleotides, primary constructs and/or mmRNA in the core.

Gels and Hydrogels

[000697] In one embodiment, the polynucleotides, primary constructs and/or mmRNA disclosed herein may be encapsulated into any hydrogel known in the art which may form a gel when injected into a subject. Hydrogels are a network of polymer chains that are hydrophilic, and are sometimes found as a colloidal gel in which water is the dispersion medium. Hydrogels are highly absorbent (they can contain over 99% water) natural or synthetic polymers. Hydrogels also possess a degree of flexibility very similar to natural tissue, due to their significant water content. The hydrogel described herein may used to encapsulate lipid nanoparticles which are biocompatible, biodegradable and/or porous.

[000698] As a non-limiting example, the hydrogel may be an aptamer-functionalized hydrogel. The aptamer-functionalized hydrogel may be programmed to release one or more polynucleotides, primary constructs and/or mmRNA using nucleic acid hybridization. (Battig et al., J. Am. Chem. Society. 2012 134:12410-12413; herein incorporated by reference in its entirety).

[000699] As another non-limiting example, the hydrogel may be a shaped as an inverted opal.

The opal hydrogels exhibit higher swelling ratios and the swelling kinetics is an order of magnitude faster as well. Methods of producing opal hydrogels and description of opal hydrogels are described in International Pub. No. WO2012148684, herein incorporated by reference in its entirety.

[000700] In yet another non-limiting example, the hydrogel may be an antibacterial hydrogel. The antibacterial hydrogel may comprise a pharmaceutical acceptable salt or organic material such as, but not limited to pharmaceutical grade and/or medical grade silver salt and aloe vera gel or extract. (International Pub. No. WO2012151438, herein incorporated by reference in its entirety).

[000701] In one embodiment, the modified mRNA may be encapsulated in a lipid nanoparticle and then the lipid nanoparticle may be encapsulated into a hyrdogel.

[000702] In one embodiment, the polynucleotides, primary constructs and/or mmRNA disclosed herein may be encapsulated into any gel known in the art. As a non-limiting example the gel may be a fluorouracil injectable gel or a fluorouracil injectable gel containing a chemical compound and/or drug known in the art. As another example, the polynucleotides, primary constructs and/or mmRNA may be encapsulated in a fluorouracil gel containing epinephrine (See e.g., Smith et al. Cancer Chemotherapty and Pharmacology, 1999 44(4):267-274; herein incorporated by reference in its entirety).

[000703] In one embodiment, the polynucleotides, primary constructs and/or mmRNA disclosed herein may be encapsulated into a fibrin gel, fibrin hydrogel or fibrin glue. In another embodiment, the polynucleotides, primary constructs and/or mmRNA may be formulated in a lipid nanoparticle or a rapidly eliminated lipid nanoparticle prior to being encapsulated into a fibrin gel, fibrin hydrogel or a fibrin glue. In yet another embodiment, the polynucleotides, primary constructs and/or mmRNA may be formulated as a lipoplex prior to being encapsulated into a fibrin gel, hydrogel or a fibrin glue. Fibrin gels, hydrogels and glues comprise two components, a fibrinogen solution and a thrombin solution which is rich in calcium (See e.g., Spicer and Mikos, Journal of Controlled Release 2010. 148: 49-55; Kidd et al. Journal of Controlled Release 2012. 157:80-85; each of which is herein incorporated by reference in its entirety). The

concentration of the components of the fibrin gel, hydrogel and/or glue can be altered to change the characteristics, the network mesh size, and/or the degradation characteristics of the gel, hydrogel and/or glue such as, but not limited to changing the release characteristics of the fibrin gel, hydrogel and/or glue. (See e.g., Spicer and Mikos, Journal of Controlled Release 2010. 148: 49-55; Kidd et al. Journal of Controlled Release 2012. 157:80-85; Catelas et al. Tissue Engineering 2008. 14:119-128; each of which is herein incorporated by reference in its entirety). This feature may be advantageous when used to deliver the modified mRNA disclosed herein. (See e.g., Kidd et al. Journal of Controlled Release 2012. 157:80-85; Catelas et al. Tissue Engineering 2008. 14:119-128; each of which is herein incorporated by reference in its entirety).

Cations and Anions

[000704] Formulations of polynucleotides, primary constructs and/or mmRNA disclosed herein may include cations or anions. In one embodiment, the formulations include metal cations such as, but not limited to, Zn2+, Ca2+, Cu2+, Mg+ and combinations thereof. As a non-limiting example, formulations may include polymers and a polynucleotides, primary constructs and/or mmRNA complexed with a metal cation (See e.g., U.S. Pat. Nos. 6,265,389 and 6,555,525, each of which is herein incorporated by reference in its entirety).

Molded Nanoparticles and Microparticles

[000705] The polynucleotides, primary constructs and/or mmRNA disclosed herein may be formulated in nanoparticles and/or microparticles. These nanoparticles and/or microparticles may be molded into any size shape and chemistry. As an example, the nanoparticles and/or microparticles may be made using the PRINT® technology by LIQUIDA TECHNOLOGIES® (Morrisville, NC) (See e.g., International Pub. No. WO2007024323; herein incorporated by reference in its entirety).

[000706] In one embodiment, the molded nanoparticles may comprise a core of the polynucleotides, primary constructs and/or mmRNA disclosed herein and a polymer shell. The polymer shell may be any of the polymers described herein and are known in the art. In an additional embodiment, the polymer shell may be used to protect the polynucleotides, primary construct and/or mmRNA in the core.

NanoJackets and NanoLiposomes

[000707] The polynucleotides, primary constructs and/or mmRNA disclosed herein may be formulated in NanoJackets and NanoLiposomes by Keystone Nano (State College, PA). NanoJackets are made of compounds that are naturally found in the body including calcium, phosphate and may also include a small amount of silicates. Nanojackets may range in size from 5 to 50 nm and may be used to deliver hydrophilic and hydrophobic compounds such as, but not limited to, polynucleotides, primary constructs and/or mmRNA.

[000708] NanoLiposomes are made of lipids such as, but not limited to, lipids which naturally occur in the body. NanoLiposomes may range in size from 60-80 nm and may be used to deliver hydrophilic and hydrophobic compounds such as, but not limited to, polynucleotides, primary constructs and/or mmRNA. In one aspect, the polynucleotides, primary constructs and/or mmRNA disclosed herein are formulated in a NanoLiposome such as, but not limited to, Ceramide NanoLiposomes.

Excipients

[000709] Pharmaceutical formulations may additionally comprise a pharmaceutically acceptable excipient, which, as used herein, includes any and all solvents, dispersion media, diluents, or other liquid vehicles, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, solid binders, lubricants and the like, as suited to the particular dosage form desired. Remington's *The Science and Practice of Pharmacy*, 21st Edition, A. R. Gennaro (Lippincott, Williams & Wilkins, Baltimore, MD, 2006; incorporated herein by reference in its entirety) discloses various excipients used in formulating pharmaceutical compositions and known techniques for the preparation thereof. Except insofar as any conventional excipient medium is incompatible with a substance or its derivatives, such as by producing any undesirable biological effect or otherwise interacting in a deleterious manner with any other component(s) of the pharmaceutical composition, its use is contemplated to be within the scope of this invention.

[000710] In some embodiments, a pharmaceutically acceptable excipient is at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% pure. In some embodiments, an excipient is approved for use in humans and for veterinary use. In some embodiments, an excipient is approved by United States Food and Drug Administration.

In some embodiments, an excipient is pharmaceutical grade. In some embodiments, an excipient meets the standards of the United States Pharmacopoeia (USP), the European Pharmacopoeia (EP), the British Pharmacopoeia, and/or the International Pharmacopoeia.

[000711] Pharmaceutically acceptable excipients used in the manufacture of pharmaceutical compositions include, but are not limited to, inert diluents, dispersing and/or granulating agents, surface active agents and/or emulsifiers, disintegrating agents, binding agents, preservatives, buffering agents, lubricating agents, and/or oils. Such excipients may optionally be included in pharmaceutical compositions.

[000712] Exemplary diluents include, but are not limited to, calcium carbonate, sodium carbonate, calcium phosphate, dicalcium phosphate, calcium sulfate, calcium hydrogen phosphate, sodium phosphate lactose, sucrose, cellulose, microcrystalline cellulose, kaolin, mannitol, sorbitol, inositol, sodium chloride, dry starch, cornstarch, powdered sugar, *etc.*, and/or combinations thereof.

[000713] Exemplary granulating and/or dispersing agents include, but are not limited to, potato starch, corn starch, tapioca starch, sodium starch glycolate, clays, alginic acid, guar gum, citrus pulp, agar, bentonite, cellulose and wood products, natural sponge, cation-exchange resins, calcium carbonate, silicates, sodium carbonate, cross-linked poly(vinyl-pyrrolidone) (crospovidone), sodium carboxymethyl starch (sodium starch glycolate), carboxymethyl cellulose, cross-linked sodium carboxymethyl cellulose (croscarmellose), methylcellulose, pregelatinized starch (starch 1500), microcrystalline starch, water insoluble starch, calcium carboxymethyl cellulose, magnesium aluminum silicate (VEEGUM®), sodium lauryl sulfate, quaternary ammonium compounds, *etc.*, and/or combinations thereof.

[000714] Exemplary surface active agents and/or emulsifiers include, but are not limited to, natural emulsifiers (e.g. acacia, agar, alginic acid, sodium alginate, tragacanth, chondrux, cholesterol, xanthan, pectin, gelatin, egg yolk, casein, wool fat, cholesterol, wax, and lecithin), colloidal clays (e.g. bentonite [aluminum silicate] and VEEGUM® [magnesium aluminum silicate]), long chain amino acid derivatives, high molecular weight alcohols (e.g. stearyl alcohol, cetyl alcohol, oleyl alcohol, triacetin monostearate, ethylene glycol distearate, glyceryl monostearate, and propylene glycol monostearate, polyvinyl alcohol), carbomers (e.g. carboxy polymethylene, polyacrylic acid, acrylic acid

polymer, and carboxyvinyl polymer), carrageenan, cellulosic derivatives (e.g. carboxymethylcellulose sodium, powdered cellulose, hydroxymethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methylcellulose, methylcellulose), sorbitan fatty acid esters (e.g. polyoxyethylene sorbitan monolaurate [TWEEN®20], polyoxyethylene sorbitan [TWEENn®60], polyoxyethylene sorbitan monooleate [TWEEN®80], sorbitan monopalmitate [SPAN®40], sorbitan monostearate [SPAN®60], sorbitan tristearate [SPAN®65], glyceryl monooleate, sorbitan monooleate [SPAN®80]), polyoxyethylene esters (e.g. polyoxyethylene monostearate [MYRJ®45], polyoxyethylene hydrogenated castor oil, polyethoxylated castor oil, polyoxymethylene stearate, and SOLUTOL®), sucrose fatty acid esters, polyethylene glycol fatty acid esters (e.g. CREMOPHOR®), polyoxyethylene ethers, (e.g. polyoxyethylene lauryl ether [BRIJ®30]), poly(vinyl-pyrrolidone), diethylene glycol monolaurate, triethanolamine oleate, sodium oleate, potassium oleate, ethyl oleate, oleic acid, ethyl laurate, sodium lauryl sulfate, PLUORINC®F 68, POLOXAMER®188, cetrimonium bromide, cetylpyridinium chloride, benzalkonium chloride, docusate sodium, etc. and/or combinations thereof.

[000715] Exemplary binding agents include, but are not limited to, starch (*e.g.* cornstarch and starch paste); gelatin; sugars (*e.g.* sucrose, glucose, dextrose, dextrin, molasses, lactose, lactitol, mannitol,); natural and synthetic gums (*e.g.* acacia, sodium alginate, extract of Irish moss, panwar gum, ghatti gum, mucilage of isapol husks, carboxymethylcellulose, methylcellulose, ethylcellulose, hydroxyethylcellulose, hydroxypropyl cellulose, hydroxypropyl methylcellulose, microcrystalline cellulose, cellulose acetate, poly(vinyl-pyrrolidone), magnesium aluminum silicate (Veegum®), and larch arabogalactan); alginates; polyethylene oxide; polyethylene glycol; inorganic calcium salts; silicic acid; polymethacrylates; waxes; water; alcohol; *etc.*; and combinations thereof.

[000716] Exemplary preservatives may include, but are not limited to, antioxidants, chelating agents, antimicrobial preservatives, antifungal preservatives, alcohol preservatives, acidic preservatives, and/or other preservatives. Exemplary antioxidants include, but are not limited to, alpha tocopherol, ascorbic acid, acorbyl palmitate, butylated hydroxyanisole, butylated hydroxytoluene, monothioglycerol, potassium metabisulfite, propionic acid, propyl gallate, sodium ascorbate, sodium bisulfite, sodium

metabisulfite, and/or sodium sulfite. Exemplary chelating agents include ethylenediaminetetraacetic acid (EDTA), citric acid monohydrate, disodium edetate, dipotassium edetate, edetic acid, fumaric acid, malic acid, phosphoric acid, sodium edetate, tartaric acid, and/or trisodium edetate. Exemplary antimicrobial preservatives include, but are not limited to, benzalkonium chloride, benzethonium chloride, benzyl alcohol, bronopol, cetrimide, cetylpyridinium chloride, chlorhexidine, chlorobutanol, chlorocresol, chloroxylenol, cresol, ethyl alcohol, glycerin, hexetidine, imidurea, phenol, phenoxyethanol, phenylethyl alcohol, phenylmercuric nitrate, propylene glycol, and/or thimerosal. Exemplary antifungal preservatives include, but are not limited to, butyl paraben, methyl paraben, ethyl paraben, propyl paraben, benzoic acid, hydroxybenzoic acid, potassium benzoate, potassium sorbate, sodium benzoate, sodium propionate, and/or sorbic acid. Exemplary alcohol preservatives include, but are not limited to, ethanol, polyethylene glycol, phenol, phenolic compounds, bisphenol, chlorobutanol, hydroxybenzoate, and/or phenylethyl alcohol. Exemplary acidic preservatives include, but are not limited to, vitamin A, vitamin C, vitamin E, beta-carotene, citric acid, acetic acid, dehydroacetic acid, ascorbic acid, sorbic acid, and/or phytic acid. Other preservatives include, but are not limited to, tocopherol, tocopherol acetate, deteroxime mesylate, cetrimide, butylated hydroxyanisol (BHA), butylated hydroxytoluened (BHT), ethylenediamine, sodium lauryl sulfate (SLS), sodium lauryl ether sulfate (SLES), sodium bisulfite, sodium metabisulfite, potassium sulfite, potassium metabisulfite, GLYDANT PLUS®, PHENONIP®, methylparaben, GERMALL®115, GERMABEN®II, NEOLONE™, KATHON™, and/or EUXYL®.

[000717] Exemplary buffering agents include, but are not limited to, citrate buffer solutions, acetate buffer solutions, phosphate buffer solutions, ammonium chloride, calcium carbonate, calcium chloride, calcium citrate, calcium glubionate, calcium gluceptate, calcium gluconate, D-gluconic acid, calcium glycerophosphate, calcium lactate, propanoic acid, calcium levulinate, pentanoic acid, dibasic calcium phosphate, phosphoric acid, tribasic calcium phosphate, calcium hydroxide phosphate, potassium acetate, potassium chloride, potassium gluconate, potassium mixtures, dibasic potassium phosphate, monobasic potassium phosphate, potassium phosphate mixtures, sodium acetate, sodium bicarbonate, sodium chloride, sodium citrate, sodium lactate, dibasic

sodium phosphate, monobasic sodium phosphate, sodium phosphate mixtures, tromethamine, magnesium hydroxide, aluminum hydroxide, alginic acid, pyrogen-free water, isotonic saline, Ringer's solution, ethyl alcohol, *etc.*, and/or combinations thereof.

[000718] Exemplary lubricating agents include, but are not limited to, magnesium stearate, calcium stearate, stearic acid, silica, talc, malt, glyceryl behanate, hydrogenated vegetable oils, polyethylene glycol, sodium benzoate, sodium acetate, sodium chloride, leucine, magnesium lauryl sulfate, sodium lauryl sulfate, *etc.*, and combinations thereof.

[000719] Exemplary oils include, but are not limited to, almond, apricot kernel, avocado, babassu, bergamot, black current seed, borage, cade, camomile, canola, caraway, carnauba, castor, cinnamon, cocoa butter, coconut, cod liver, coffee, corn, cotton seed, emu, eucalyptus, evening primrose, fish, flaxseed, geraniol, gourd, grape seed, hazel nut, hyssop, isopropyl myristate, jojoba, kukui nut, lavandin, lavender, lemon, litsea cubeba, macademia nut, mallow, mango seed, meadowfoam seed, mink, nutmeg, olive, orange, orange roughy, palm, palm kernel, peach kernel, peanut, poppy seed, pumpkin seed, rapeseed, rice bran, rosemary, safflower, sandalwood, sasquana, savoury, sea buckthorn, sesame, shea butter, silicone, soybean, sunflower, tea tree, thistle, tsubaki, vetiver, walnut, and wheat germ oils. Exemplary oils include, but are not limited to, butyl stearate, caprylic triglyceride, capric triglyceride, cyclomethicone, diethyl sebacate, dimethicone 360, isopropyl myristate, mineral oil, octyldodecanol, oleyl alcohol, silicone oil, and/or combinations thereof.

[000720] Excipients such as cocoa butter and suppository waxes, coloring agents, coating agents, sweetening, flavoring, and/or perfuming agents can be present in the composition, according to the judgment of the formulator.

Delivery

[000721] The present disclosure encompasses the delivery of polynucleotides, primary constructs or mmRNA for any of therapeutic, pharmaceutical, diagnostic or imaging by any appropriate route taking into consideration likely advances in the sciences of drug delivery. Delivery may be naked or formulated.

Naked Delivery

[000722] The polynucleotides, primary constructs or mmRNA of the present invention may be delivered to a cell naked. As used herein in, "naked" refers to delivering

polynucleotides, primary constructs or mmRNA free from agents which promote transfection. For example, the polynucleotides, primary constructs or mmRNA delivered to the cell may contain no modifications. The naked polynucleotides, primary constructs or mmRNA may be delivered to the cell using routes of administration known in the art and described herein.

Formulated Delivery

[000723] The polynucleotides, primary constructs or mmRNA of the present invention may be formulated, using the methods described herein. The formulations may contain polynucleotides, primary constructs or mmRNA which may be modified and/or unmodified. The formulations may further include, but are not limited to, cell penetration agents, a pharmaceutically acceptable carrier, a delivery agent, a bioerodible or biocompatible polymer, a solvent, and a sustained-release delivery depot. The formulated polynucleotides, primary constructs or mmRNA may be delivered to the cell using routes of administration known in the art and described herein.

[000724] The compositions may also be formulated for direct delivery to an organ or tissue in any of several ways in the art including, but not limited to, direct soaking or bathing, via a catheter, by gels, powder, ointments, creams, gels, lotions, and/or drops, by using substrates such as fabric or biodegradable materials coated or impregnated with the compositions, and the like.

Administration

[000725] The polynucleotides, primary constructs or mmRNA of the present invention may be administered by any route which results in a therapeutically effective outcome. These include, but are not limited to enteral, gastroenteral, epidural, oral, transdermal, epidural (peridural), intracerebral (into the cerebrum), intracerebroventricular (into the cerebral ventricles), epicutaneous (application onto the skin), intradermal, (into the skin itself), subcutaneous (under the skin), nasal administration (through the nose), intravenous (into a vein), intraarterial (into an artery), intramuscular (into a muscle), intracardiac (into the heart), intraosseous infusion (into the bone marrow), intrathecal (into the spinal canal), intraperitoneal, (infusion or injection into the peritoneum), intravesical infusion, intravitreal, (through the eye), intracavernous injection, (into the base of the penis), intravaginal administration, intrauterine, extra-amniotic

administration, transdermal (diffusion through the intact skin for systemic distribution), transmucosal (diffusion through a mucous membrane), insufflation (snorting), sublingual, sublabial, enema, eye drops (onto the conjunctiva), or in ear drops. In specific embodiments, compositions may be administered in a way which allows them cross the blood-brain barrier, vascular barrier, or other epithelial barrier.Non-limiting routes of administration for the polynucleotides, primary constructs or mmRNA of the present invention are described below.

Parenteral and Injectible Administration

[000726] Liquid dosage forms for parenteral administration include, but are not limited to, pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups, and/or elixirs. In addition to active ingredients, liquid dosage forms may comprise inert diluents commonly used in the art such as, for example, water or other solvents, solubilizing agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof. Besides inert diluents, oral compositions can include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, and/or perfuming agents. In certain embodiments for parenteral administration, compositions are mixed with solubilizing agents such as CREMOPHOR®, alcohols, oils, modified oils, glycols, polysorbates, cyclodextrins, polymers, and/or combinations thereof.

[000727] Injectable preparations, for example, sterile injectable aqueous or oleaginous suspensions may be formulated according to the known art using suitable dispersing agents, wetting agents, and/or suspending agents. Sterile injectable preparations may be sterile injectable solutions, suspensions, and/or emulsions in nontoxic parenterally acceptable diluents and/or solvents, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, U.S.P., and isotonic sodium chloride solution. Sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil can

be employed including synthetic mono- or diglycerides. Fatty acids such as oleic acid can be used in the preparation of injectables.

[000728] Injectable formulations can be sterilized, for example, by filtration through a bacterial-retaining filter, and/or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable medium prior to use.

[000729] In order to prolong the effect of an active ingredient, it is often desirable to slow the absorption of the active ingredient from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material with poor water solubility. The rate of absorption of the drug then depends upon its rate of dissolution which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle. Injectable depot forms are made by forming microencapsule matrices of the drug in biodegradable polymers such as polylactide-polyglycolide. Depending upon the ratio of drug to polymer and the nature of the particular polymer employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations are prepared by entrapping the drug in liposomes or microemulsions which are compatible with body tissues.

Rectal and Vaginal Administration

[000730] Compositions for rectal or vaginal administration are typically suppositories which can be prepared by mixing compositions with suitable non-irritating excipients such as cocoa butter, polyethylene glycol or a suppository wax which are solid at ambient temperature but liquid at body temperature and therefore melt in the rectum or vaginal cavity and release the active ingredient.

Oral Administration

[000731] Liquid dosage forms for oral administration include, but are not limited to, pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups, and/or elixirs. In addition to active ingredients, liquid dosage forms may comprise inert diluents commonly used in the art such as, for example, water or other solvents, solubilizing agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl

carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof. Besides inert diluents, oral compositions can include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, and/or perfuming agents. In certain embodiments for parenteral administration, compositions are mixed with solubilizing agents such as CREMOPHOR®, alcohols, oils, modified oils, glycols, polysorbates, cyclodextrins, polymers, and/or combinations thereof.

[000732] Solid dosage forms for oral administration include capsules, tablets, pills, powders, and granules. In such solid dosage forms, an active ingredient is mixed with at least one inert, pharmaceutically acceptable excipient such as sodium citrate or dicalcium phosphate and/or fillers or extenders (*e.g.* starches, lactose, sucrose, glucose, mannitol, and silicic acid), binders (*e.g.* carboxymethylcellulose, alginates, gelatin, polyvinylpyrrolidinone, sucrose, and acacia), humectants (*e.g.* glycerol), disintegrating agents (*e.g.* agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate), solution retarding agents (*e.g.* paraffin), absorption accelerators (*e.g.* quaternary ammonium compounds), wetting agents (*e.g.* cetyl alcohol and glycerol monostearate), absorbents (*e.g.* kaolin and bentonite clay), and lubricants (*e.g.* talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate), and mixtures thereof. In the case of capsules, tablets and pills, the dosage form may comprise buffering agents.

Topical or Transdermal Administration

[000733] As described herein, compositions containing the polynucleotides, primary constructs or mmRNA of the invention may be formulated for administration topically. The skin may be an ideal target site for delivery as it is readily accessible. Gene expression may be restricted not only to the skin, potentially avoiding nonspecific toxicity, but also to specific layers and cell types within the skin.

[000734] The site of cutaneous expression of the delivered compositions will depend on the route of nucleic acid delivery. Three routes are commonly considered to deliver polynucleotides, primary constructs or mmRNA to the skin: (i) topical application (e.g.

for local/regional treatment and/or cosmetic applications); (ii) intradermal injection (e.g. for local/regional treatment and/or cosmetic applications); and (iii) systemic delivery (e.g. for treatment of dermatologic diseases that affect both cutaneous and extracutaneous regions). Polynucleotides, primary constructs or mmRNA can be delivered to the skin by several different approaches known in the art. Most topical delivery approaches have been shown to work for delivery of DNA, such as but not limited to, topical application of non-cationic liposome–DNA complex, cationic liposome–DNA complex, particle-mediated (gene gun), puncture-mediated gene transfections, and viral delivery approaches. After delivery of the nucleic acid, gene products have been detected in a number of different skin cell types, including, but not limited to, basal keratinocytes, sebaceous gland cells, dermal fibroblasts and dermal macrophages.

[000735] In one embodiment, the invention provides for a variety of dressings (e.g., wound dressings) or bandages (e.g., adhesive bandages) for conveniently and/or effectively carrying out methods of the present invention. Typically dressing or bandages may comprise sufficient amounts of pharmaceutical compositions and/or polynucleotides, primary constructs or mmRNA described herein to allow a user to perform multiple treatments of a subject(s).

[000736] In one embodiment, the invention provides for the polynucleotides, primary constructs or mmRNA compositions to be delivered in more than one injection.

[000737] In one embodiment, before topical and/or transdermal administration at least one area of tissue, such as skin, may be subjected to a device and/or solution which may increase permeability. In one embodiment, the tissue may be subjected to an abrasion device to increase the permeability of the skin (see U.S. Patent Publication No. 20080275468, herein incorporated by reference in its entirety). In another embodiment, the tissue may be subjected to an ultrasound enhancement device. An ultrasound enhancement device may include, but is not limited to, the devices described in U.S. Publication No. 20040236268 and U.S. Patent Nos. 6,491,657 and 6,234,990; each of which are herein incorporated by reference in their entireties. Methods of enhancing the permeability of tissue are described in U.S. Publication Nos. 20040171980 and 20040236268 and U.S. Pat. No. 6,190,315; each of which are herein incorporated by reference in their entireties.

[000738] In one embodiment, a device may be used to increase permeability of tissue before delivering formulations of modified mRNA described herein. The permeability of skin may be measured by methods known in the art and/or described in U.S. Patent No. 6,190,315, herein incorporated by reference in its entirety. As a non-limiting example, a modified mRNA formulation may be delivered by the drug delivery methods described in U.S. Patent No. 6,190,315, herein incorporated by reference in its entirety.

[000739] In another non-limiting example tissue may be treated with a eutectic mixture of local anesthetics (EMLA) cream before, during and/or after the tissue may be subjected to a device which may increase permeability. Katz et al. (Anesth Analg (2004); 98:371-76; herein incorporated by reference in its entirety) showed that using the EMLA cream in combination with a low energy, an onset of superficial cutaneous analgesia was seen as fast as 5 minutes after a pretreatment with a low energy ultrasound.

[000740] In one embodiment, enhancers may be applied to the tissue before, during, and/or after the tissue has been treated to increase permeability. Enhancers include, but are not limited to, transport enhancers, physical enhancers, and cavitation enhancers. Non-limiting examples of enhancers are described in U.S. Patent No. 6,190,315, herein incorporated by reference in its entirety.

[000741] In one embodiment, a device may be used to increase permeability of tissue before delivering formulations of modified mRNA described herein, which may further contain a substance that invokes an immune response. In another non-limiting example, a formulation containing a substance to invoke an immune response may be delivered by the methods described in U.S. Publication Nos. 20040171980 and 20040236268; each of which are herein incorporated by reference in their entireties.

[000742] Dosage forms for topical and/or transdermal administration of a composition may include ointments, pastes, creams, lotions, gels, powders, solutions, sprays, inhalants and/or patches. Generally, an active ingredient is admixed under sterile conditions with a pharmaceutically acceptable excipient and/or any needed preservatives and/or buffers as may be required.

[000743] Additionally, the present invention contemplates the use of transdermal patches, which often have the added advantage of providing controlled delivery of a compound to the body. Such dosage forms may be prepared, for example, by dissolving

and/or dispensing the compound in the proper medium. Alternatively or additionally, rate may be controlled by either providing a rate controlling membrane and/or by dispersing the compound in a polymer matrix and/or gel.

[000744] Formulations suitable for topical administration include, but are not limited to, liquid and/or semi liquid preparations such as liniments, lotions, oil in water and/or water in oil emulsions such as creams, ointments and/or pastes, and/or solutions and/or suspensions.

[000745] Topically-administrable formulations may, for example, comprise from about 0.1% to about 10% (w/w) active ingredient, although the concentration of active ingredient may be as high as the solubility limit of the active ingredient in the solvent. Formulations for topical administration may further comprise one or more of the additional ingredients described herein.

Depot Administration

[000746] As described herein, in some embodiments, the composition is formulated in depots for extended release. Generally, a specific organ or tissue (a "target tissue") is targeted for administration.

[000747] In some aspects of the invention, the polynucleotides, primary constructs or mmRNA are spatially retained within or proximal to a target tissue. Provided are method of providing a composition to a target tissue of a mammalian subject by contacting the target tissue (which contains one or more target cells) with the composition under conditions such that the composition, in particular the nucleic acid component(s) of the composition, is substantially retained in the target tissue, meaning that at least 10, 20, 30, 40, 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, 99, 99.9, 99.99 or greater than 99.99% of the composition is retained in the target tissue. Advantageously, retention is determined by measuring the amount of the nucleic acid present in the composition that enters one or more target cells. For example, at least 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 85, 90, 95, 96, 97, 98, 99, 99.9, 99.99 or greater than 99.99% of the nucleic acids administered to the subject are present intracellularly at a period of time following administration. For example, intramuscular injection to a mammalian subject is performed using an aqueous composition containing a ribonucleic acid and a transfection reagent, and retention of the

composition is determined by measuring the amount of the ribonucleic acid present in the muscle cells.

[000748] Aspects of the invention are directed to methods of providing a composition to a target tissue of a mammalian subject, by contacting the target tissue (containing one or more target cells) with the composition under conditions such that the composition is substantially retained in the target tissue. The composition contains an effective amount of a polynucleotides, primary constructs or mmRNA such that the polypeptide of interest is produced in at least one target cell. The compositions generally contain a cell penetration agent, although "naked" nucleic acid (such as nucleic acids without a cell penetration agent or other agent) is also contemplated, and a pharmaceutically acceptable carrier.

[000749] In some circumstances, the amount of a protein produced by cells in a tissue is desirably increased. Preferably, this increase in protein production is spatially restricted to cells within the target tissue. Thus, provided are methods of increasing production of a protein of interest in a tissue of a mammalian subject. A composition is provided that contains polynucleotides, primary constructs or mmRNA characterized in that a unit quantity of composition has been determined to produce the polypeptide of interest in a substantial percentage of cells contained within a predetermined volume of the target tissue.

[000750] In some embodiments, the composition includes a plurality of different polynucleotides, primary constructs or mmRNA, where one or more than one of the polynucleotides, primary constructs or mmRNA encodes a polypeptide of interest. Optionally, the composition also contains a cell penetration agent to assist in the intracellular delivery of the composition. A determination is made of the dose of the composition required to produce the polypeptide of interest in a substantial percentage of cells contained within the predetermined volume of the target tissue (generally, without inducing significant production of the polypeptide of interest in tissue adjacent to the predetermined volume, or distally to the target tissue). Subsequent to this determination, the determined dose is introduced directly into the tissue of the mammalian subject.

[000751] In one embodiment, the invention provides for the polynucleotides, primary constructs or mmRNA to be delivered in more than one injection or by split dose injections.

[000752] In one embodiment, the invention may be retained near target tissue using a small disposable drug reservoir, patch pump or osmotic pump. Non-limiting examples of patch pumps include those manufactured and/or sold by BD® (Franklin Lakes, NJ), Insulet Corporation (Bedford, MA), SteadyMed Therapeutics (San Francisco, CA), Medtronic (Minneapolis, MN) (e.g., MiniMed), UniLife (York, PA), Valeritas (Bridgewater, NJ), and SpringLeaf Therapeutics (Boston, MA). A non-limiting example of an osmotic pump include those manufactured by DURECT® (Cupertino, CA) (e.g., DUROS® and ALZET ®).

Pulmonary Administration

[000753] A pharmaceutical composition may be prepared, packaged, and/or sold in a formulation suitable for pulmonary administration via the buccal cavity. Such a formulation may comprise dry particles which comprise the active ingredient and which have a diameter in the range from about 0.5 nm to about 7 nm or from about 1 nm to about 6 nm. Such compositions are suitably in the form of dry powders for administration using a device comprising a dry powder reservoir to which a stream of propellant may be directed to disperse the powder and/or using a self propelling solvent/powder dispensing container such as a device comprising the active ingredient dissolved and/or suspended in a low-boiling propellant in a sealed container. Such powders comprise particles wherein at least 98% of the particles by weight have a diameter greater than 0.5 nm and at least 95% of the particles by number have a diameter less than 7 nm. Alternatively, at least 95% of the particles by weight have a diameter greater than 1 nm and at least 90% of the particles by number have a diameter less than 6 nm. Dry powder compositions may include a solid fine powder diluent such as sugar and are conveniently provided in a unit dose form.

[000754] Low boiling propellants generally include liquid propellants having a boiling point of below 65 °F at atmospheric pressure. Generally the propellant may constitute 50% to 99.9% (w/w) of the composition, and active ingredient may constitute 0.1% to 20% (w/w) of the composition. A propellant may further comprise additional ingredients

such as a liquid non-ionic and/or solid anionic surfactant and/or a solid diluent (which may have a particle size of the same order as particles comprising the active ingredient).

[000755] As a non-limiting example, the polynucleotides, primary constructs and/or mmRNA described herein may be formulated for pulmonary delivery by the methods described in U.S. Pat. No. 8,257,685; herein incorporated by reference in its entirety.

[000756] Pharmaceutical compositions formulated for pulmonary delivery may provide an active ingredient in the form of droplets of a solution and/or suspension. Such formulations may be prepared, packaged, and/or sold as aqueous and/or dilute alcoholic solutions and/or suspensions, optionally sterile, comprising active ingredient, and may conveniently be administered using any nebulization and/or atomization device. Such formulations may further comprise one or more additional ingredients including, but not limited to, a flavoring agent such as saccharin sodium, a volatile oil, a buffering agent, a surface active agent, and/or a preservative such as methylhydroxybenzoate. Droplets provided by this route of administration may have an average diameter in the range from about 0.1 nm to about 200 nm.

Intranasal, nasal and buccal Administration

[000757] Formulations described herein as being useful for pulmonary delivery are useful for intranasal delivery of a pharmaceutical composition. Another formulation suitable for intranasal administration is a coarse powder comprising the active ingredient and having an average particle from about 0.2 μm to 500 μm. Such a formulation is administered in the manner in which snuff is taken, *i.e.* by rapid inhalation through the nasal passage from a container of the powder held close to the nose.

[000758] Formulations suitable for nasal administration may, for example, comprise from about as little as 0.1% (w/w) and as much as 100% (w/w) of active ingredient, and may comprise one or more of the additional ingredients described herein. A pharmaceutical composition may be prepared, packaged, and/or sold in a formulation suitable for buccal administration. Such formulations may, for example, be in the form of tablets and/or lozenges made using conventional methods, and may, for example, 0.1% to 20% (w/w) active ingredient, the balance comprising an orally dissolvable and/or degradable composition and, optionally, one or more of the additional ingredients described herein. Alternately, formulations suitable for buccal administration may

comprise a powder and/or an aerosolized and/or atomized solution and/or suspension comprising active ingredient. Such powdered, aerosolized, and/or aerosolized formulations, when dispersed, may have an average particle and/or droplet size in the range from about 0.1 nm to about 200 nm, and may further comprise one or more of any additional ingredients described herein.

Ophthalmic Administration

[000759] A pharmaceutical composition may be prepared, packaged, and/or sold in a formulation suitable for ophthalmic administration. Such formulations may, for example, be in the form of eye drops including, for example, a 0.1/1.0% (w/w) solution and/or suspension of the active ingredient in an aqueous or oily liquid excipient. Such drops may further comprise buffering agents, salts, and/or one or more other of any additional ingredients described herein. Other ophthalmically-administrable formulations which are useful include those which comprise the active ingredient in microcrystalline form and/or in a liposomal preparation. Ear drops and/or eye drops are contemplated as being within the scope of this invention. A multilayer thin film device may be prepared to contain a pharmaceutical composition for delivery to the eye and/or surrounding tissue.

Payload Administration: Detectable Agents and Therapeutic Agents

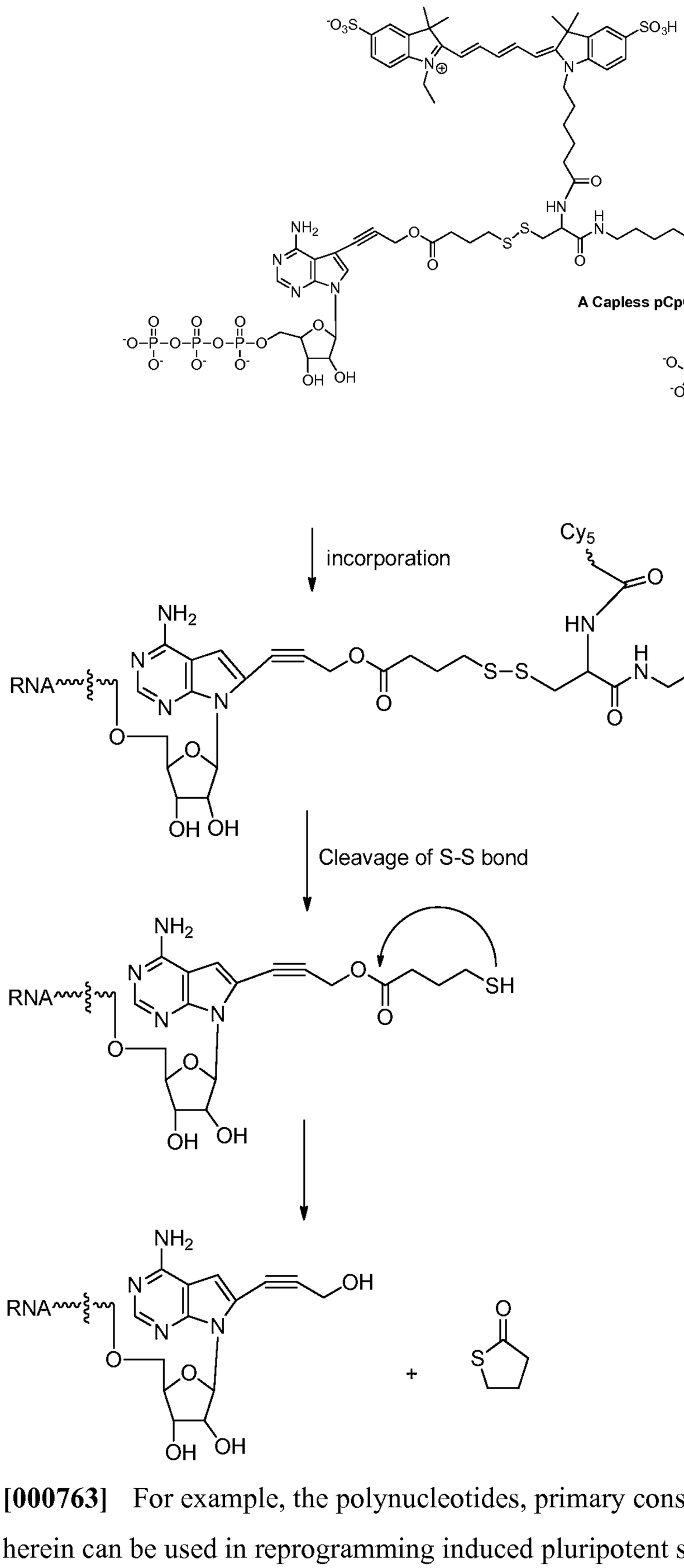
[000760] The polynucleotides, primary constructs or mmRNA described herein can be used in a number of different scenarios in which delivery of a substance (the "payload") to a biological target is desired, for example delivery of detectable substances for detection of the target, or delivery of a therapeutic agent. Detection methods can include, but are not limited to, both imaging *in vitro* and *in vivo* imaging methods, *e.g.*, immunohistochemistry, bioluminescence imaging (BLI), Magnetic Resonance Imaging (MRI), positron emission tomography (PET), electron microscopy, X-ray computed tomography, Raman imaging, optical coherence tomography, absorption imaging, thermal imaging, fluorescence reflectance imaging, fluorescence microscopy, fluorescence molecular tomographic imaging, nuclear magnetic resonance imaging, X-ray imaging, ultrasound imaging, photoacoustic imaging, lab assays, or in any situation where tagging/staining/imaging is required.

[000761] The polynucleotides, primary constructs or mmRNA can be designed to include both a linker and a payload in any useful orientation. For example, a linker

having two ends is used to attach one end to the payload and the other end to the nucleobase, such as at the C-7 or C-8 positions of the deaza-adenosine or deaza-guanosine or to the N-3 or C-5 positions of cytosine or uracil. The polynucleotide of the invention can include more than one payload (e.g., a label and a transcription inhibitor), as well as a cleavable linker. In one embodiment, the modified nucleotide is a modified 7-deaza-adenosine triphosphate, where one end of a cleavable linker is attached to the C7 position of 7-deaza-adenine, the other end of the linker is attached to an inhibitor (e.g., to the C5 position of the nucleobase on a cytidine), and a label (e.g., Cy5) is attached to the center of the linker (see, e.g., compound 1 of A*pCp C5 Parg Capless in Fig. 5 and columns 9 and 10 of U.S. Pat. No. 7,994,304, incorporated herein by reference). Upon incorporation of the modified 7-deaza-adenosine triphosphate to an encoding region, the resulting polynucleotide having a cleavable linker attached to a label and an inhibitor (e.g., a polymerase inhibitor). Upon cleavage of the linker (e.g., with reductive conditions to reduce a linker having a cleavable disulfide moiety), the label and inhibitor are released. Additional linkers and payloads (e.g., therapeutic agents, detectable labels, and cell penetrating payloads) are described herein.

[000762] Scheme 12 below depicts an exemplary modified nucleotide wherein the nucleobase, adenine, is attached to a linker at the C-7 carbon of 7-deaza adenine. In addition, Scheme 12 depicts the modified nucleotide with the linker and payload, e.g., a detectable agent, incorporated onto the 3' end of the mRNA. Disulfide cleavage and 1,2-addition of the thiol group onto the propargyl ester releases the detectable agent. The remaining structure (depicted, for example, as pApC5Parg in Scheme 12) is the inhibitor. The rationale for the structure of the modified nucleotides is that the tethered inhibitor sterically interferes with the ability of the polymerase to incorporate a second base. Thus, it is critical that the tether be long enough to affect this function and that the inhibiter be in a stereochemical orientation that inhibits or prohibits second and follow on nucleotides into the growing polynucleotide strand.

Scheme 12



[000763] For example, the polynucleotides, primary constructs or mmRNA described herein can be used in reprogramming induced pluripotent stem cells (iPS cells), which

can directly track cells that are transfected compared to total cells in the cluster. In another example, a drug that may be attached to the polynucleotides, primary constructs or mmRNA via a linker and may be fluorescently labeled can be used to track the drug *in vivo*, *e.g.* intracellularly. Other examples include, but are not limited to, the use of a polynucleotides, primary constructs or mmRNA in reversible drug delivery into cells.

[000764] The polynucleotides, primary constructs or mmRNA described herein can be used in intracellular targeting of a payload, *e.g.*, detectable or therapeutic agent, to specific organelle. Exemplary intracellular targets can include, but are not limited to, the nuclear localization for advanced mRNA processing, or a nuclear localization sequence (NLS) linked to the mRNA containing an inhibitor.

[000765] In addition, the polynucleotides, primary constructs or mmRNA described herein can be used to deliver therapeutic agents to cells or tissues, *e.g.*, in living animals. For example, the polynucleotides, primary constructs or mmRNA described herein can be used to deliver highly polar chemotherapeutics agents to kill cancer cells. The polynucleotides, primary constructs or mmRNA attached to the therapeutic agent through a linker can facilitate member permeation allowing the therapeutic agent to travel into a cell to reach an intracellular target.

[000766] In one example, the linker is attached at the 2'-position of the ribose ring and/or at the 3' and/or 5' positionof the polynucleotides, primary constructs mmRNA (See e.g., International Pub. No. WO2012030683, herein incorporated by reference in its entirety). The linker may be any linker disclosed herein, known in the art and/or disclosed in International Pub. No. WO2012030683, herein incorporated by reference in its entirety.

[000767] In another example, the polynucleotides, primary constructs or mmRNA can be attached to the polynucleotides, primary constructs or mmRNA a viral inhibitory peptide (VIP) through a cleavable linker. The cleavable linker can release the VIP and dye into the cell. In another example, the polynucleotides, primary constructs or mmRNA can be attached through the linker to an ADP-ribosylate, which is responsible for the actions of some bacterial toxins, such as cholera toxin, diphtheria toxin, and pertussis toxin. These toxin proteins are ADP-ribosyltransferases that modify target proteins in human cells. For example, cholera toxin ADP-ribosylates G proteins modifies

human cells by causing massive fluid secretion from the lining of the small intestine, which results in life-threatening diarrhea.

[000768] In some embodiments, the payload may be a therapeutic agent such as a cytotoxin, radioactive ion, chemotherapeutic, or other therapeutic agent. A cytotoxin or cytotoxic agent includes any agent that may be detrimental to cells. Examples include, but are not limited to, taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, teniposide, vincristine, vinblastine, colchicine, doxorubicin, daunorubicin, dihydroxyanthracinedione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, puromycin, maytansinoids, *e.g.*, maytansinol (see U.S. Pat. No. 5,208,020 incorporated herein in its entirety), rachelmycin (CC-1065, see U.S. Pat. Nos. 5,475,092, 5,585,499, and 5,846,545, all of which are incorporated herein by reference), and analogs or homologs thereof. Radioactive ions include, but are not limited to iodine (*e.g.*, iodine 125 or iodine 131), strontium 89, phosphorous, palladium, cesium, iridium, phosphate, cobalt, yttrium 90, samarium 153, and praseodymium. Other therapeutic agents include, but are not limited to, antimetabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g., mechlorethamine, thiotepa chlorambucil, rachelmycin (CC-1065), melphalan, carmustine (BSNU), lomustine (CCNU), cyclophosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (e.g., daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (e.g., dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (e.g., vincristine, vinblastine, taxol and maytansinoids).

[000769] In some embodiments, the payload may be a detectable agent, such as various organic small molecules, inorganic compounds, nanoparticles, enzymes or enzyme substrates, fluorescent materials, luminescent materials (e.g., luminol), bioluminescent materials (e.g., luciferase, luciferin, and aequorin), chemiluminescent materials, radioactive materials (e.g., ^{18}F, ^{67}Ga, ^{81m}Kr, ^{82}Rb, ^{111}In, ^{123}I, ^{133}Xe, ^{201}Tl, ^{125}I, ^{35}S, ^{14}C, ^{3}H, or ^{99m}Tc (e.g., as pertechnetate (technetate(VII), TcO_4^-)), and contrast agents (e.g., gold (*e.g.*, gold nanoparticles), gadolinium (e.g., chelated Gd), iron oxides (e.g., superparamagnetic iron oxide (SPIO), monocrystalline iron oxide nanoparticles

(MIONs), and ultrasmall superparamagnetic iron oxide (USPIO)), manganese chelates (e.g., Mn-DPDP), barium sulfate, iodinated contrast media (iohexol), microbubbles, or perfluorocarbons). Such optically-detectable labels include for example, without limitation, 4-acetamido-4'-isothiocyanatostilbene-2,2′disulfonic acid; acridine and derivatives (e.g., acridine and acridine isothiocyanate); 5-(2′-aminoethyl)aminonaphthalene-1-sulfonic acid (EDANS); 4-amino-N-[3-vinylsulfonyl)phenyl]naphthalimide-3,5 disulfonate; N-(4-anilino-l-naphthyl)maleimide; anthranilamide; BODIPY; Brilliant Yellow; coumarin and derivatives (e.g., coumarin, 7-amino-4-methylcoumarin (AMC, Coumarin 120), and 7-amino-4-trifluoromethylcoumarin (Coumarin 151)); cyanine dyes; cyanosine; 4',6-diaminidino-2-phenylindole (DAPI); 5′ 5"-dibromopyrogallol-sulfonaphthalein (Bromopyrogallol Red); 7-diethylamino-3-(4'-isothiocyanatophenyl)-4-methylcoumarin; diethylenetriamine pentaacetate; 4,4'-diisothiocyanatodihydro-stilbene-2,2′-disulfonic acid; 4,4'-diisothiocyanatostilbene-2,2′-disulfonic acid; 5-[dimethylamino]-naphthalene-1-sulfonyl chloride (DNS, dansylchloride); 4-dimethylaminophenylazophenyl-4'-isothiocyanate (DABITC); eosin and derivatives (e.g., eosin and eosin isothiocyanate); erythrosin and derivatives (e.g., erythrosin B and erythrosin isothiocyanate); ethidium; fluorescein and derivatives (e.g., 5-carboxyfluorescein (FAM), 5-(4,6-dichlorotriazin-2-yl)aminofluorescein (DTAF), 2′,7'-dimethoxy-4'5′-dichloro-6-carboxyfluorescein, fluorescein, fluorescein isothiocyanate, X-rhodamine-5-(and-6)-isothiocyanate (QFITC or XRITC), and fluorescamine); 2-[2-[3-[[1,3-dihydro-1,1-dimethyl-3-(3-sulfopropyl)-2H-benz[e]indol-2-ylidene]ethylidene]-2-[4-(ethoxycarbonyl)-1-piperazinyl]-1-cyclopenten-1-yl]ethenyl]-1,1-dimethyl-3-(3-sulforpropyl)-1H-benz[e]indolium hydroxide, inner salt, compound with n,n-diethylethanamine(1:1) (IR144); 5-chloro-2-[2-[3-[(5-chloro-3-ethyl-2(3H)-benzothiazol- ylidene)ethylidene]-2-(diphenylamino)-1-cyclopenten-1-yl]ethenyl]-3-ethyl benzothiazolium perchlorate (IR140); Malachite Green isothiocyanate; 4-methylumbelliferone orthocresolphthalein; nitrotyrosine; pararosaniline; Phenol Red; B-phycoerythrin; o-phthaldialdehyde; pyrene and derivatives(e.g., pyrene, pyrene butyrate, and succinimidyl 1-pyrene); butyrate quantum dots; Reactive Red 4 (CIBACRON™ Brilliant Red 3B-A); rhodamine and derivatives (e.g., 6-carboxy-X-rhodamine (ROX), 6-carboxyrhodamine (R6G), lissamine rhodamine

B sulfonyl chloride rhodarnine (Rhod), rhodamine B, rhodamine 123, rhodamine X isothiocyanate, sulforhodamine B, sulforhodamine 101, sulfonyl chloride derivative of sulforhodamine 101 (Texas Red), N,N,N',N'tetramethyl-6-carboxyrhodamine (TAMRA) tetramethyl rhodamine, and tetramethyl rhodamine isothiocyanate (TRITC)); riboflavin; rosolic acid; terbium chelate derivatives; Cyanine-3 (Cy3); Cyanine-5 (Cy5); cyanine-5.5 (Cy5.5), Cyanine-7 (Cy7); IRD 700; IRD 800; Alexa 647; La Jolta Blue; phthalo cyanine; and naphthalo cyanine.

[000770] In some embodiments, the detectable agent may be a non-detectable pre-cursor that becomes detectable upon activation (e.g., fluorogenic tetrazine-fluorophore constructs (e.g., tetrazine-BODIPY FL, tetrazine-Oregon Green 488, or tetrazine-BODIPY TMR-X) or enzyme activatable fluorogenic agents (e.g., PROSENSE® (VisEn Medical))). In vitro assays in which the enzyme labeled compositions can be used include, but are not limited to, enzyme linked immunosorbent assays (ELISAs), immunoprecipitation assays, immunofluorescence, enzyme immunoassays (EIA), radioimmunoassays (RIA), and Western blot analysis.

Combinations

[000771] The polynucleotides, primary constructs or mmRNA may be used in combination with one or more other therapeutic, prophylactic, diagnostic, or imaging agents. By "in combination with," it is not intended to imply that the agents must be administered at the same time and/or formulated for delivery together, although these methods of delivery are within the scope of the present disclosure. Compositions can be administered concurrently with, prior to, or subsequent to, one or more other desired therapeutics or medical procedures. In general, each agent will be administered at a dose and/or on a time schedule determined for that agent. In some embodiments, the present disclosure encompasses the delivery of pharmaceutical, prophylactic, diagnostic, or imaging compositions in combination with agents that may improve their bioavailability, reduce and/or modify their metabolism, inhibit their excretion, and/or modify their distribution within the body. As a non-limiting example, the nucleic acids or mmRNA may be used in combination with a pharmaceutical agent for the treatment of cancer or to control hyperproliferative cells. In U.S. Pat. No. 7,964,571, herein incorporated by reference in its entirety, a combination therapy for the treatment of solid primary or

metastasized tumor is described using a pharmaceutical composition including a DNA plasmid encoding for interleukin-12 with a lipopolymer and also administering at least one anticancer agent or chemotherapeutic. Further, the nucleic acids and mmRNA of the present invention that encodes anti-proliferative molecules may be in a pharmaceutical composition with a lipopolymer (see e.g., U.S. Pub. No. 20110218231, herein incorporated by reference in its entirety, claiming a pharmaceutical composition comprising a DNA plasmid encoding an anti-proliferative molecule and a lipopolymer) which may be administered with at least one chemotherapeutic or anticancer agent.

[000772] It will further be appreciated that therapeutically, prophylactically, diagnostically, or imaging active agents utilized in combination may be administered together in a single composition or administered separately in different compositions. In general, it is expected that agents utilized in combination with be utilized at levels that do not exceed the levels at which they are utilized individually. In some embodiments, the levels utilized in combination will be lower than those utilized individually. In one embodiment, the combinations, each or together may be administered according to the split dosing regimens described herein.

Dosing

[000773] The present invention provides methods comprising administering modified mRNAs and their encoded proteins or complexes in accordance with the invention to a subject in need thereof. Nucleic acids, proteins or complexes, or pharmaceutical, imaging, diagnostic, or prophylactic compositions thereof, may be administered to a subject using any amount and any route of administration effective for preventing, treating, diagnosing, or imaging a disease, disorder, and/or condition (*e.g.*, a disease, disorder, and/or condition relating to working memory deficits). The exact amount required will vary from subject to subject, depending on the species, age, and general condition of the subject, the severity of the disease, the particular composition, its mode of administration, its mode of activity, and the like. Compositions in accordance with the invention are typically formulated in dosage unit form for ease of administration and uniformity of dosage. It will be understood, however, that the total daily usage of the compositions of the present invention may be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective,

prophylactically effective, or appropriate imaging dose level for any particular patient will depend upon a variety of factors including the disorder being treated and the severity of the disorder; the activity of the specific compound employed; the specific composition employed; the age, body weight, general health, sex and diet of the patient; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific compound employed; and like factors well known in the medical arts.

[000774] In certain embodiments, compositions in accordance with the present invention may be administered at dosage levels sufficient to deliver from about 0.0001 mg/kg to about 100 mg/kg, from about 0.001 mg/kg to about 0.05 mg/kg, from about 0.005 mg/kg to about 0.05 mg/kg, from about 0.001 mg/kg to about 0.005 mg/kg, from about 0.05 mg/kg to about 0.5 mg/kg, from about 0.01 mg/kg to about 50 mg/kg, from about 0.1 mg/kg to about 40 mg/kg, from about 0.5 mg/kg to about 30 mg/kg, from about 0.01 mg/kg to about 10 mg/kg, from about 0.1 mg/kg to about 10 mg/kg, or from about 1 mg/kg to about 25 mg/kg, of subject body weight per day, one or more times a day, to obtain the desired therapeutic, diagnostic, prophylactic, or imaging effect. The desired dosage may be delivered three times a day, two times a day, once a day, every other day, every third day, every week, every two weeks, every three weeks, or every four weeks. In certain embodiments, the desired dosage may be delivered using multiple administrations (*e.g.*, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, or more administrations). When multiple administrations are employed, split dosing regimens such as those described herein may be used.

[000775] According to the present invention, it has been discovered that administration of mmRNA in split-dose regimens produce higher levels of proteins in mammalian subjects. As used herein, a "split dose" is the division of single unit dose or total daily dose into two or more doses, e.g, two or more administrations of the single unit dose. As used herein, a "single unit dose" is a dose of any therapeutic administed in one dose/at one time/single route/single point of contact, i.e., single administration event. As used herein, a "total daily dose" is an amount given or prescribed in 24 hr period. It may be administered as a single unit dose. In one embodiment, the mmRNA of the present

invention are administed to a subject in split doses. The mmRNA may be formulated in buffer only or in a formulation described herein.

Dosage Forms

[000776] A pharmaceutical composition described herein can be formulated into a dosage form described herein, such as a topical, intranasal, intratracheal, or injectable (e.g., intravenous, intraocular, intravitreal, intramuscular, intracardiac, intraperitoneal, subcutaneous).

Liquid dosage forms

[000777] Liquid dosage forms for parenteral administration include, but are not limited to, pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups, and/or elixirs. In addition to active ingredients, liquid dosage forms may comprise inert diluents commonly used in the art including, but not limited to, water or other solvents, solubilizing agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof. In certain embodiments for parenteral administration, compositions may be mixed with solubilizing agents such as CREMOPHOR®, alcohols, oils, modified oils, glycols, polysorbates, cyclodextrins, polymers, and/or combinations thereof.

Injectable

[000778] Injectable preparations, for example, sterile injectable aqueous or oleaginous suspensions may be formulated according to the known art and may include suitable dispersing agents, wetting agents, and/or suspending agents. Sterile injectable preparations may be sterile injectable solutions, suspensions, and/or emulsions in nontoxic parenterally acceptable diluents and/or solvents, for example, a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed include, but are not limited to, water, Ringer's solution, U.S.P., and isotonic sodium chloride solution. Sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono-

or diglycerides. Fatty acids such as oleic acid can be used in the preparation of injectables.

Injectable formulations can be sterilized, for example, by filtration through a bacterial-retaining filter, and/or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable medium prior to use.

In order to prolong the effect of an active ingredient, it may be desirable to slow the absorption of the active ingredient from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material with poor water solubility. The rate of absorption of the polynucleotide, primary construct or mmRNA then depends upon its rate of dissolution which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally administered polynucleotide, primary construct or mmRNA may be accomplished by dissolving or suspending the polynucleotide, primary construct or mmRNA in an oil vehicle. Injectable depot forms are made by forming microencapsule matrices of the polynucleotide, primary construct or mmRNA in biodegradable polymers such as polylactide-polyglycolide. Depending upon the ratio of polynucleotide, primary construct or mmRNA to polymer and the nature of the particular polymer employed, the rate of polynucleotide, primary construct or mmRNA release can be controlled. Examples of other biodegradable polymers include, but are not limited to, poly(orthoesters) and poly(anhydrides). Depot injectable formulations may be prepared by entrapping the polynucleotide, primary construct or mmRNA in liposomes or microemulsions which are compatible with body tissues.

Pulmonary

[000779] Formulations described herein as being useful for pulmonary delivery may also be used for intranasal delivery of a pharmaceutical composition. Another formulation suitable for intranasal administration may be a coarse powder comprising the active ingredient and having an average particle from about 0.2 μm to 500 μm. Such a formulation may be administered in the manner in which snuff is taken, i.e. by rapid inhalation through the nasal passage from a container of the powder held close to the nose.

[000780] Formulations suitable for nasal administration may, for example, comprise from about as little as 0.1% (w/w) and as much as 100% (w/w) of active ingredient, and may comprise one or more of the additional ingredients described herein. A pharmaceutical composition may be prepared, packaged, and/or sold in a formulation suitable for buccal administration. Such formulations may, for example, be in the form of tablets and/or lozenges made using conventional methods, and may, for example, contain about 0.1% to 20% (w/w) active ingredient, where the balance may comprise an orally dissolvable and/or degradable composition and, optionally, one or more of the additional ingredients described herein. Alternately, formulations suitable for buccal administration may comprise a powder and/or an aerosolized and/or atomized solution and/or suspension comprising active ingredient. Such powdered, aerosolized, and/or aerosolized formulations, when dispersed, may have an average particle and/or droplet size in the range from about 0.1 nm to about 200 nm, and may further comprise one or more of any additional ingredients described herein.

[000781] General considerations in the formulation and/or manufacture of pharmaceutical agents may be found, for example, in Remington: The Science and Practice of Pharmacy 21st ed., Lippincott Williams & Wilkins, 2005 (incorporated herein by reference in its entirety).

Coatings or Shells

[000782] Solid dosage forms of tablets, dragees, capsules, pills, and granules can be prepared with coatings and shells such as enteric coatings and other coatings well known in the pharmaceutical formulating art. They may optionally comprise opacifying agents and can be of a composition that they release the active ingredient(s) only, or preferentially, in a certain part of the intestinal tract, optionally, in a delayed manner. Examples of embedding compositions which can be used include polymeric substances and waxes. Solid compositions of a similar type may be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols and the like.

Properties of Pharmaceutical Compositions

[000783] The pharmaceutical compositions described herein can be characterized by one or more of bioavailability, therapeutic window and/or volume of distribution.

Bioavailability

[000784] The polynucleotides, primary constructs or mmRNA, when formulated into a composition with a delivery agent as described herein, can exhibit an increase in bioavailability as compared to a composition lacking a delivery agent as described herein. As used herein, the term "bioavailability" refers to the systemic availability of a given amount of polynucleotides, primary constructs or mmRNA administered to a mammal. Bioavailability can be assessed by measuring the area under the curve (AUC) or the maximum serum or plasma concentration (C_{max}) of the unchanged form of a compound following administration of the compound to a mammal. AUC is a determination of the area under the curve plotting the serum or plasma concentration of a compound along the ordinate (Y-axis) against time along the abscissa (X-axis). Generally, the AUC for a particular compound can be calculated using methods known to those of ordinary skill in the art and as described in G. S. Banker, Modern Pharmaceutics, Drugs and the Pharmaceutical Sciences, v. 72, Marcel Dekker, New York, Inc., 1996, herein incorporated by reference in its entirety.

[000785] The C_{max} value is the maximum concentration of the compound achieved in the serum or plasma of a mammal following administration of the compound to the mammal. The C_{max} value of a particular compound can be measured using methods known to those of ordinary skill in the art. The phrases "increasing bioavailability" or "improving the pharmacokinetics," as used herein mean that the systemic availability of a first polynucleotide, primary construct or mmRNA, measured as AUC, C_{max}, or C_{min} in a mammal is greater, when co-administered with a delivery agent as described herein, than when such co-administration does not take place. In some embodiments, the bioavailability of the polynucleotide, primary construct or mmRNA can increase by at least about 2%, at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or about 100%.

Therapeutic Window

[000786] The polynucleotides, primary constructs or mmRNA, when formulated into a composition with a delivery agent as described herein, can exhibit an increase in the therapeutic window of the administered polynucleotide, primary construct or mmRNA composition as compared to the therapeutic window of the administered polynucleotide, primary construct or mmRNA composition lacking a delivery agent as described herein. As used herein "therapeutic window" refers to the range of plasma concentrations, or the range of levels of therapeutically active substance at the site of action, with a high probability of eliciting a therapeutic effect. In some embodiments, the therapeutic window of the polynucleotide, primary construct or mmRNA when co-administered with a delivery agent as described herein can increase by at least about 2%, at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or about 100%.

Volume of Distribution

[000787] The polynucleotides, primary constructs or mmRNA, when formulated into a composition with a delivery agent as described herein, can exhibit an improved volume of distribution (V_{dist}), e.g., reduced or targeted, relative to a composition lacking a delivery agent as described herein. The volume of distribution (Vdist) relates the amount of the drug in the body to the concentration of the drug in the blood or plasma. As used herein, the term "volume of distribution" refers to the fluid volume that would be required to contain the total amount of the drug in the body at the same concentration as in the blood or plasma: Vdist equals the amount of drug in the body/concentration of drug in blood or plasma. For example, for a 10 mg dose and a plasma concentration of 10 mg/L, the volume of distribution would be 1 liter. The volume of distribution reflects the extent to which the drug is present in the extravascular tissue. A large volume of distribution reflects the tendency of a compound to bind to the tissue components compared with plasma protein binding. In a clinical setting, Vdist can be used to determine a loading dose to achieve a steady state concentration. In some embodiments, the volume of distribution of the polynucleotide, primary construct or mmRNA when co-

administered with a delivery agent as described herein can decrease at least about 2%, at least about 5%, at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%.

Biological Effect

[000788] In one embodiment, the biological effect of the modified mRNA delivered to the animals may be categorized by analyzing the protein expression in the animals. The protein expression may be determined from analyzing a biological sample collected from a mammal administered the modified mRNA of the present invention. In one embodiment, the expression protein encoded by the modified mRNA administered to the mammal of at least 50 pg/ml may be preferred. For example, a protein expression of 50-200 pg/ml for the protein encoded by the modified mRNA delivered to the mammal may be seen as a therapeutically effective amount of protein in the mammal.

Detection of Modified Nucleic Acids by Mass Spectrometry

[000789] Mass spectrometry (MS) is an analytical technique that can provide structural and molecular mass/concentration information on molecules after their conversion to ions. The molecules are first ionized to acquire positive or negative charges and then they travel through the mass analyzer to arrive at different areas of the detector according to their mass/charge (m/z) ratio.

[000790] Mass spectrometry is performed using a mass spectrometer which includes an ion source for ionizing the fractionated sample and creating charged molecules for further analysis. For example ionization of the sample may be performed by electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), photoionization, electron ionization, fast atom bombardment (FAB)/liquid secondary ionization (LSIMS), matrix assisted laser desorption/ionization (MALDI), field ionization, field desorption, thermospray/plasmaspray ionization, and particle beam ionization. The skilled artisan will understand that the choice of ionization method can be determined based on the analyte to be measured, type of sample, the type of detector, the choice of positive versus negative mode, etc.

[000791] After the sample has been ionized, the positively charged or negatively charged ions thereby created may be analyzed to determine a mass-to-charge ratio (i.e., m/z). Suitable analyzers for determining mass-to-charge ratios include quadropole analyzers, ion traps analyzers, and time-of-flight analyzers. The ions may be detected using several detection modes. For example, selected ions may be detected (i.e., using a selective ion monitoring mode (SIM)), or alternatively, ions may be detected using a scanning mode, e.g., multiple reaction monitoring (MRM) or selected reaction monitoring (SRM).

[000792] Liquid chromatography-multiple reaction monitoring (LC-MS/MRM) coupled with stable isotope labeled dilution of peptide standards has been shown to be an effective method for protein verification (e.g., Keshishian et al., Mol Cell Proteomics 2009 8: 2339-2349; Kuhn et al., Clin Chem 2009 55:1108-1117; Lopez et al., Clin Chem 2010 56:281-290; each of which are herein incorporated by reference in its entirety). Unlike untargeted mass spectrometry frequently used in biomarker discovery studies, targeted MS methods are peptide sequence–based modes of MS that focus the full analytical capacity of the instrument on tens to hundreds of selected peptides in a complex mixture. By restricting detection and fragmentation to only those peptides derived from proteins of interest, sensitivity and reproducibility are improved dramatically compared to discovery-mode MS methods. This method of mass spectrometry-based multiple reaction monitoring (MRM) quantitation of proteins can dramatically impact the discovery and quantitation of biomarkers via rapid, targeted, multiplexed protein expression profiling of clinical samples.

[000793] In one embodiment, a biological sample which may contain at least one protein encoded by at least one modified mRNA of the present invention may be analyzed by the method of MRM-MS. The quantification of the biological sample may further include, but is not limited to, isotopically labeled peptides or proteins as internal standards.

[000794] According to the present invention, the biological sample, once obtained from the subject, may be subjected to enzyme digestion. As used herein, the term "digest" means to break apart into shorter peptides. As used herein, the phrase "treating a sample to digest proteins" means manipulating a sample in such a way as to break down proteins

in a sample. These enzymes include, but are not limited to, trypsin, endoproteinase Glu-C and chymotrypsin. In one embodiment, a biological sample which may contain at least one protein encoded by at least one modified mRNA of the present invention may be digested using enzymes.

[000795] In one embodiment, a biological sample which may contain protein encoded by modified mRNA of the present invention may be analyzed for protein using electrospray ionization. Electrospray ionization (ESI) mass spectrometry (ESIMS) uses electrical energy to aid in the transfer of ions from the solution to the gaseous phase before they are analyzed by mass spectrometry. Samples may be analyzed using methods known in the art (e.g., Ho et al., Clin Biochem Rev. 2003 24(1):3-12; herein incorporated by reference in its entirety). The ionic species contained in solution may be transferred into the gas phase by dispersing a fine spray of charge droplets, evaporating the solvent and ejecting the ions from the charged droplets to generate a mist of highly charged droplets. The mist of highly charged droplets may be analyzed using at least 1, at least 2, at least 3 or at least 4 mass analyzers such as, but not limited to, a quadropole mass analyzer. Further, the mass spectrometry method may include a purification step. As a non-limiting example, the first quadrapole may be set to select a single m/z ratio so it may filter out other molecular ions having a different m/z ratio which may eliminate complicated and time-consuming sample purification procedures prior to MS analysis.

[000796] In one embodiment, a biological sample which may contain protein encoded by modified mRNA of the present invention may be analyzed for protein in a tandem ESIMS system (e.g., MS/MS). As non-limiting examples, the droplets may be analyzed using a product scan (or daughter scan) a precursor scan (parent scan) a neutral loss or a multiple reaction monitoring.

[000797] In one embodiment, a biological sample which may contain protein encoded by modified mRNA of the present invention may be analyzed using matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MALDIMS). MALDI provides for the nondestructive vaporization and ionization of both large and small molecules, such as proteins. In MALDI analysis, the analyte is first co-crystallized with a large molar excess of a matrix compound, which may also include, but is not limited to, an ultraviolet absorbing weak organic acid. Non-limiting examples of matrices used in MALDI are α-

cyano-4-hydroxycinnamic acid, 3,5-dimethoxy-4-hydroxycinnamic acid and 2,5-dihydroxybenzoic acid. Laser radiation of the analyte-matrix mixture may result in the vaporization of the matrix and the analyte. The laser induced desorption provides high ion yields of the intact analyte and allows for measurement of compounds with high accuracy. Samples may be analyzed using methods known in the art (e.g., Lewis, Wei and Siuzdak, Encyclopedia of Analytical Chemistry 2000:5880-5894; herein incorporated by reference in its entirety). As non-limiting examples, mass analyzers used in the MALDI analysis may include a linear time-of-flight (TOF), a TOF reflectron or a Fourier transform mass analyzer.

[000798] In one embodiment, the analyte-matrix mixture may be formed using the dried-droplet method. A biologic sample is mixed with a matrix to create a saturated matrix solution where the matrix-to-sample ratio is approximately 5000:1. An aliquot (approximately 0.5-2.0 uL) of the saturated matrix solution is then allowed to dry to form the analyte-matrix mixture.

[000799] In one embodiment, the analyte-matrix mixture may be formed using the thin-layer method. A matrix homogeneous film is first formed and then the sample is then applied and may be absorbed by the matrix to form the analyte-matrix mixture.

[000800] In one embodiment, the analyte-matrix mixture may be formed using the thick-layer method. A matrix homogeneous film is formed with a nitro-cellulose matrix additive. Once the uniform nitro-cellulose matrix layer is obtained the sample is applied and absorbed into the matrix to form the analyte-matrix mixture.

[000801] In one embodiment, the analyte-matrix mixture may be formed using the sandwich method. A thin layer of matrix crystals is prepared as in the thin-layer method followed by the addition of droplets of aqueous trifluoroacetic acid, the sample and matrix. The sample is then absorbed into the matrix to form the analyte-matrix mixture.

V. Uses of Polynucleotides, primary constructs and mmRNA of the Invention

[000802] The polynucleotides, primary constructs and mmRNA of the present invention are designed, in preferred embodiments, to provide for avoidance or evasion of deleterious bio-responses such as the immune response and/or degradation pathways, overcoming the threshold of expression and/or improving protein production capacity, improved expression rates or translation efficiency, improved drug or protein half life

and/or protein concentrations, optimized protein localization, to improve one or more of the stability and/or clearance in tissues, receptor uptake and/or kinetics, cellular access by the compositions, engagement with translational machinery, secretion efficiency (when applicable), accessibility to circulation, and/or modulation of a cell's status, function and/or activity.

Therapeutics

Therapeutic Agents

[000803] The polynucleotides, primary constructs or mmRNA of the present invention, such as modified nucleic acids and modified RNAs, and the proteins translated from them described herein can be used as therapeutic or prophylactic agents. They are provided for use in medicine. For example, a polynucleotide, primary construct or mmRNA described herein can be administered to a subject, wherein the polynucleotide, primary construct or mmRNA is translated *in vivo* to produce a therapeutic or prophylactic polypeptide in the subject. Provided are compositions, methods, kits, and reagents for diagnosis, treatment or prevention of a disease or condition in humans and other mammals. The active therapeutic agents of the invention include polynucleotides, primary constructs or mmRNA, cells containing polynucleotides, primary constructs or mmRNA or polypeptides translated from the polynucleotides, primary constructs or mmRNA.

[000804] In certain embodiments, provided herein are combination therapeutics containing one or more polynucleotide, primary construct or mmRNA containing translatable regions that encode for a protein or proteins that boost a mammalian subject's immunity along with a protein that induces antibody-dependent cellular toxicity. For example, provided herein are therapeutics containing one or more nucleic acids that encode trastuzumab and granulocyte-colony stimulating factor (G-CSF). In particular, such combination therapeutics are useful in Her2+ breast cancer patients who develop induced resistance to trastuzumab. (See, e.g., Albrecht, Immunotherapy. 2(6):795-8 (2010)).

[000805] Provided herein are methods of inducing translation of a recombinant polypeptide in a cell population using the polynucleotide, primary construct or mmRNA described herein. Such translation can be *in vivo*, *ex vivo*, *in culture*, or *in vitro*. The cell population is contacted with an effective amount of a composition containing a nucleic

acid that has at least one nucleoside modification, and a translatable region encoding the recombinant polypeptide. The population is contacted under conditions such that the nucleic acid is localized into one or more cells of the cell population and the recombinant polypeptide is translated in the cell from the nucleic acid.

[000806] An "effective amount" of the composition is provided based, at least in part, on the target tissue, target cell type, means of administration, physical characteristics of the nucleic acid (e.g., size, and extent of modified nucleosides), and other determinants. In general, an effective amount of the composition provides efficient protein production in the cell, preferably more efficient than a composition containing a corresponding unmodified nucleic acid. Increased efficiency may be demonstrated by increased cell transfection (i.e., the percentage of cells transfected with the nucleic acid), increased protein translation from the nucleic acid, decreased nucleic acid degradation (as demonstrated, e.g., by increased duration of protein translation from a modified nucleic acid), or reduced innate immune response of the host cell.

[000807] Aspects of the invention are directed to methods of inducing *in vivo* translation of a recombinant polypeptide in a mammalian subject in need thereof. Therein, an effective amount of a composition containing a nucleic acid that has at least one structural or chemical modification and a translatable region encoding the recombinant polypeptide is administered to the subject using the delivery methods described herein. The nucleic acid is provided in an amount and under other conditions such that the nucleic acid is localized into a cell of the subject and the recombinant polypeptide is translated in the cell from the nucleic acid. The cell in which the nucleic acid is localized, or the tissue in which the cell is present, may be targeted with one or more than one rounds of nucleic acid administration.

[000808] In certain embodiments, the administered polynucleotide, primary construct or mmRNA directs production of one or more recombinant polypeptides that provide a functional activity which is substantially absent in the cell, tissue or organism in which the recombinant polypeptide is translated. For example, the missing functional activity may be enzymatic, structural, or gene regulatory in nature. In related embodiments, the administered polynucleotide, primary construct or mmRNA directs production of one or more recombinant polypeptides that increases (e.g., synergistically) a functional activity

which is present but substantially deficient in the cell in which the recombinant polypeptide is translated.

[000809] In other embodiments, the administered polynucleotide, primary construct or mmRNA directs production of one or more recombinant polypeptides that replace a polypeptide (or multiple polypeptides) that is substantially absent in the cell in which the recombinant polypeptide is translated. Such absence may be due to genetic mutation of the encoding gene or regulatory pathway thereof. In some embodiments, the recombinant polypeptide increases the level of an endogenous protein in the cell to a desirable level; such an increase may bring the level of the endogenous protein from a subnormal level to a normal level or from a normal level to a super-normal level.

[000810] Alternatively, the recombinant polypeptide functions to antagonize the activity of an endogenous protein present in, on the surface of, or secreted from the cell. Usually, the activity of the endogenous protein is deleterious to the subject; for example, due to mutation of the endogenous protein resulting in altered activity or localization. Additionally, the recombinant polypeptide antagonizes, directly or indirectly, the activity of a biological moiety present in, on the surface of, or secreted from the cell. Examples of antagonized biological moieties include lipids (e.g., cholesterol), a lipoprotein (e.g., low density lipoprotein), a nucleic acid, a carbohydrate, a protein toxin such as shiga and tetanus toxins, or a small molecule toxin such as botulinum, cholera, and diphtheria toxins. Additionally, the antagonized biological molecule may be an endogenous protein that exhibits an undesirable activity, such as a cytotoxic or cytostatic activity.

[000811] The recombinant proteins described herein may be engineered for localization within the cell, potentially within a specific compartment such as the nucleus, or are engineered for secretion from the cell or translocation to the plasma membrane of the cell.

[000812] In some embodiments, modified mRNAs and their encoded polypeptides in accordance with the present invention may be used for treatment of any of a variety of diseases, disorders, and/or conditions, including but not limited to one or more of the following: autoimmune disorders (*e.g.* diabetes, lupus, multiple sclerosis, psoriasis, rheumatoid arthritis); inflammatory disorders (*e.g.* arthritis, pelvic inflammatory disease); infectious diseases (*e.g.* viral infections (*e.g.*, HIV, HCV, RSV), bacterial infections,

fungal infections, sepsis); neurological disorders (*e.g.* Alzheimer's disease, Huntington's disease; autism; Duchenne muscular dystrophy); cardiovascular disorders (*e.g.* atherosclerosis, hypercholesterolemia, thrombosis, clotting disorders, angiogenic disorders such as macular degeneration); proliferative disorders (*e.g.* cancer, benign neoplasms); respiratory disorders (*e.g.* chronic obstructive pulmonary disease); digestive disorders (*e.g.* inflammatory bowel disease, ulcers); musculoskeletal disorders (*e.g.* fibromyalgia, arthritis); endocrine, metabolic, and nutritional disorders (*e.g.* diabetes, osteoporosis); urological disorders (*e.g.* renal disease); psychological disorders (*e.g.* depression, schizophrenia); skin disorders (*e.g.* wounds, eczema); blood and lymphatic disorders (*e.g.* anemia, hemophilia); *etc.*

[000813] Diseases characterized by dysfunctional or aberrant protein activity include cystic fibrosis, sickle cell anemia, epidermolysis bullosa, amyotrophic lateral sclerosis, and glucose-6-phosphate dehydrogenase deficiency. The present invention provides a method for treating such conditions or diseases in a subject by introducing nucleic acid or cell-based therapeutics containing the polynucleotide, primary construct or mmRNA provided herein, wherein the polynucleotide, primary construct or mmRNA encode for a protein that antagonizes or otherwise overcomes the aberrant protein activity present in the cell of the subject. Specific examples of a dysfunctional protein are the missense mutation variants of the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which produce a dysfunctional protein variant of CFTR protein, which causes cystic fibrosis.

[000814] Diseases characterized by missing (or substantially diminished such that proper (normal or physiological protein function does not occur) protein activity include cystic fibrosis, Niemann-Pick type C, β thalassemia major, Duchenne muscular dystrophy, Hurler Syndrome, Hunter Syndrome, and Hemophilia A. Such proteins may not be present, or are essentially non-functional. The present invention provides a method for treating such conditions or diseases in a subject by introducing nucleic acid or cell-based therapeutics containing the polynucleotide, primary construct or mmRNA provided herein, wherein the polynucleotide, primary construct or mmRNA encode for a protein that replaces the protein activity missing from the target cells of the subject. Specific examples of a dysfunctional protein are the nonsense mutation variants of the

cystic fibrosis transmembrane conductance regulator (CFTR) gene, which produce a nonfunctional protein variant of CFTR protein, which causes cystic fibrosis.

[000815] Thus, provided are methods of treating cystic fibrosis in a mammalian subject by contacting a cell of the subject with a polynucleotide, primary construct or mmRNA having a translatable region that encodes a functional CFTR polypeptide, under conditions such that an effective amount of the CTFR polypeptide is present in the cell. Preferred target cells are epithelial, endothelial and mesothelial cells, such as the lung, and methods of administration are determined in view of the target tissue; i.e., for lung delivery, the RNA molecules are formulated for administration by inhalation.

[000816] In another embodiment, the present invention provides a method for treating hyperlipidemia in a subject, by introducing into a cell population of the subject with a modified mRNA molecule encoding Sortilin, a protein recently characterized by genomic studies, thereby ameliorating the hyperlipidemia in a subject. The *SORT1* gene encodes a trans-Golgi network (TGN) transmembrane protein called Sortilin. Genetic studies have shown that one of five individuals has a single nucleotide polymorphism, rs12740374, in the 1p13 locus of the SORT1 gene that predisposes them to having low levels of low-density lipoprotein (LDL) and very-low-density lipoprotein (VLDL). Each copy of the minor allele, present in about 30% of people, alters LDL cholesterol by 8 mg/dL, while two copies of the minor allele, present in about 5% of the population, lowers LDL cholesterol 16 mg/dL. Carriers of the minor allele have also been shown to have a 40% decreased risk of myocardial infarction. Functional *in vivo* studies in mice describes that overexpression of *SORT1* in mouse liver tissue led to significantly lower LDL-cholesterol levels, as much as 80% lower, and that silencing SORT1 increased LDL cholesterol approximately 200% (Musunuru K et al. From noncoding variant to phenotype via *SORT1* at the 1p13 cholesterol locus. *Nature* 2010; 466: 714-721).

[000817] In another embodiment, the present invention provides a method for treating hematopoietic disorders, cardiovascular disease, oncology, diabetes, cystic fibrosis, neurological diseases, inborn errors of metabolism, skin and systemic disorders, and blindness. The identity of molecular targets to treat these specific diseases has been described (Templeton ed., Gene and Cell Therapy: Therapeutic Mechanisms and

Strategies, 3rd Edition, Bota Raton, FL:CRC Press; herein incorporated by reference in its entirety).

[000818] Provided herein, are methods to prevent infection and/or sepsis in a subject at risk of developing infection and/or sepsis, the method comprising administering to a subject in need of such prevention a composition comprising a polynucleotide, primary construct or mmRNA precursor encoding an anti-microbial polypeptide (*e.g.*, an anti-bacterial polypeptide), or a partially or fully processed form thereof in an amount sufficient to prevent infection and/or sepsis. In certain embodiments, the subject at risk of developing infection and/or sepsis may be a cancer patient. In certain embodiments, the cancer patient may have undergone a conditioning regimen. In some embodiments, the conditioning regiment may include, but is not limited to, chemotherapy, radiation therapy, or both. As a non-limiting example, a polynucleotide, primary construct or mmRNA can encode Protein C, its zymogen or prepro-protein, the activated form of Protein C (APC) or variants of Protein C which are known in the art. The polynucleotides, primary constructs or mmRNA may be chemically modified and delivered to cells. Non-limiting examples of polypeptides which may be encoded within the chemically modified mRNAs of the present invention include those taught in US Patents 7,226,999; 7,498,305; 6,630,138 each of which is incorporated herein by reference in its entirety. These patents teach Protein C like molecules, variants and derivatives, any of which may be encoded within the chemically modified molecules of the present invention.

[000819] Further provided herein, are methods to treat infection and/or sepsis in a subject, the method comprising administering to a subject in need of such treatment a composition comprising a polynucleotide, primary construct or mmRNA precursor encoding an anti-microbial polypeptide (*e.g.*, an anti-bacterial polypeptide), *e.g.*, an anti-microbial polypeptide described herein, or a partially or fully processed form thereof in an amount sufficient to treat an infection and/or sepsis. In certain embodiments, the subject in need of treatment is a cancer patient. In certain embodiments, the cancer patient has undergone a conditioning regimen. In some embodiments, the conditioning regiment may include, but is not limited to, chemotherapy, radiation therapy, or both.

[000820] In certain embodiments, the subject may exhibits acute or chronic microbial infections (*e.g.*, bacterial infections). In certain embodiments, the subject may have received or may be receiving a therapy. In certain embodiments, the therapy may include, but is not limited to, radiotherapy, chemotherapy, steroids, ultraviolet radiation, or a combination thereof. In certain embodiments, the patient may suffer from a microvascular disorder. In some embodiments, the microvascular disorder may be diabetes. In certain embodiments, the patient may have a wound. In some embodiments, the wound may be an ulcer. In a specific embodiment, the wound may be a diabetic foot ulcer. In certain embodiments, the subject may have one or more burn wounds. In certain embodiments, the administration may be local or systemic. In certain embodiments, the administration may be subcutaneous. In certain embodiments, the administration may be intravenous. In certain embodiments, the administration may be oral. In certain embodiments, the administration may be topical. In certain embodiments, the administration may be by inhalation. In certain embodiments, the administration may be rectal. In certain embodiments, the administration may be vaginal.

[000821] Other aspects of the present disclosure relate to transplantation of cells containing polynucleotide, primary construct, or mmRNA to a mammalian subject. Administration of cells to mammalian subjects is known to those of ordinary skill in the art, and include, but is not limited to, local implantation (e.g., topical or subcutaneous administration), organ delivery or systemic injection (e.g., intravenous injection or inhalation), and the formulation of cells in pharmaceutically acceptable carrier. Such compositions containing polynucleotide, primary construct, or mmRNA can be formulated for administration intramuscularly, transarterially, intraperitoneally, intravenously, intranasally, subcutaneously, endoscopically, transdermally, or intrathecally. In some embodiments, the composition may be formulated for extended release.

[000822] The subject to whom the therapeutic agent may be administered suffers from or may be at risk of developing a disease, disorder, or deleterious condition. Provided are methods of identifying, diagnosing, and classifying subjects on these bases, which may include clinical diagnosis, biomarker levels, genome-wide association studies (GWAS), and other methods known in the art.

Rare Liver Diseases or Disorders

Progressive Familial Intrahepatic Cholestasis (PFIC)

[000823] In one embodiment, the rare liver disease or disorder polynucleotide, primary construct, or mmRNA of the present invention may be used to treat progressive familial intrahepatic cholestasis (PFIC). The term "progressive familial intrahepatic cholestasis" or "PFIC" as used herein refers to a liver disorder that can lead to liver failure. PFIC is characterized by autosomal recessive defects in bile formation and hepatocellular cholestasis. As used herein, "hepatocellular" is a term used to describe something that affects or pertains to liver cells (also referred to as hepatocytes). As used herein, the term "cholestasis" refers to a condition characterized by slowed or disrupted flow of bile from the liver. As used herein, the term "bile" refers to a liquid substance produced by the liver comprising water, bile salts, mucus, fats inorganic salts and cholesterol, which aids in the emulsification and digestion of dietary fats.

[000824] There are three known types of PFIC (PFIC-1, PFIC-2 and PFIC-3), and they have been traced to mutations in genes encoding proteins involved in the hepatocellular transport system and in the formation of bile.

[000825] PFIC-1 and PFIC-2 are typically diagnosed in infancy but diagnosis may occur during the prenatal period or the neonatal period. As used herein, the term "prenatal" refers to the period of time in the life of an organism that occurs before birth. As used herein, the term "neonatal" refers to a period of time in the life of an organism that occurs after birth. In humans, the neonatal period may include a period of time from birth to about 1 month, to about 3 months or to about 6 months after birth. As used herein, the term "infancy" refers to a period in the life of an organism that occurs between birth and childhood. In humans, infancy may include the period of time from birth to about 1 year of life, to about 2 years of life, to about 3 years of life or to about 4 years of life.

[000826] PFIC-3 may be diagnosed during the prenatal period, during the neonatal period or during infancy. In some cases, PFIC-3 may be able evade diagnosis until childhood or young adulthood. As used herein, the term "childhood" refers to the period of time in the life of an organism that occurs after infancy and before young adulthood. In humans, childhood may include the period of time from about 2 years of age to about 10

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JUMBO APPLICATIONS/PATENTS

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Claims

We claim:

1. An isolated polynucleotide comprising;

(a) a first region of linked nucleosides, said first region encoding a polypeptide of interest, said polypeptide of interest selected from the group consisting of SEQ ID NOs 769-1392;

(b) a first flanking region located at the 5′ terminus of said first region comprising;

(i) a sequence of linked nucleosides selected from the group consisting of the native 5′ UTR of any of the nucleic acids that encode any of SEQ ID NOs 769-1392, SEQ ID NOs: 1-4 and functional variants thereof; and

(ii) at least one 5′ terminal cap;

(c) a second flanking region located at the 3′ terminus of said first region comprising;

(i') a sequence of linked nucleosides selected from the group consisting of the native 3′ UTR of any of the nucleic acids that encode any of SEQ ID NOs 769-1392, SEQ ID NOs 5-21 and functional variants thereof; and

(ii') a 3′ tailing sequence of linked nucleosides.

2. The isolated polynucleotide of claim 1 wherein the first region of linked nucleosides comprises at least an open reading frame of a nucleic acid sequence, wherein the nucleic acid sequence selected from the group consisting of SEQ ID NOs: 1393-21423, 21435-21438, 21442-21443, 21449, 21451-21473, 21521-21542, 21639-21642, 21671-21679, 21681, 21682, 21684, 21686, 21687, 21724-21726, 21728 and 21730-21738.

3. The isolated polynucleotide of claim 1, wherein the 3′tailing sequence of linked nucleosides is selected from the group consisting of a poly-A tail of approximately 160 nucleotides and a polyA-G quartet.

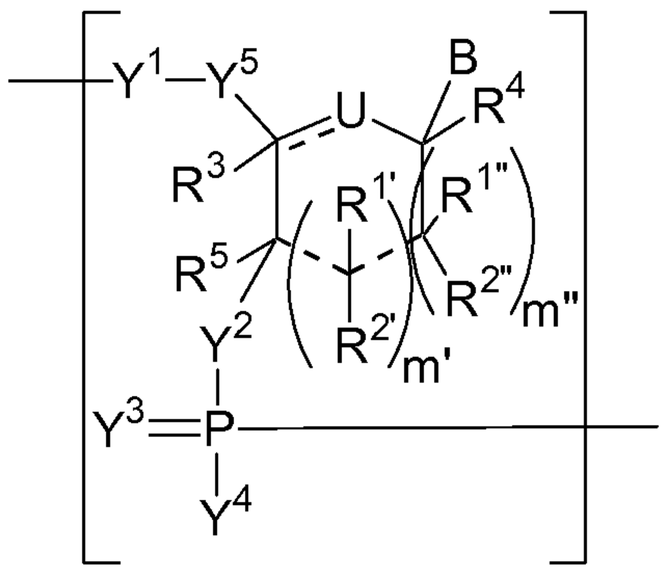
4. The isolated polynucleotide of any one of claims 1-3 which is purified.

5. The isolated polynucleotide of any one of claims 1-4, wherein the at least one 5′ terminal cap is selected from the group consisting of Cap0, Cap1, ARCA, inosine, N1-methyl-guanosine, 2′fluoro-guanosine, 7-deaza-guanosine, 8-oxo-guanosine, 2-amino-guanosine, LNA-guanosine, and 2-azido-guanosine.

6. The isolated polynucleotide of any preceding claim, wherein at least one of said linked nucleosides comprises at least one modification as compared to the chemical structure of an A, G, U or C ribonucleotide.

7. The isolated polynucleotide of claim 6, wherein at least one said modification is located in a nucleoside base and/or sugar portion.

8. The isolated polynucleotide of any one of claims 1-7, wherein said first region comprises n number of linked nucleosides having Formula (Ia):



(Ia), or a pharmaceutically acceptable salt or stereoisomer thereof,

wherein

U is O, S, $N(R^U)_{nu}$, or $C(R^U)_{nu}$, wherein nu is an integer from 0 to 2 and each R^U is, independently, H, halo, or optionally substituted alkyl;

$\overset{---}{=}$ is a single or double bond;

--- is a single bond or absent;

each of $R^{1'}$, $R^{2'}$, $R^{1''}$, $R^{2''}$, R^3, R^4, and R^5 is, independently, H, halo, hydroxy, thiol, optionally substituted alkyl, optionally substituted alkoxy, optionally substituted

alkenyloxy, optionally substituted alkynyloxy, optionally substituted aminoalkoxy, optionally substituted alkoxyalkoxy, optionally substituted hydroxyalkoxy, optionally substituted amino, azido, optionally substituted aryl, optionally substituted aminoalkyl, or absent; wherein the combination of R^3 with one or more of $R^{1'}$, $R^{1''}$, $R^{2'}$, $R^{2''}$, or R^5 can join together to form optionally substituted alkylene or optionally substituted heteroalkylene and, taken together with the carbons to which they are attached, provide an optionally substituted heterocyclyl; wherein the combination of R^5 with one or more of $R^{1'}$, $R^{1''}$, $R^{2'}$, or $R^{2''}$ can join together to form optionally substituted alkylene or optionally substituted heteroalkylene and, taken together with the carbons to which they are attached, provide an optionally substituted heterocyclyl; and wherein the combination of R^4 and one or more of $R^{1'}$, $R^{1''}$, $R^{2'}$, $R^{2''}$, R^3, or R^5 can join together to form optionally substituted alkylene or optionally substituted heteroalkylene and, taken together with the carbons to which they are attached, provide an optionally substituted heterocyclyl;

each of Y^1, Y^2, and Y^3, is, independently, O, S, $-NR^{N1}-$, optionally substituted alkylene, or optionally substituted heteroalkylene, wherein R^{N1} is H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted aryl, or absent;

each Y^4 is, independently, H, hydroxy, thiol, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted thioalkoxy, optionally substituted alkoxyalkoxy, or optionally substituted amino;

each Y^5 is, independently, O, S, optionally substituted alkylene, or optionally substituted heteroalkylene;

n is an integer from 1 to 100,000; and

B is a nucleobase, wherein the combination of B and $R^{1'}$, the combination of B and $R^{2'}$, the combination of B and $R^{1''}$, or the combination of B and $R^{2''}$ can, taken together with the carbons to which they are attached, optionally form a bicyclic group or wherein the combination of B, $R^{1''}$, and R^3 or the combination of B, $R^{2''}$, and R^3 can optionally form a tricyclic or tetracyclic group.

9. The isolated polynucleotide of claim 8, wherein B is not pseudouridine (ψ) or 5-methyl-cytidine (m^5C).

10. The isolated polynucleotide of any one of claims 8-9, wherein

U is O or $C(R^U)_{nu}$, wherein nu is an integer from 1 to 2 and each R^U is, independently, H, halo, or optionally substituted alkyl;

each of R^1, $R^{1'}$, $R^{1''}$, R^2, $R^{2'}$, and $R^{2''}$, if present, is, independently, H, halo, hydroxy, optionally substituted alkyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted aminoalkoxy, optionally substituted alkoxyalkoxy, optionally substituted amino, azido, optionally substituted aryl, or optionally substituted aminoalkyl;

each of R^3 and R^4 is, independently, H, halo, hydroxy, optionally substituted alkyl, or optionally substituted alkoxyalkoxy;

each of Y^1, Y^2, and Y^3, is, independently, O, S, $-NR^{N1}-$, optionally substituted alkylene, or optionally substituted heteroalkylene, wherein R^{N1} is H, optionally substituted alkyl, optionally substituted alkenyl, or optionally substituted alkynyl;

each Y^4 is, independently, H, hydroxy, thiol, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted thioalkoxy, or optionally substituted amino;

each Y^5 is, independently, O or optionally substituted alkylene; and

n is an integer from 10 to 10,000.

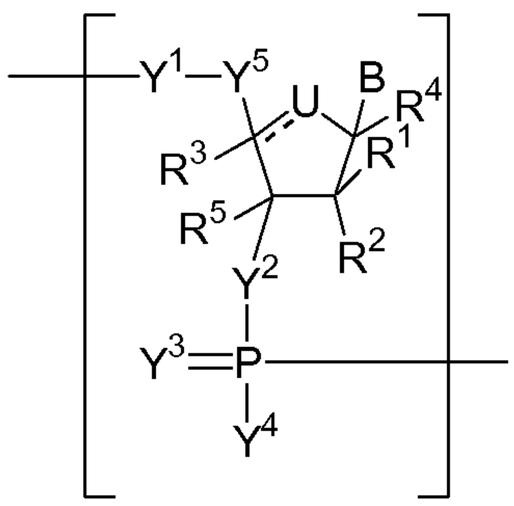
11. The isolated polynucleotide of claim 10, wherein each of R^1, $R^{1'}$, and $R^{1''}$, if present, is H.

12. The isolated polynucleotide of claim 11, wherein each of R^2, $R^{2'}$, and $R^{2''}$, if present, is, independently, H, halo, hydroxy, optionally substituted alkoxy, or optionally substituted alkoxyalkoxy.

13. The isolated polynucleotide of claim 10, wherein each of R^2, $R^{2'}$, and $R^{2''}$, if present, is H.

14. The isolated polynucleotide of claim 13, wherein each of R^1, $R^{1'}$, and $R^{1''}$, if present, is, independently, H, halo, hydroxy, optionally substituted alkoxy, or optionally substituted alkoxyalkoxy.

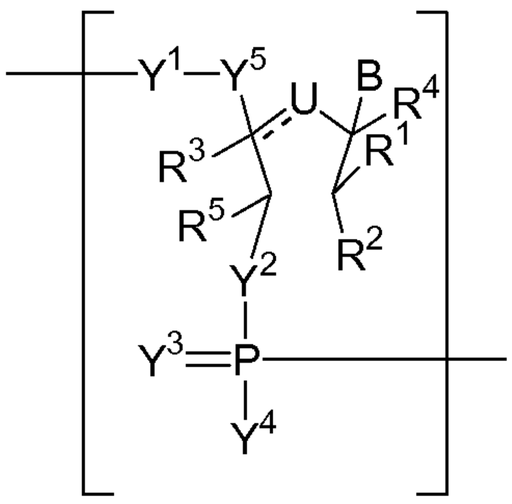
15. The isolated polynucleotide of claim 8, wherein said first region comprises n number of linked nucleotides having Formula (IIa):



(IIa), or a pharmaceutically acceptable salt or stereoisomer thereof.

16. isolated The polypeptide of claim 15, wherein said first region comprises n number of linked nucleosides having Formula (IIb) or (IIc), or a pharmaceutically acceptable salt thereof.

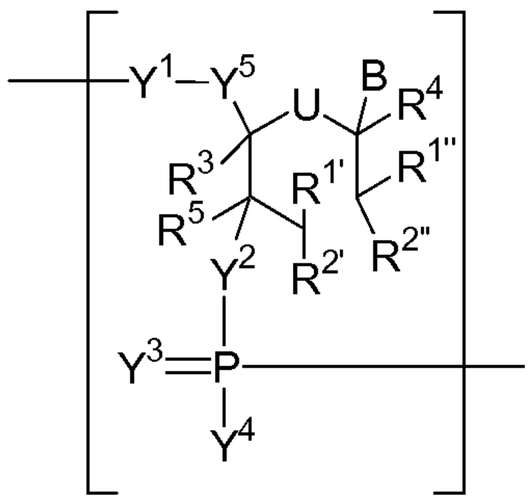
17. The isolated polynucleotide of claim 8, wherein said first region comprises n number of linked nucleosides having Formula (IId):



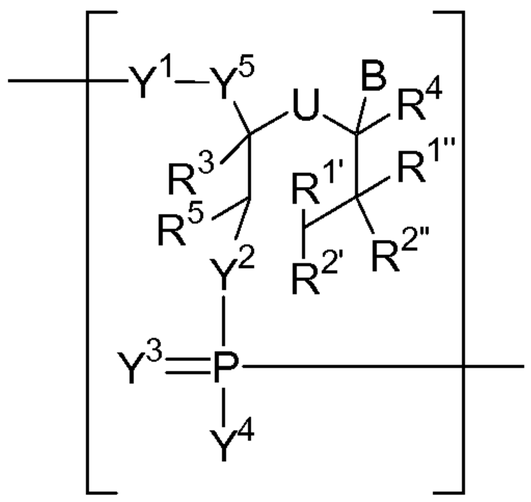
(IId), or a pharmaceutically acceptable salt or stereoisomer thereof.

18. The isolated polypeptide of claim 17, wherein said first region comprises n number of linked nucleosides having Formula (IIe) or (IIf), or a pharmaceutically acceptable salt thereof.

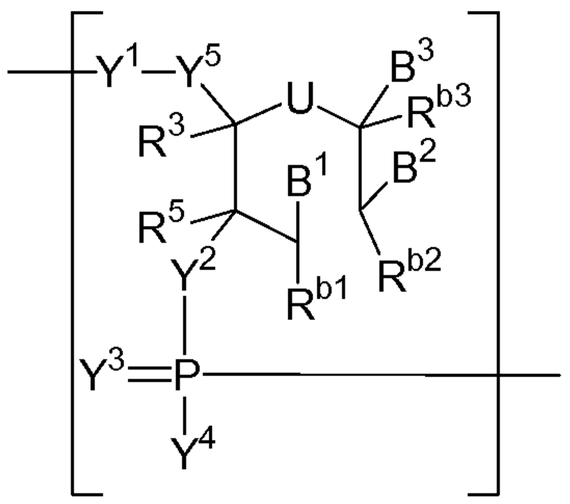
19. The isolated polynucleotide of claim 8, wherein said first region comprises n number of linked nucleotides, each of said linked nucleotides independently having one of Formulas (IIg)-(IIj):



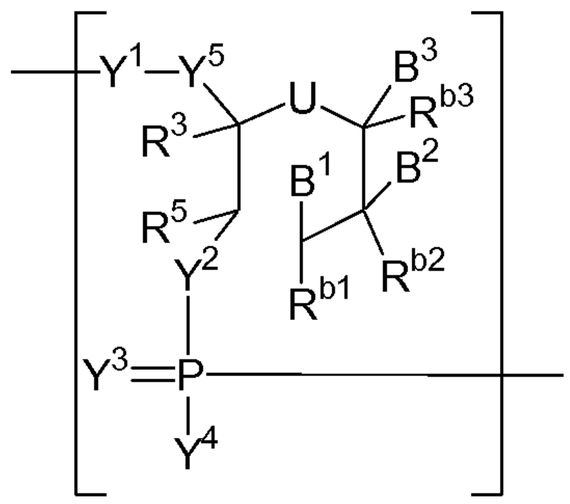
(IIg),



(IIh),

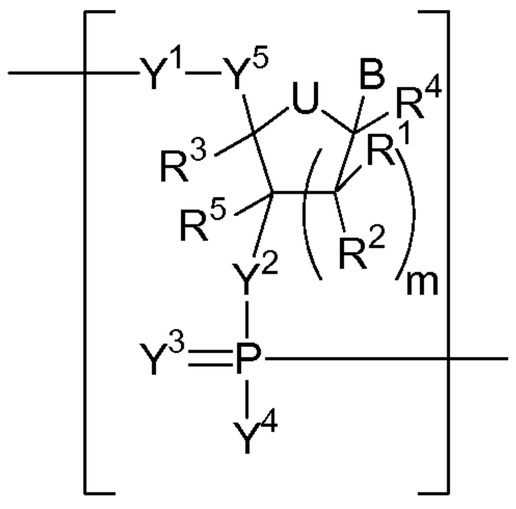


(IIi), or



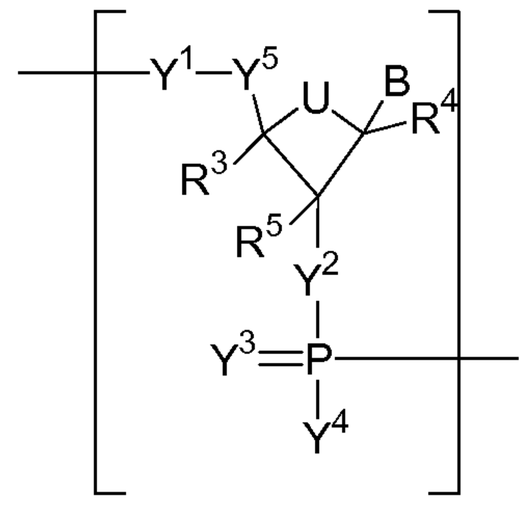
(IIj), or a pharmaceutically acceptable salt or stereoisomer thereof.

20. The isolated polynucleotide of claim 8, wherein said first region comprises n number of linked nucleosides having Formula (IIk):



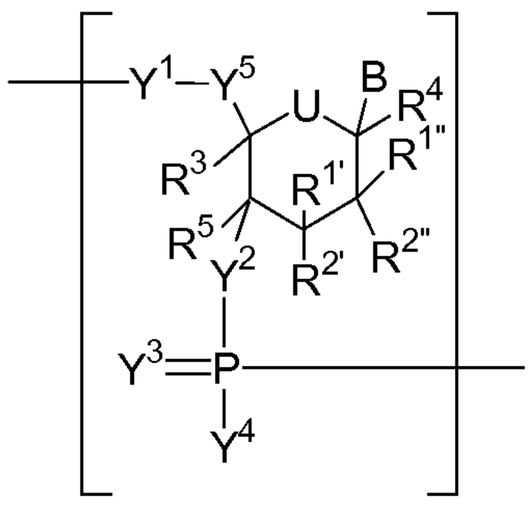
(IIk) , or a pharmaceutically acceptable salt or stereoisomer thereof.

21. The isolated polynucleotide of claim 20, wherein said first region comprises n number of linked nucleosides having Formula (III):



(III), or a pharmaceutically acceptable salt or stereoisomer thereof.

22. The isolated polynucleotide of claim 20, wherein said first region comprises n number of linked nucleosides having Formula (IIm):



(IIm), or a pharmaceutically acceptable salt or stereoisomer thereof,

wherein

each of $R^{1'}$, $R^{1''}$, $R^{2'}$, and $R^{2''}$ is, independently, H, halo, hydroxy, optionally substituted alkyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted aminoalkoxy, optionally substituted alkoxyalkoxy, or absent; and wherein the combination of $R^{2'}$ and R^3 or the combination of $R^{2''}$ and R^3 can be taken together to form optionally substituted alkylene or optionally substituted heteroalkylene.

23. The isolated polynucleotide of any one of claims 11-22, wherein

U is O or $C(R^U)_{nu}$, wherein nu is an integer from 1 to 2 and each R^U is, independently, H, halo, or optionally substituted alkyl;

each of R^1 and R^2 is, independently, H, halo, hydroxy, optionally substituted alkyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted aminoalkoxy, optionally substituted alkoxyalkoxy, optionally substituted amino, azido, optionally substituted aryl, or optionally substituted aminoalkyl;

each of R^3 and R^4 is, independently, H or optionally substituted alkyl;

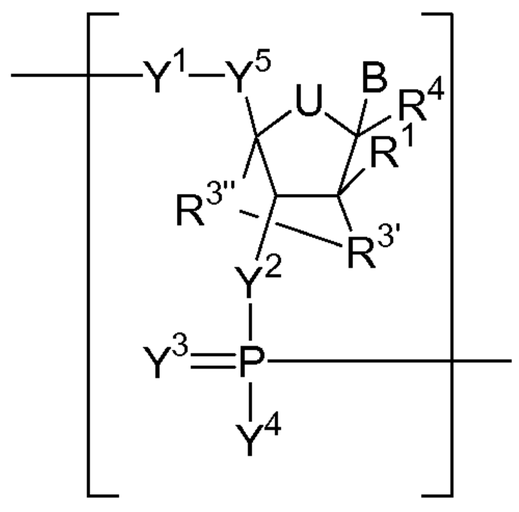
each of Y^1, Y^2, and Y^3, is, independently, O, S, $-NR^{N1}-$, optionally substituted alkylene, or optionally substituted heteroalkylene, wherein R^{N1} is H, optionally substituted alkyl, optionally substituted alkenyl, or optionally substituted alkynyl;

each Y^4 is, independently, H, hydroxy, thiol, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted thioalkoxy, or optionally substituted amino;

each Y^5 is, independently, O or optionally substituted alkylene; and

n is an integer from 10 to 10,000.

24. The isolated polynucleotide of claim 8, wherein said first region comprises n number of linked nucleosides having Formula (IIn):



(IIn), or a pharmaceutically acceptable salt or stereoisomer thereof,

wherein

U is O or $C(R^U)_{nu}$, wherein nu is an integer from 1 to 2 and each R^U is, independently, H, halo, or optionally substituted alkyl;

each of R^1 and R^4 is, independently, H, halo, hydroxy, optionally substituted alkyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted aminoalkoxy, optionally substituted

alkoxyalkoxy, optionally substituted amino, azido, optionally substituted aryl, or optionally substituted aminoalkyl;

$R^{3'}$ is O, S, or -NR^{N1}-, wherein R^{N1} is H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, or optionally substituted aryl;

$R^{3''}$ is optionally substituted alkylene or optionally substituted heteroalkylene;

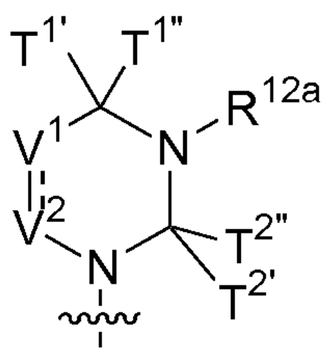
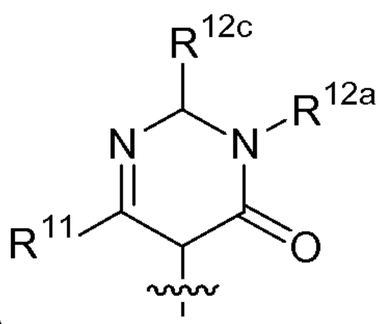
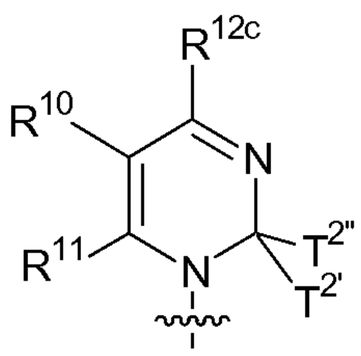
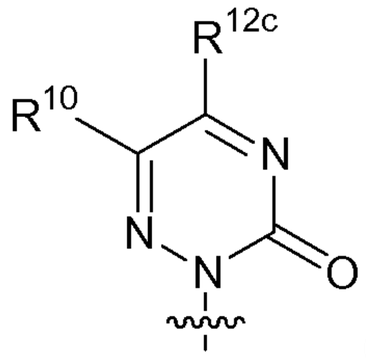
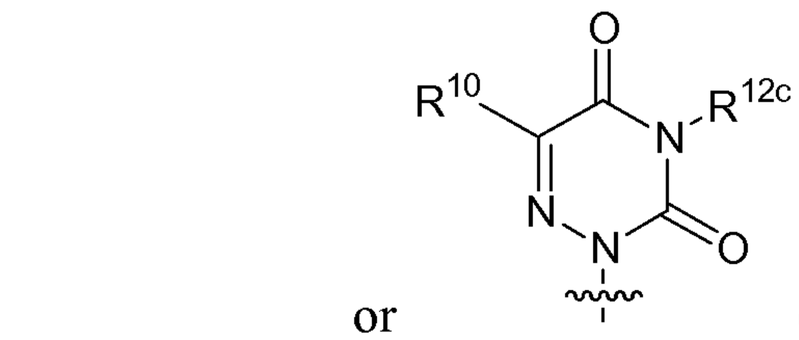
each of Y^1, Y^2, and Y^3, is, independently, O, S, -NR^{N1}-, optionally substituted alkylene, or optionally substituted heteroalkylene, wherein R^{N1} is H, optionally substituted alkyl, optionally substituted alkenyl, or optionally substituted alkynyl;

each Y^4 is, independently, H, hydroxy, thiol, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted thioalkoxy, or optionally substituted amino;

each Y^5 is, independently, O, S, optionally substituted alkylene (e.g., methylene), or optionally substituted heteroalkylene; and

n is an integer from 10 to 10,000.

25. The isolated polynucleotide of any one of claims 8-24, wherein in said n number of B has, each B independently has a formula selected from Formula (b1)-(b5):


(b1),

(b2),

(b3),

(b4),

or

(b5), or a pharmaceutically acceptable salt or stereoisomer thereof,

wherein

 connecting V^1 and V^2 in formula (b1) is a single or double bond;

each of $T^{1'}$, $T^{1''}$, $T^{2'}$, and $T^{2''}$ is, independently, H, optionally substituted alkyl, optionally substituted alkoxy, or optionally substituted thioalkoxy, or the combination of $T^{1'}$ and $T^{1''}$ or the combination of $T^{2'}$ and $T^{2''}$ join together to form O (oxo), S (thio), or Se (seleno);

each of V^1 and V^2 is, independently, O, S, $N(R^{Vb})_{nv}$, or $C(R^{Vb})_{nv}$, wherein nv is an integer from 0 to 2 and each R^{Vb} is, independently, H, halo, optionally substituted amino acid, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted alkoxy, optionally substituted alkenyloxy, or optionally substituted alkynyloxy;

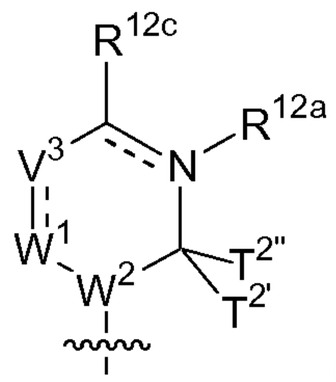
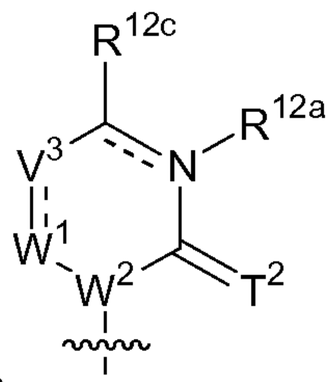
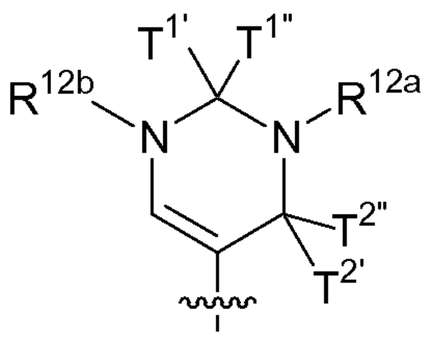
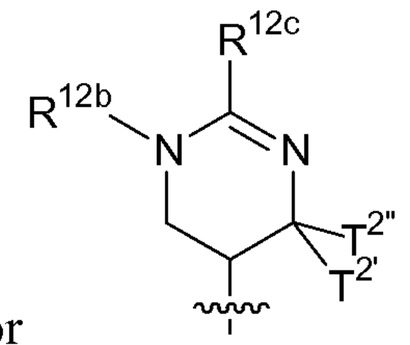
R^{10} is H, halo, optionally substituted amino acid, hydroxy, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted aminoalkyl, optionally substituted alkoxy, optionally substituted alkoxycarbonylalkyl, optionally substituted alkoxycarbonylalkoxy, optionally substituted carboxyalkoxy, optionally substituted carboxyalkyl, or optionally substituted carbamoylalkyl;

R^{11} is H or optionally substituted alkyl;

R^{12a} is H, optionally substituted alkyl, or optionally substituted aminoalkyl; and

R^{12c} is H, halo, optionally substituted alkyl, optionally substituted alkoxy, optionally substituted thioalkoxy, optionally substituted amino, or optionally substituted aminoalkyl.

26. The isolated polynucleotide of any one of claims 8-25, wherein n number of B has Formula (b6)-(b9):


(b6),

(b7),

(b8), or

(b9), or a pharmaceutically acceptable salt or stereoisomer thereof,

wherein

⚌ is a single or double bond;

each of $T^{1'}$, $T^{1''}$, $T^{2'}$, and $T^{2''}$ is, independently, H, optionally substituted alkyl, optionally substituted alkoxy, or optionally substituted thioalkoxy, or the combination of $T^{1'}$ and $T^{1''}$ or the combination of $T^{2'}$ and $T^{2''}$ join together to form O (oxo), S (thio), or Se (seleno);

each of W^1 and W^2 is, independently, $N(R^{Wa})_{nw}$ or $C(R^{Wa})_{nw}$, wherein nw is an integer from 0 to 2 and each R^{Wa} is, independently, H, optionally substituted alkyl, or optionally substituted alkoxy;

each V^3 is, independently, O, S, $N(R^{Va})_{nv}$, or $C(R^{Va})_{nv}$, wherein nv is an integer from 0 to 2 and each R^{Va} is, independently, H, halo, optionally substituted amino acid, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted heterocyclyl, optionally substituted alkheterocyclyl, optionally substituted alkoxy, optionally substituted alkenyloxy, or optionally substituted alkynyloxy, and wherein R^{Va} and R^{12c} taken together with the carbon atoms to which they are attached can form optionally substituted cycloalkyl, optionally substituted aryl, or optionally substituted heterocyclyl;

R^{12a} is H, optionally substituted alkyl, optionally substituted aminoalkyl, or absent;

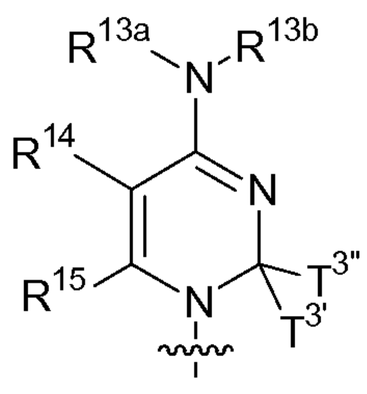
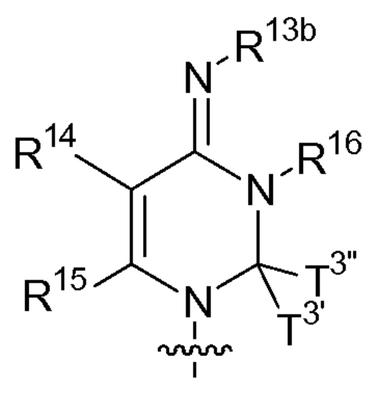
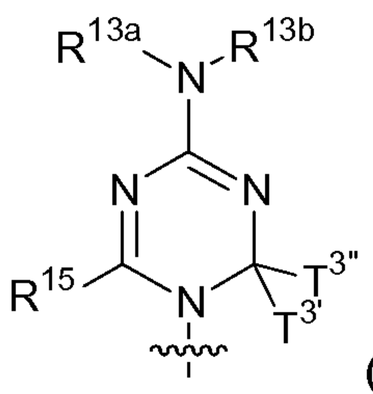
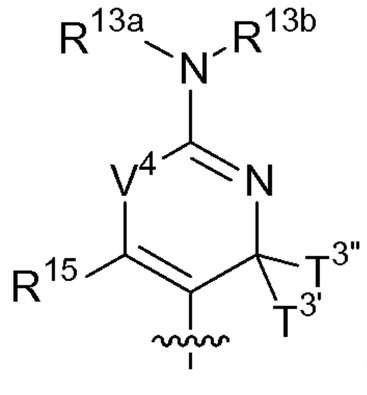
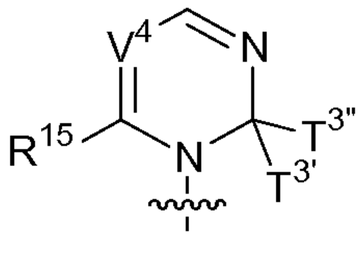
R^{12b} is H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted alkaryl, optionally substituted heterocyclyl, optionally substituted alkheterocyclyl, or optionally substituted amino acid, wherein the combination of R^{12b} and $T^{1'}$ or the combination of R^{12b} and R^{12c} can join together to form optionally substituted heterocyclyl; and

R^{12c} is H, halo, optionally substituted alkyl, optionally substituted alkoxy, optionally substituted thioalkoxy, optionally substituted amino, or optionally substituted aminoalkyl.

27. The isolated polynucleotide of claim 26, wherein R^{12a}, R^{12b}, R^{12c}, or R^{Va} is substituted with $-(CH_2)_{s2}(OCH_2CH_2)_{s1}(CH_2)_{s3}OR'$, wherein s1 is an integer from 1 to 10, each of s2 and s3, independently, is an integer from 0 to 10, and R' is H or $C_{1\text{-}20}$ alkyl); or $-NR^{N1}(CH_2)_{s2}(CH_2CH_2O)_{s1}(CH_2)_{s3}NR^{N1}$, wherein s1 is an integer from 1 to 10, each of s2

and s3, independently, is an integer from 0 to 10, and each R^{N1} is, independently, hydrogen or optionally substituted C_{1-6} alkyl.

28. The isolated polynucleotide of any one of claims 8-27, wherein n number of B has Formula (b10)-(b14):


(b10),

(b11),

(b12),

(b13), or

(b14), or a pharmaceutically acceptable salt or stereoisomer thereof,

wherein

each of $T^{3'}$ and $T^{3''}$ is, independently, H, optionally substituted alkyl, optionally substituted alkoxy, or optionally substituted thioalkoxy, or the combination of $T^{3'}$ and $T^{3''}$ join together to form O (oxo), S (thio), or Se (seleno);

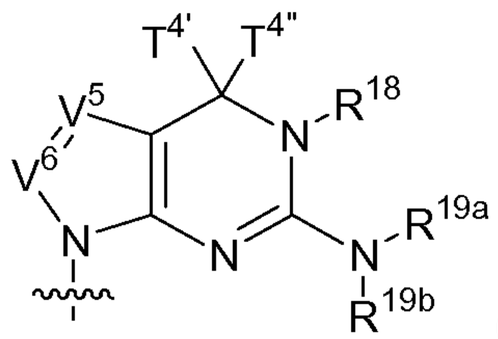
each V^4 is, independently, O, S, $N(R^{Vc})_{nv}$, or $C(R^{Vc})_{nv}$, wherein nv is an integer from 0 to 2 and each R^{Vc} is, independently, H, halo, optionally substituted amino acid, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted heterocyclyl, optionally substituted alkheterocyclyl, or optionally substituted alkynyloxy, wherein the combination of R^{13b} and R^{Vc} can be taken together to form optionally substituted heterocyclyl;

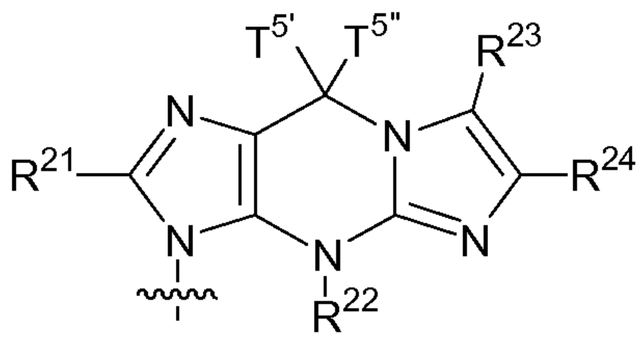
each of R^{13a} and R^{13b} is, independently, H, optionally substituted acyl, optionally substituted alkyl, or optionally substituted alkoxy, wherein the combination of R^{13b} and R^{14} can be taken together to form optionally substituted heterocyclyl;

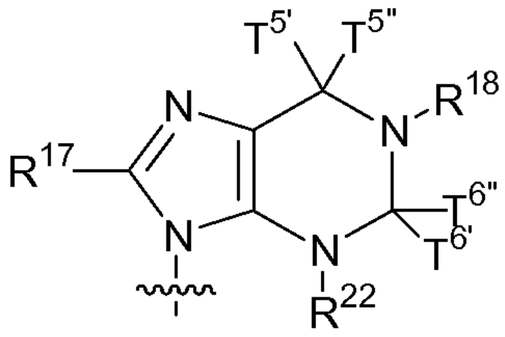
each R^{14} is, independently, H, halo, hydroxy, thiol, optionally substituted acyl, optionally substituted amino acid, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted hydroxyalkyl, optionally substituted alkoxy, optionally substituted alkenyloxy, optionally substituted alkynyloxy, optionally substituted aminoalkoxy, optionally substituted alkoxyalkoxy, optionally substituted amino, azido, optionally substituted aryl, optionally substituted heterocyclyl, optionally substituted alkheterocyclyl, or optionally substituted aminoalkyl; and

each of R^{15} and R^{16} is, independently, H, optionally substituted alkyl, optionally substituted alkenyl, or optionally substituted alkynyl.

29. The isolated polynucleotide of any one of claims 7-27, wherein n number of B has Formula (b15)-(b17):


(b15),


(b16), or


(b17), or a pharmaceutically acceptable salt or stereoisomer thereof,

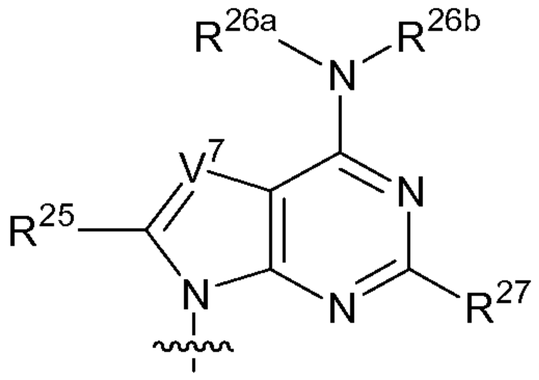
wherein

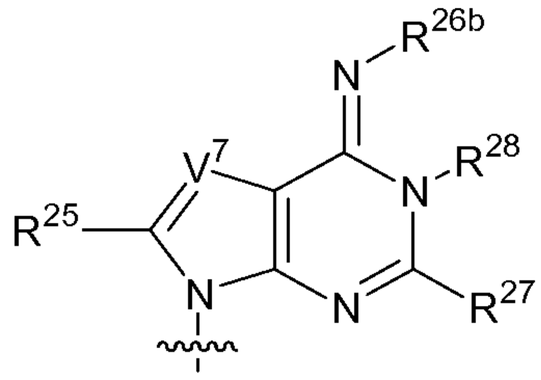
each of $T^{4'}$, $T^{4''}$, $T^{5'}$, $T^{5''}$, $T^{6'}$, and $T^{6''}$ is, independently, H, optionally substituted alkyl, or optionally substituted alkoxy, and wherein the combination of $T^{4'}$ and $T^{4''}$ or the combination of $T^{5'}$ and $T^{5''}$ or the combination of $T^{6'}$ and $T^{6''}$ together form O, S, or Se;

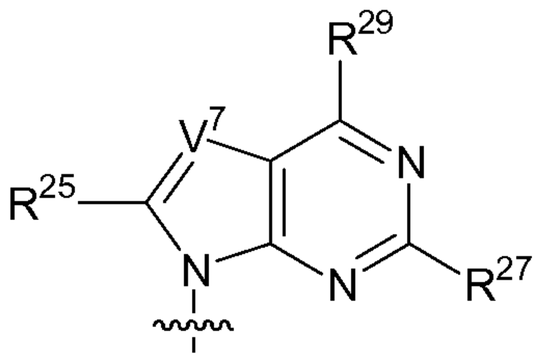
each of V^5 and V^6 is, independently, O, S, $N(R^{Vd})_{nv}$, or $C(R^{Vd})_{nv}$, wherein nv is an integer from 0 to 2 and each R^{Vd} is, independently, H, halo, optionally substituted amino acid, cyano, amidine, optionally substituted aminoalkyl, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted alkoxy, optionally substituted alkenyloxy, or optionally substituted alkynyloxy; and

each of R^{17}, R^{18}, R^{19a}, R^{19b}, R^{21}, R^{22}, R^{23}, and R^{24} is, independently, H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, or optionally substituted amino acid.

30. The polynucleotide of any one of claims 8-29, wherein n number of B has Formula (b18)-(b20):


(b18),


(b19), or


(b20), or a pharmaceutically acceptable salt or stereoisomer thereof,

wherein

each V^7 is, independently, O, S, $N(R^{Ve})_{nv}$, or $C(R^{Ve})_{nv}$, wherein nv is an integer from 0 to 2 and each R^{Ve} is, independently, H, halo, optionally substituted amino acid, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted alkoxy, optionally substituted alkenyloxy, or optionally substituted alkynyloxy;

each R^{25} is, independently, H, optionally substituted alkyl, optionally substituted alkenyl, or optionally substituted alkynyl;

each of R^{26a} and R^{26b} is, independently, H, optionally substituted acyl, optionally substituted amino acid, optionally substituted carbamoylalkyl, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted hydroxyalkyl, optionally substituted hydroxyalkenyl, or optionally substituted alkoxy;

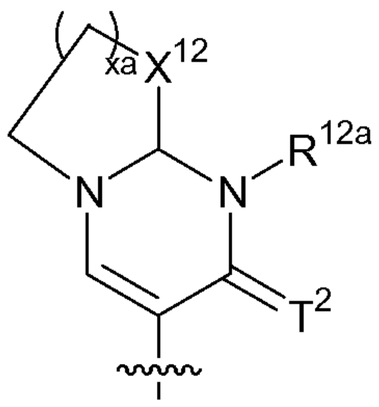
each R^{27} is, independently, H, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted alkoxy, or optionally substituted amino;

each R^{28} is, independently, H, optionally substituted alkyl, optionally substituted alkenyl, or optionally substituted alkynyl; and

each R^{29} is, independently, H, optionally substituted acyl, optionally substituted amino acid, optionally substituted carbamoylalkyl, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted hydroxyalkyl, optionally substituted hydroxyalkenyl, optionally substituted alkoxy, or optionally substituted amino.

31. The isolated polynucleotide of claim 30, wherein R^{26a}, R^{26b}, or R^{29} is substituted with $-(CH_2)_{s2}(OCH_2CH_2)_{s1}(CH_2)_{s3}OR'$, wherein s1 is an integer from 1 to 10, each of s2 and s3, independently, is an integer from 0 to 10, and R' is H or C_{1-20} alkyl); or $-NR^{N1}(CH_2)_{s2}(CH_2CH_2O)_{s1}(CH_2)_{s3}NR^{N1}$, wherein s1 is an integer from 1 to 10, each of s2 and s3, independently, is an integer from 0 to 10, and each R^{N1} is, independently, hydrogen or optionally substituted C_{1-6} alkyl.

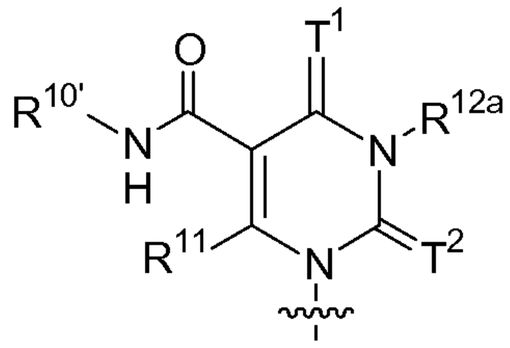
32. The isolated polynucleotide of any one of claims 8-31, wherein n number of B has Formula (b21):



(b21), or a pharmaceutically acceptable salt or stereoisomer thereof,

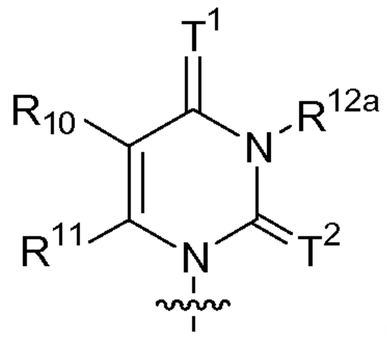
wherein X^{12} is, independently, O, S, optionally substituted alkylene, or optionally substituted heteroalkylene; xa is an integer from 0 to 3; R^{12a} is H, optionally substituted alkyl, optionally substituted aminoalkyl, or absent; and T^2 is O, S, or Se.

33. The isolated polynucleotide of any one of claims 8-32, wherein n number of B has Formula (b22):



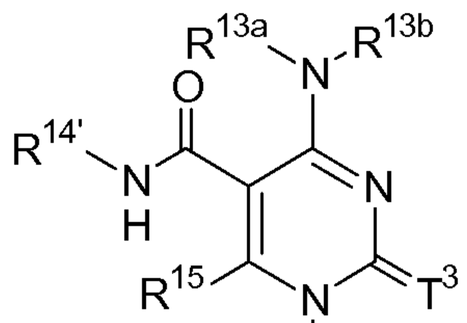
(b22), or a pharmaceutically acceptable salt or stereoisomer thereof, wherein $R^{10'}$ is, independently, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted aryl, optionally substituted heterocyclyl, optionally substituted aminoalkyl, optionally substituted alkoxy, optionally substituted alkoxycarbonylalkyl, optionally substituted alkoxycarbonylalkoxy, optionally substituted carboxyalkoxy, optionally substituted carboxyalkyl, or optionally substituted carbamoylalkyl; R^{11} is H or optionally substituted alkyl; R^{12a} is H, optionally substituted alkyl, optionally substituted aminoalkyl, or absent; and each of T^1 and T^2 is, independently, O, S, or Se.

34. The isolated polynucleotide of any one of claims 8-33, wherein n number of B has Formula (b23):



(b23), wherein R^{10} is optionally substituted heterocyclyl or optionally substituted aryl; R^{11} is H or optionally substituted alkyl; R^{12a} is H, optionally substituted alkyl, optionally substituted aminoalkyl, or absent; and each of T^1 and T^2 is, independently, O, S, or Se.

35. The isolated polynucleotide of any one of claims 8-34, wherein n number of B has Formula (b24):



(b24), wherein

T^3 is O, S, or Se;

each of R^{13a} and R^{13b} is, independently, H, optionally substituted acyl, optionally substituted alkyl, or optionally substituted alkoxy, wherein the combination of R^{13b} and R^{14} can be taken together to form optionally substituted heterocyclyl;

$R^{14'}$ is, independently, optionally substituted alkyl, optionally substituted alkenyl, optionally substituted alkynyl, optionally substituted aryl, optionally substituted heterocyclyl, optionally substituted alkheterocyclyl, optionally substituted alkaryl, optionally substituted aminoalkyl, optionally substituted alkoxy, optionally substituted alkoxycarbonylalkyl, optionally substituted alkoxycarbonylalkoxy, optionally substituted carboxyalkoxy, optionally substituted carboxyalkyl, or optionally substituted carbamoylalkyl; and

each R^{15} is, independently, H, optionally substituted alkyl, optionally substituted alkenyl, or optionally substituted alkynyl.

36. The isolated polynucleotide of any one of claims 1-35, further comprising a targeting moiety, wherein said targeting moiety is covalently bound to said polynucleotide.

37. The isolated polynucleotide of claim 36, wherein said targeting moiety is an antibody, thyrotropin, melanotropin, lectin, glycoprotein, surfactant protein A, Mucin carbohydrate, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-gulucosamine multivalent mannose, multivalent fucose, glycosylated polyaminoacids, multivalent galactose, transferrin, bisphosphonate, polyglutamate,

polyaspartate, a lipid, cholesterol, a steroid, bile acid, folate, vitamin B12, biotin, an RGD peptide, an RGD peptide mimetic, or an aptamer.

38. A pharmaceutical composition comprising the isolated polynucleotide of any one of claims 1-37.

39. A pharmaceutical composition comprising the isolated polynucleotide of any one of claims 1-37 and a pharmaceutically acceptable excipient.

40. The pharmaceutical composition of claim 39, wherein the excipient is selected from a solvent, aqueous solvent, non-aqueous solvent, dispersion media, diluent, dispersion, suspension aid, surface active agent, isotonic agent, thickening or emulsifying agent, preservative, lipid, lipidoids liposome, lipid nanoparticle, core-shell nanoparticles, polymer, lipoplexe peptide, protein, cell, hyaluronidase, and mixtures thereof.

41. The pharmaceutical composition of claim 40, where the pharmaceutical composition comprises a lipid and wherein said lipid is selected from DLin-DMA, DLin-K-DMA, DLin-KC2-DMA, 98N12-5, C12-200, DLin-MC3-DMA, DODMA, DSDMA, DLenDMA, reLNPs, PLGA and PEGylated lipids and mixtures thereof.

42. A method of producing a polypeptide of interest in a mammalian cell, tissue or organism comprising administering to said cell, tissue or organism the isolated polynucleotide of any one of claims 1-37 or the pharmaceutical composition of any of claims 38-41.

43. The method of claim 42, wherein the isolated polynucleotide is formulated.

44. The method of claim 43, wherein the formulation comprises a lipid which is selected from one of DLin-DMA, DLin-K-DMA, DLin-KC2-DMA, 98N12-5, C12-200, DLin-MC3-DMA, DODMA, DSDMA, DLenDMA, reLNPs, PLGA, PEGylated lipids and mixtures or combinations thereof.

45. The method of claim 44, wherein the isolated polynucleotide is administered at a total daily dose of between 1 ug and 150 ug.

46. The method of claim 45, wheriein administration is by injection.

47. The method of claim 46, wherein administration is intradermal or subcutaneous or intramuscular or intravitreal.

48. The method of claim 45, wherein levels of the polypeptide of interest in the serum of the mammal are at least 50 pg/mL at least two hours after administration.

49. The method of claim 45, wherein the levels of the polypeptide of interest in the serum of the mammal remain above 50 pg/mL for at least 72 hours after administration.

50. The method of claim 49, wherein the levels of the polypeptide of interest in the serum of the mammal remain above 60 pg/mL for at least 72 hours after administration.

51. The method of claim 44, wherein the resulting polynucleotide formulation has a mean particle size of 80 nm-160 nm, a PDI of between 0.02 and 0.20 and a lipid to polnucleotide ratio (wt/wt) of between 10-20.

52. A method for producing an increased level of a polypeptide of interest selected from the group consisting of SEQ ID NOs 769-1392 in a mammalian cell, tissue or organism, comprising administering to said cell, tissue or organism a total daily dose of the isolated polynucleotide of any one of claims 4-37 or the pharmaceutical composition of any one of claims 38-41 in two or more equal or unequal split doses.

53. The method of claim 52, wherein the level of the polypeptide produced in response to said administration is greater than the levels produced by administering the same total daily dose of the isolated polynucleotide or pharmaceutical composition as a

single administration.

54. The method of claim 52, wherein the mammalian organism is a human patient in need of an increased level of the polypeptide of interest.

55. The method of claim 54, wherein the increased level of the polypeptide of interest is detectable in a bodily fluid of said patient.

56. The method of claim 55, wherein the bodily fluid is selected from the group consisting of peripheral blood, serum, plasma, ascites, urine, cerebrospinal fluid (CSF), sputum, saliva, bone marrow, synovial fluid, aqueous humor, amniotic fluid, cerumen, breast milk, broncheoalveolar lavage fluid, semen, prostatic fluid, cowper's fluid or pre-ejaculatory fluid, sweat, fecal matter, hair, tears, cyst fluid, pleural and peritoneal fluid, pericardial fluid, lymph, chyme, chyle, bile, interstitial fluid, menses, pus, sebum, vomit, vaginal secretions, mucosal secretion, stool water, pancreatic juice, lavage fluids from sinus cavities, bronchopulmonary aspirates, blastocyl cavity fluid, and umbilical cord blood.

57. The method of claim 56, wherein the bodily fluid is serum and the polpeptide per unit drug (PUD) is greater than 1.

58. The method of claim 57, wherein the dose splitting factor (DSF) is greater than 4.

59. The method of claim 55, wherein administration is transdermal.

60. The method of claim 59, wherein transdermal administration comprises utilization of one or more members selected from the group consisting of a patch, cream, ointment, mechanical device, needle, sponge, depot and fabric.

61. The method of claim 59, wherein administration is according to a dosing regimen which occurs over the course of hours, days, weeks, months, or years.

62. The method of claim 52, wherein said two or more split doses comprise a first dose of the polynucleotide or pharmaceutical composition administered at a time T1 followed by a second dose of the polynucleotide or pharmaceutical composition administered at a time T2, wherein said time T1 and said time T2 are separated by no more than 1 minute and wherein said first dose and said second dose are administered in amounts that result in higher levels of the polypeptide of interest in said subject than if the amounts of polynucleotide or pharmaceutical composition were administered together in a single unit dose.

63. The method of claim 62, further comprising administering a plurality of doses of said polynucleotide or pharmaceutical composition, Nx at times Tn, wherein x and n are independently selected from 3 to about 1000 and where the time between Tn and Tn+1 is separated by increments of no more than 10 seconds.

64. The method of claim 63, wherein administration occurs by direct injection.

65. The method of claim 64, wherein direct injection is selected from the group consisting of intravenous, intradermal, subcutaneous, intramuscular and intravitreal.

66. The method of claim 64, wherein said first dose is administered proximal to said second or plurality of doses.

67. The method of claim 64, wherein said first dose is administered distal to said second or plurality of doses.

68. The method of claim 64, wherein the distance between the site of injection of said first dose and the site of injection of any second or plurality of doses is from about 1mm to about 10 cm.

69. The method of claim 64, wherein injection is made at a depth of from 0.1mm to about 1cm.

70. The method of claim 65, wherein direct injection is achieved by using one or more devices selected from multineedle injection systems, catheter or lumen systems, and ultrasound, electrical or radiation based systems.

71. The method of claim 63, wherein the amount polynucleotide or pharmaceutical composition administered in any dose is substantially equal.

72. The method of claim 63, wherein time T1 and time T2 are separated by no more than 30 seconds.

73. The method of claim 63, wherein time T1 and time T2 are separated by no more than 10 seconds.

74. The method of claim 63, wherein the first dose, the second dose and any of a plurality of doses are administered at substantially the same time.

75. The method of claim 63, wherein the single unit dose is between about 10 mg/kg and about 500 mg/kg.

76. The method of claim 63, wherein the single unit dose is between about 1.0 mg/kg and about 10 mg/kg.

77. The method of claim 63, wherein the single unit dose is between about 0.001 mg/kg and about 1.0 mg/kg.

78. A method of preparing a lipid nanopartical formulation of a polynucleotide encoding a polypeptide of interest comprising rapidly injecting a first ethanolic solution into a second aqueous solution wherein,

(a) said first ethanolic solution comprises a mixture of lipid:DSPC: Cholesterol: PEG-c-DOMG to yield a molar ratio of 50:10:38.5:1.5 and having a final lipid concentration of approximately 25mM, and

(b) said second aqueous solution comprises a sodium citrate buffered solution of the polynucleotide encoding the polypeptide of interest having a concentration of 1-2 mg/mL and a pH of approximately 3,

wherein the rapid injection results in a suspension containing 33% ethanol and a total lipid to polynucleotide weight ratio of at least 10:1.

79. The method of claim 78, wherein the rapid injection is performed either manually (MI) or by the aid of a syringe pump (SP).

80. The method of claim 79, further comprising dialyzing the resultant suspension against phosphate buffered saline (PBS) at pH 7.4.

81. The method of claim 80, wherein dialysis is performed more than once.

82. The method of claim 81, further comprising filtering the dialyzed suspension through a 0.2 μm sterile filter.

83. The method of any of claims 78-82, wherein the lipid is selected from the group consisting of DLin-DMA, DLin-K-DMA, DLin-KC2-DMA, 98N12-5, C12-200, DLin-MC3-DMA, DODMA, DSDMA, DLenDMA, reLNPs, PLGA and PEGylated lipids.

84. The method of any of claims 78-83, wherein the polynucleotide is selected from the polynucleotide of any of claims 1-37.

85. A lipid nanopartical formulation of a polynucleotide encoding a polypeptide of interest produced by the method of any of claims 78-84 and having a particle size of 80 nm-160 nm, a PDI of between 0.02 and 0.20 and a lipid to polnucleotide ratio (wt/wt) of between 10-30.

86. A lipid nanopartical formulation of claim 85, wherein the polynucleotide is selected from the polynucleotide of any of claims 1-37.

87. A reLNP formulation of a polynucleotide said polynucleotide encoding a polypeptide of interest.

88. A reLNP formulation of claim 87, wherein the polynucleotide is selected from the polynucleotide of any of claims 1-36.

89. A sustained release formulation of a polynucleotide said polynucleotide encoding a polypeptide of interest.

90. A sustained release formulation of claim 89, wherein the polynucleotide is selected from the polynucleotide of any of claims 1-36.

91. A polynucleotide encoding a fusion protein said fusion protein comprising a first polypeptide and a second polypeptide.

92. The polynucleotide of claim 91 where the first polypeptide is selected from the group consisting of Fc receptor, Fab fragment, Fab' fragment, $F(ab')_2$ fragment, Fv fragment, IgA domain, IgD domain, IgE domain, IgD domain, IgM domain, IgV domain, IgC1 domain, IgC2 domain and IgI domain and the second polypeptide is a polypeptide of interest.

FIGURE 1

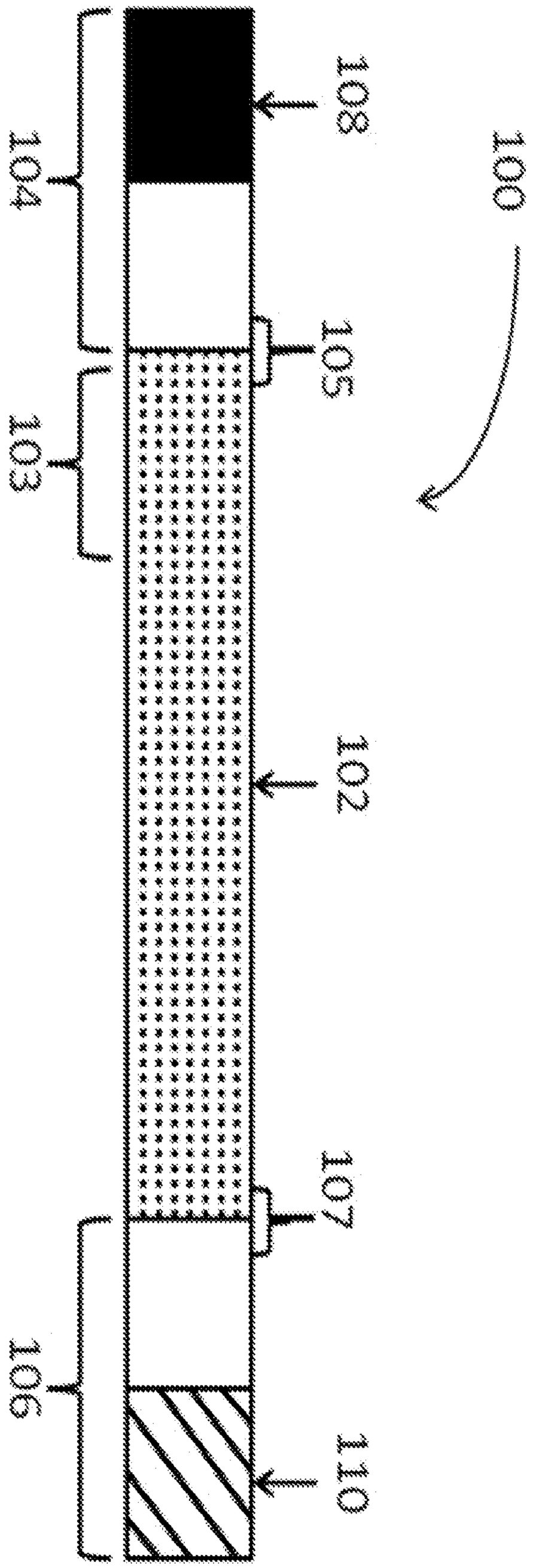
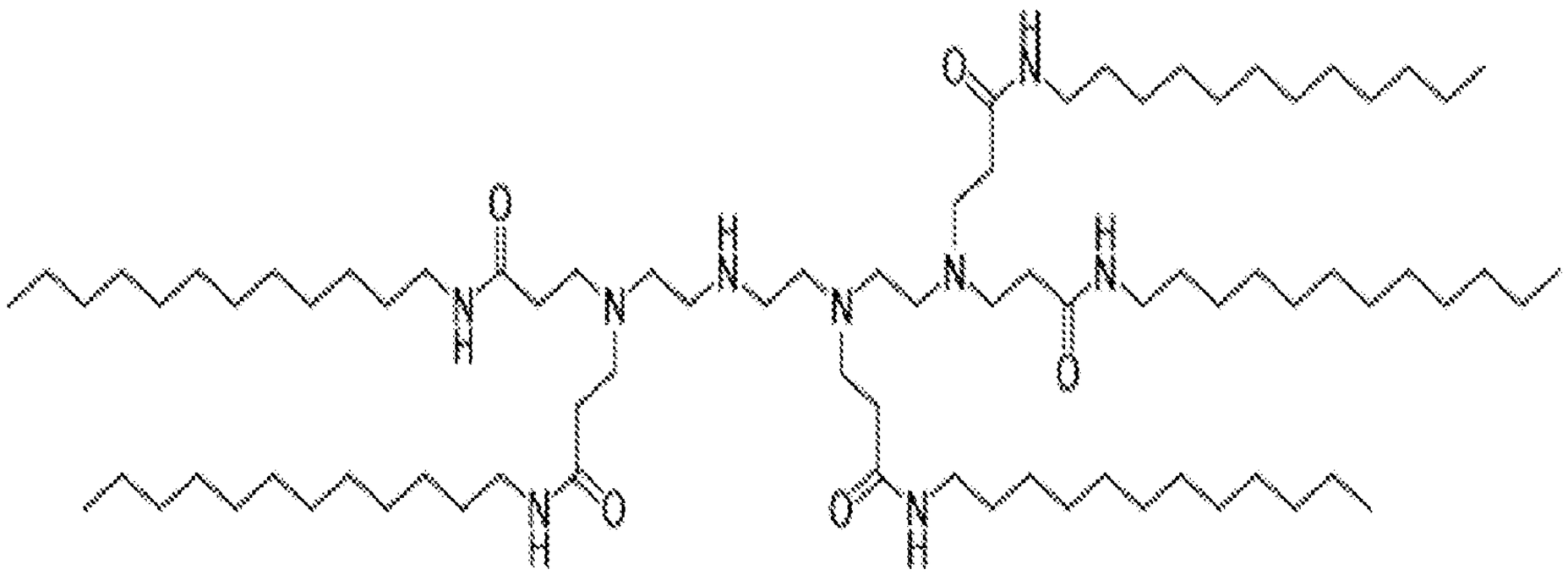
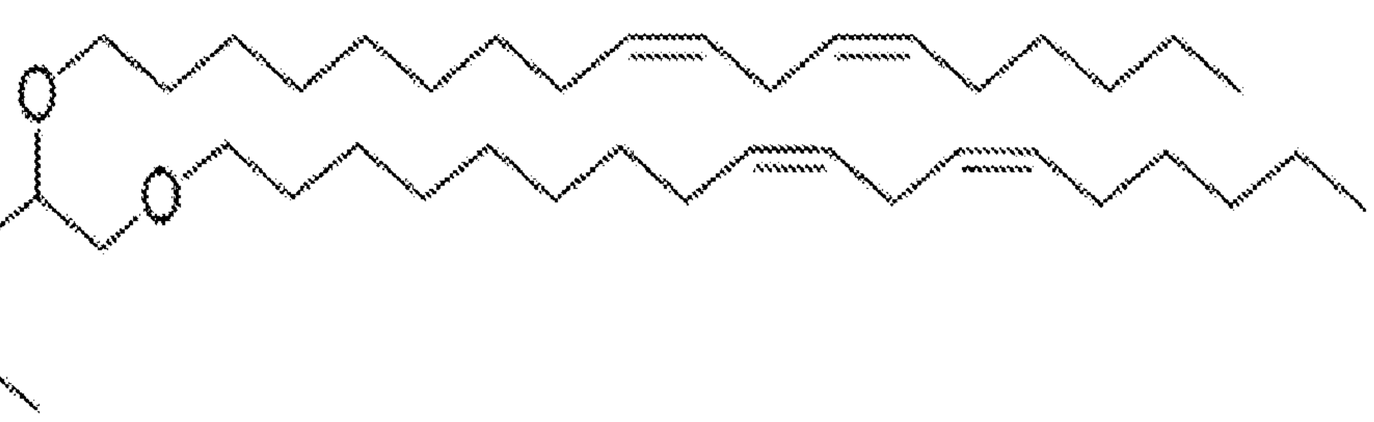


FIGURE 2

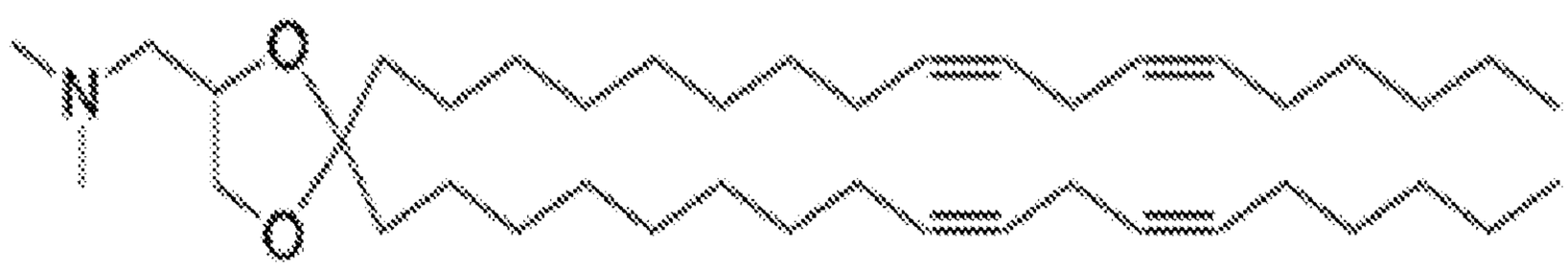
98N12-5 (TETA5-LAP)



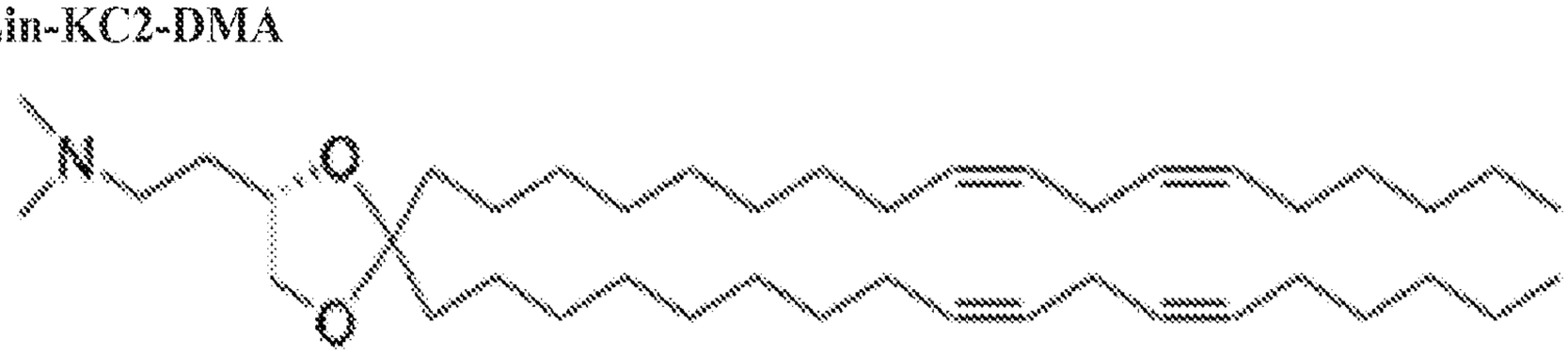
DLin-DMA



DLin-K-DMA (2,2-Dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane)



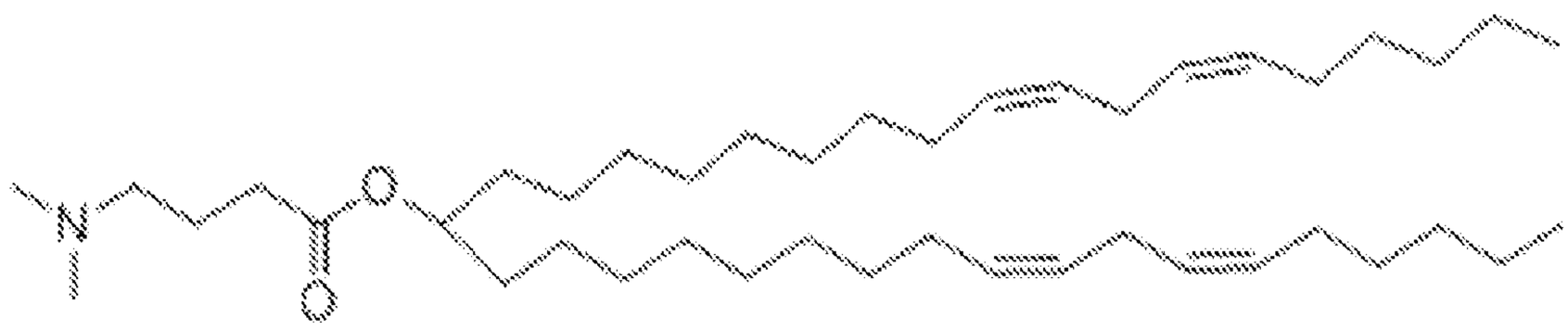
DLin-KC2-DMA



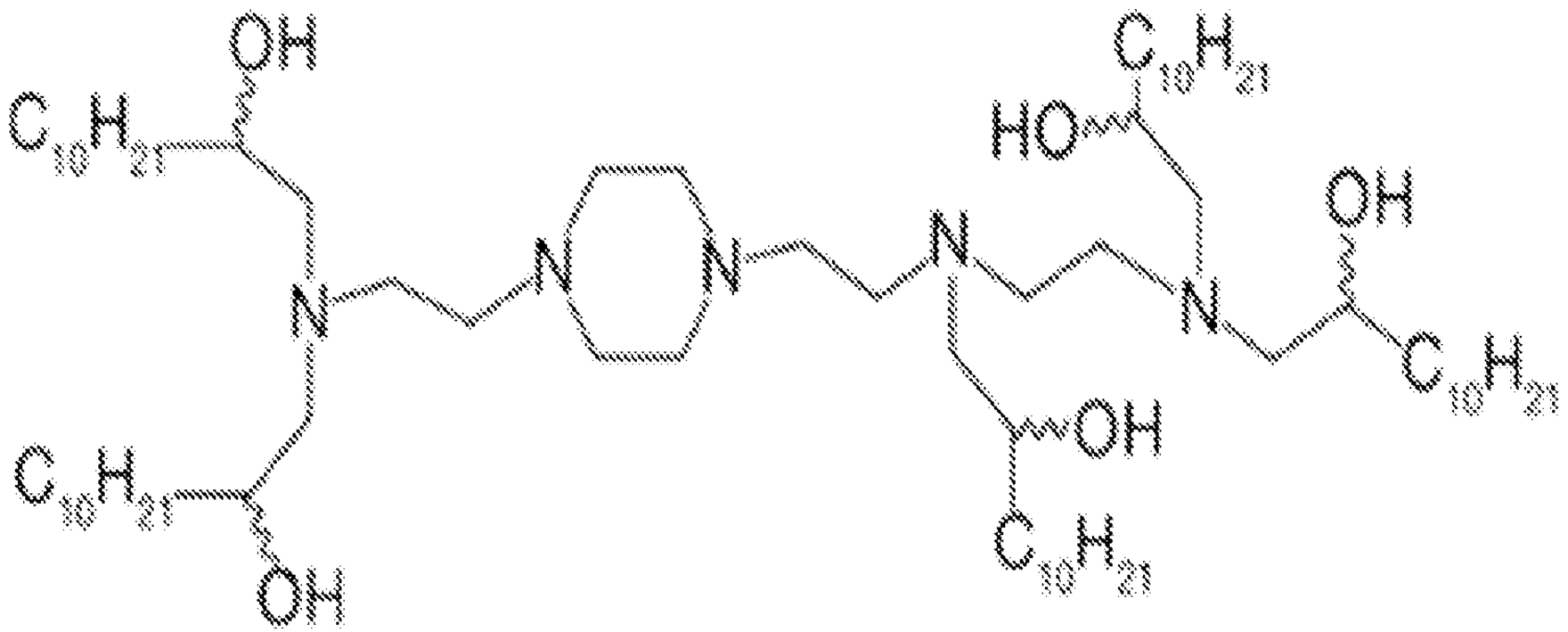
PRIOR ART

FIGURE 2 CONT.

DLin-MC3-DMA

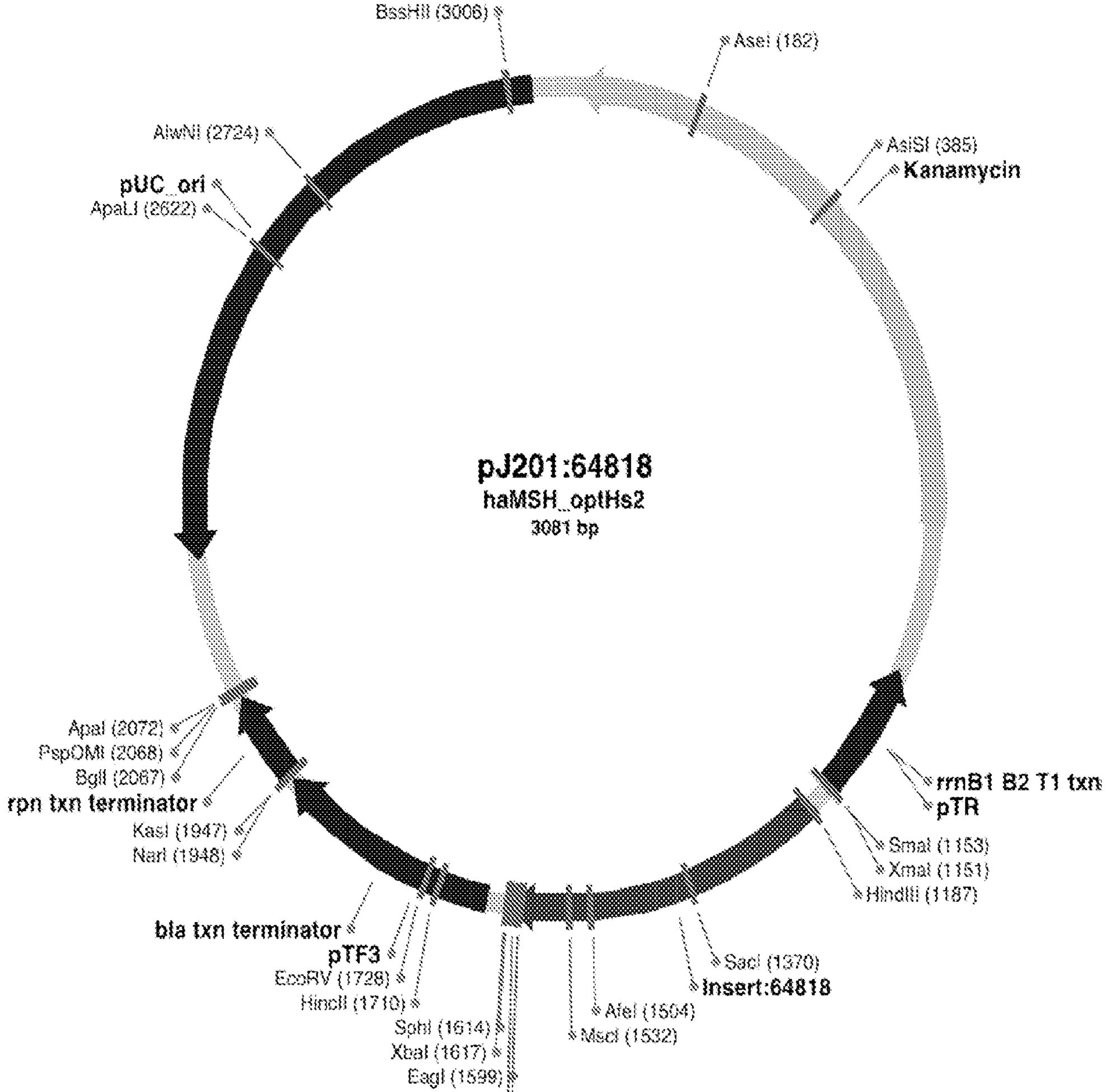


C12-200



PRIOR ART

FIGURE 3



PRIOR ART

FIGURE 4

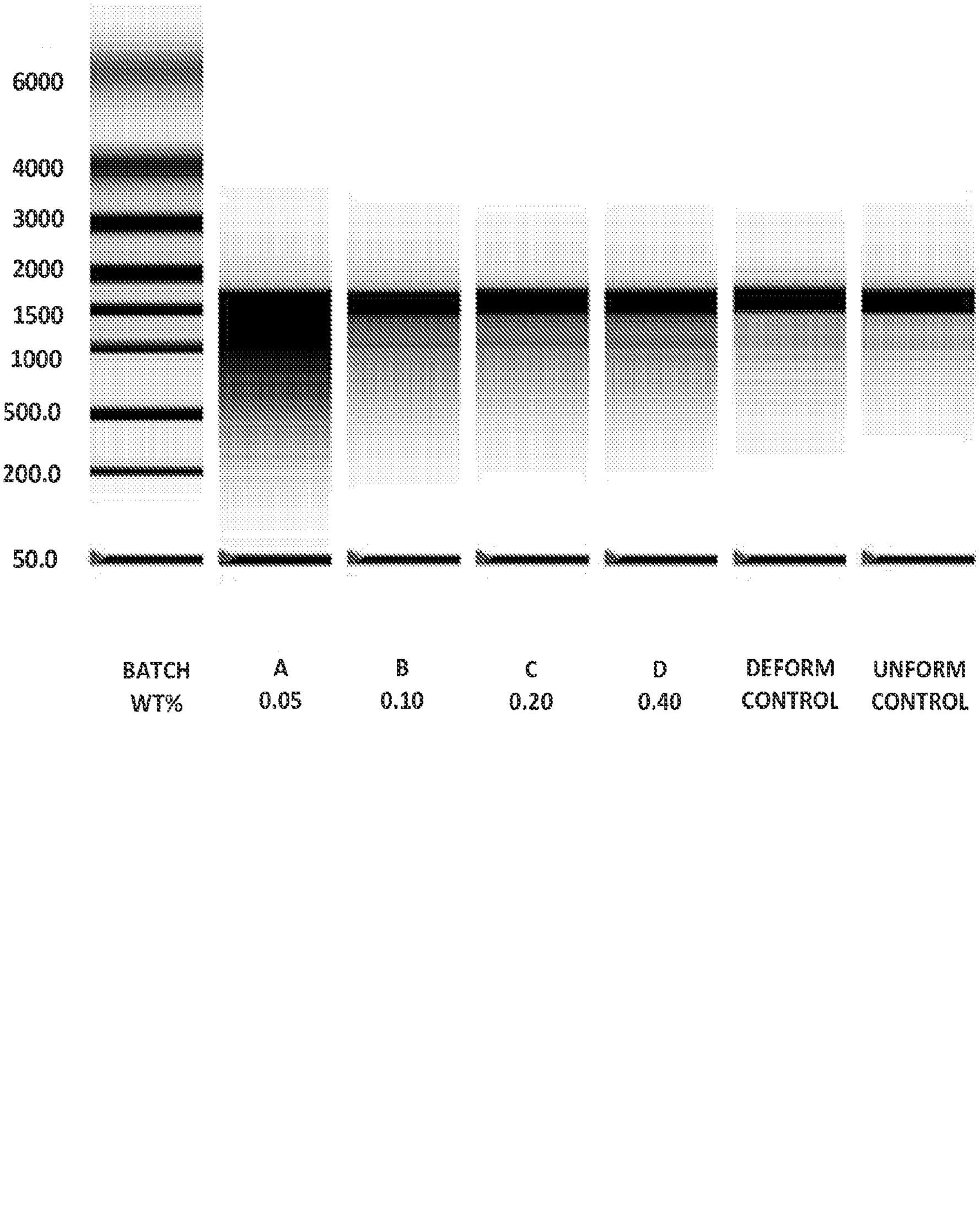

6000
4000
3000
2000
1500
1000
500.0
200.0
50.0
BATCH
WT%
A
0.05
B
0.10
C
0.20
D
0.40
DEFORM CONTROL
UNFORM CONTROL

FIGURE 5

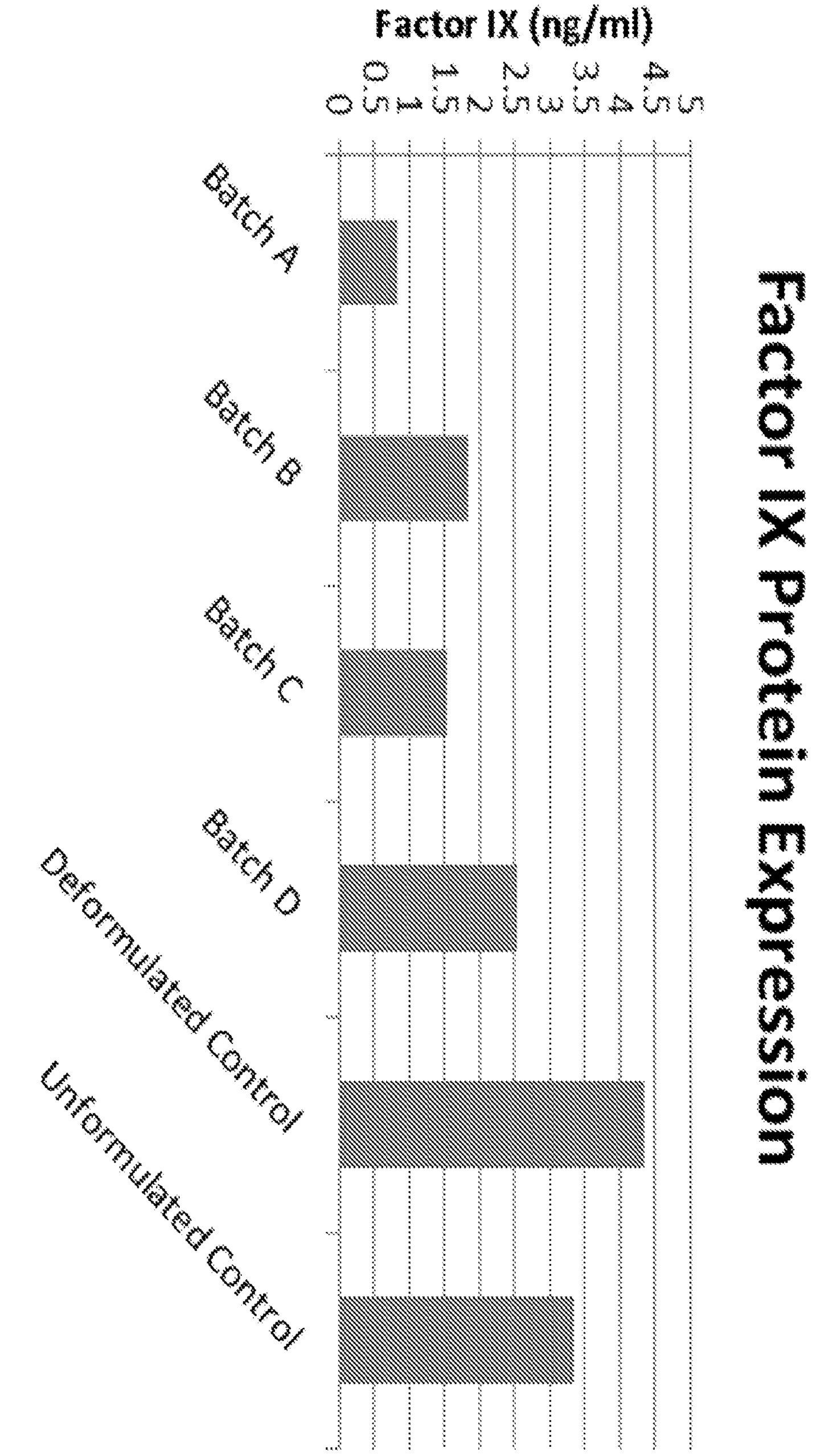

Factor IX (ng/ml)
0
1
2
3
4
Factor IX Protein Expression
Batch A
Batch B
Batch C
Batch D
Deformulated Control
Unformulated Control

FIGURE 6

A.

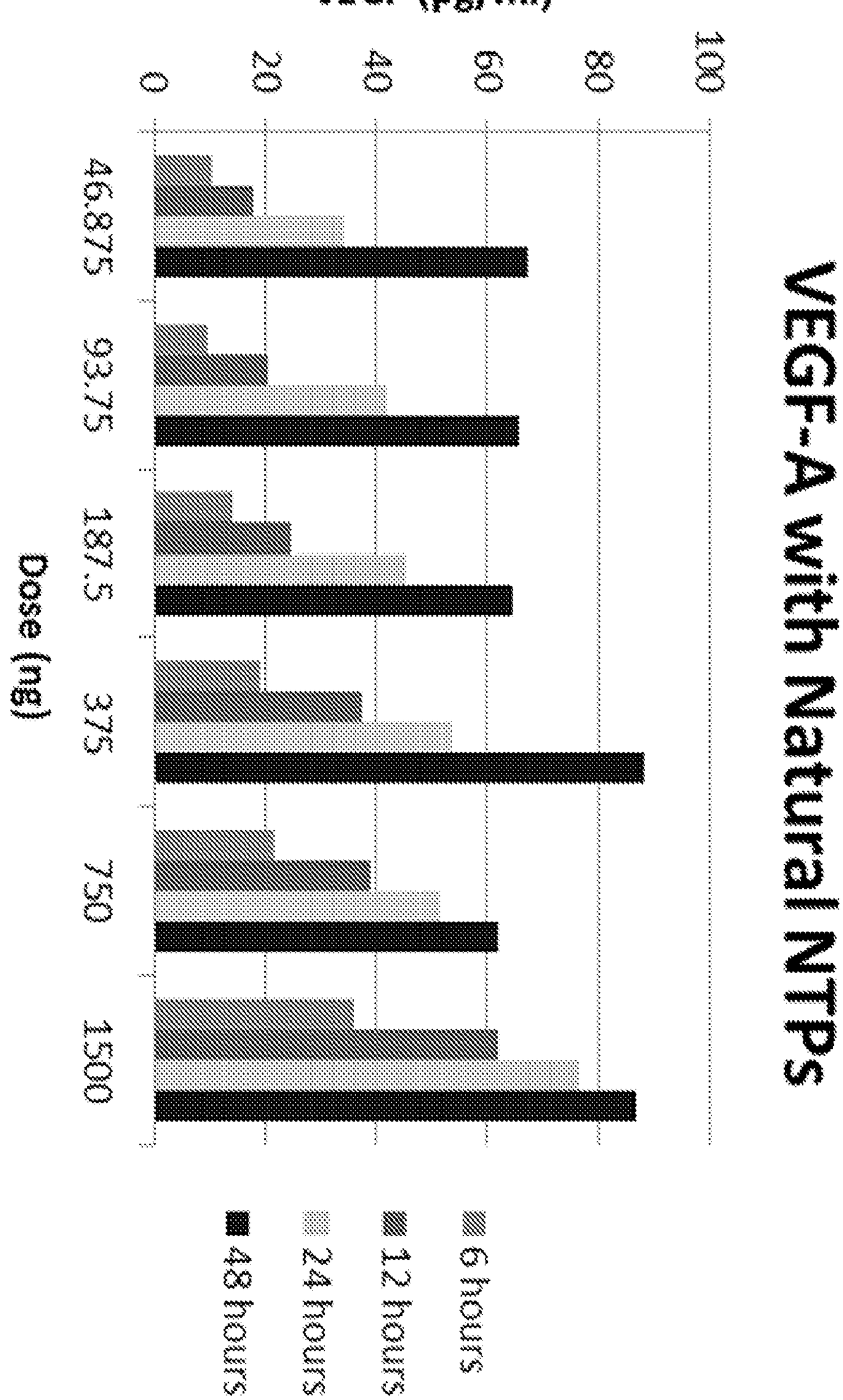

VEGF (pg/ml)
0
200
400
600
800
1000
VEGF-A with Natural NTPs
Dose (ng)
46.875
93.75
187.5
375
750
1500
6 hours
12 hours
24 hours
48 hours

FIGURE 6 CONT.

B.

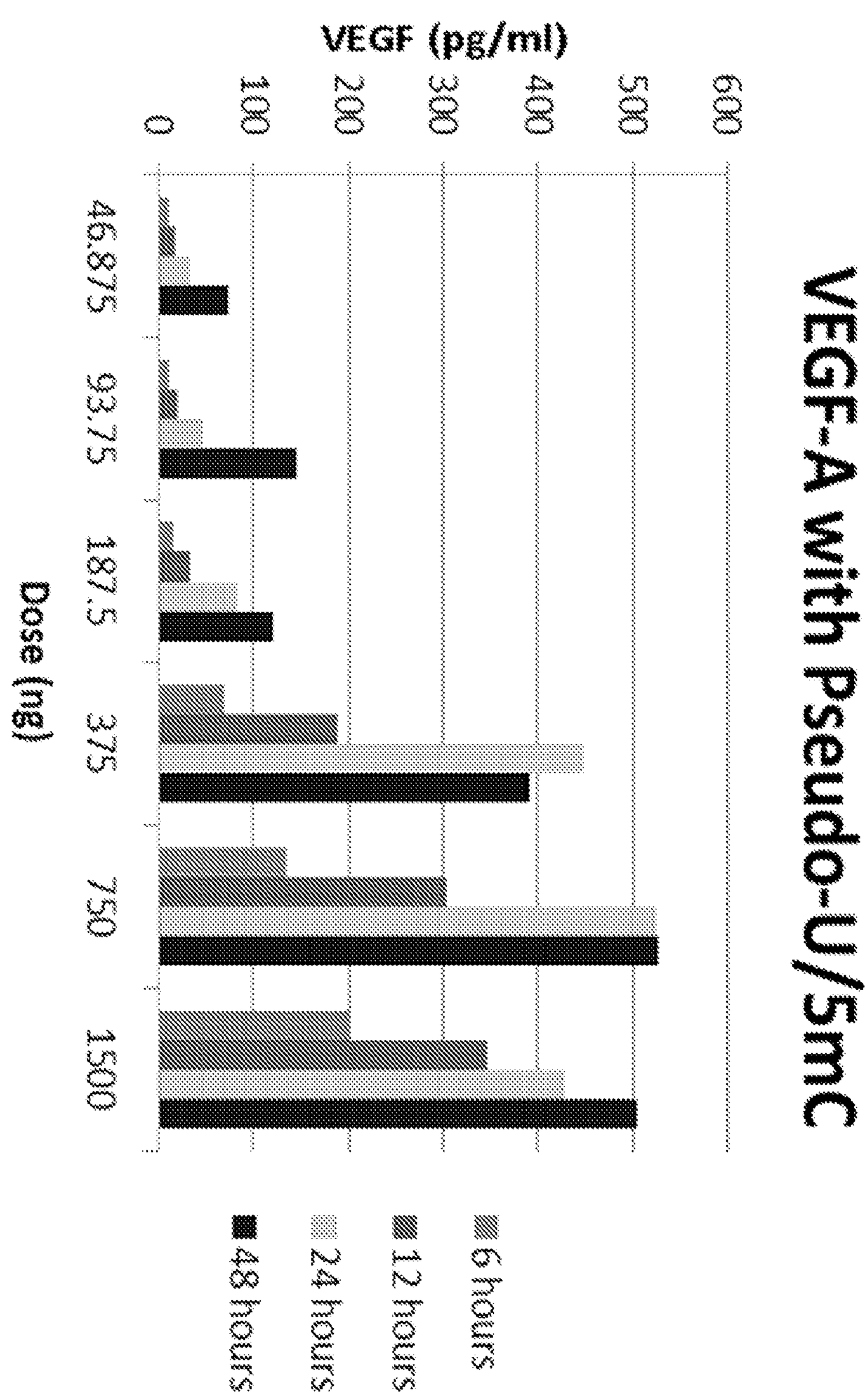


FIGURE 6 CONT.

C.

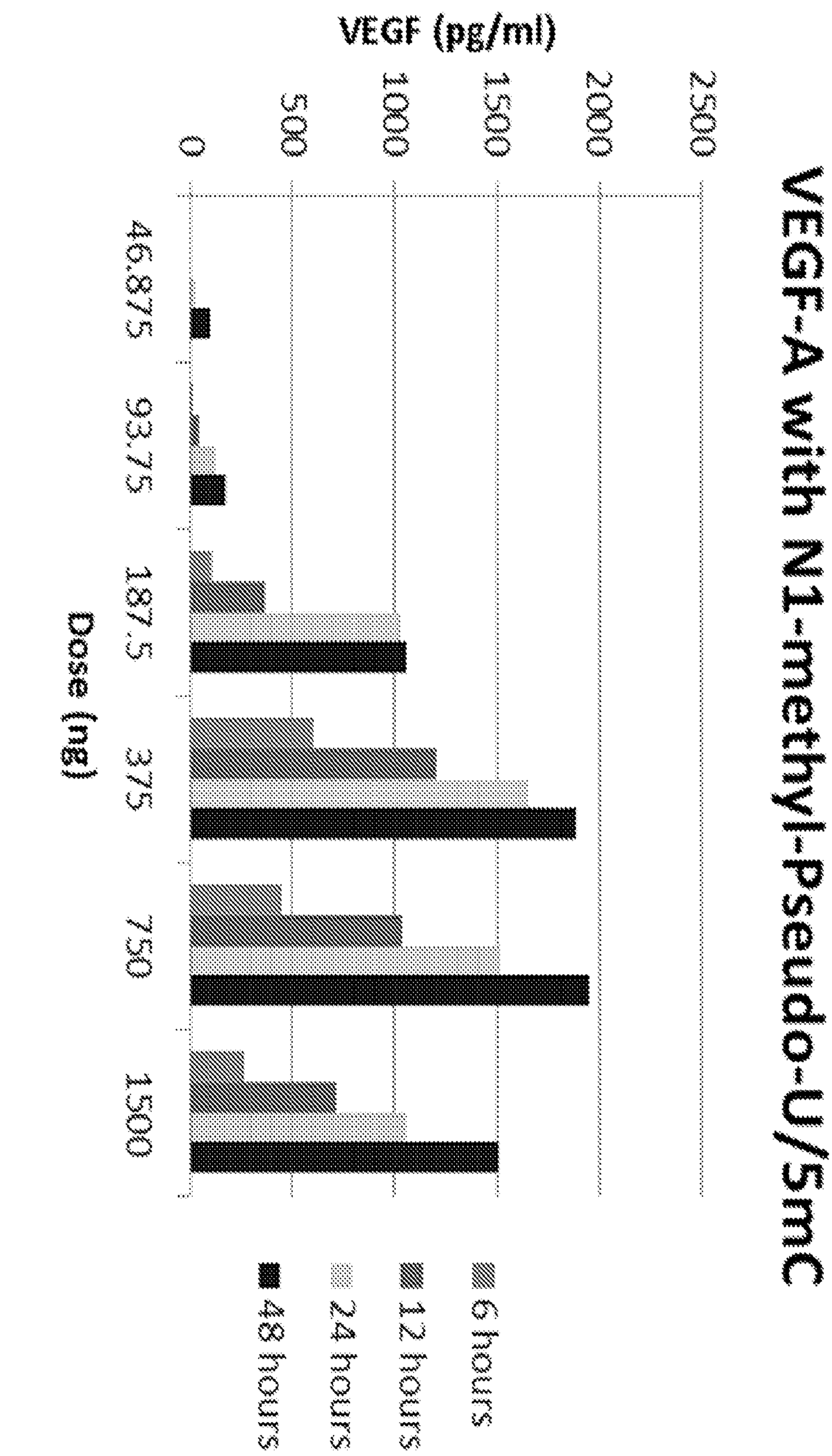


FIGURE 7

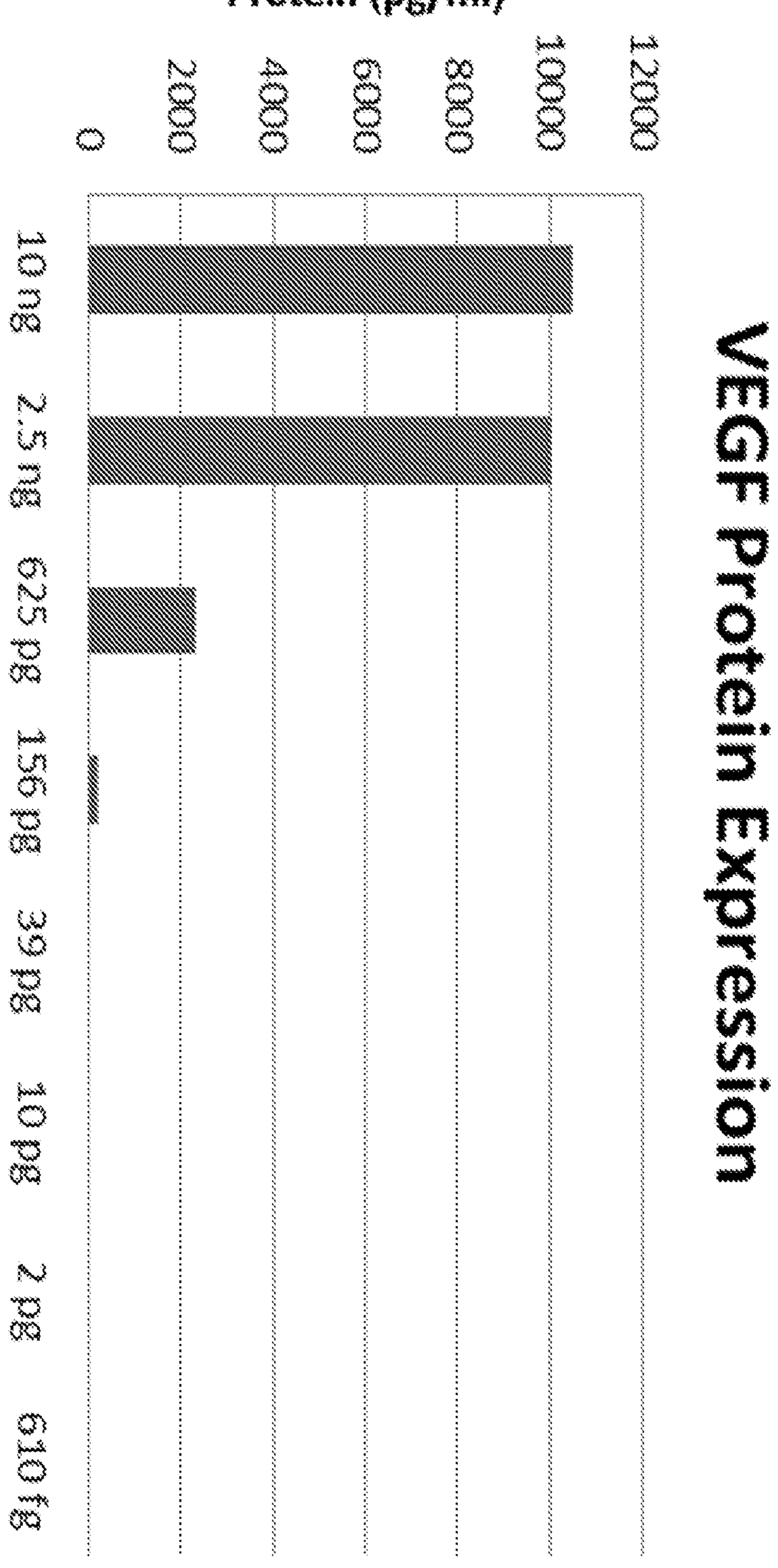

Protein (pg/ml)
0
2000
4000
6000
8000
10000
12000
VEGF Protein Expression
Amount Transfected
10 ng
2.5 ng
625 pg
156 pg
39 pg
10 pg
2 pg
610 pg

FIGURE 8

A.

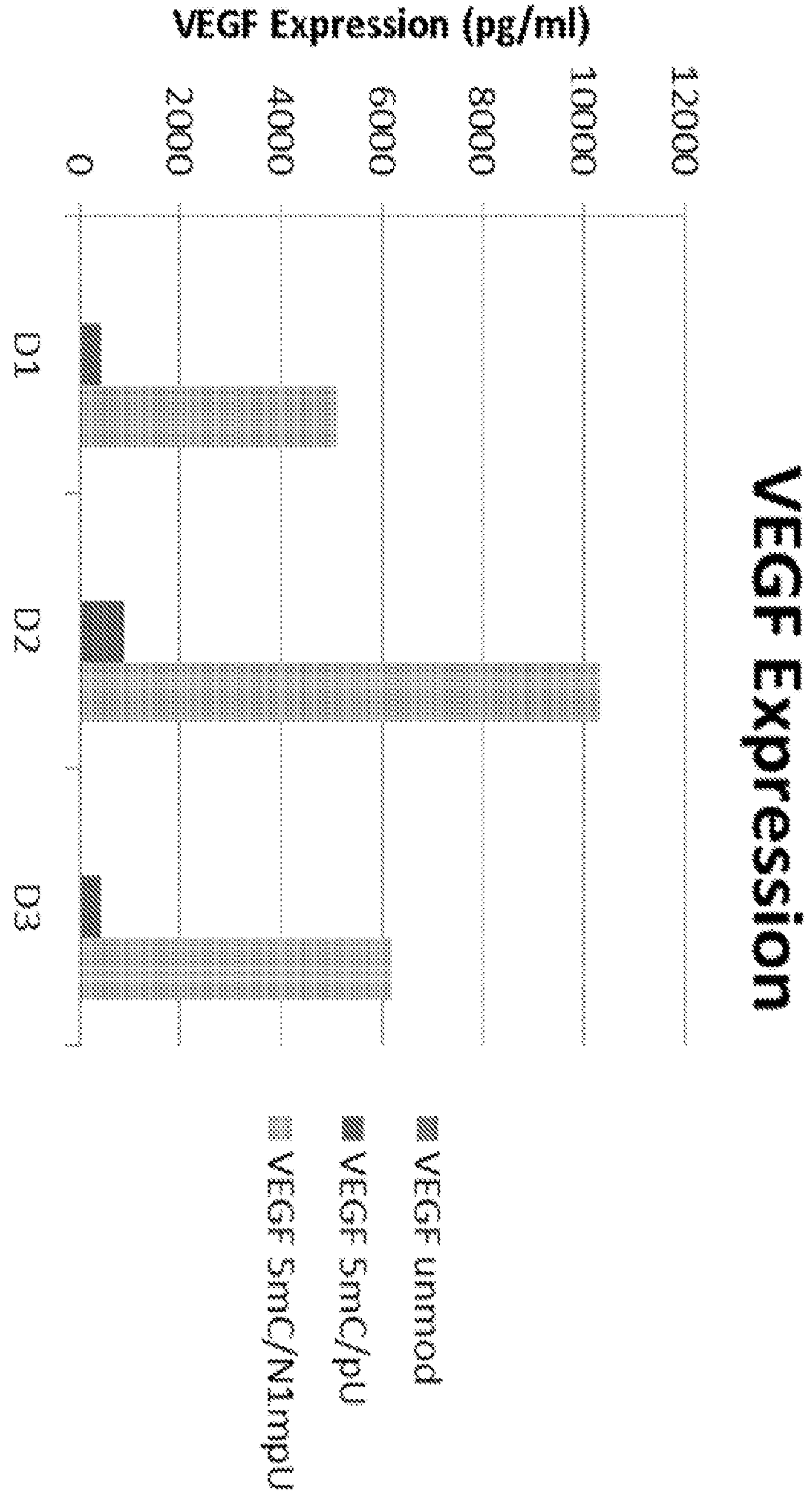

VEGF Expression (pg/ml)
0
2000
4000
6000
8000
10000
12000
D1
D2
D3
VEGF mmol/pu
VEGF pd/pus
VEGF mmol/pus

FIGURE 8 CONT.

B.

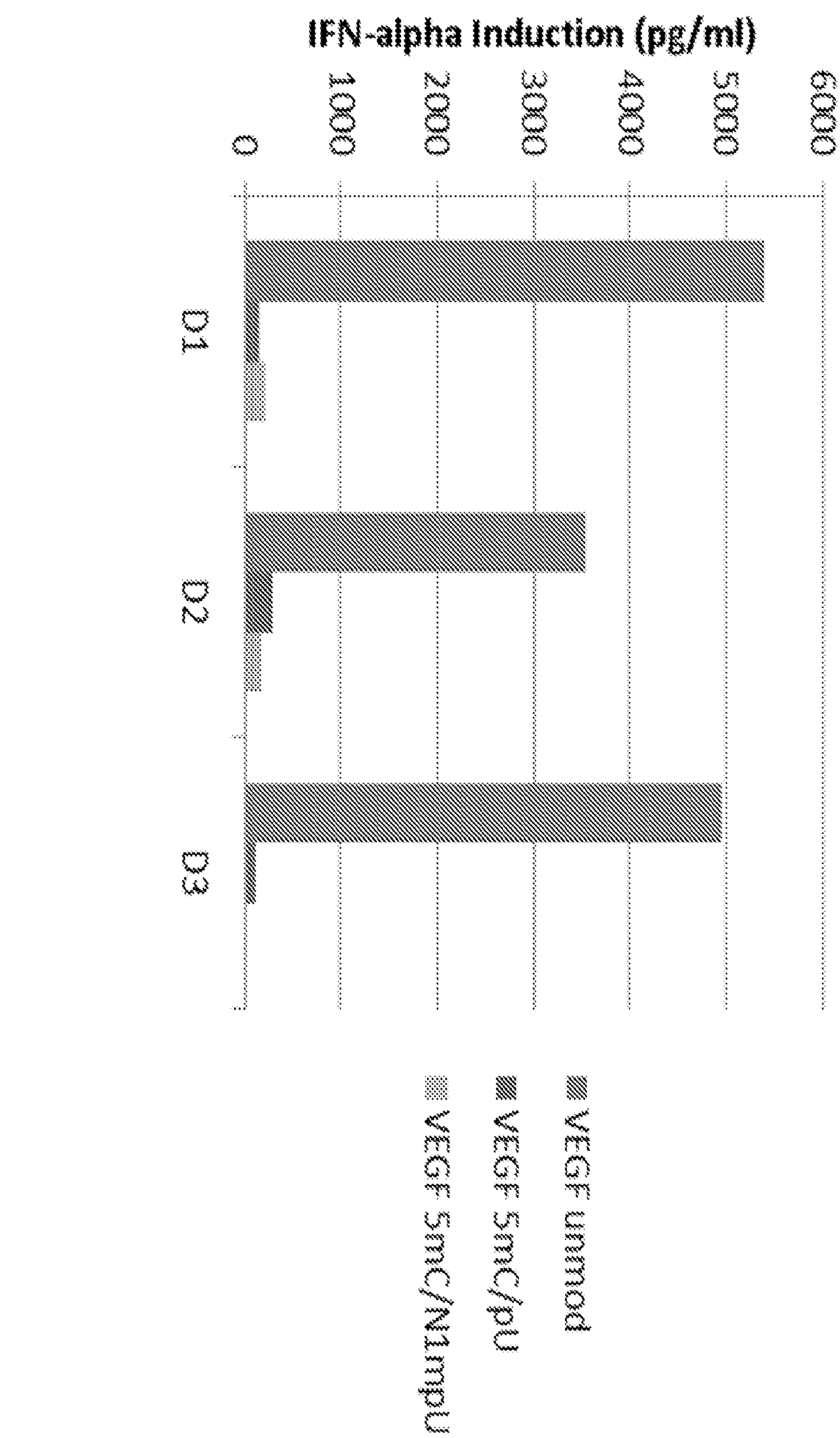


FIGURE 9

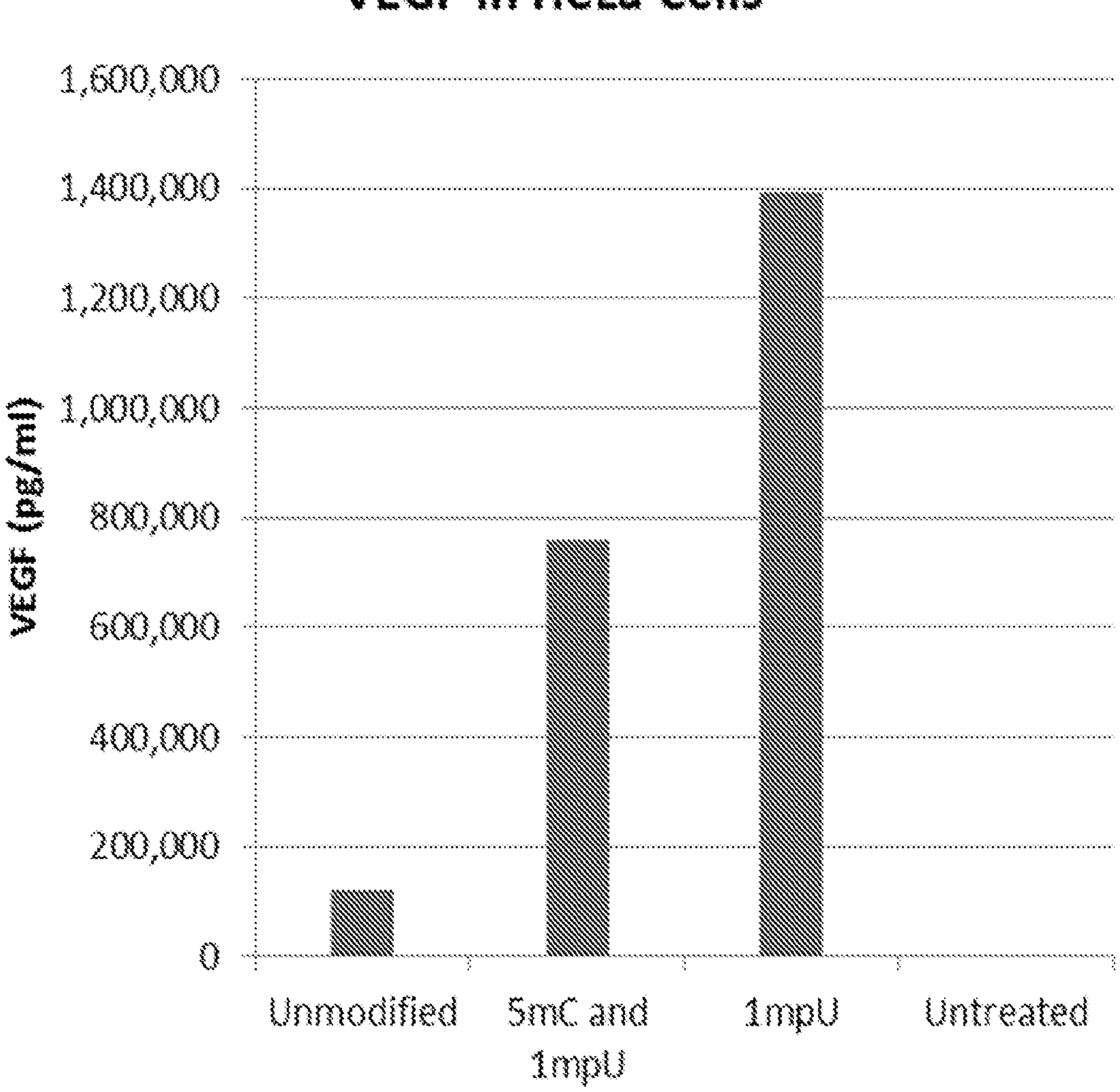

VEGF in HeLa Cells
VEGF (pg/ml)
1,600,000
1,400,000
1,200,000
1,000,000
800,000
600,000
400,000
200,000
0
Unmodified
5mC and 1mpU
1mpU
Untreated

FIGURE 10

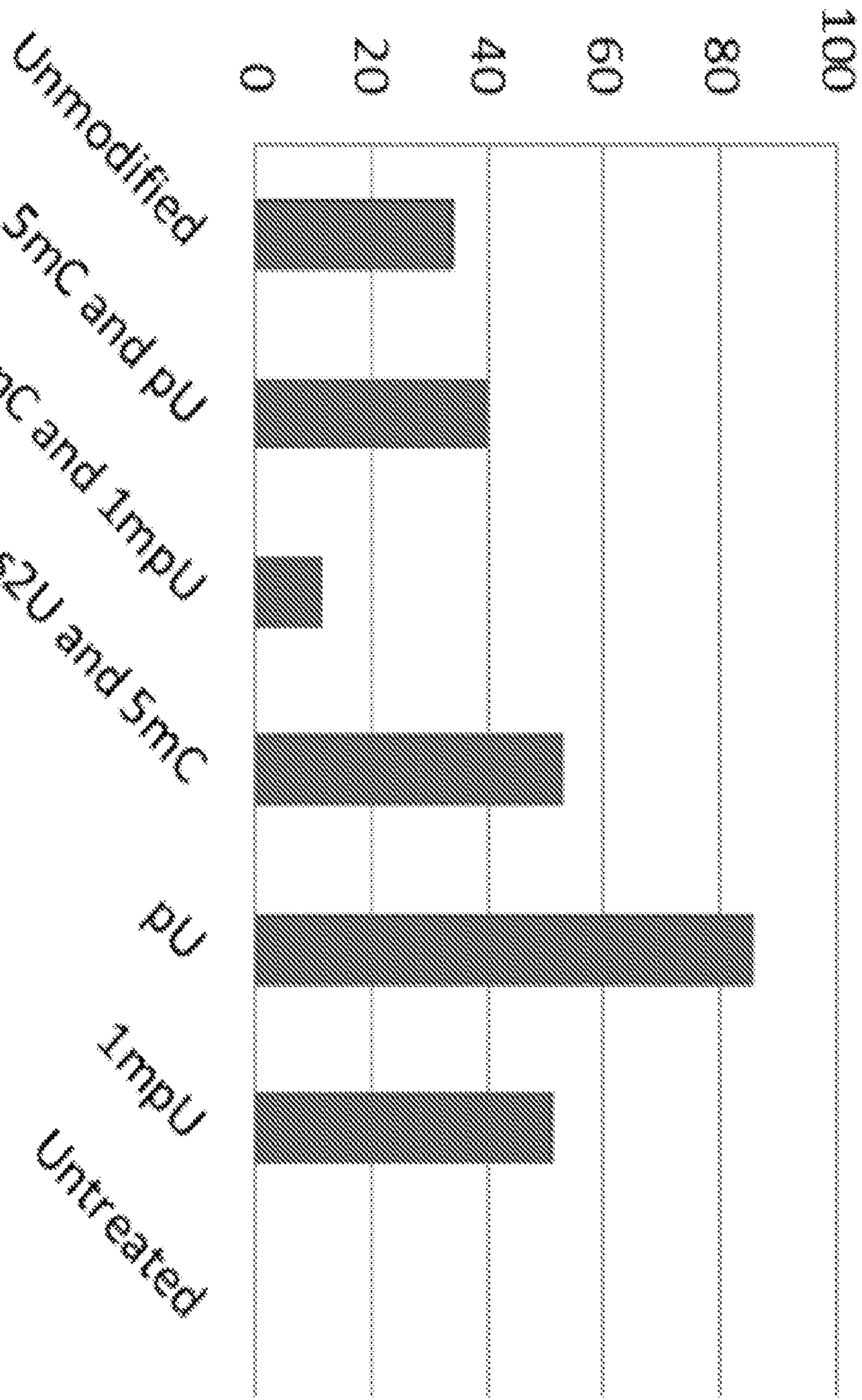

VEGF (pg/ml)
0
20
40
60
80
100
VEGF Lipoplexes in Mice
Untreated
1mpU
pU
5mC and pU
5mC and 1mpU
s2U and 5mC
Unmodified

FIGURE 11

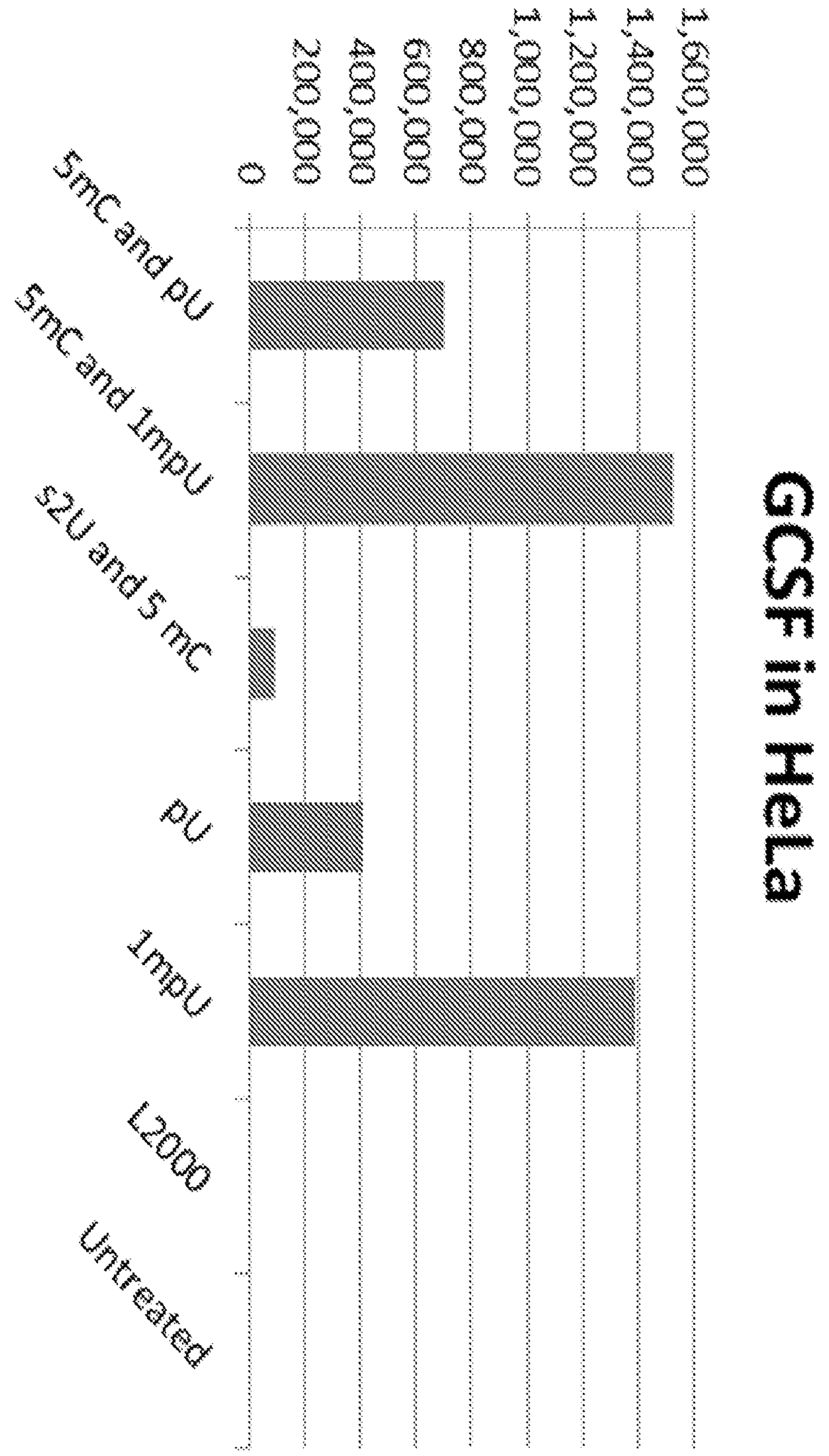

GCSF (pg/ml)
0
200,000
400,000
600,000
800,000
1,000,000
1,200,000
1,400,000
1,600,000
GCSF in Hela
5mC and pU
5mC and 1mpU
s2U and 5 mC
pU
1mpU
L2000
Untreated

FIGURE 12

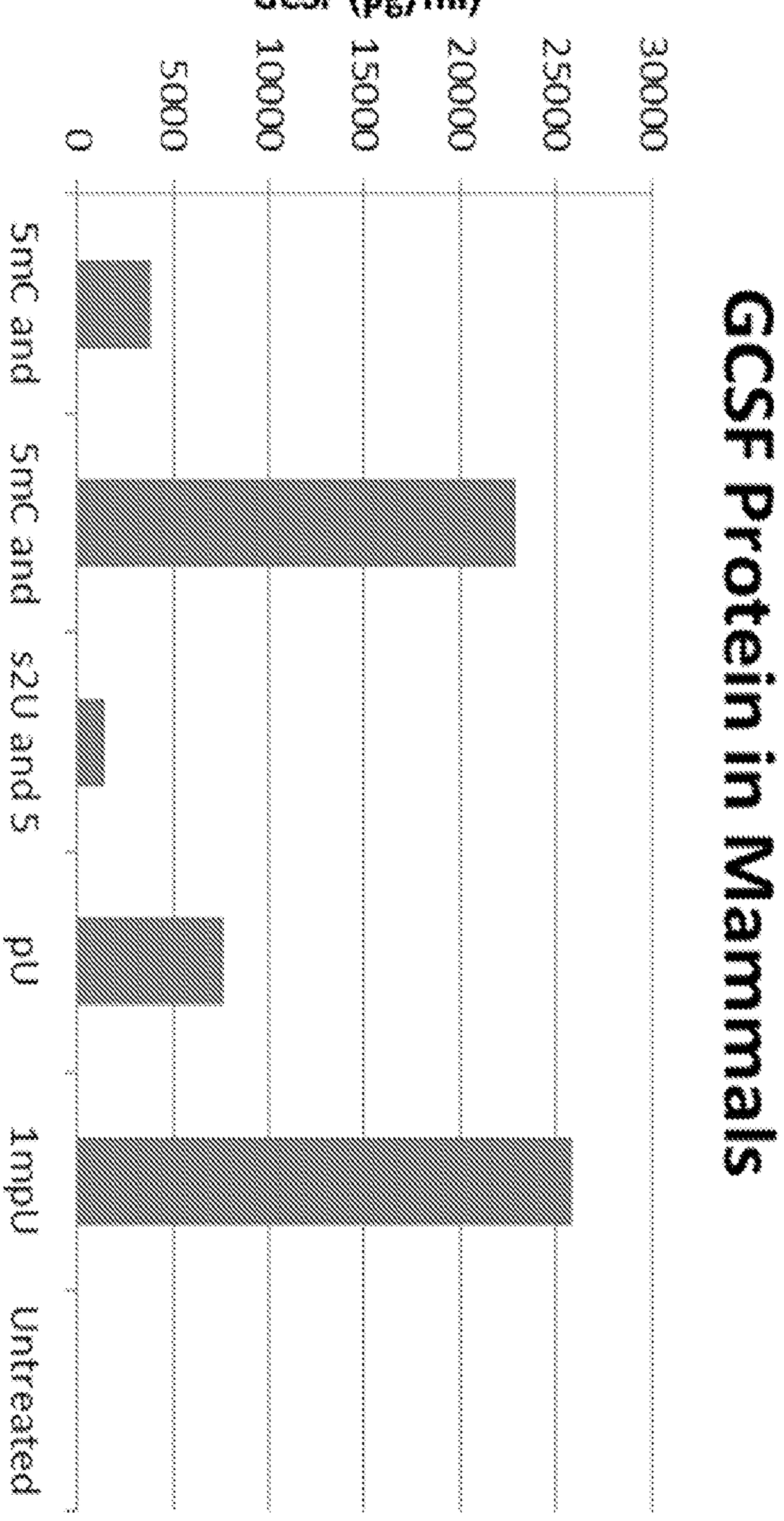

GCSF (pg/ml)
0
5000
10000
15000
20000
25000
30000
GCSF Protein in Mammals
unteated
1mpu
5mc and 1mpu
nd
5mc and nd
mc
5mc and 5hmc

FIGURE 13

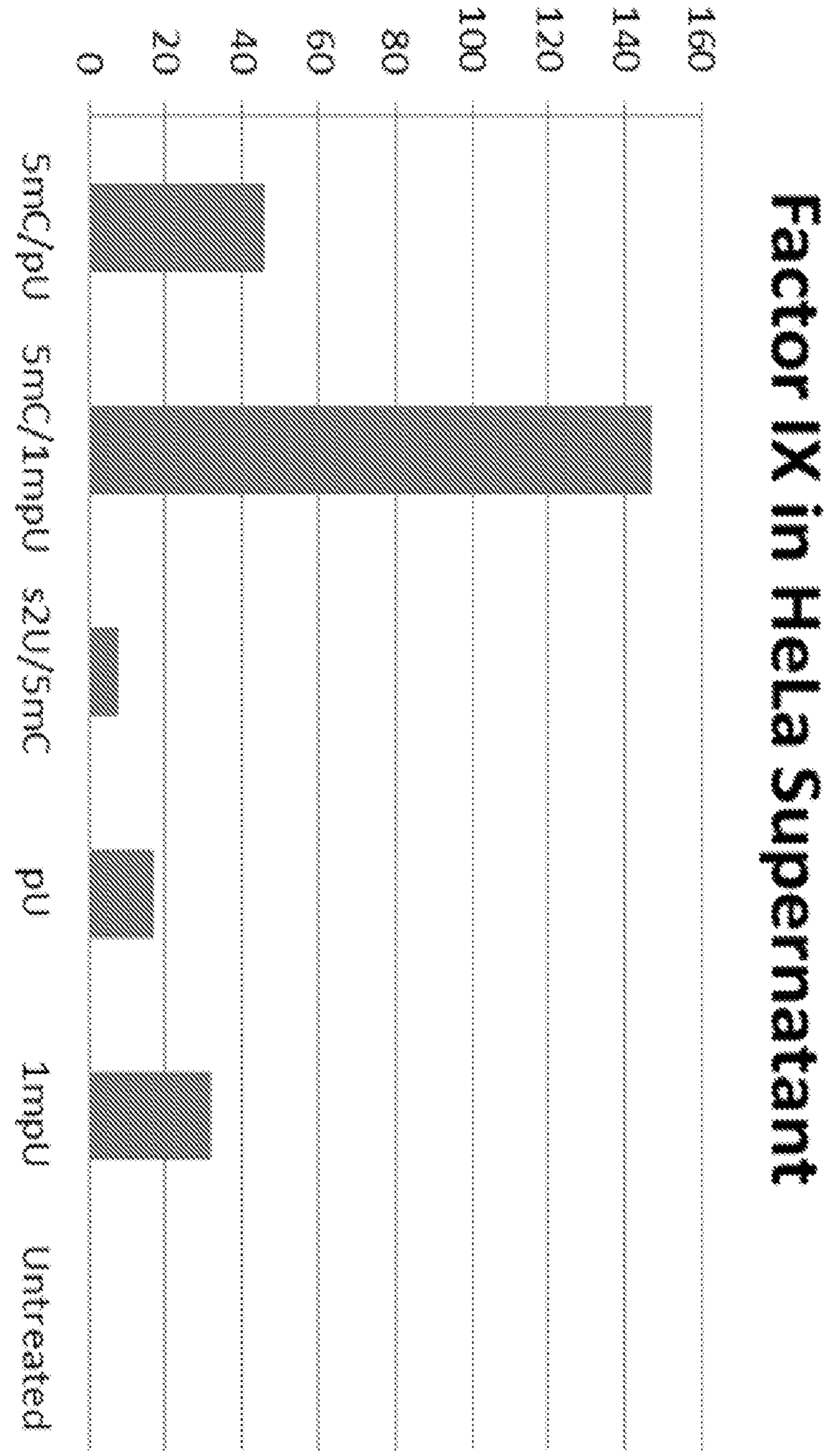

Factor IX (ng/ml)
0
20
40
60
80
100
120
140
160
Factor IX in HeLa Supernatant
untreated
1mpu
pu
2mpu
5mc/pu
5mc/1mpu
5mc/2pu

FIGURE 14

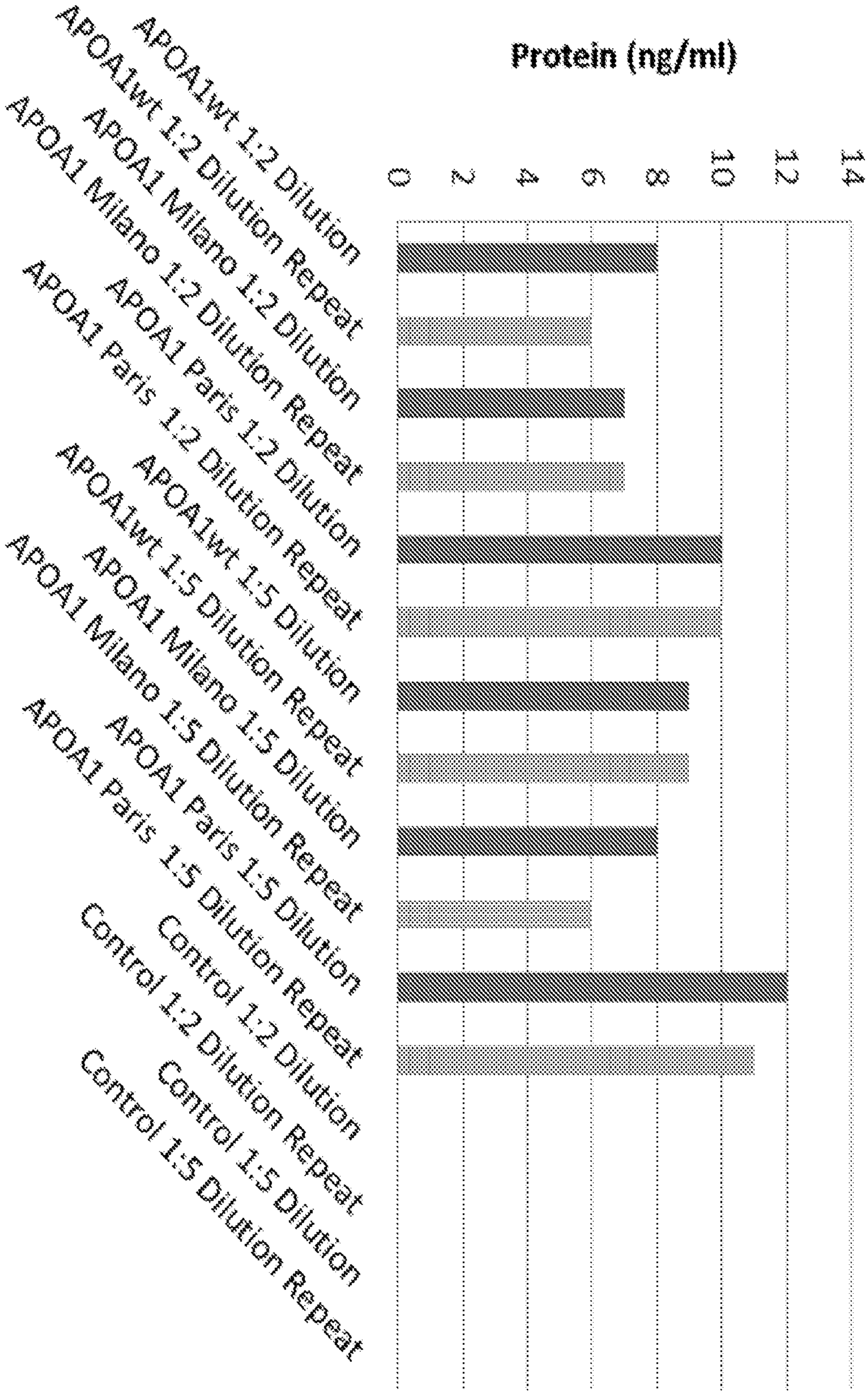

Human APOA1 in Hela Cells
Protein (ng/ml)
0
2
4
6
8
10
12
14
APOA1wt 1:2 Dilution
APOA1wt 1:2 Dilution Repeat
APOA1 Milano 1:2 Dilution
APOA1 Milano 1:2 Dilution Repeat
APOA1 Paris 1:2 Dilution
APOA1 Paris 1:2 Dilution Repeat
APOA1wt 1:5 Dilution
APOA1wt 1:5 Dilution Repeat
APOA1 Milano 1:5 Dilution
APOA1 Milano 1:5 Dilution Repeat
APOA1 Paris 1:5 Dilution
APOA1 Paris 1:5 Dilution Repeat
Control 1:2 Dilution
Control 1:2 Dilution Repeat
Control 1:5 Dilution
Control 1:5 Dilution Repeat

FIGURE 15

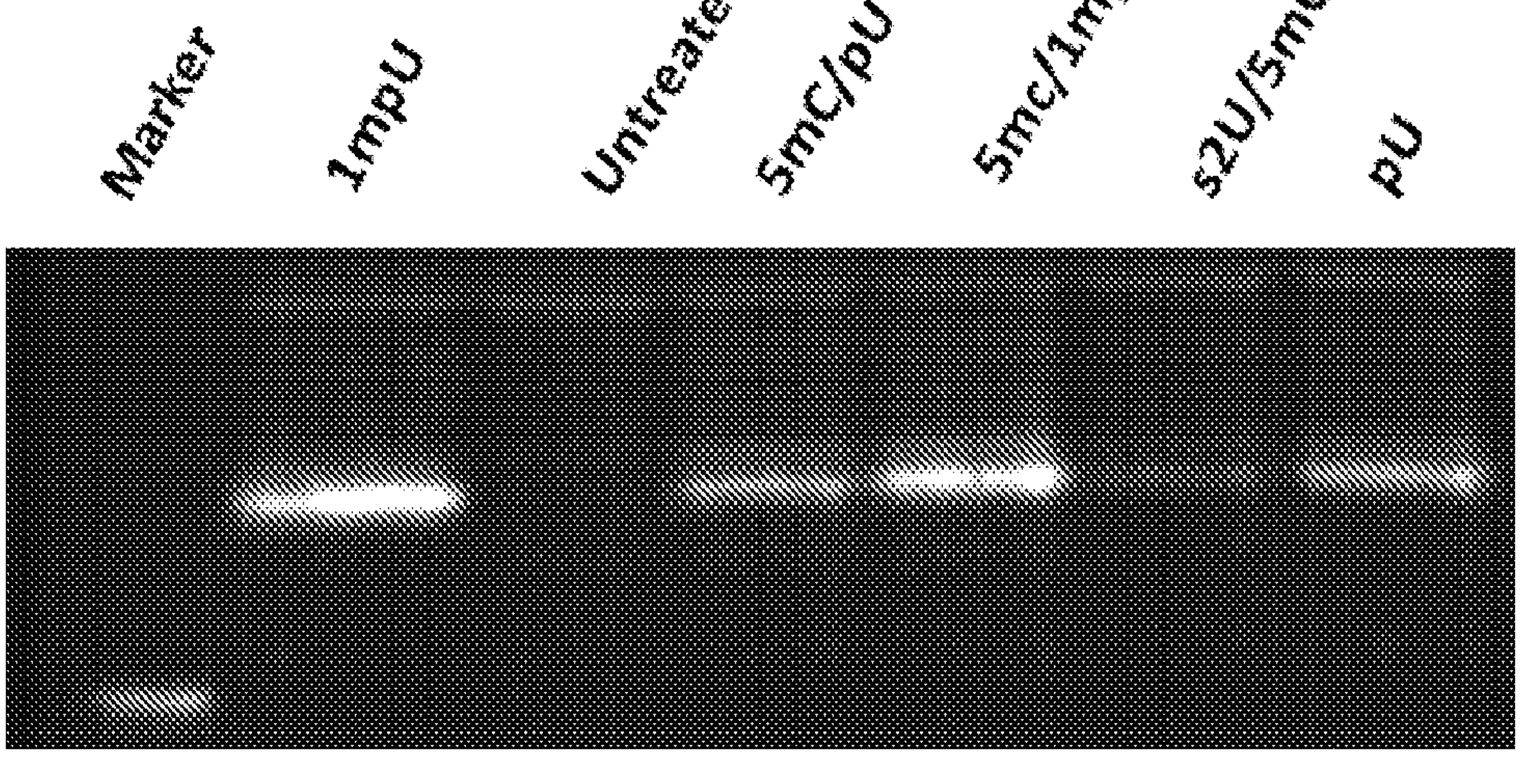


FIGURE 16

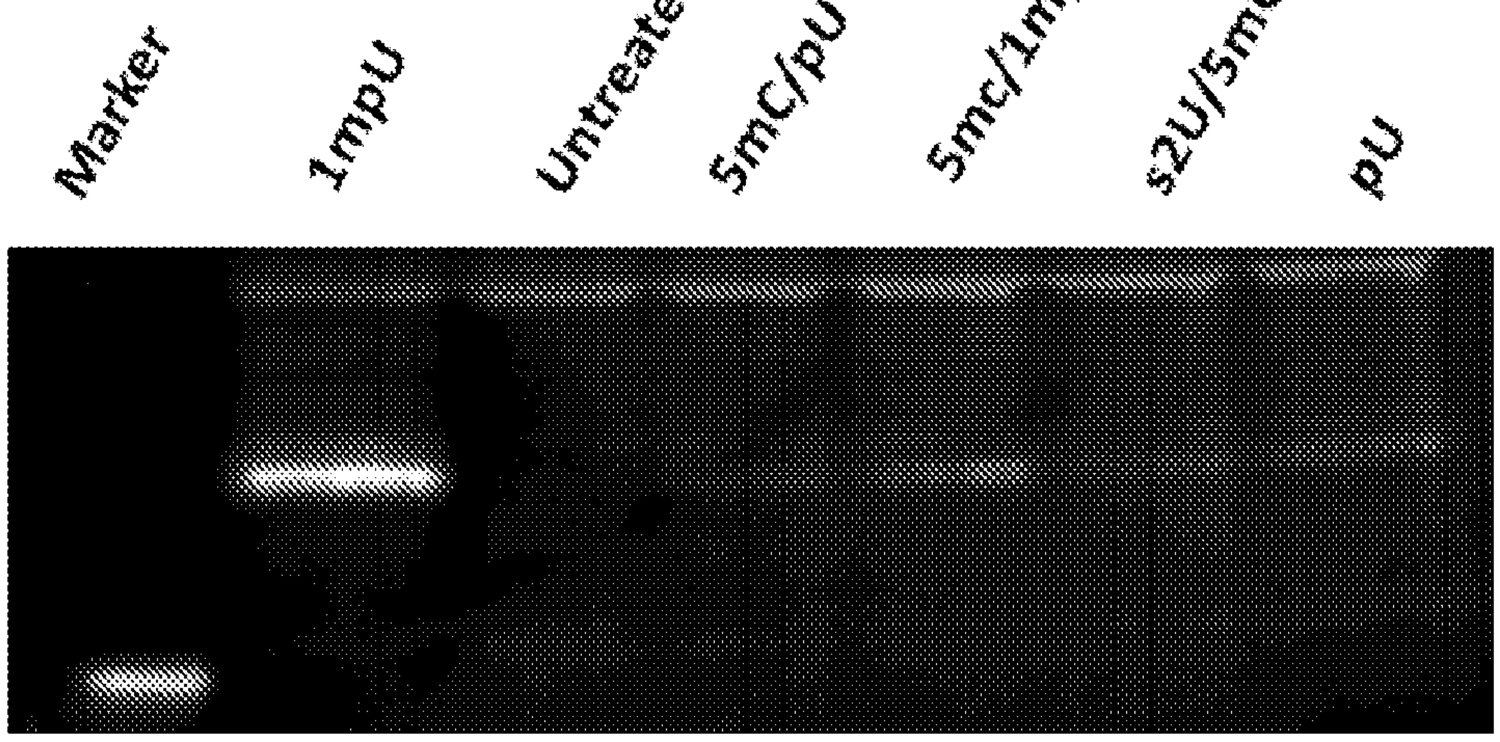


FIGURE 17

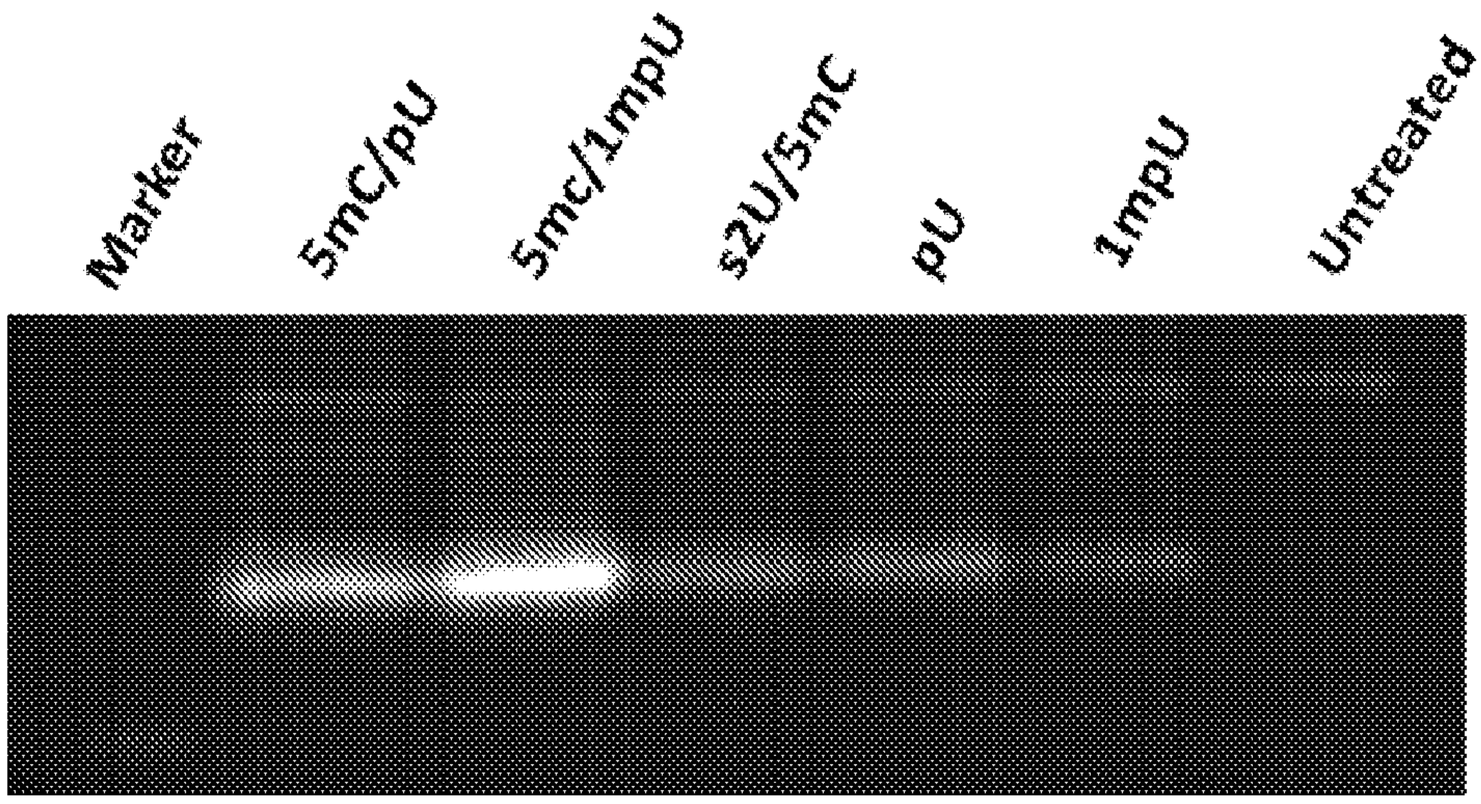


FIGURE 18

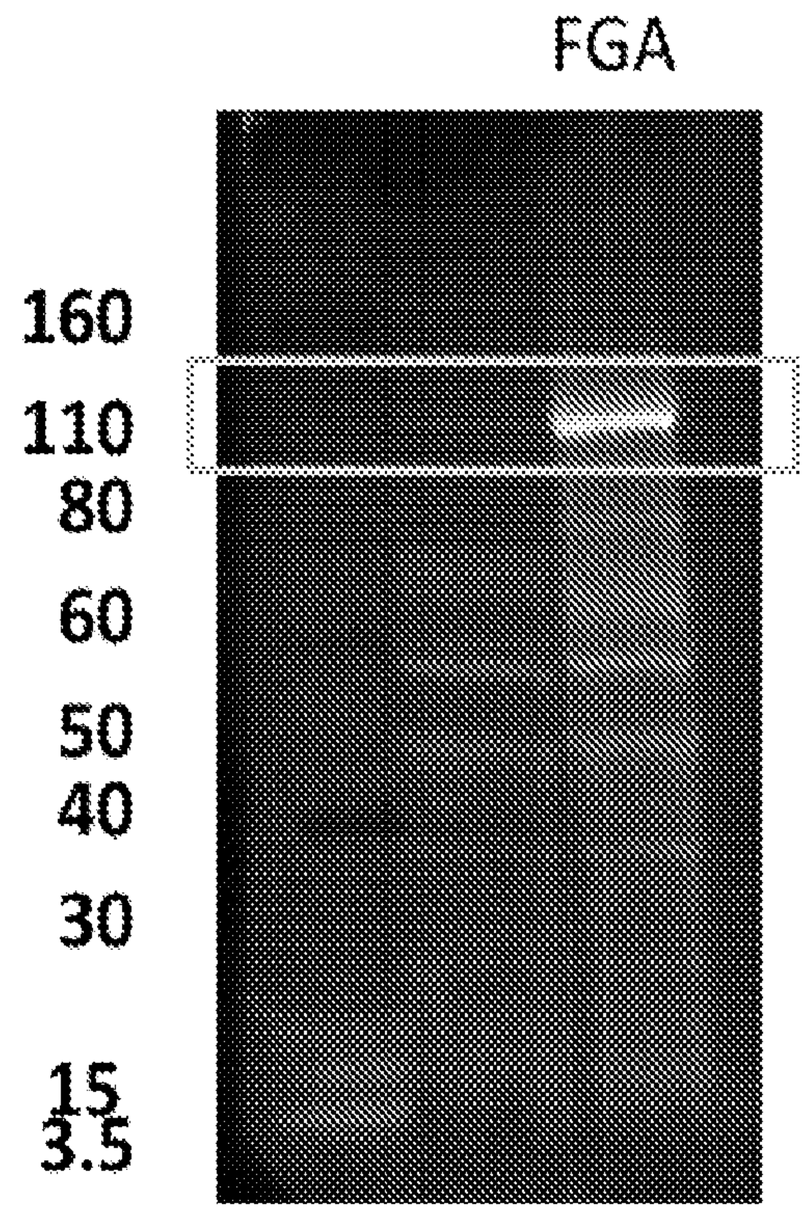


FIGURE 19

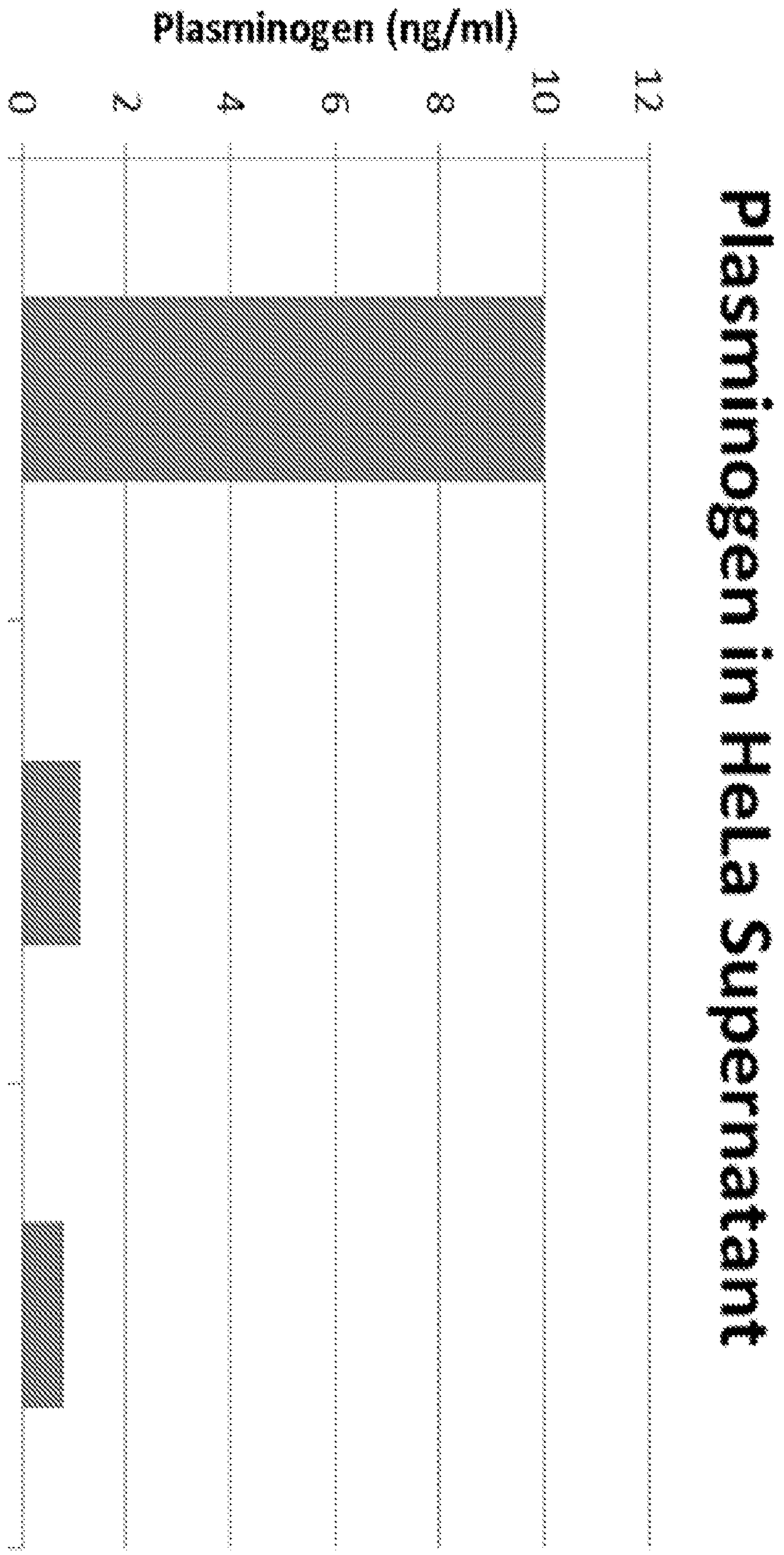

Plasminogen (ng/ml)
0
2
4
6
8
10
12
Plasminogen in HeLa Supernatant
Plasminogen
Epo
Untreated

FIGURE 20

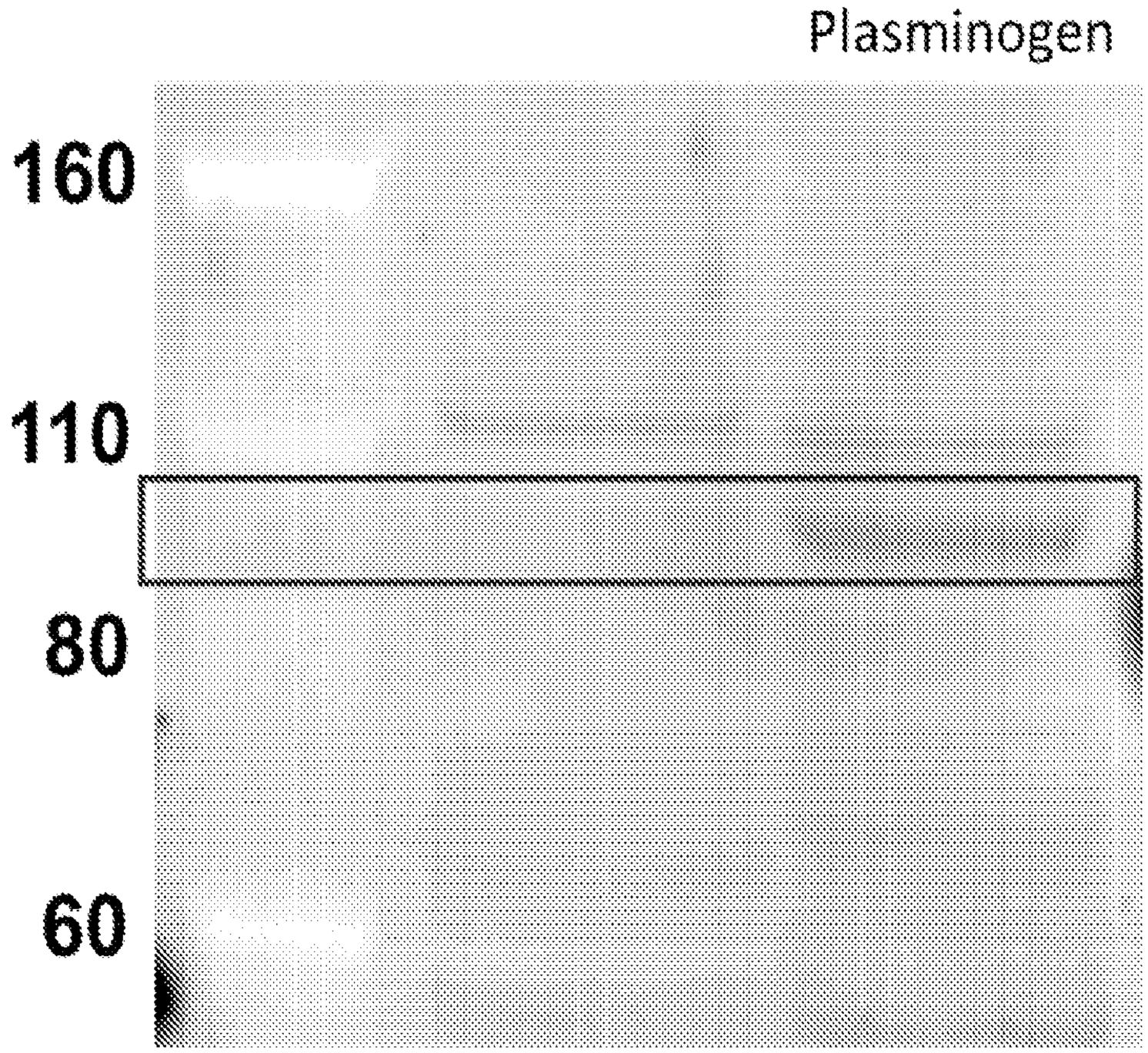


FIGURE 21

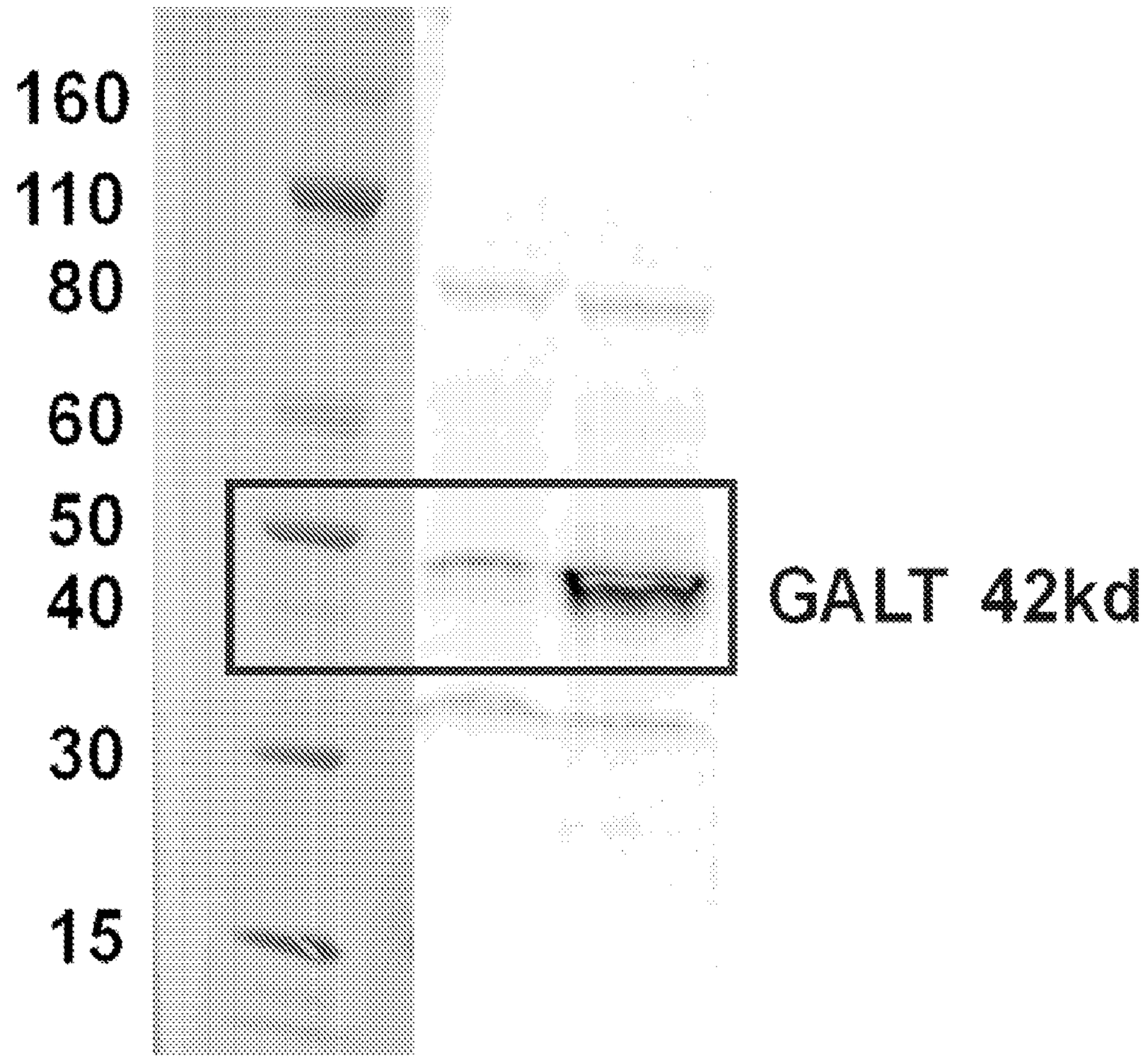


FIGURE 22

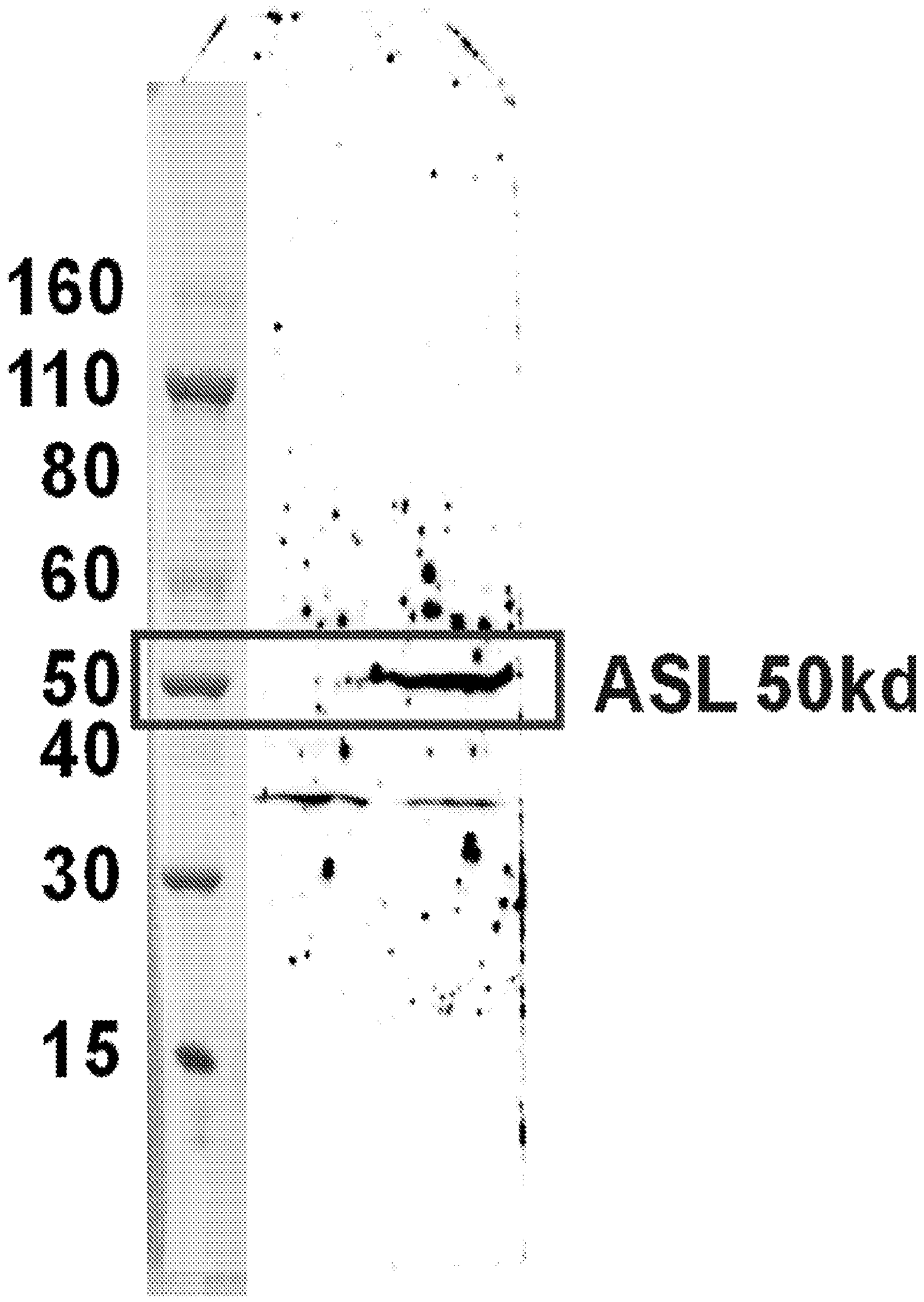


FIGURE 23

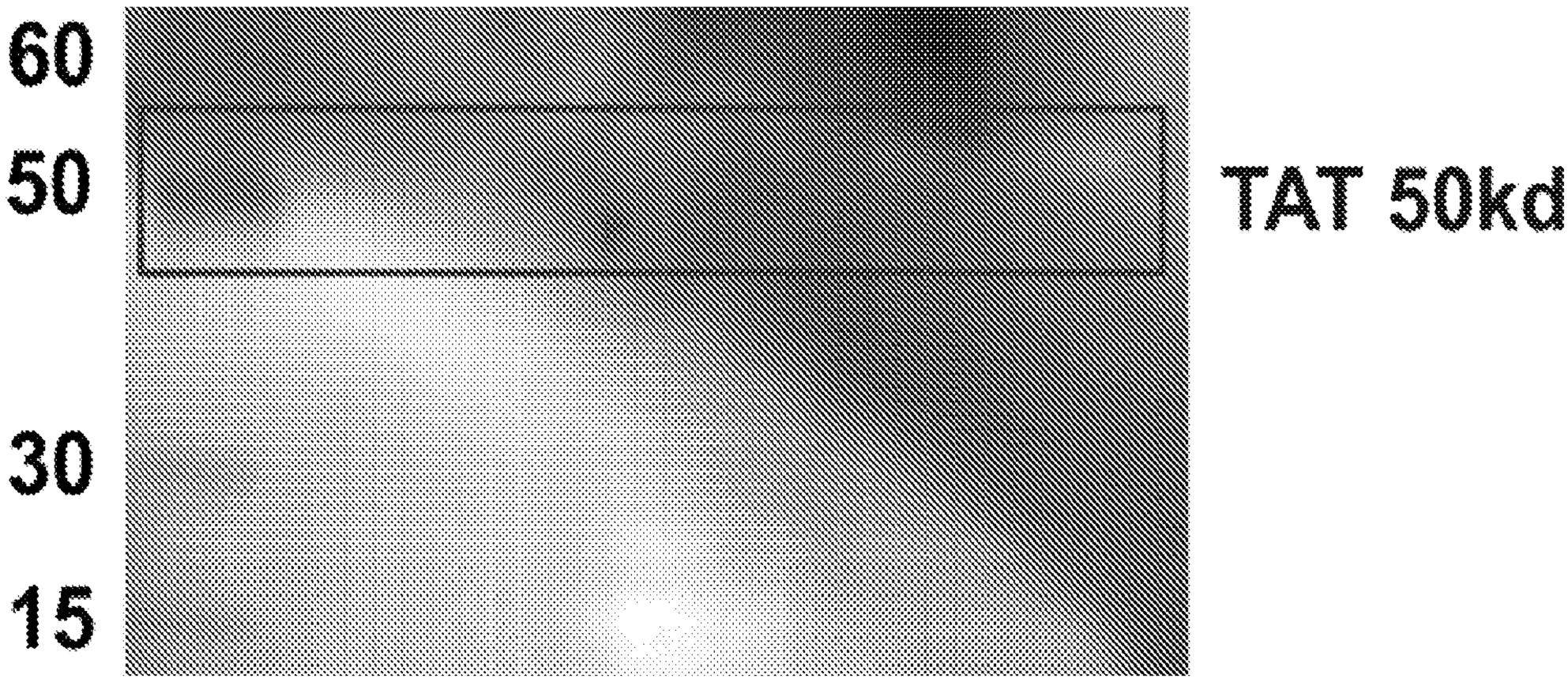


FIGURE 24

FIGURE 25

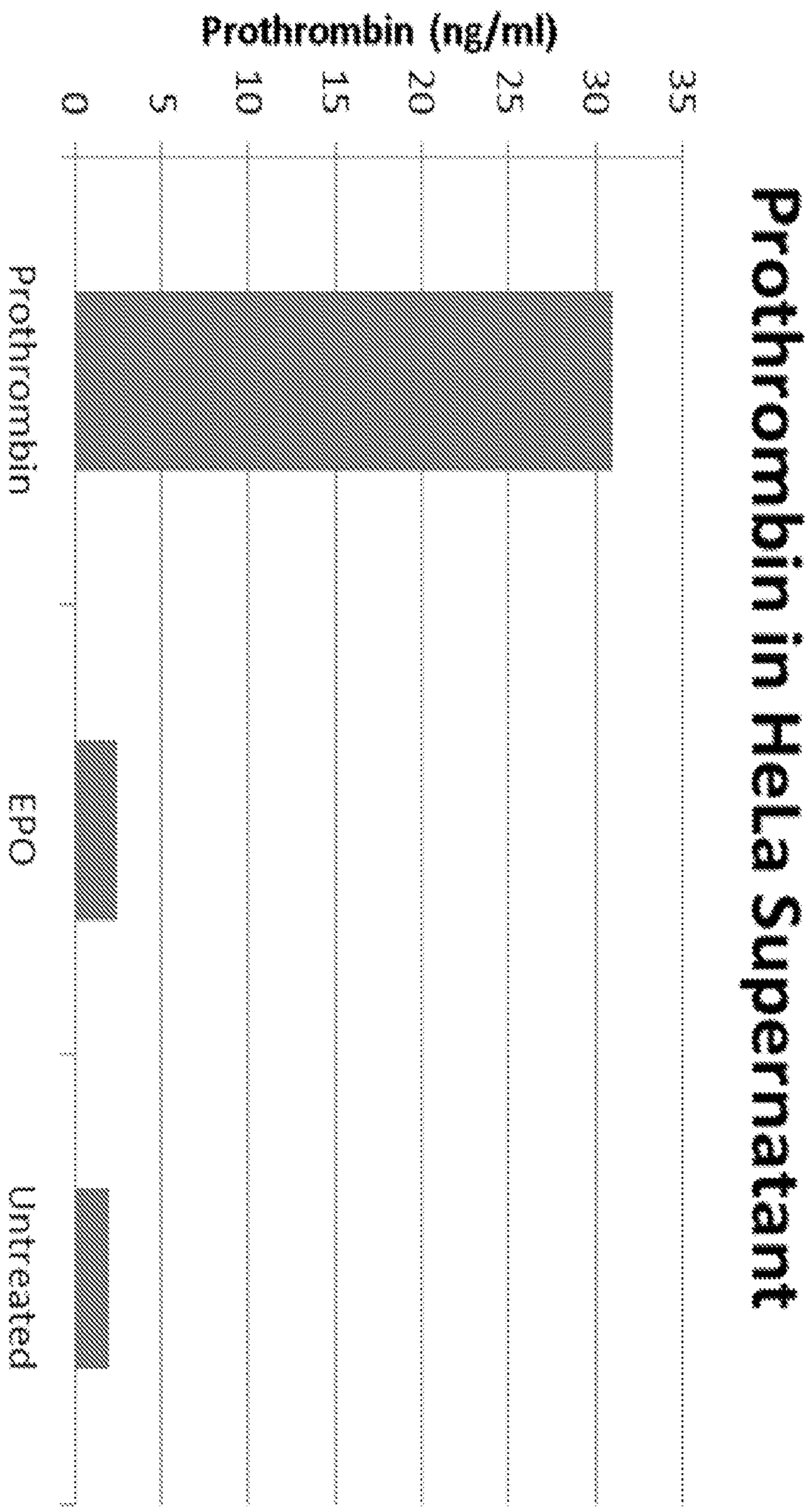

Prothrombin (ng/ml)
0
5
10
15
20
25
30
35
Prothrombin in HeLa Supernatant
prothrombin
EPO
Untreated

FIGURE 26

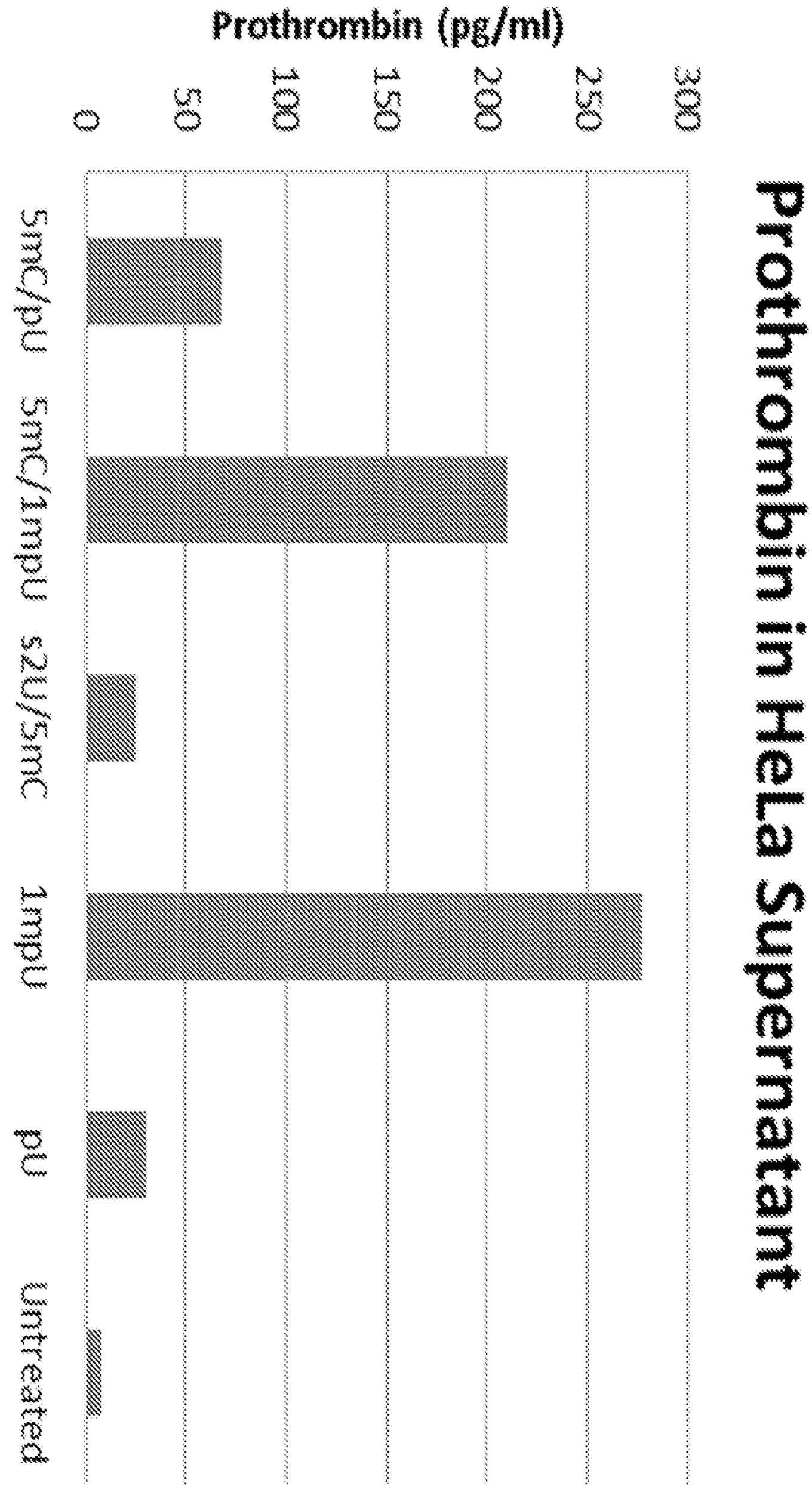

Prothrombin in HeLa Supernatant
Prothrombin (pg/ml)
0
50
100
150
200
250
300
untreated
pu
1mpu
smc/pu
smc/1mpu
s2u/smc

FIGURE 27

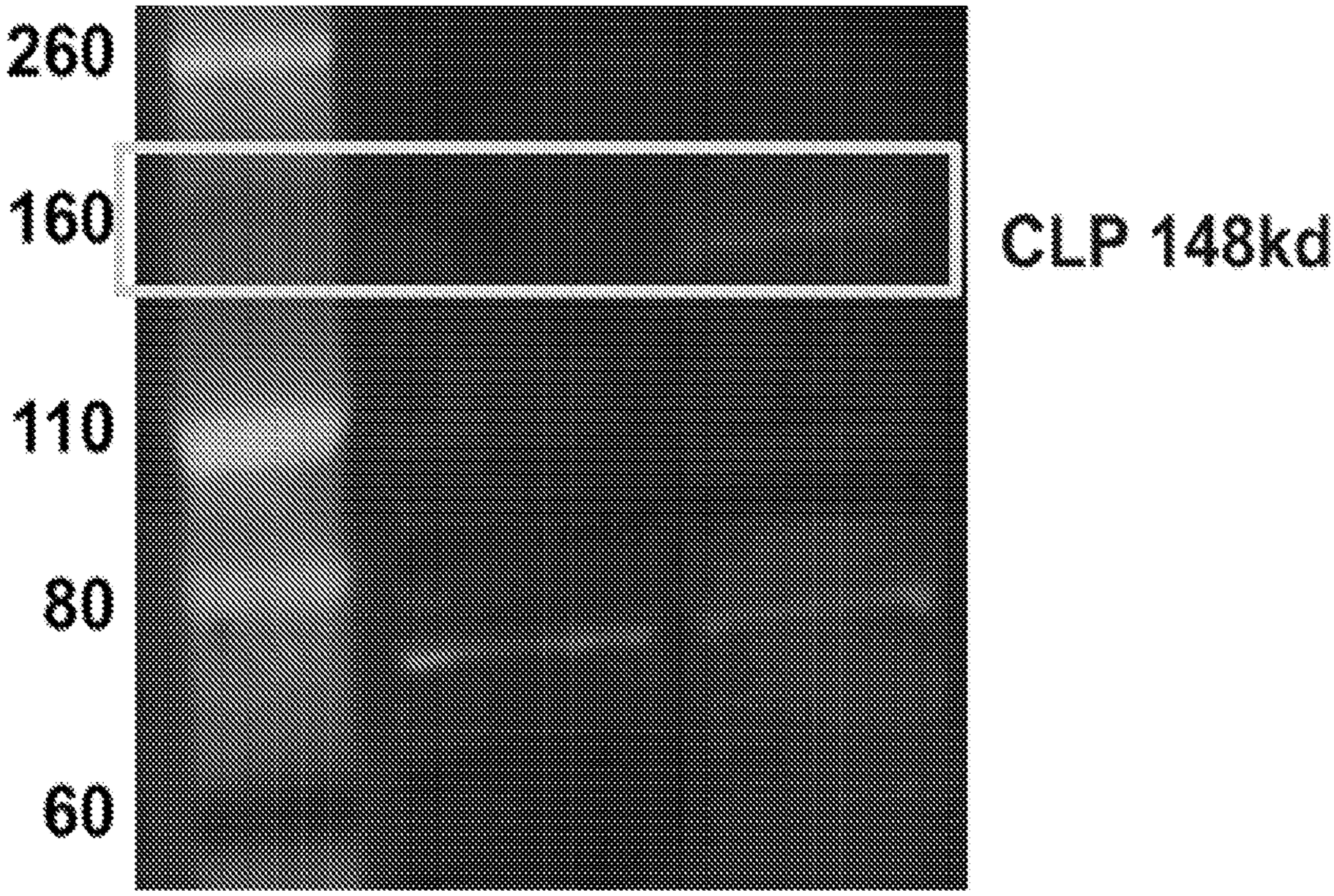


FIGURE 28

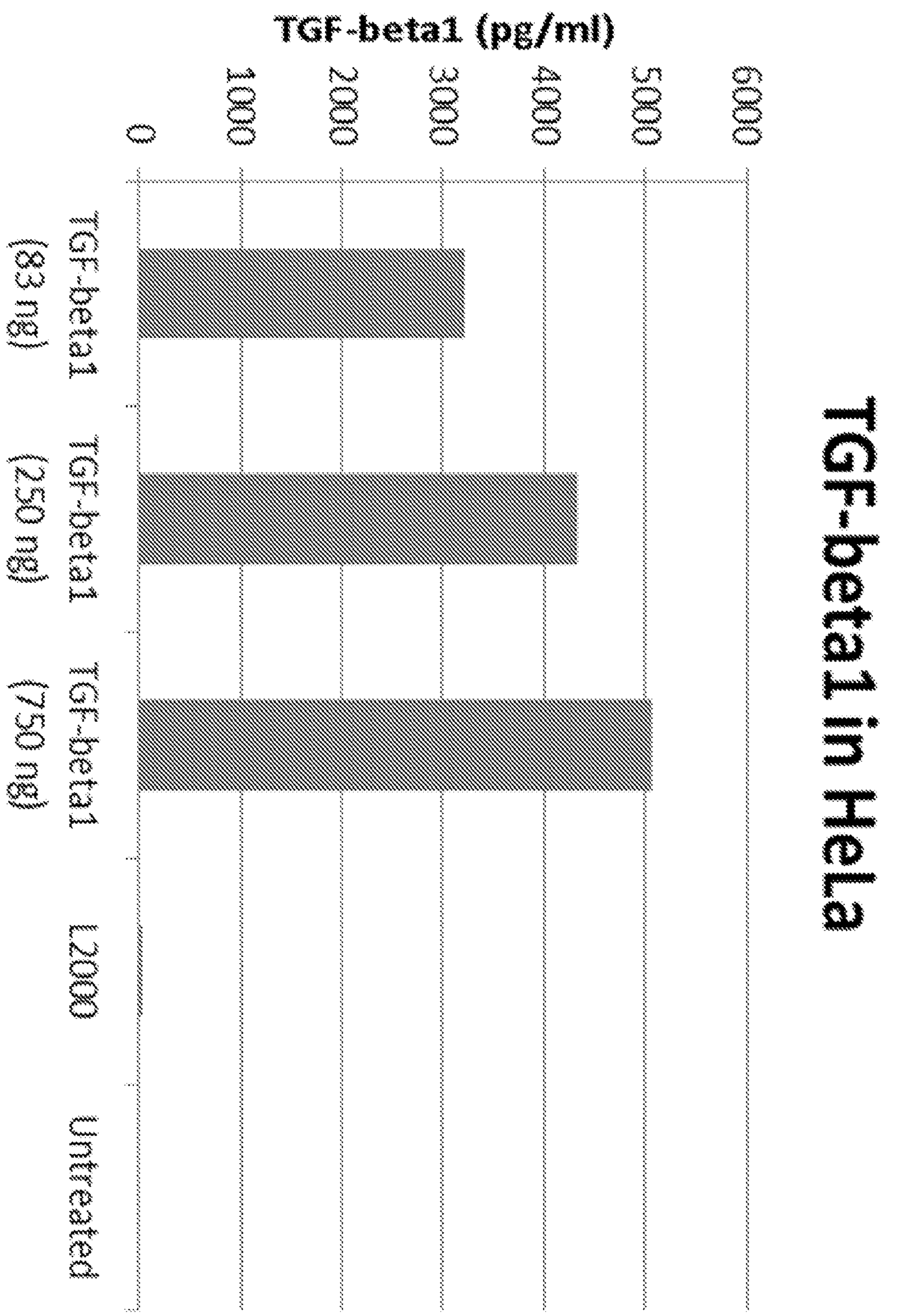

TGF-beta1 (pg/ml)
0
1000
2000
3000
4000
5000
6000
TGF-beta1 in Hela
TGF-beta1 (83 ng)
TGF-beta1 (250 ng)
TGF-beta1 (750 ng)
1200
Untreated

FIGURE 29

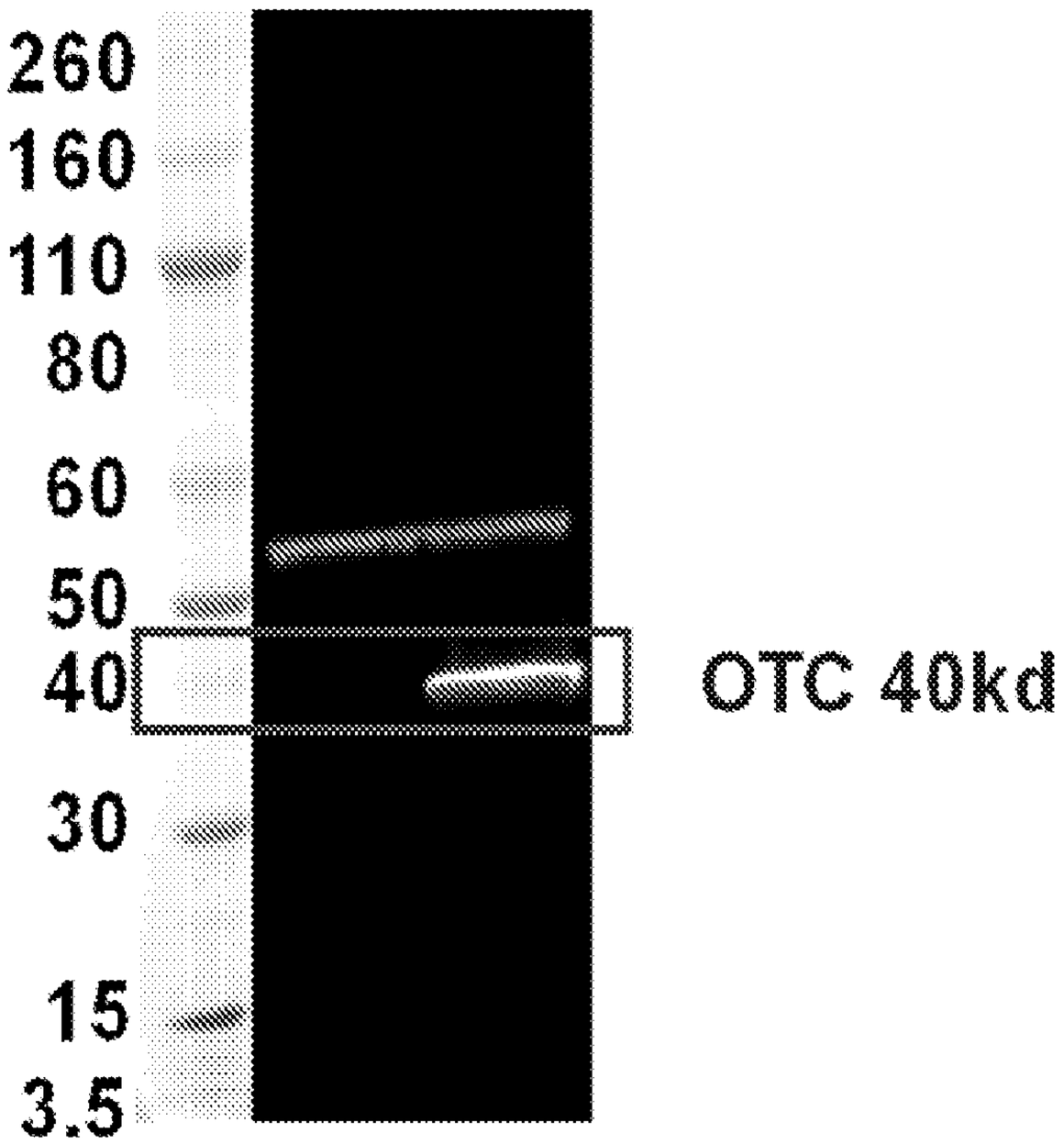


FIGURE 30

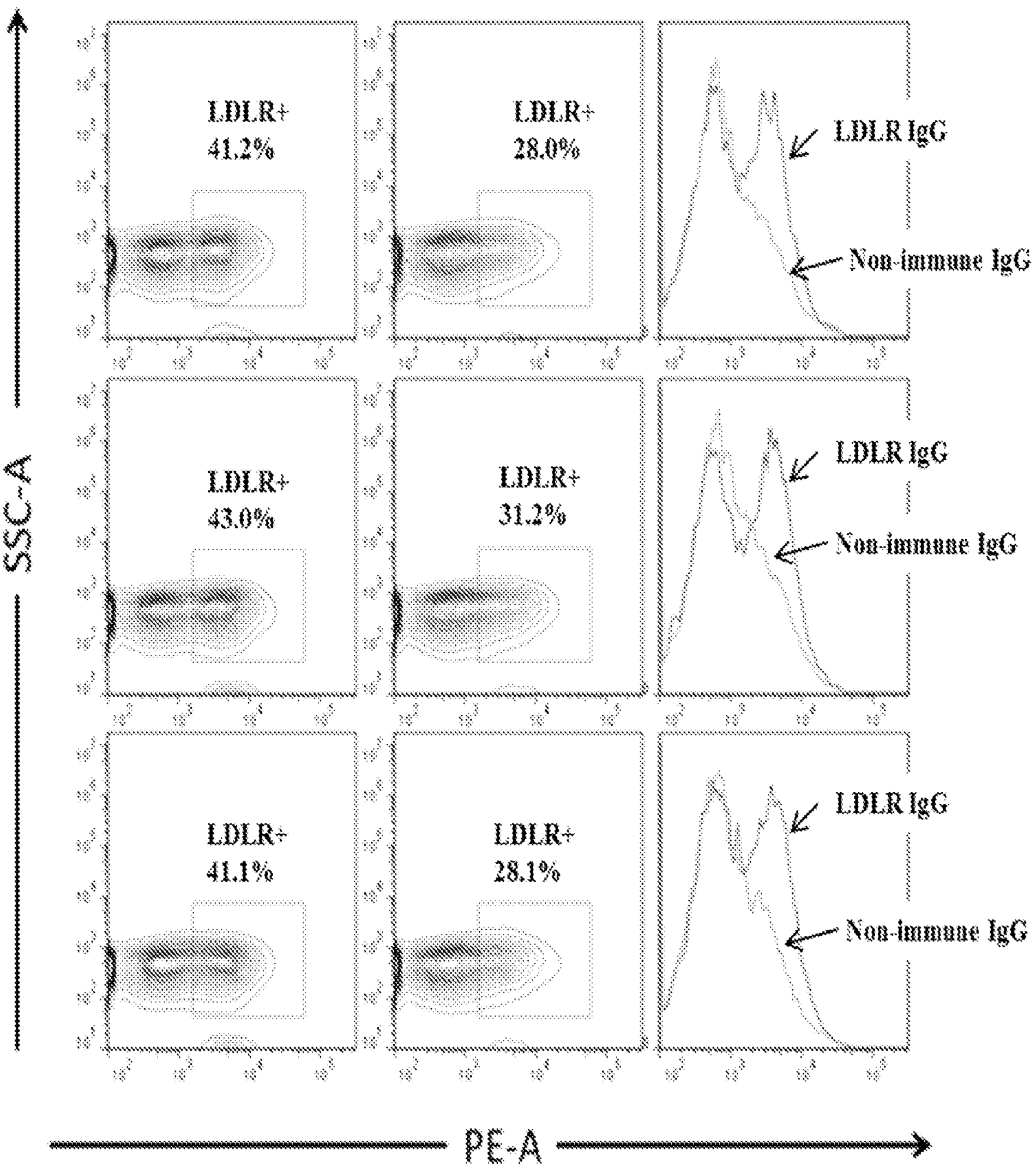

LDLR
LDLR+
41.2%
LDLR+
28.0%
LDLR IgG
Non-immune IgG
LDLR+
43.0%
LDLR+
31.2%
LDLR IgG
Non-immune IgG
LDLR+
41.1%
LDLR+
28.1%
LDLR IgG
Non-immune IgG
SSC-A
PE-A

FIGURE 31


Replicate 1
Replicate 2
Replicate 3
180
116
90
66
40
12
UGT1A1

FIGURE 32

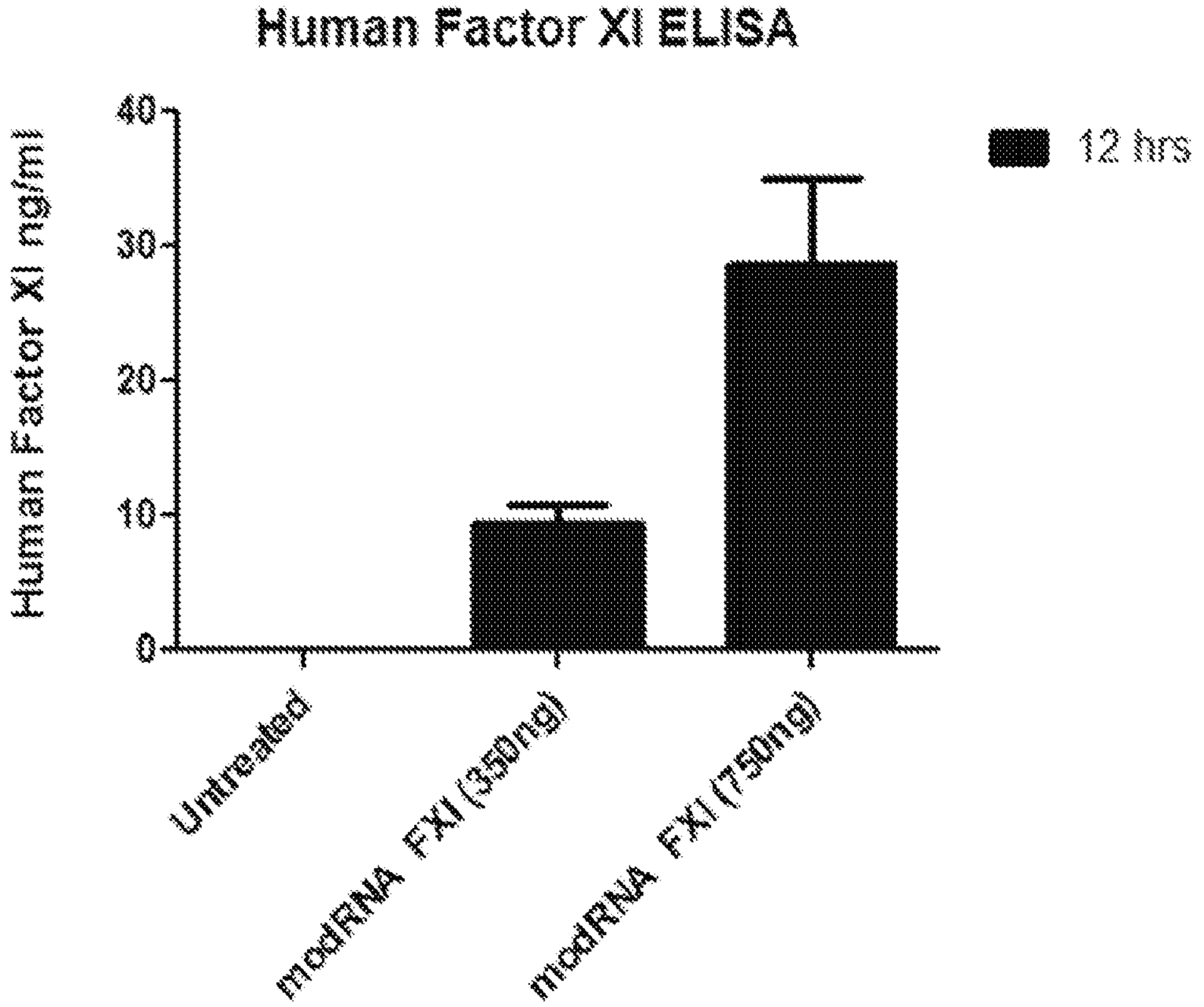

Human Factor XI ELISA
Human Factor XI ng/ml
0
10
20
30
40
12 hrs
Untreated
FXI (350ng)
FXI (750ng)
mock RNA
mock RNA

FIGURE 33

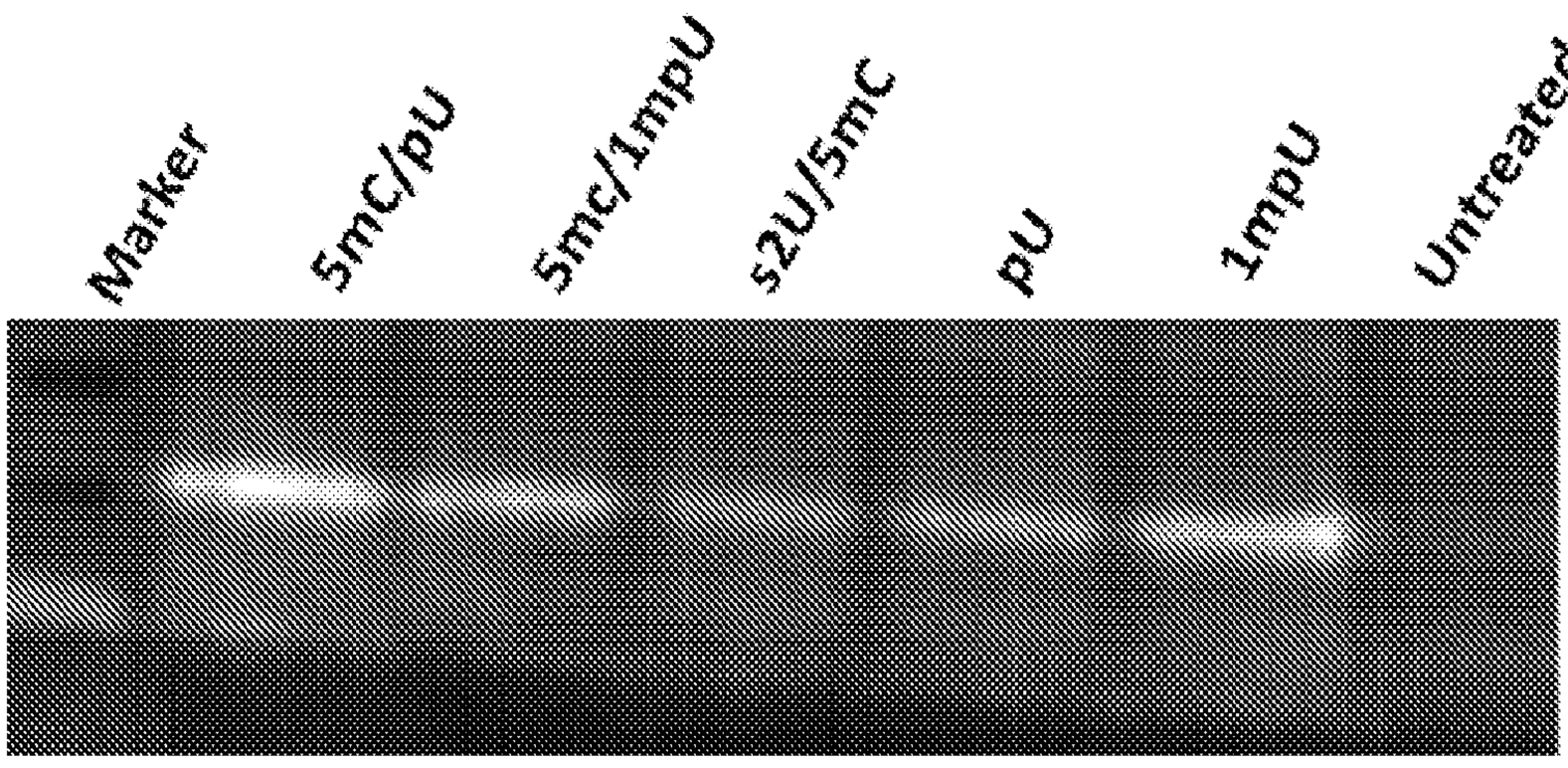


FIGURE 34

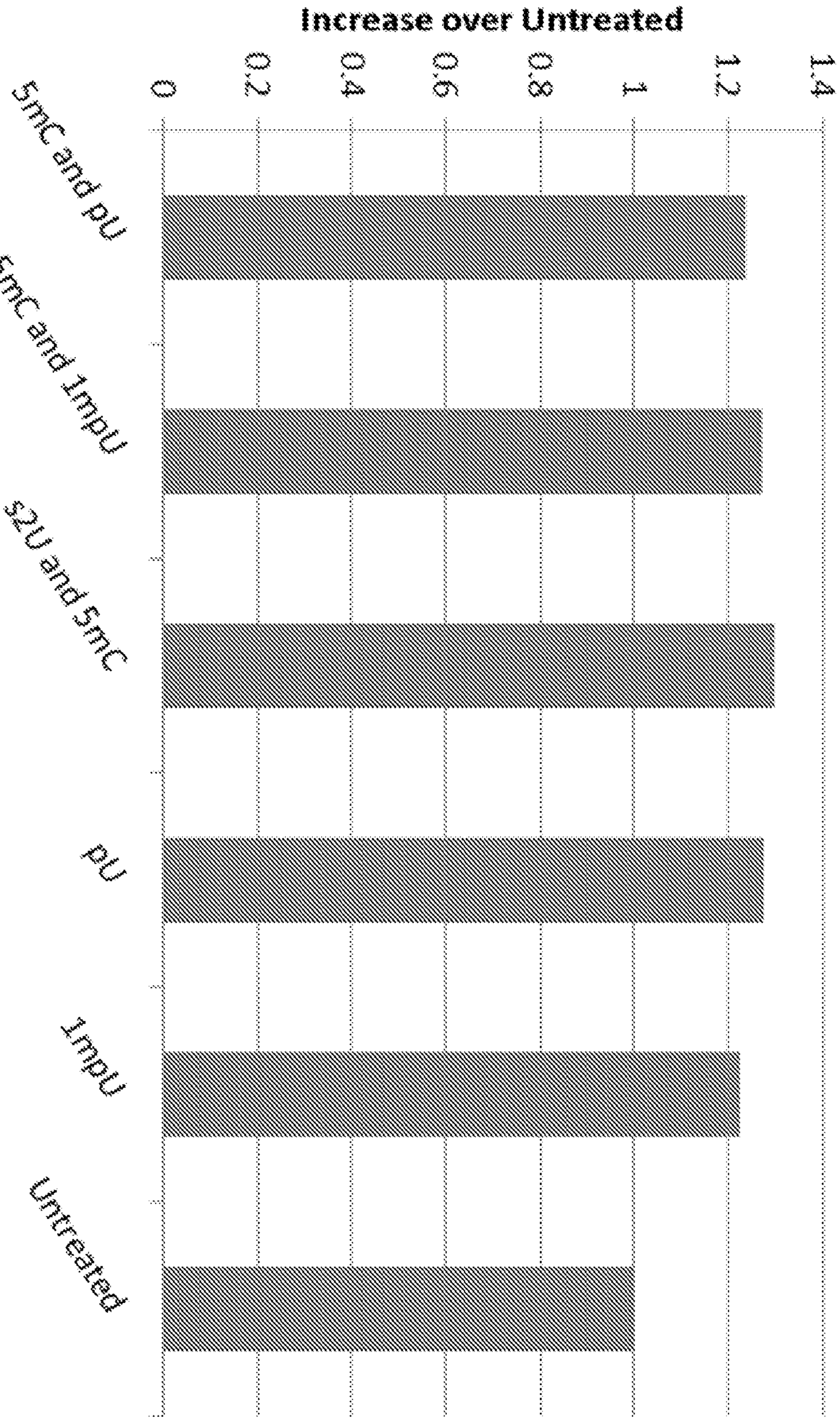

Increase over Untreated
0
0.2
0.4
0.6
0.8
1
1.2
1.4
Factor VIII in Hela
5mC and pU
5mC and 1mpU
s2U and 5mC
pU
1mpU
Untreated

FIGURE 35

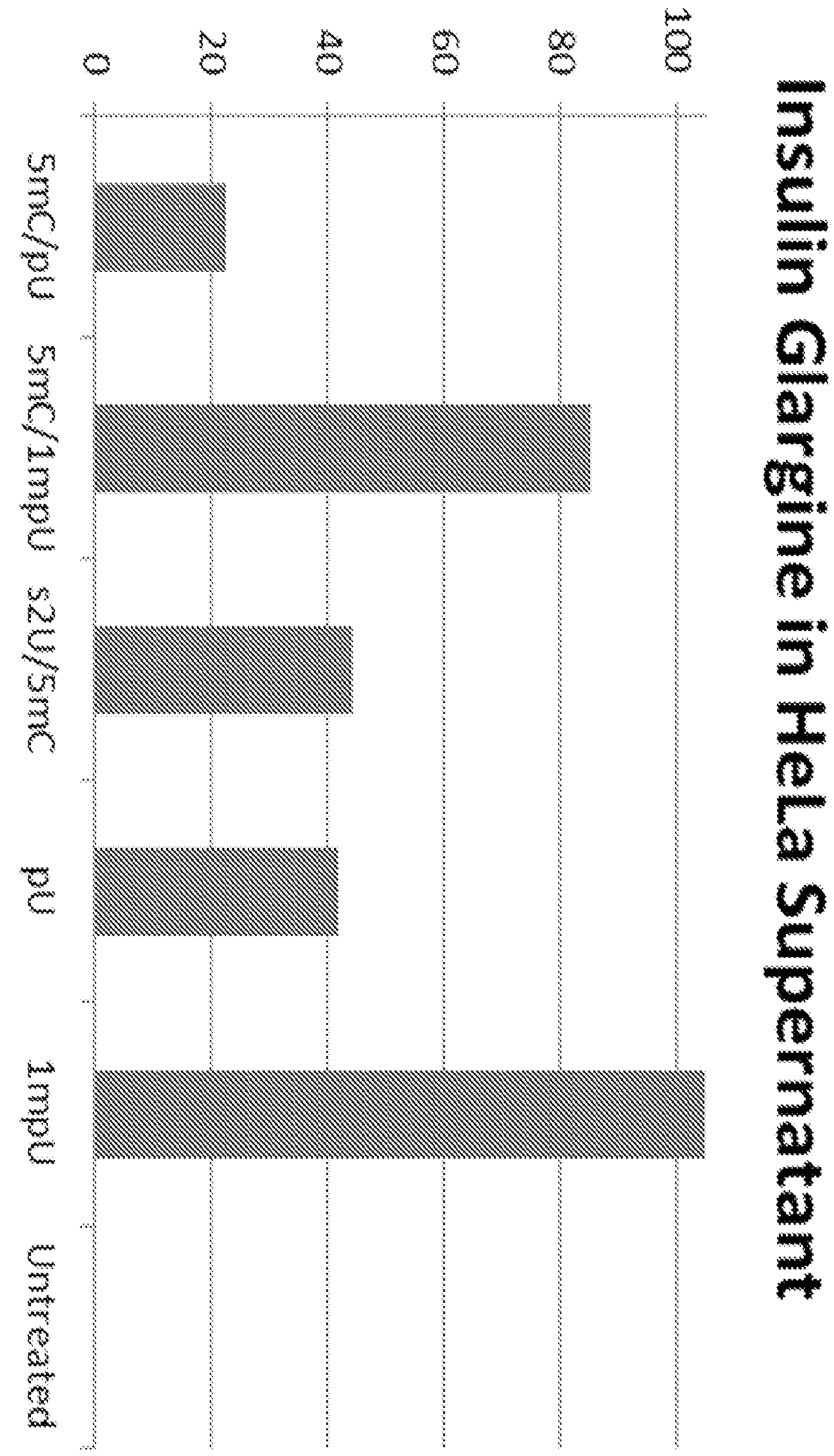

Insulin (mIU/ml)
0
20
40
60
80
100
Insulin Glargine in Healthy Subjects Supernatant
Untreated
1mpu
nd
2mpu
5mpu
10mpu

FIGURE 36

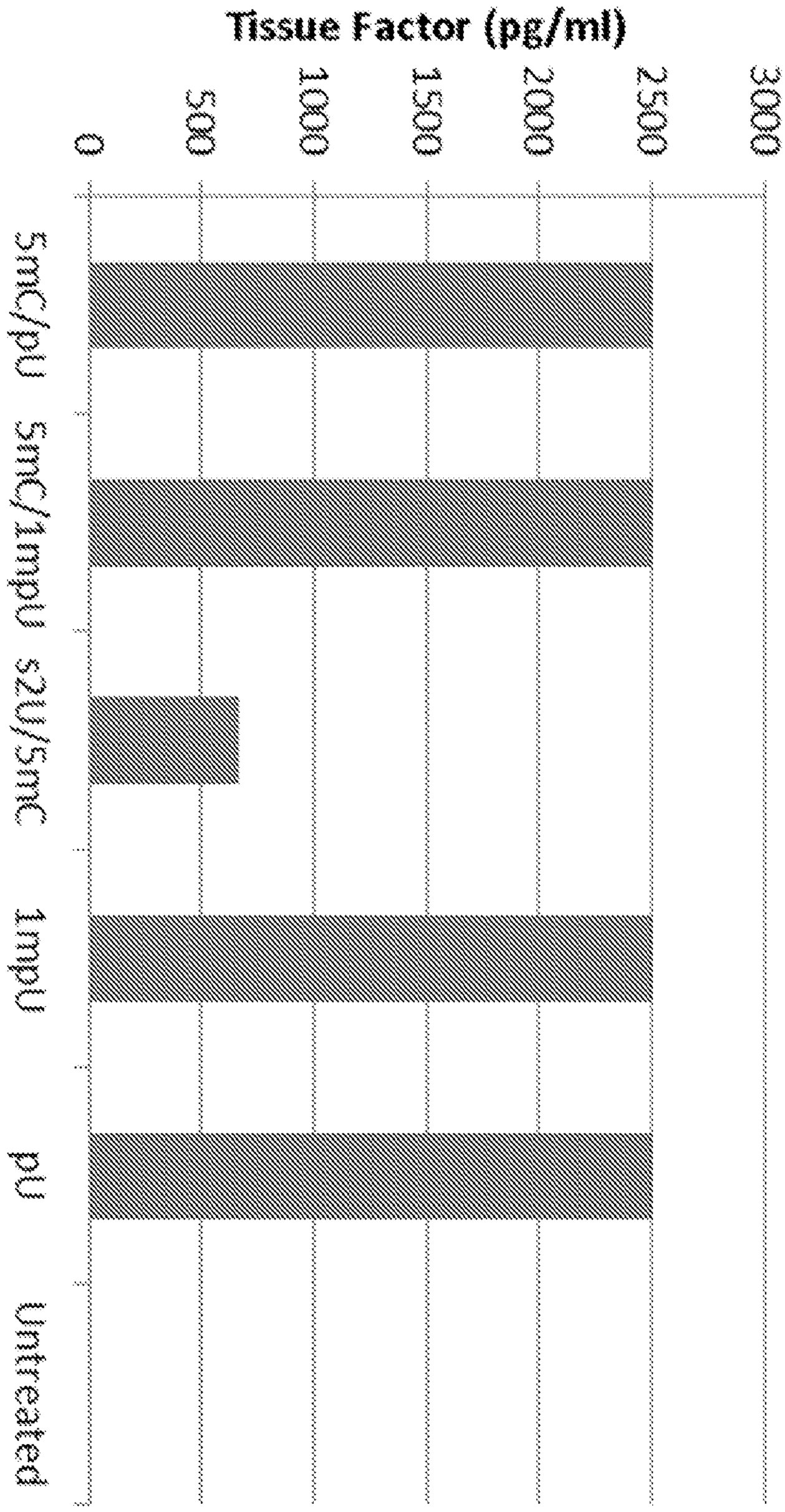

Tissue Factor (pg/ml)
0
500
1000
1500
2000
2500
3000
Tissue Factor in HeLa
smc/pu
smc/tmpu
s2u/snc
tmpu
pu
Untreated

FIGURE 37

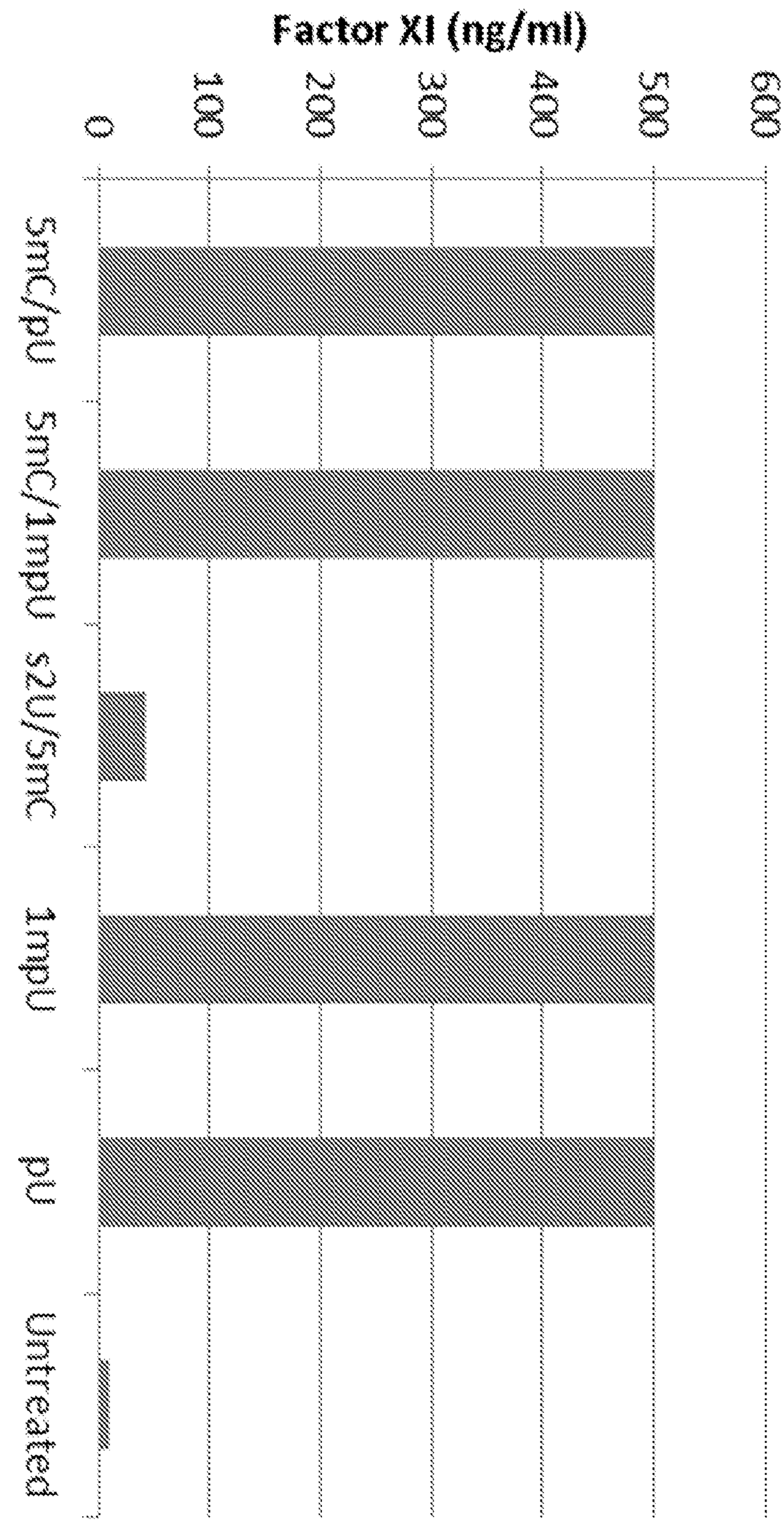

Factor XI (ng/ml)
0
100
200
300
400
500
600
Factor XI in Hela
5mC/pu
5mC/1mpu
s2u/5mc
1mpu
pu
Untreated

FIGURE 38

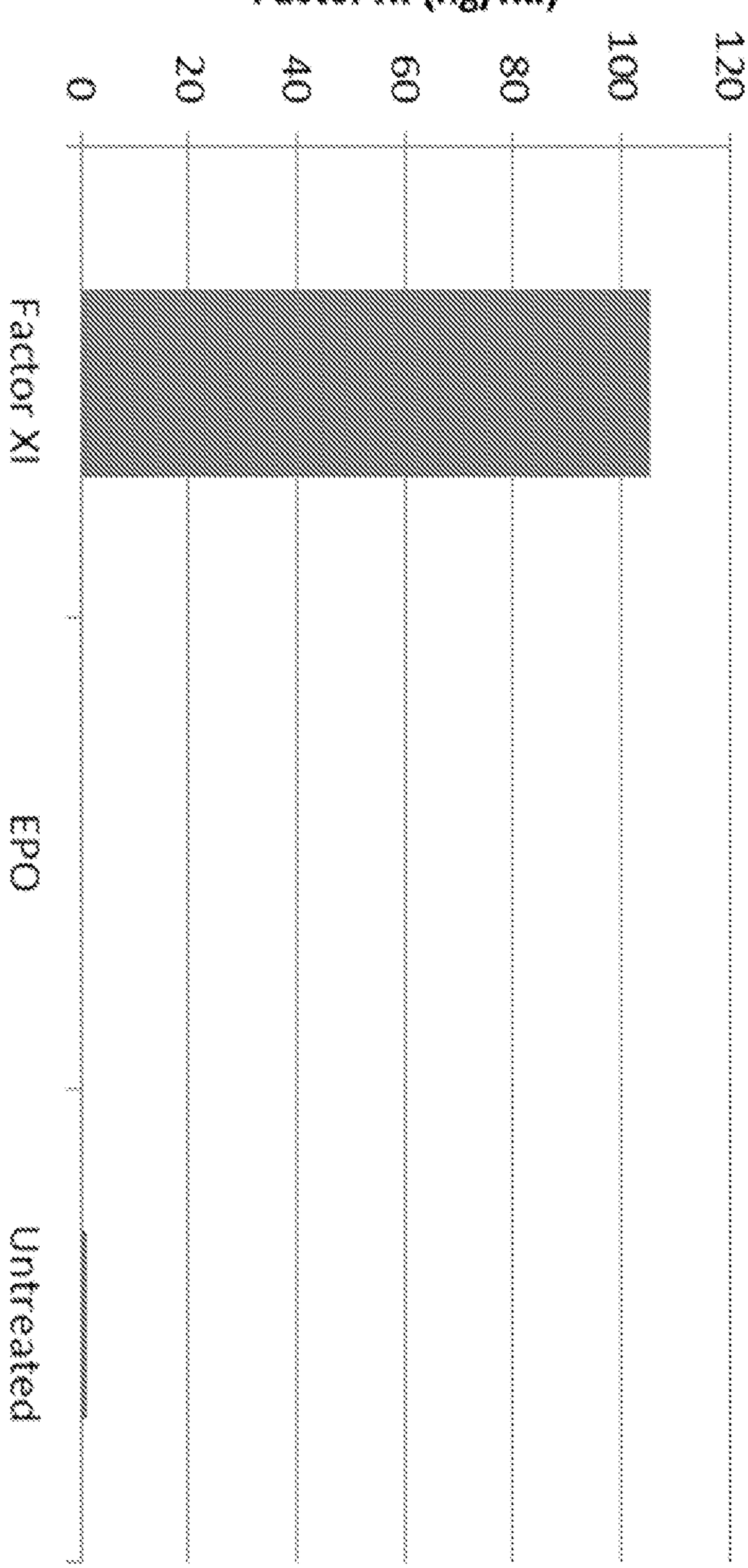

Factor XI (ng/ml)
0
20
40
60
80
100
120
Factor XI in Hela
Factor XI
EPO
Untreated

FIGURE 39

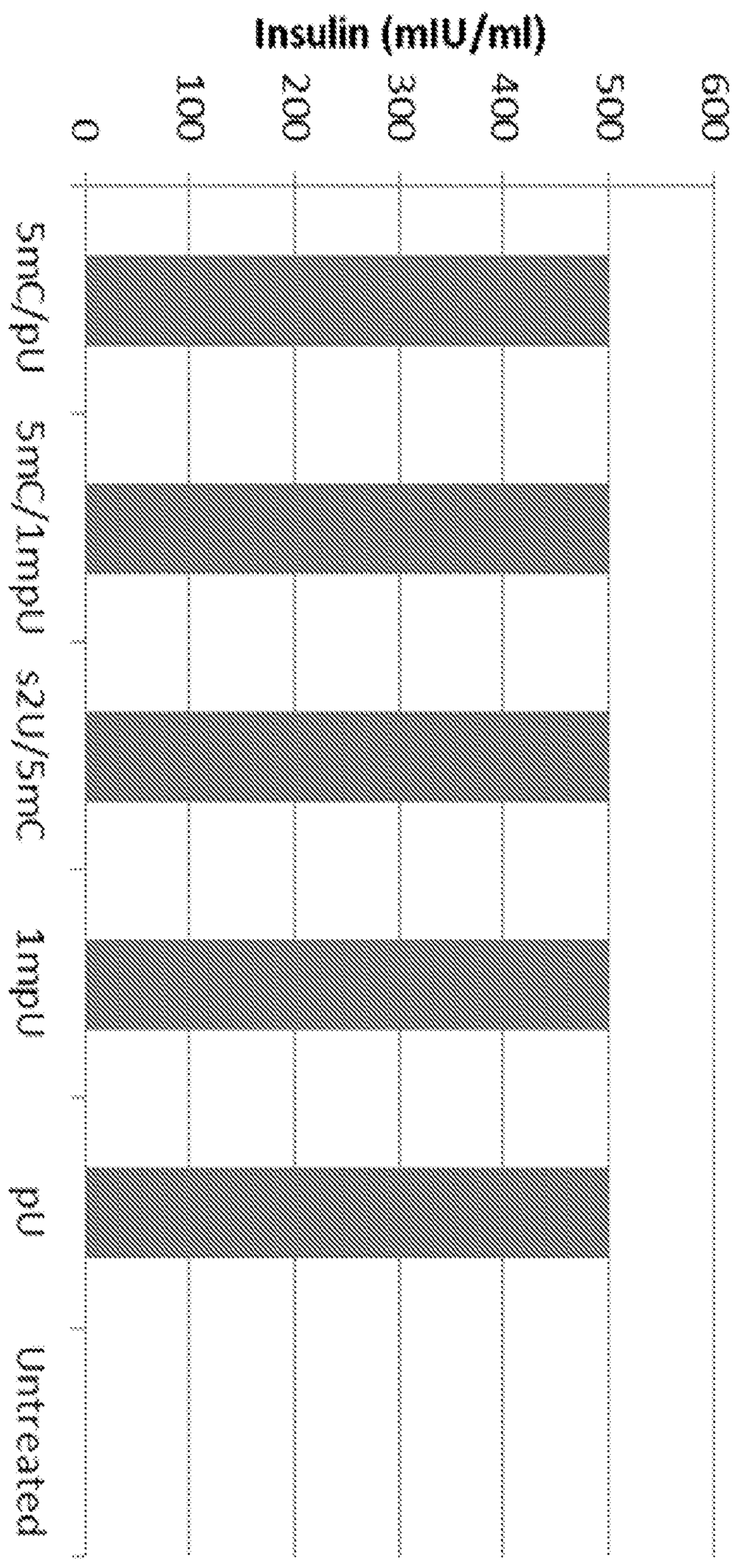

Insulin (mIU/ml)
0
100
200
300
400
500
600
Insulin Aspart
untreated
nd pu
tmpu
smc/pu smc/1mpu s2u/smc

FIGURE 40

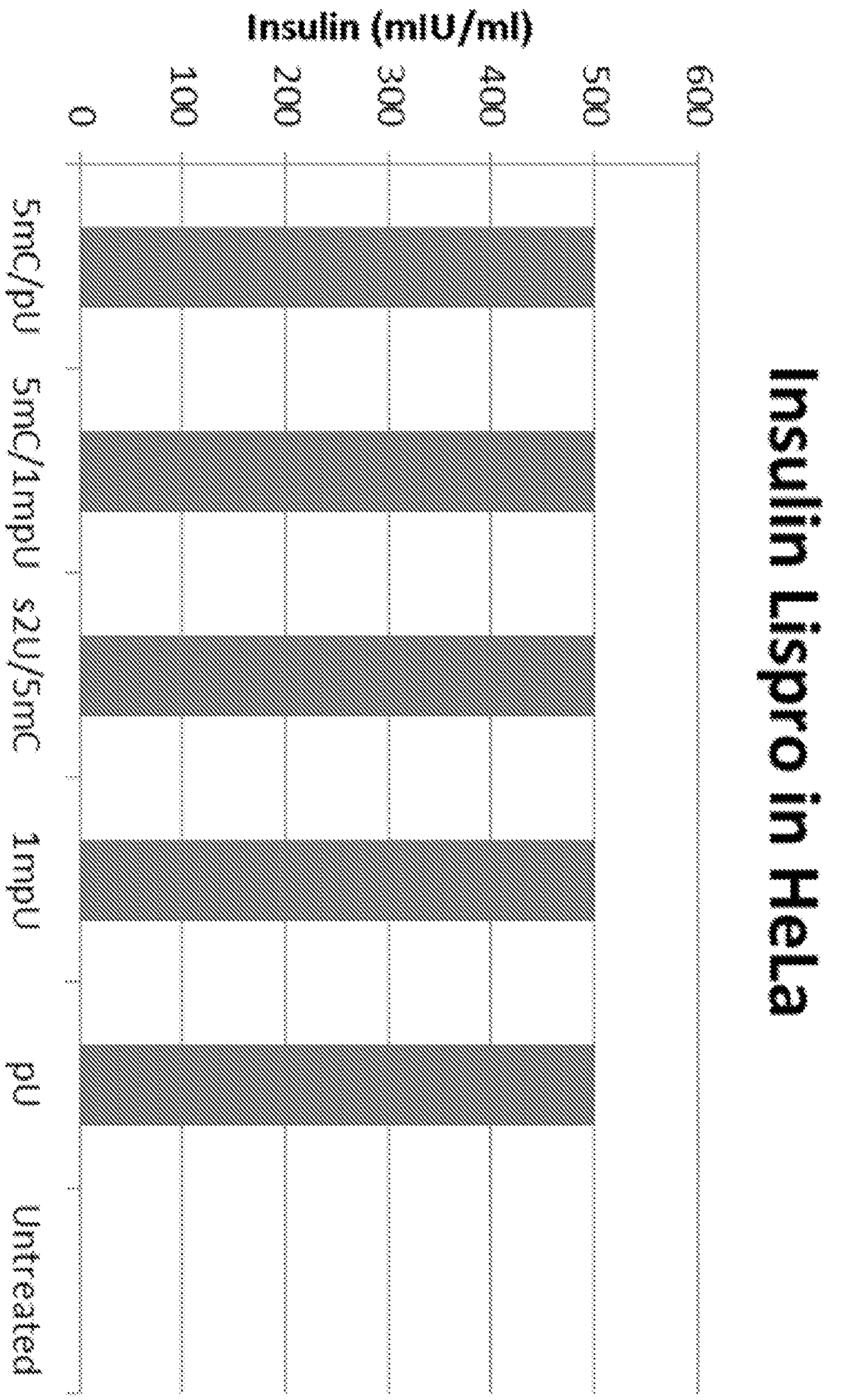

Insulin (mIU/ml)
0
100
200
300
400
500
600
Insulin Lispro in Hela
Untreated
nd
1mpu
nd
2su/5mc
5mc/1mpu
5mc/pu

FIGURE 41

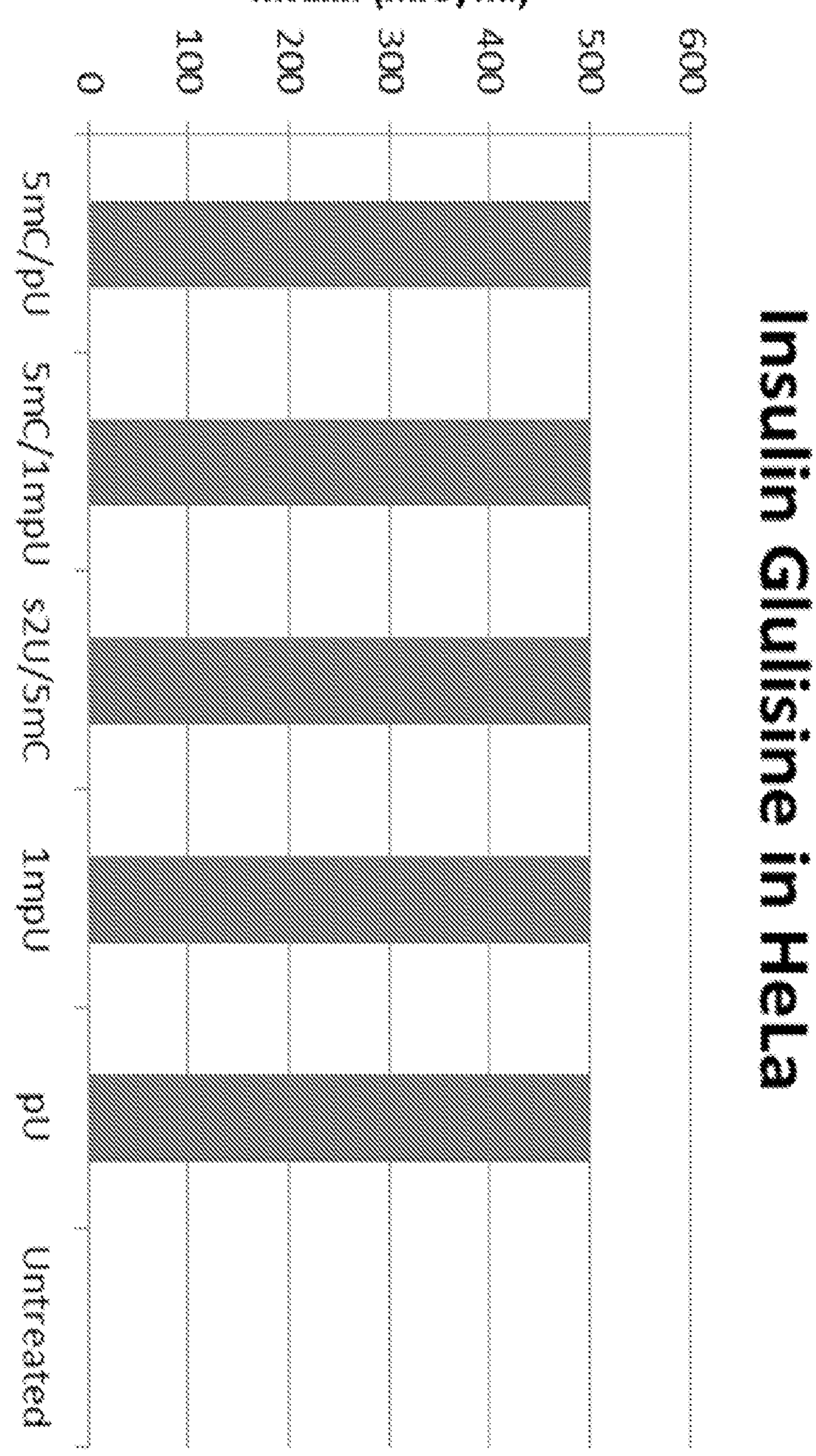

Insulin (mIU/ml)
0
100
200
300
400
500
600
Insulin Glulisine in Hela
untreated
nd
1mpu
5mc/pu
5mc/1mpu
52u/5mc

FIGURE 42

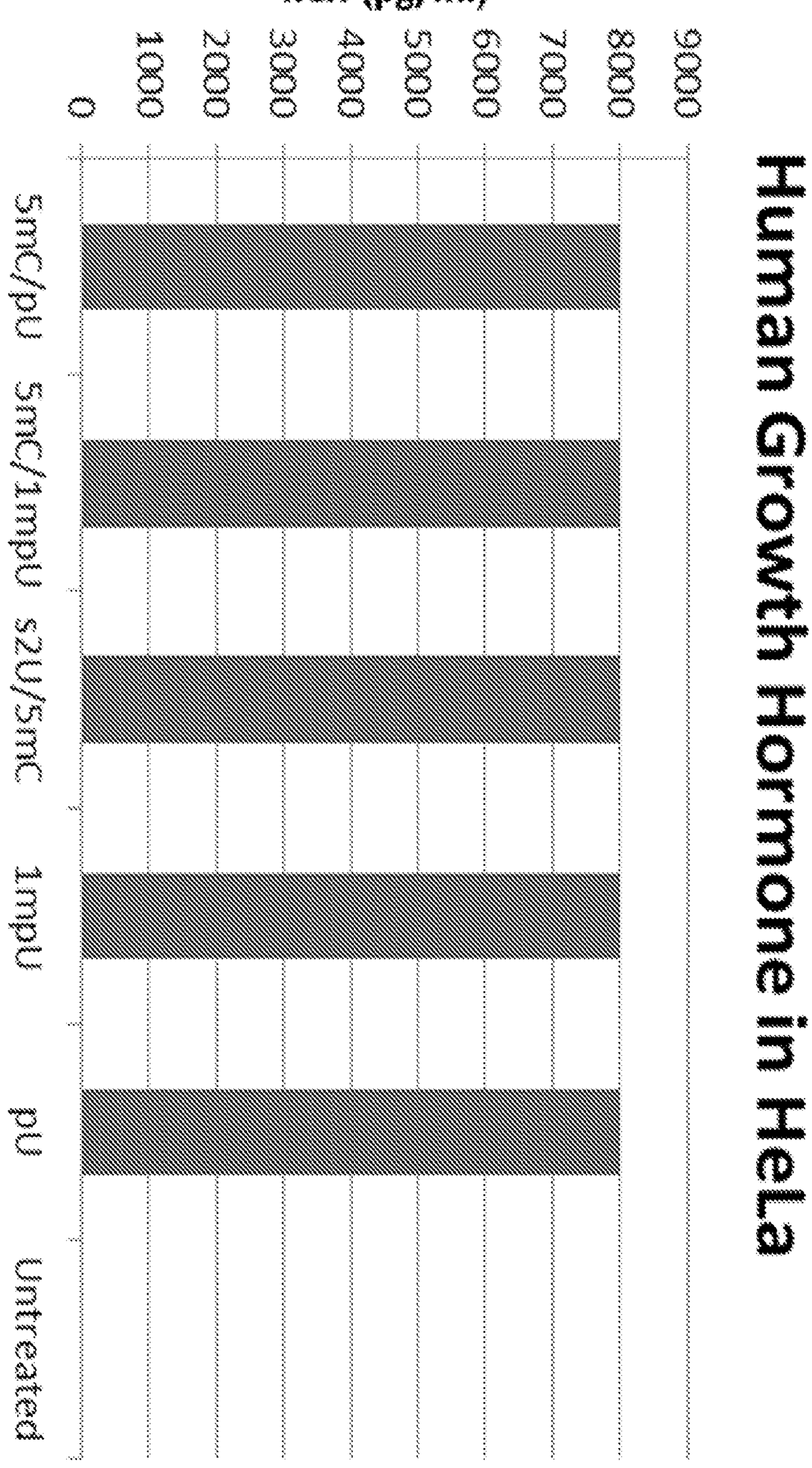
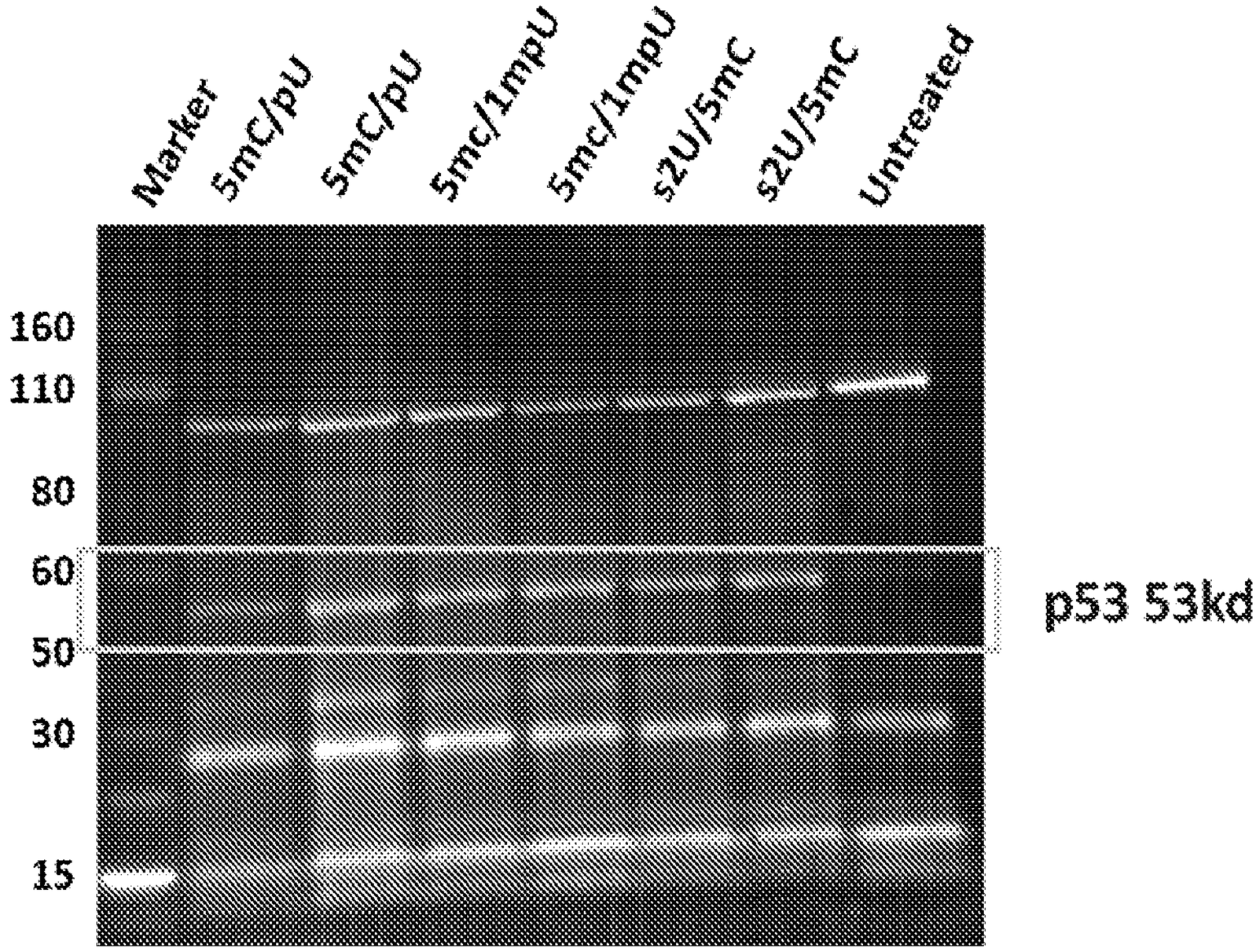

hCH (pg/ml)
0
1000
2000
3000
4000
5000
6000
7000
8000
9000
Human Growth Hormone in HeLa
Untreated
pu
5mC/1mpu
5mC/pu
1mpu
5mC
s2U/5mC

FIGURE 43

A.


Marker
5mC/pU
5mC/pU
5mc/1mpU
5mc/1mpU
s2U/5mC
s2U/5mC
Untreated
160
110
80
60
50
30
15
p53 53kd

B.

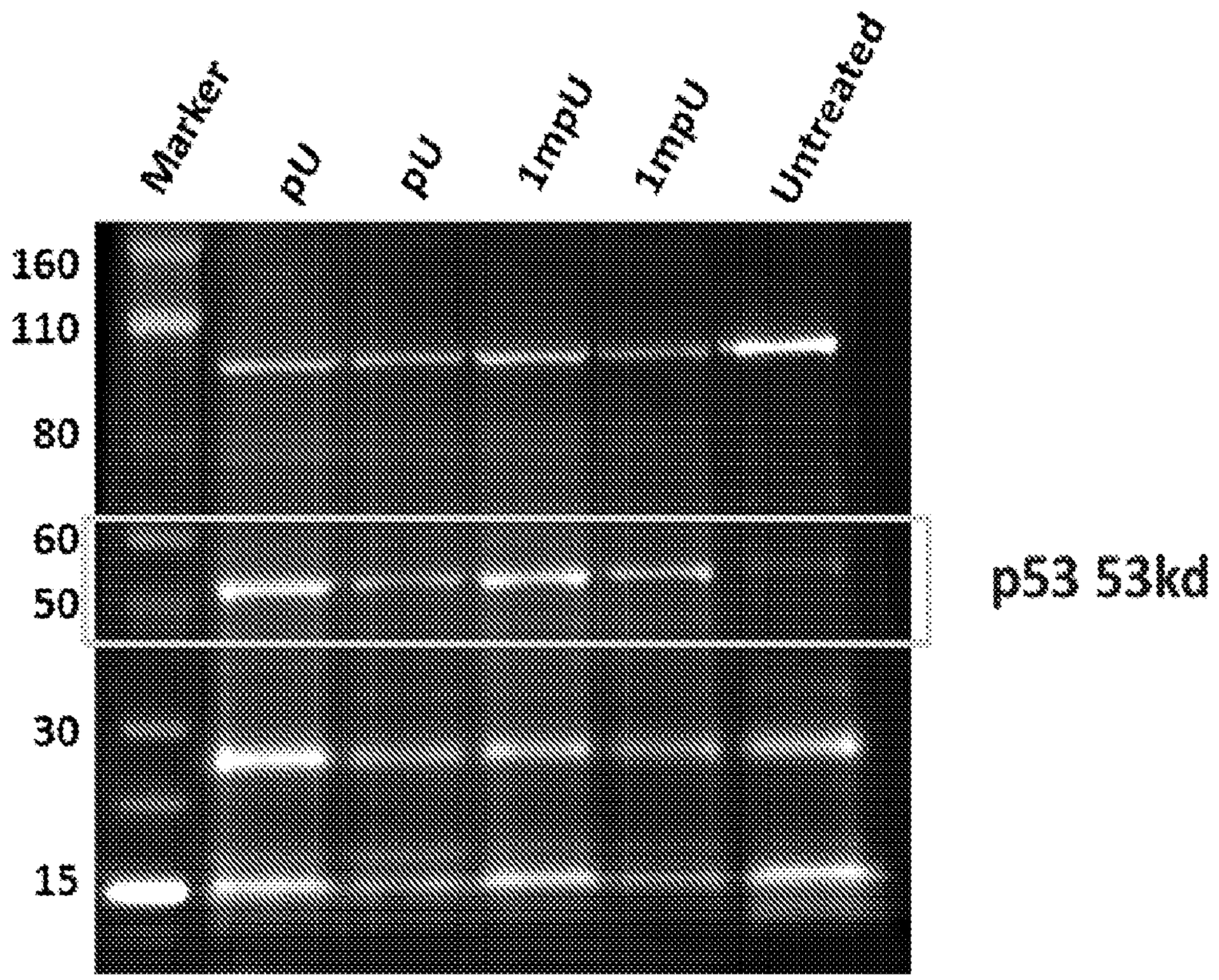
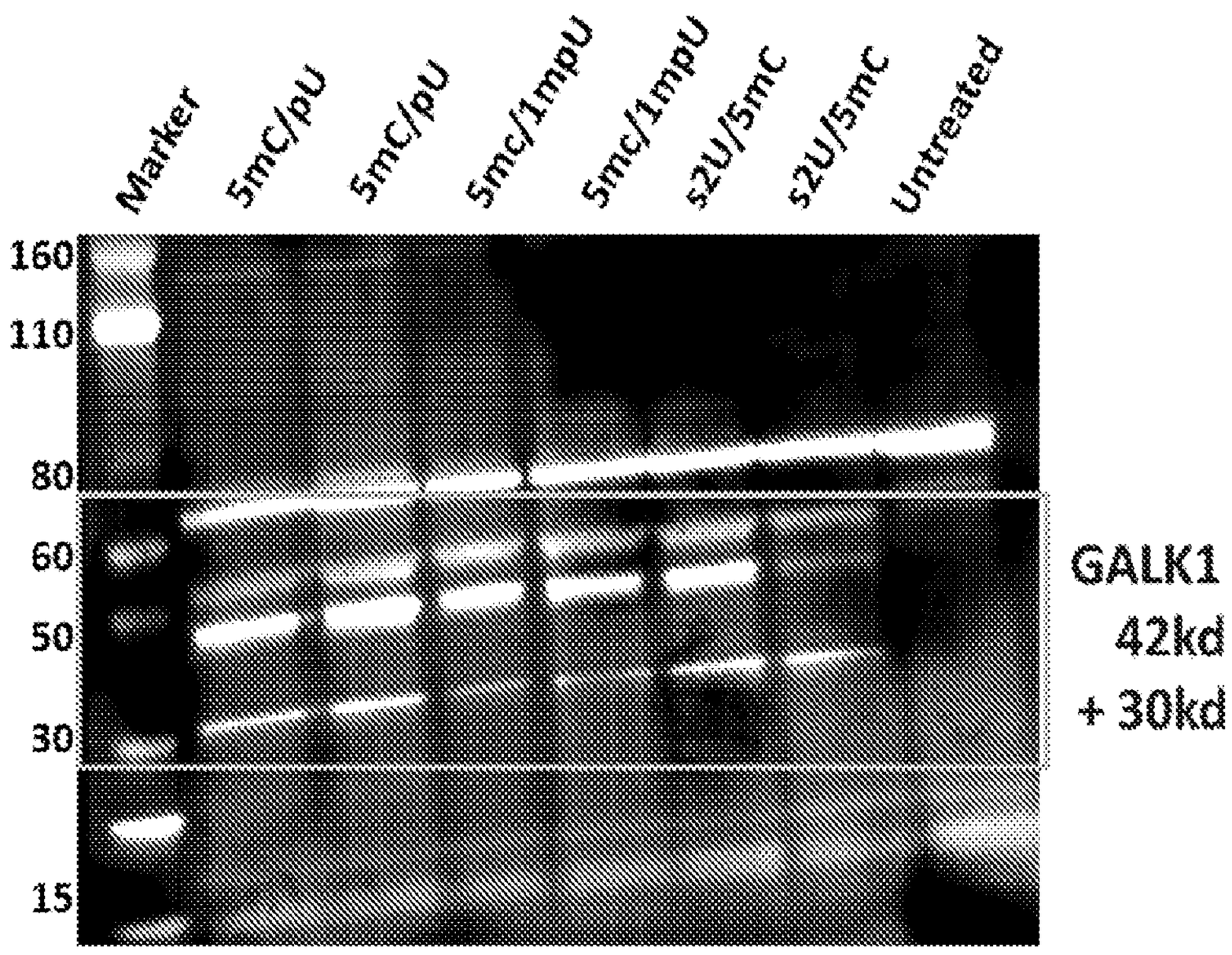

Marker
pU
pU
1mpU
1mpU
Untreated
160
110
80
60
50
30
15
p53 53kd

FIGURE 44



FIGURE 45

A.


Marker
5mC/pU
5mC/pU
5mc/1mpU
5mc/1mpU
s2U/5mC
s2U/5mC
Untreated
160
110
80
60
50
30
15
GALK1
42kd
+ 30kd

B.

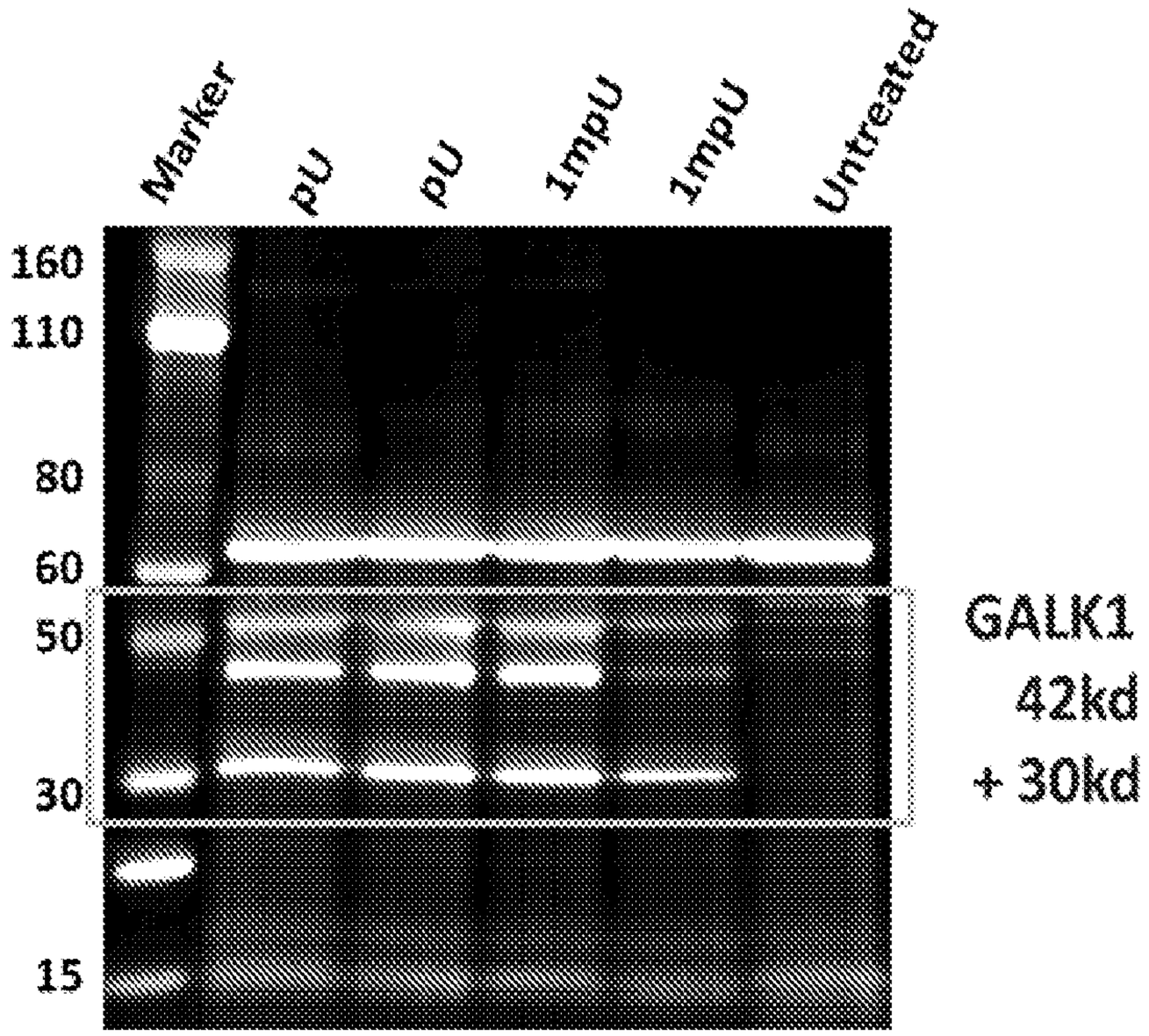

Marker
pU
pU
1mpU
1mpU
Untreated
160
110
80
60
50
30
15
GALK1
42kd
+ 30kd

FIGURE 46

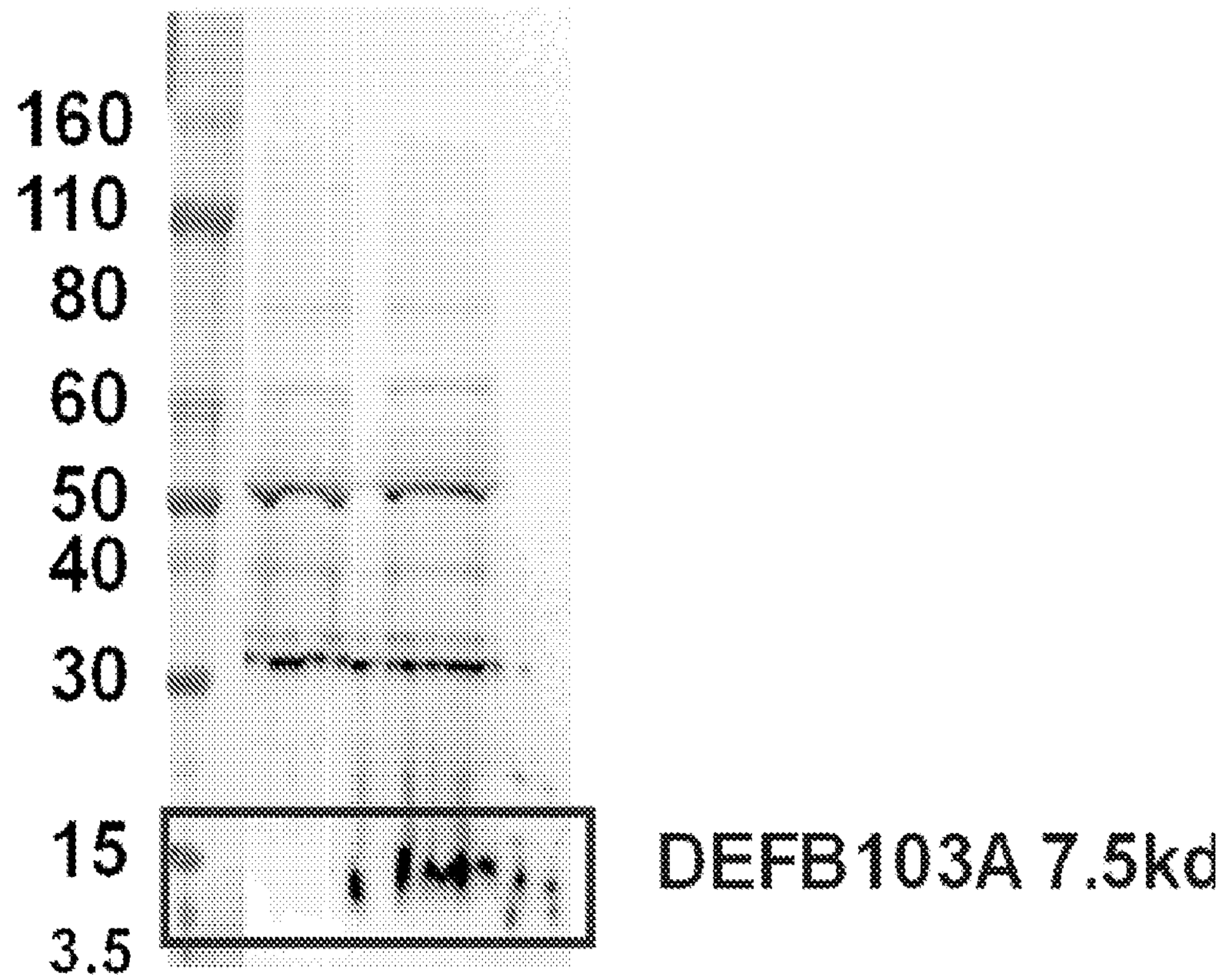


FIGURE 47

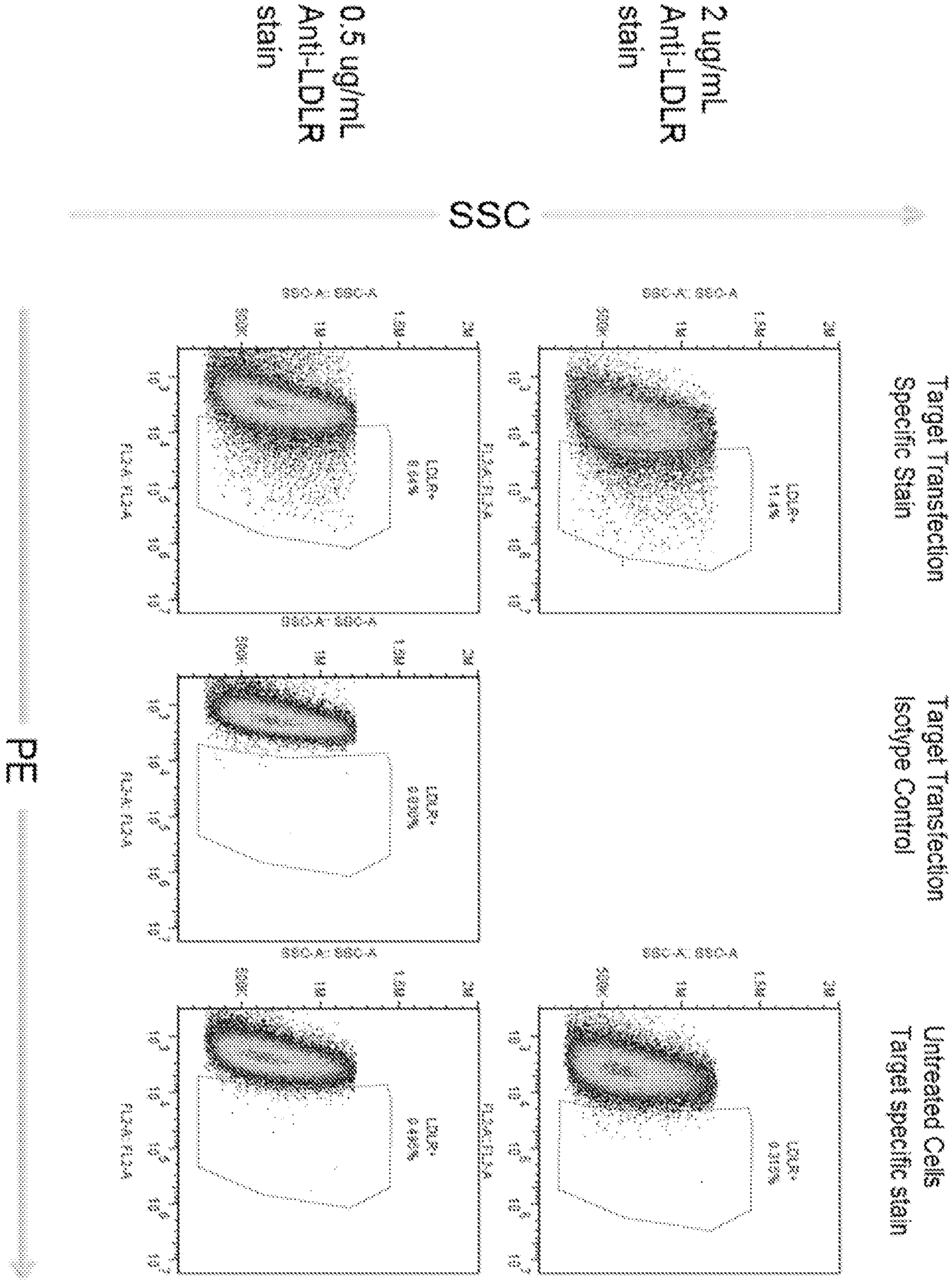

2 ug/mL Anti-LDLR stain
0.5 ug/mL Anti-LDLR stain
Untreated Cells
Target specific stain
Target Transfection Specific Stain
Target Transfection Isotype Control
SSC
PE
SSC-A: SSC-A
FL24-A: FL24-A
LDLR+
11.4%
LDLR+
8.94%
LDLR+
6.68%
LDLR+
0.33%

FIGURE 48

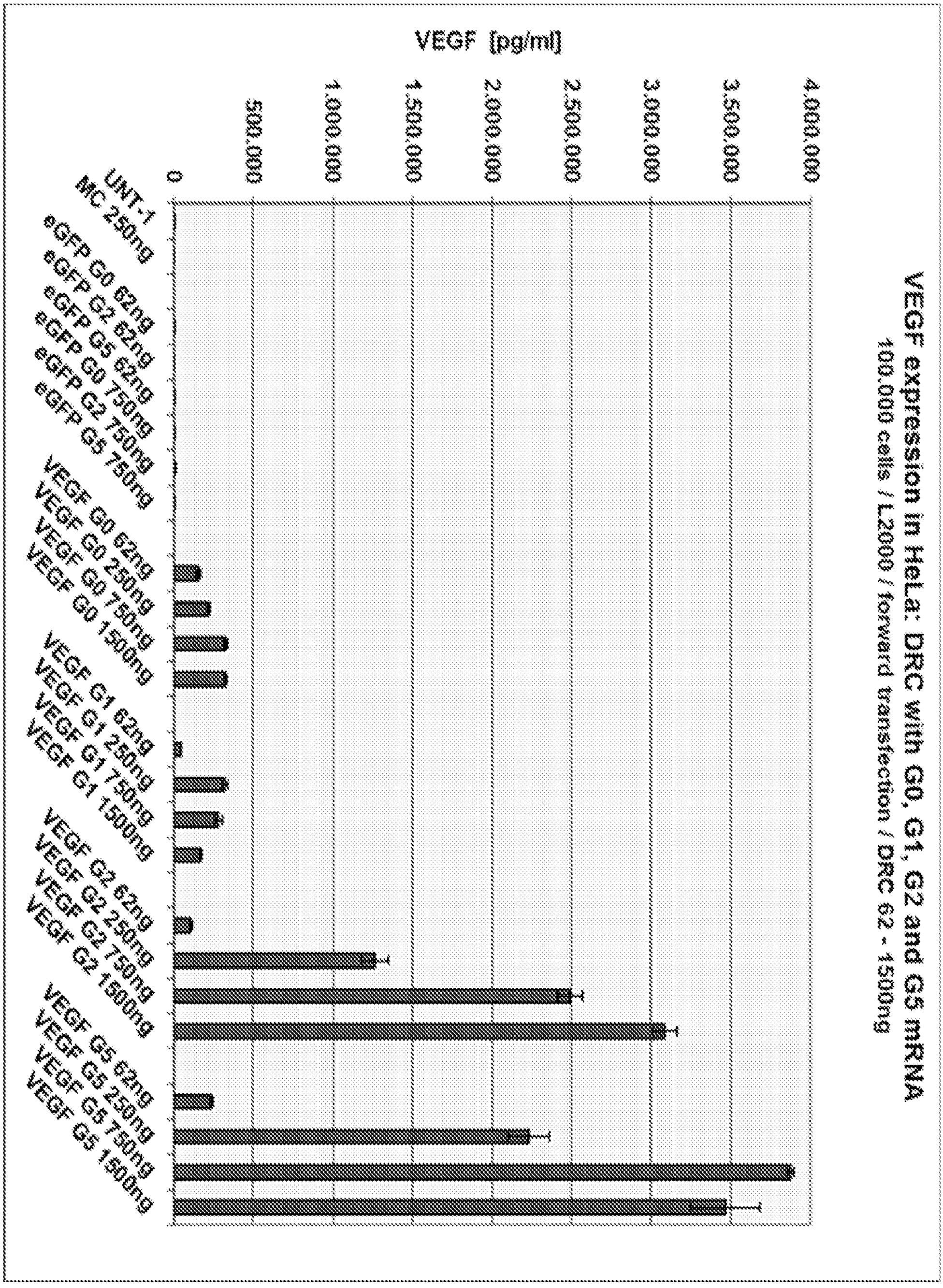

VEGF [pg/ml]
VEGF expression in HeLa: DRC with G0, G1, G2 and G5 mRNA
100.000 cells / L2000 / forward transfection / DRC 62 - 1500ng
0
500.000
1.000.000
1.500.000
2.000.000
2.500.000
3.000.000
3.500.000
4.000.000
UNT-1
MC 250ng
eGFP G0 62ng
eGFP G2 62ng
eGFP G5 62ng
eGFP G0 750ng
eGFP G2 750ng
eGFP G5 750ng
VEGF G0 62ng
VEGF G0 250ng
VEGF G0 750ng
VEGF G0 1500ng
VEGF G1 62ng
VEGF G1 250ng
VEGF G1 750ng
VEGF G1 1500ng
VEGF G2 62ng
VEGF G2 250ng
VEGF G2 750ng
VEGF G2 1500ng
VEGF G5 62ng
VEGF G5 250ng
VEGF G5 750ng
VEGF G5 1500ng

FIGURE 49

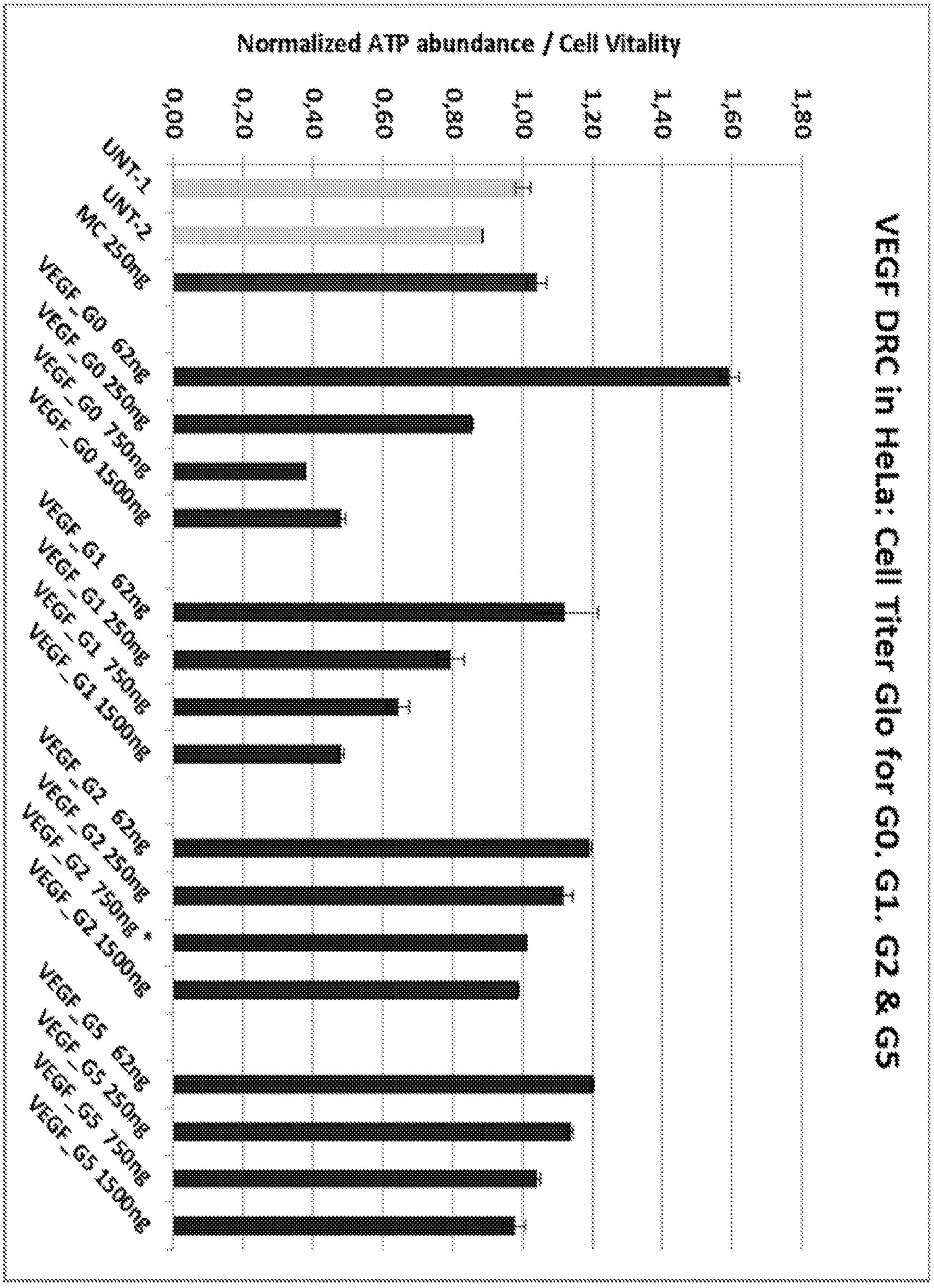

Normalized ATP abundance / Cell Vitality
0,00
0,20
0,40
0,60
0,80
1,00
1,20
1,40
1,60
1,80
VEGF DRC in HeLa: Cell Titer Glo for G0, G1, G2 & G5
UNT-1
UNT-2
MC 250nM
VEGF_G0 62nM
VEGF_G0 250nM
VEGF_G0 750nM
VEGF_G0 1500nM
VEGF_G1 62nM
VEGF_G1 250nM
VEGF_G1 750nM
VEGF_G1 1500nM
VEGF_G2 62nM
VEGF_G2 250nM
VEGF_G2 750nM *
VEGF_G2 1500nM
VEGF_G5 62nM
VEGF_G5 250nM
VEGF_G5 750nM
VEGF_G5 1500nM

FIGURE 50

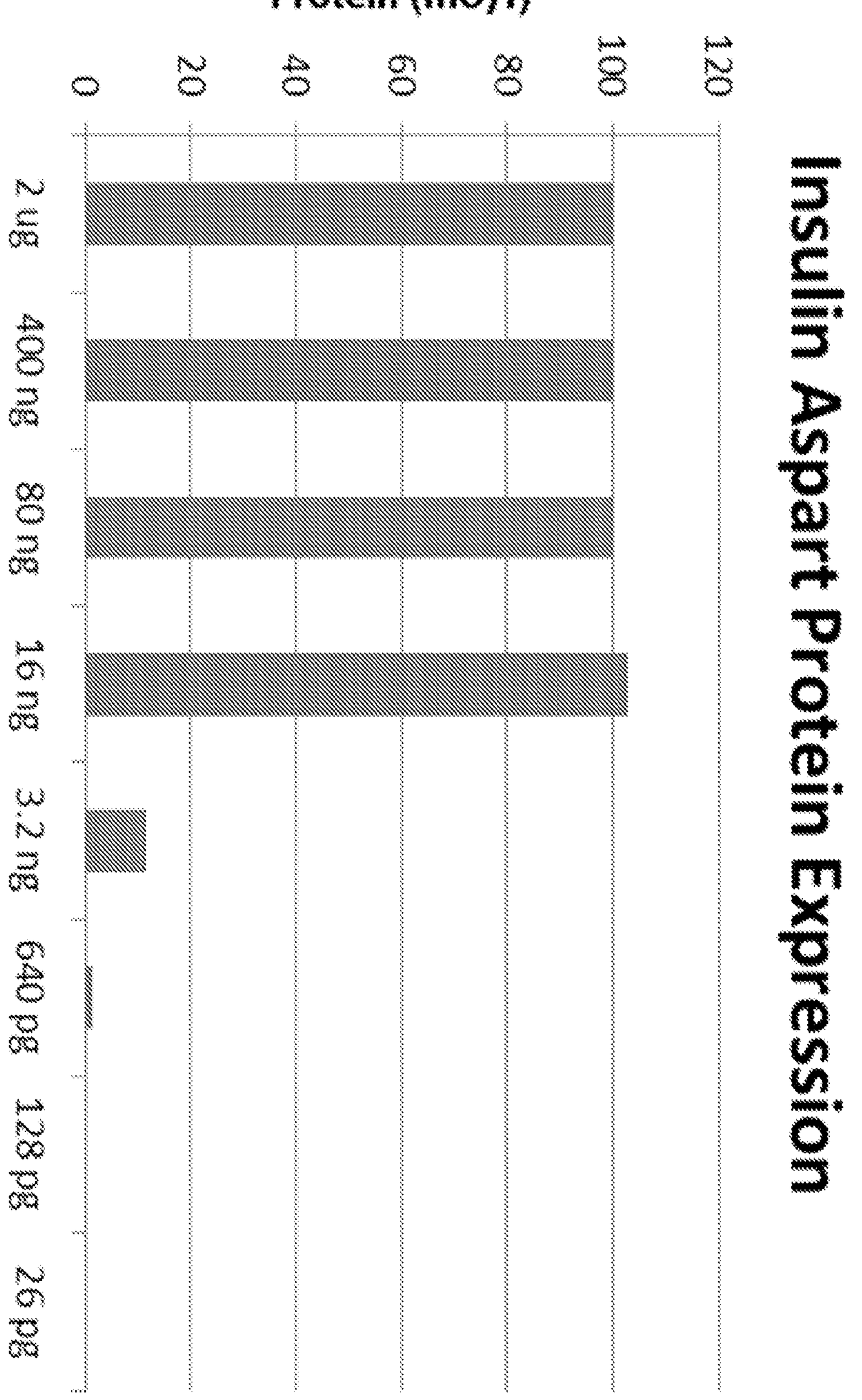

Protein (mU/l)
0
20
40
60
80
100
120
Insulin Aspart: Protein Expression
2 ug
400 ng
80 ng
16 ng
3.2 ng
640 pg
128 pg
26 pg

FIGURE 51

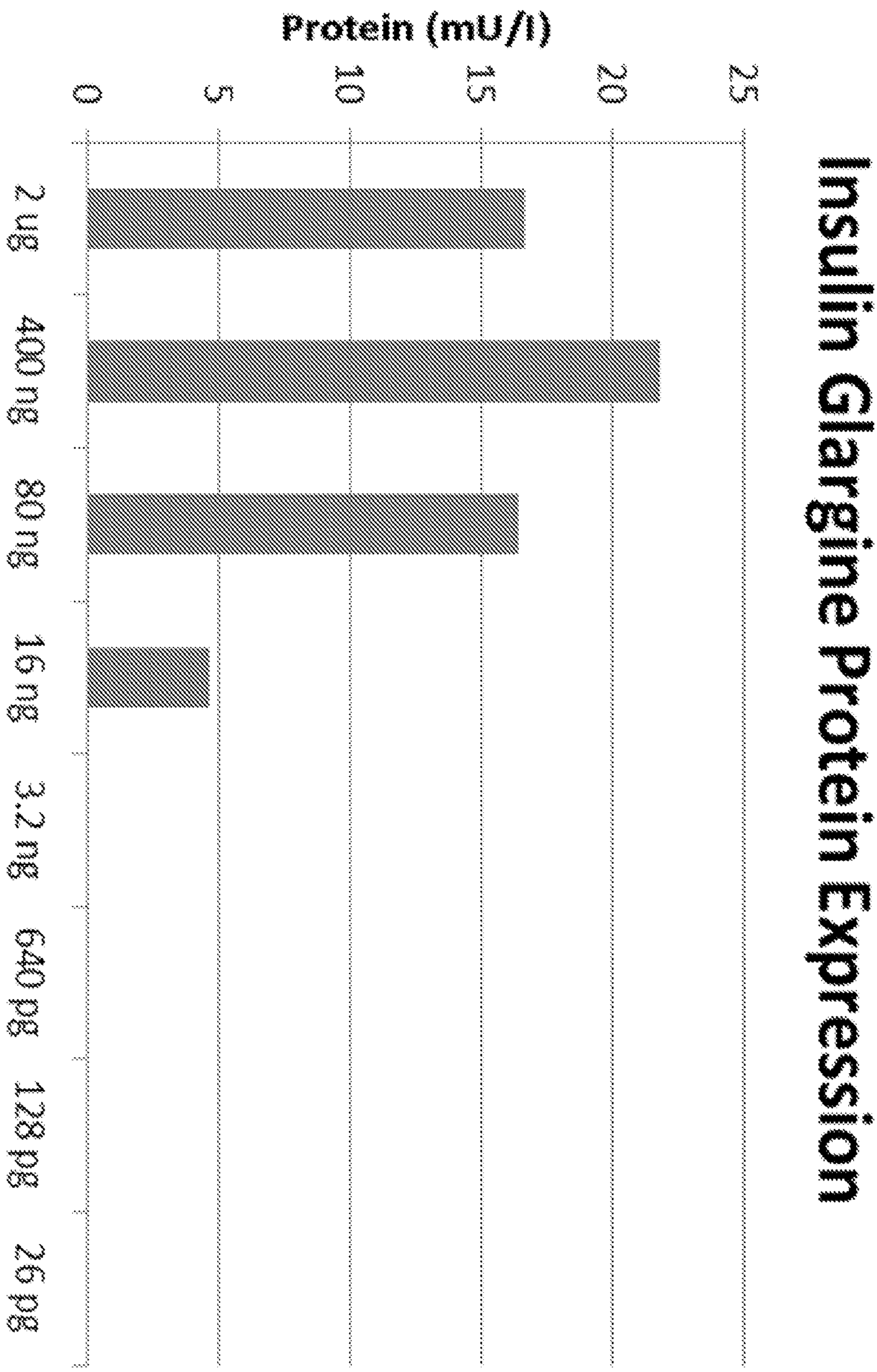

Insulin Glargine Protein Expression
Protein (mU/l)
0
5
10
15
20
25
2 ng
400 ng
80 ng
16 ng
3.2 ng
640 pg
128 pg
25 pg

FIGURE 52

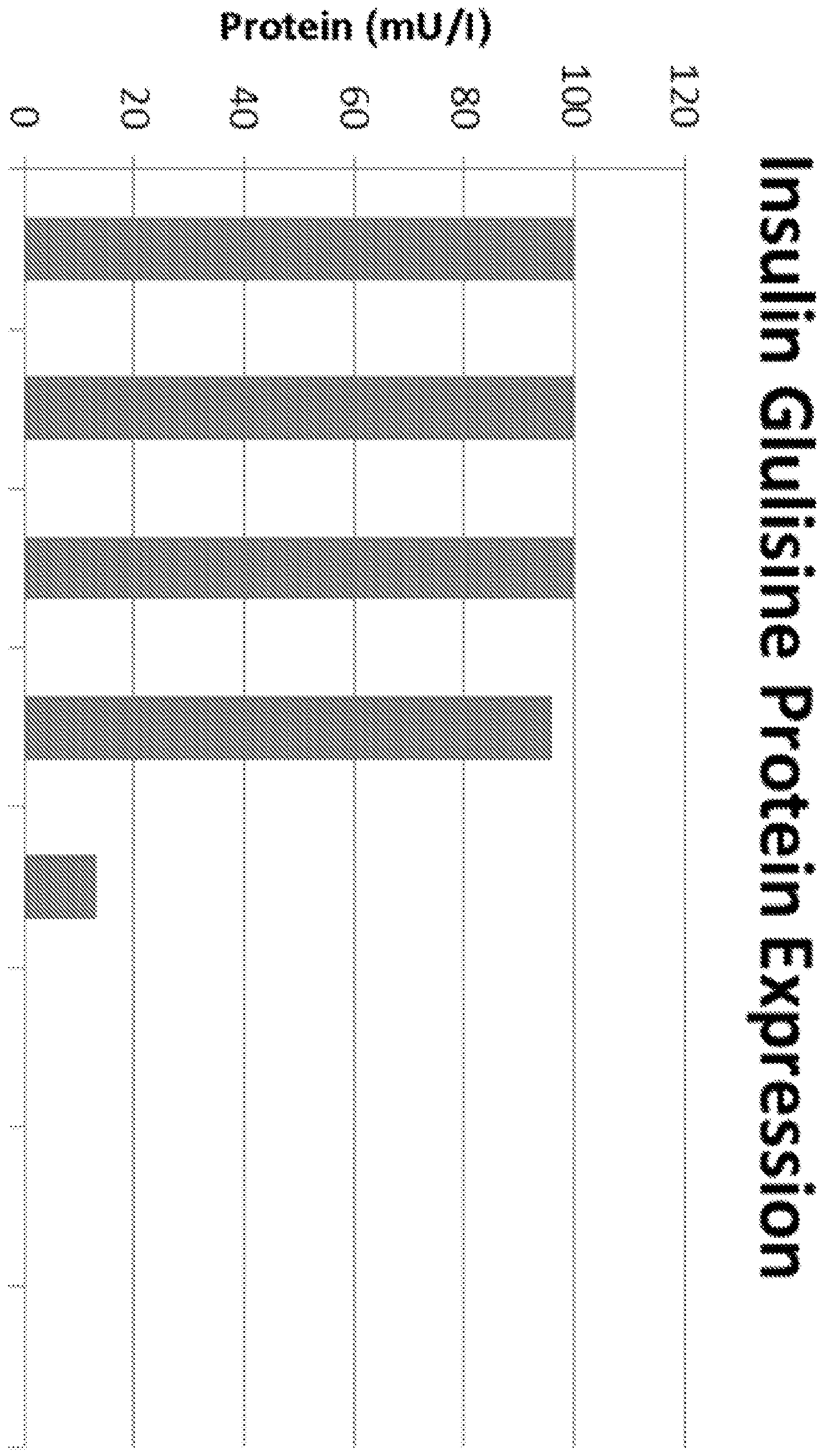

Protein (mU/l)
0
20
40
60
80
100
120
Insulin Glulisine
Protein Expression
2 ug
400 ng
80 ng
16 ng
3.2 ng
640 pg
128 pg
26 pg

FIGURE 53

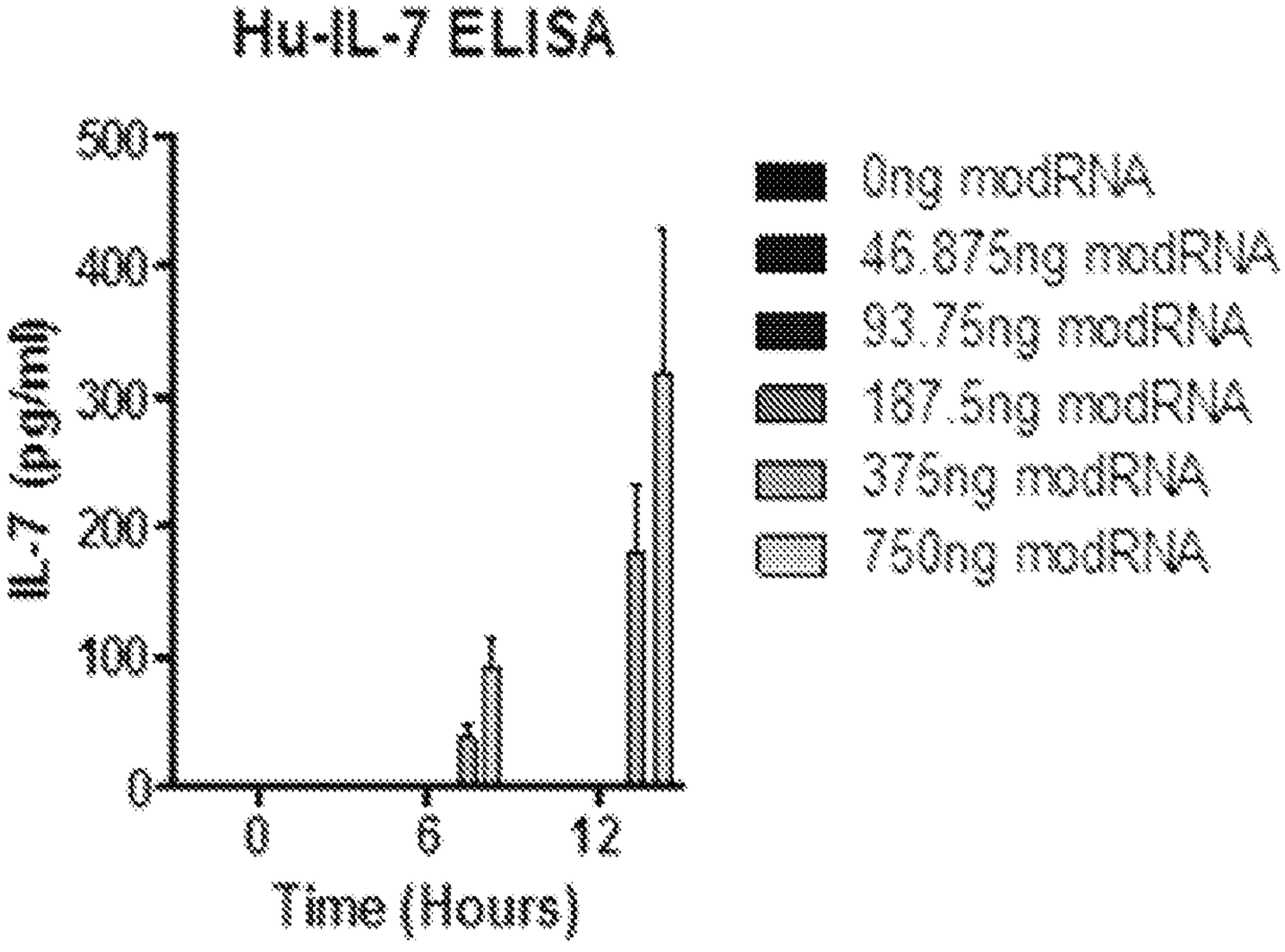
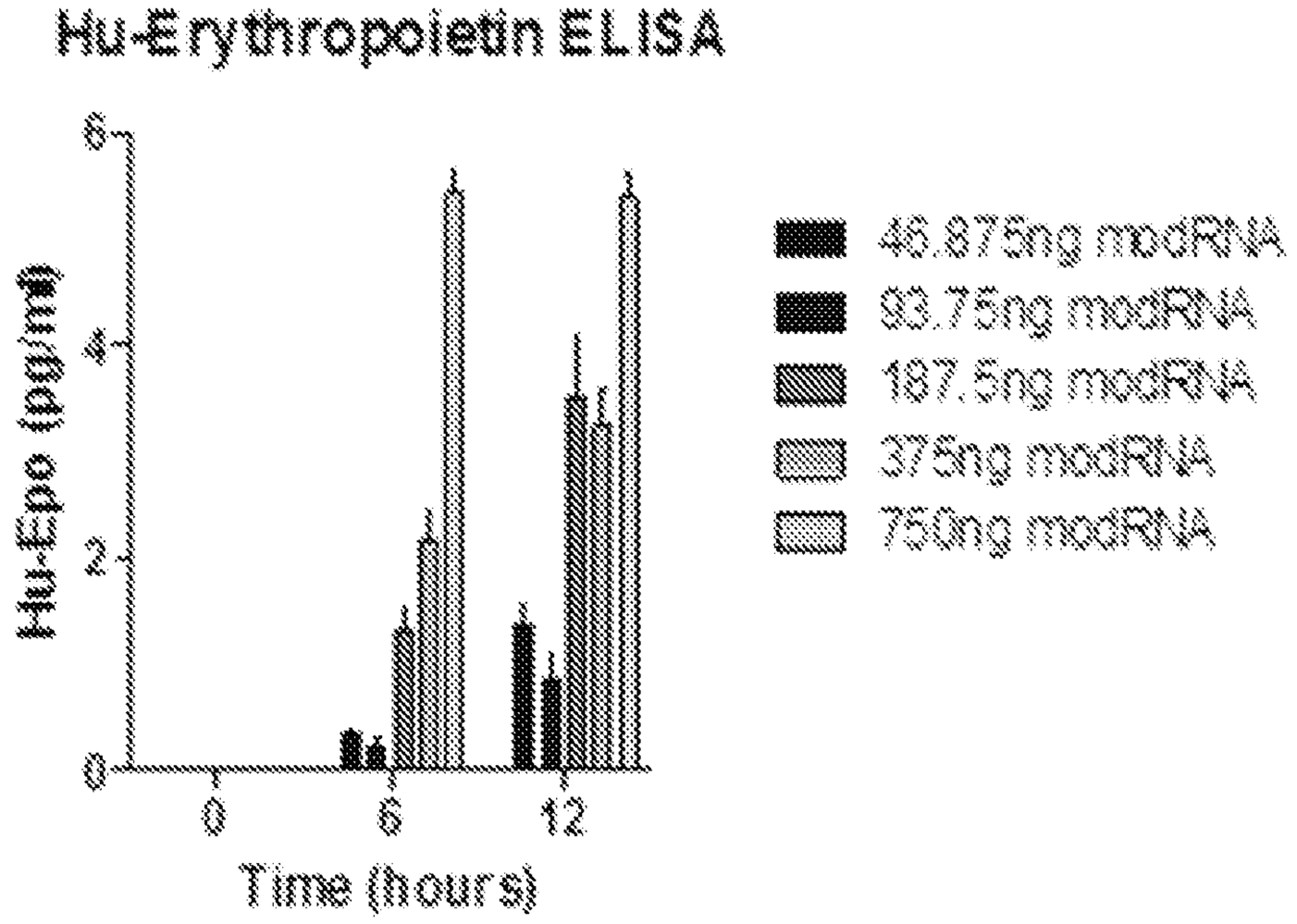

Hu-IL-7 ELISA
IL-7 (pg/ml)
500
400
300
200
100
0
0
6
12
Time (Hours)
0ng modRNA
46.875ng modRNA
93.75ng modRNA
187.5ng modRNA
375ng modRNA
750ng modRNA

FIGURE 54


Hu-Erythropoietin ELISA
Hu-Epo (pg/ml)
6
4
2
0
0
6
12
Time (hours)
46.875ng modRNA
93.75ng modRNA
187.5ng modRNA
375ng modRNA
750ng modRNA

EXAMPLE 55

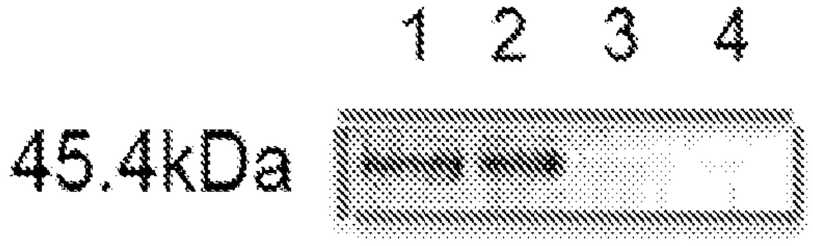


FIGURE 56

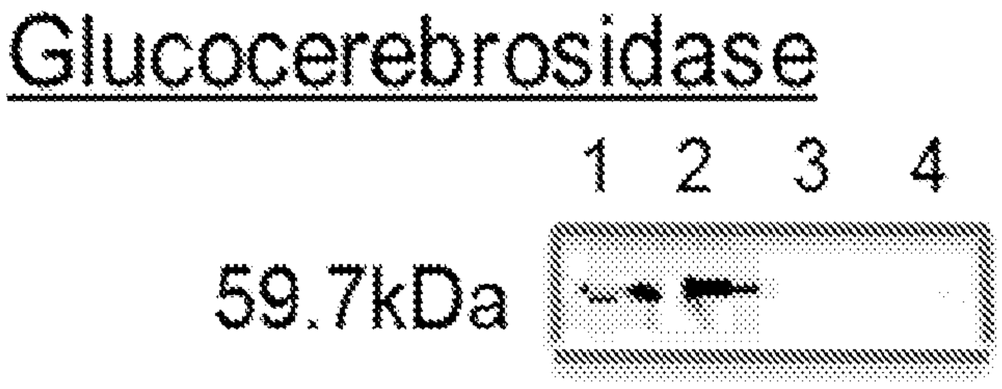


FIGURE 57

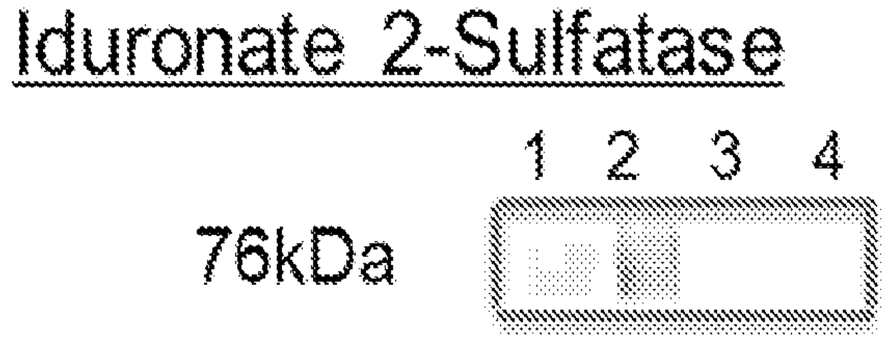


FIGURE 58

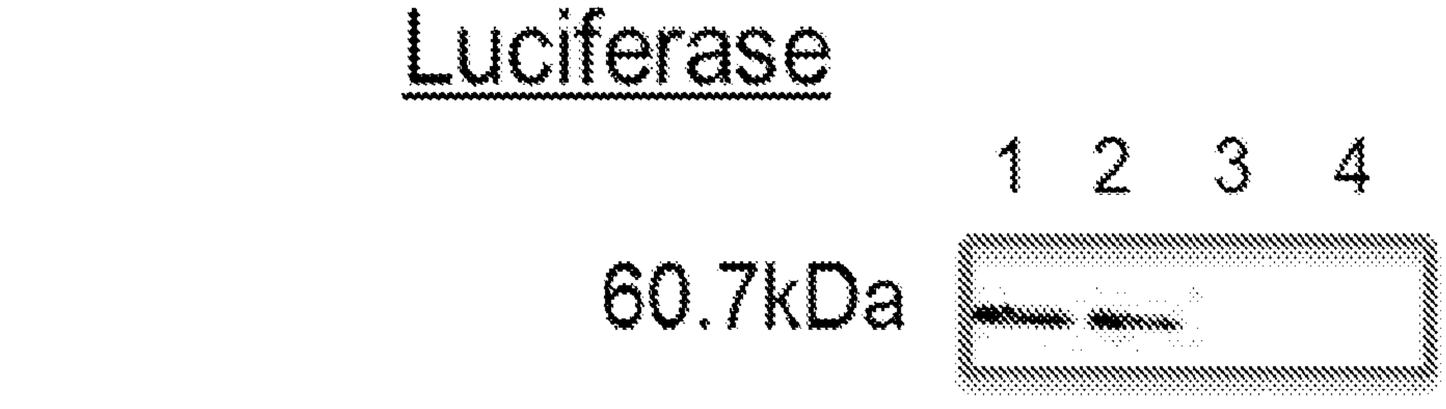


FIGURE 59

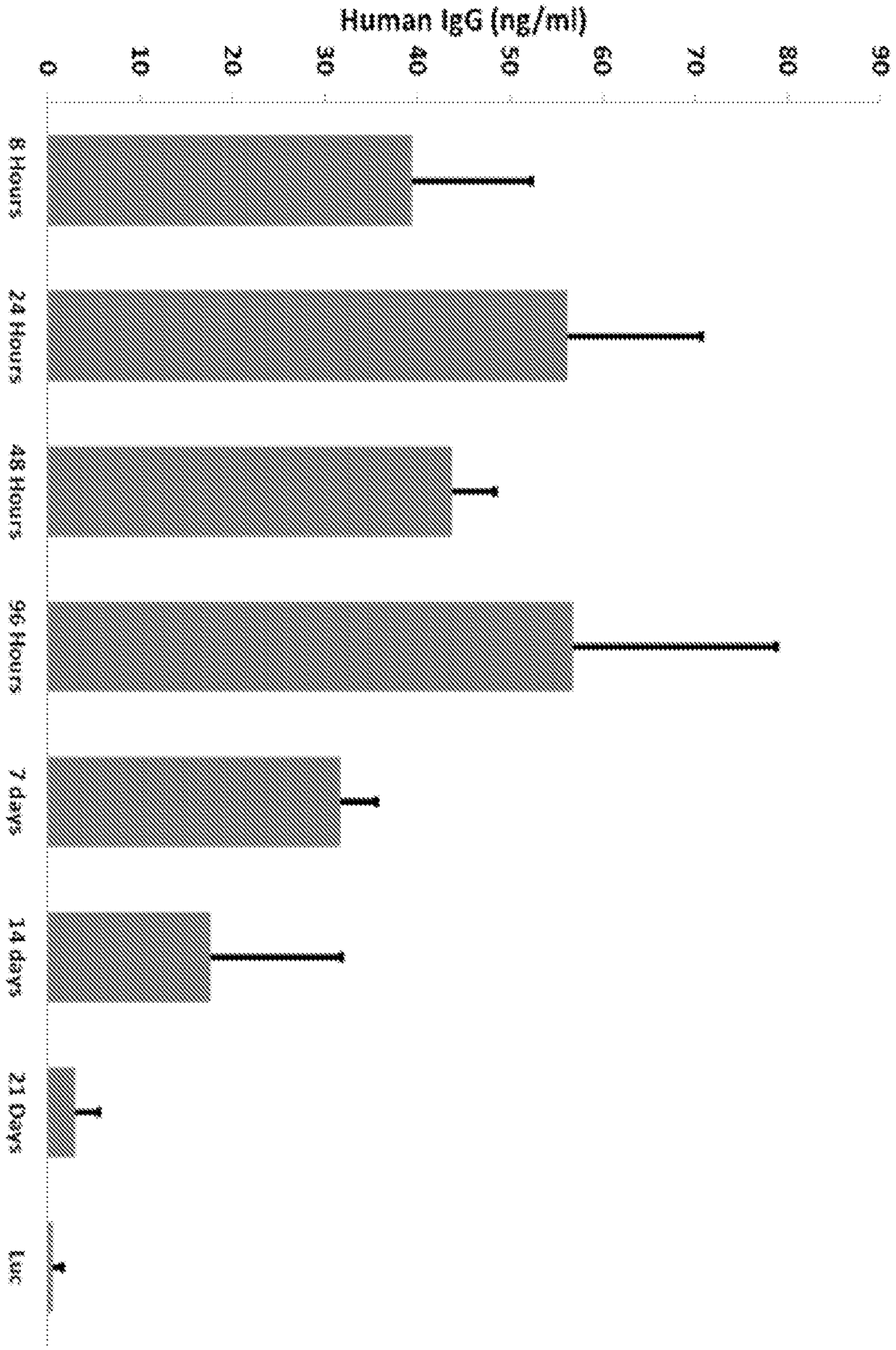

Human IgG (ng/ml)
0
10
20
30
40
50
60
70
80
90
96 hours
48 hours
24 hours
7 days
14 days
21 Days
3rd

FIGURE 60

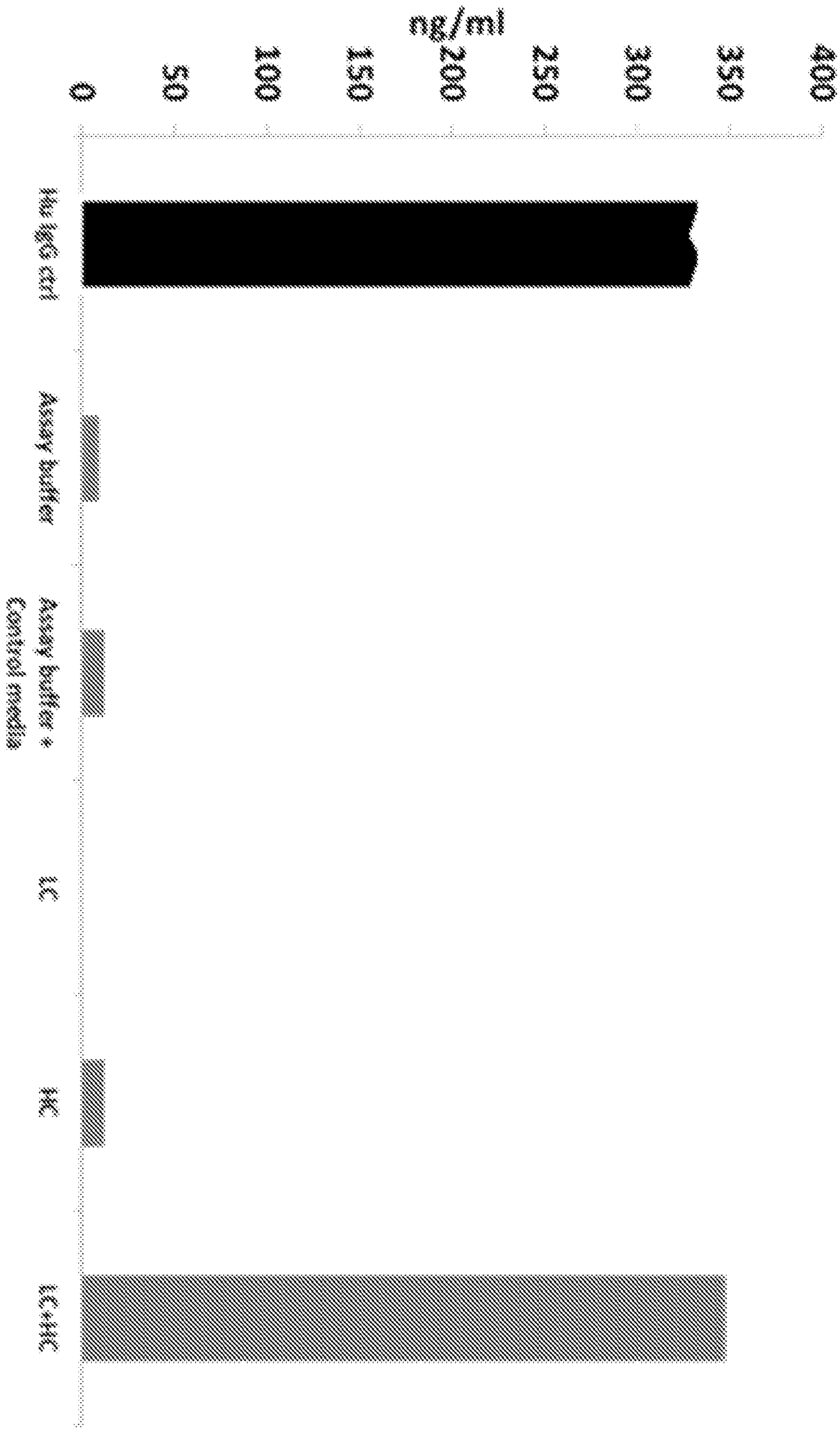

ng/ml
0
25
100
250
500
750
1000
1250
Away buffer
Away buffer
Control mAb
EC+HC

FIGURE 61

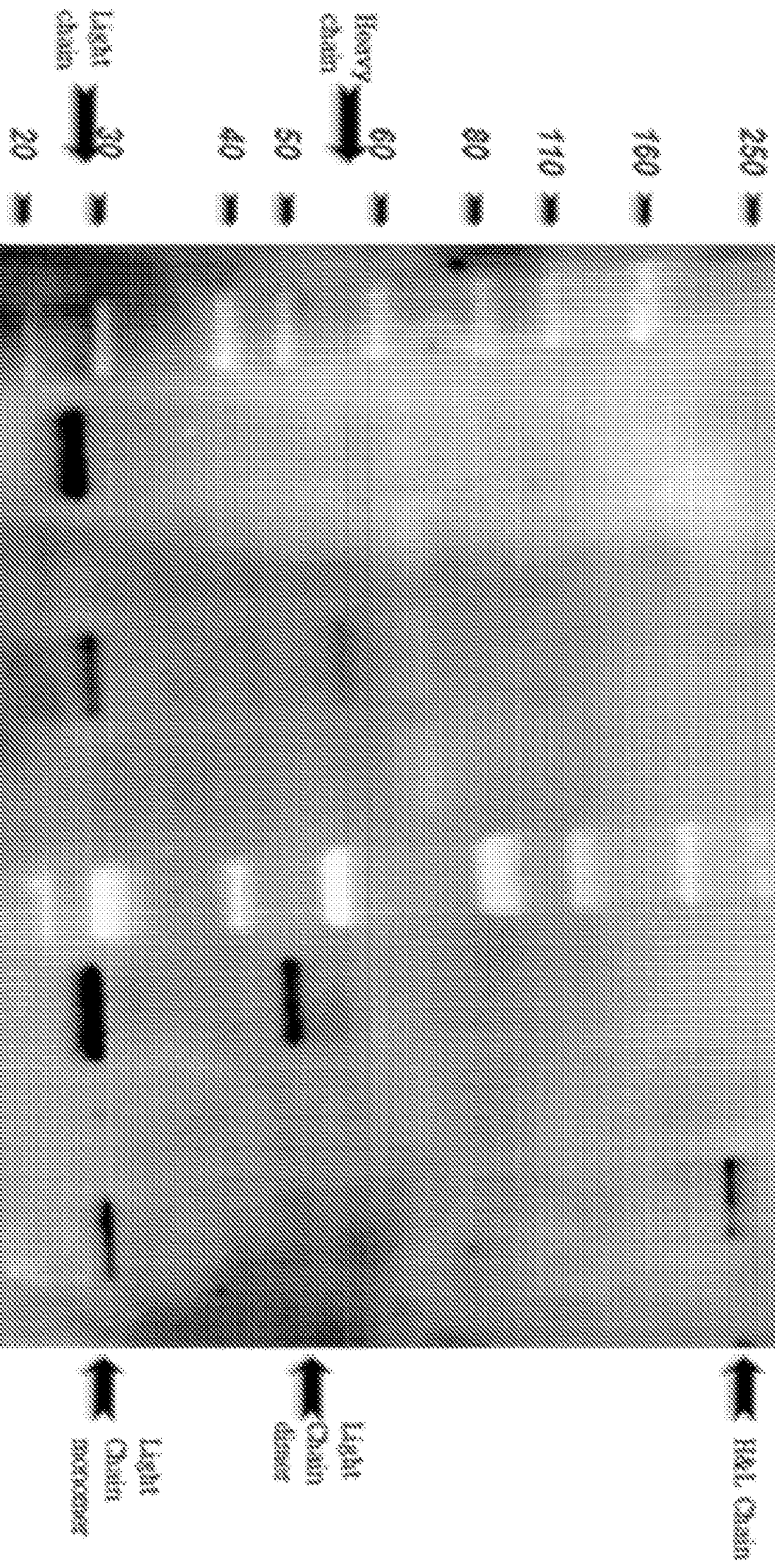

Ctrl
Ctrl
Ctrl

FIGURE 62

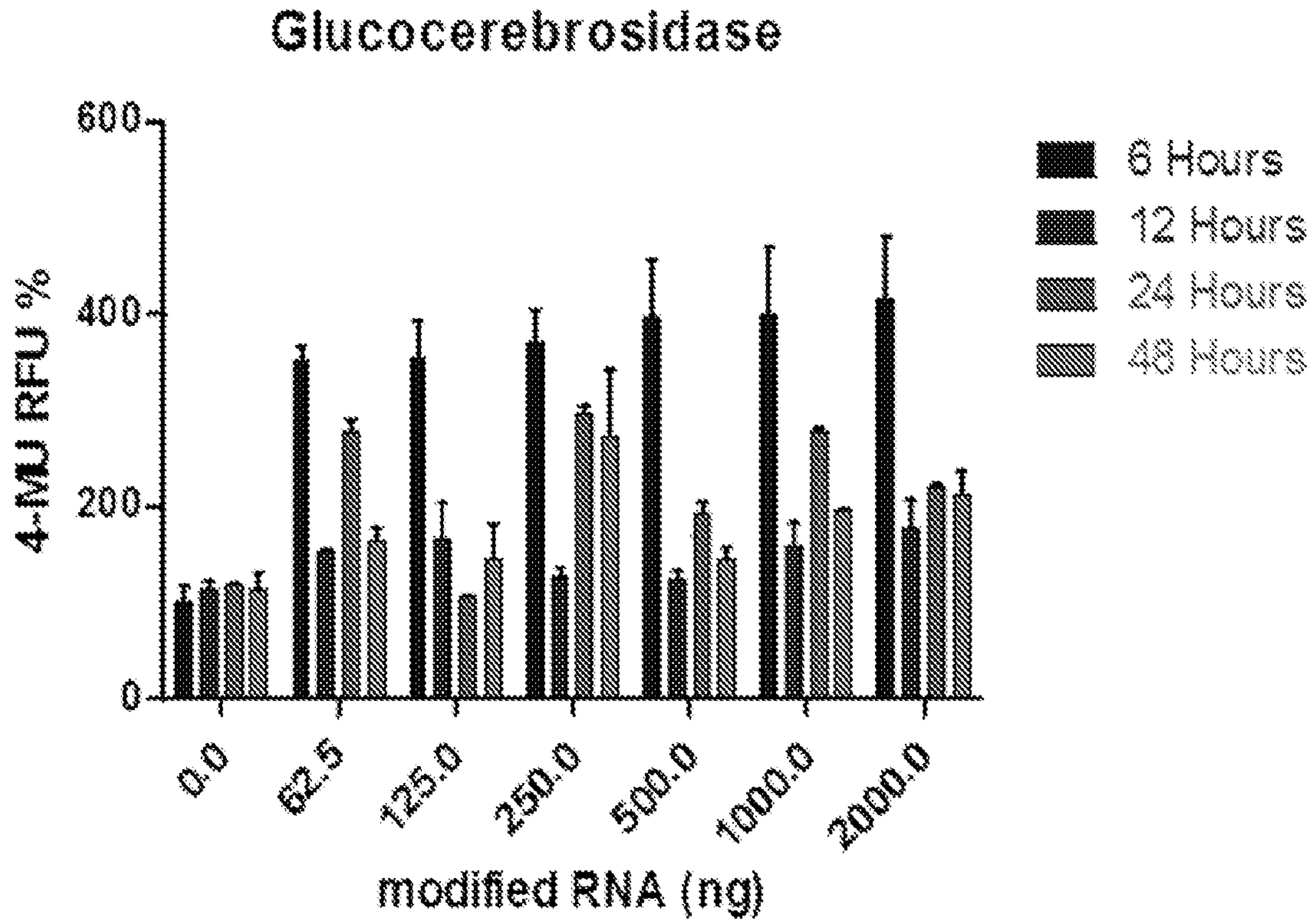

Glucocerebrosidase
600
400
200
0
4-MU RFU %
0.0
62.5
125.0
250.0
500.0
1000.0
2000.0
modified RNA (ng)
6 Hours
12 Hours
24 Hours
48 Hours

FIGURE 63

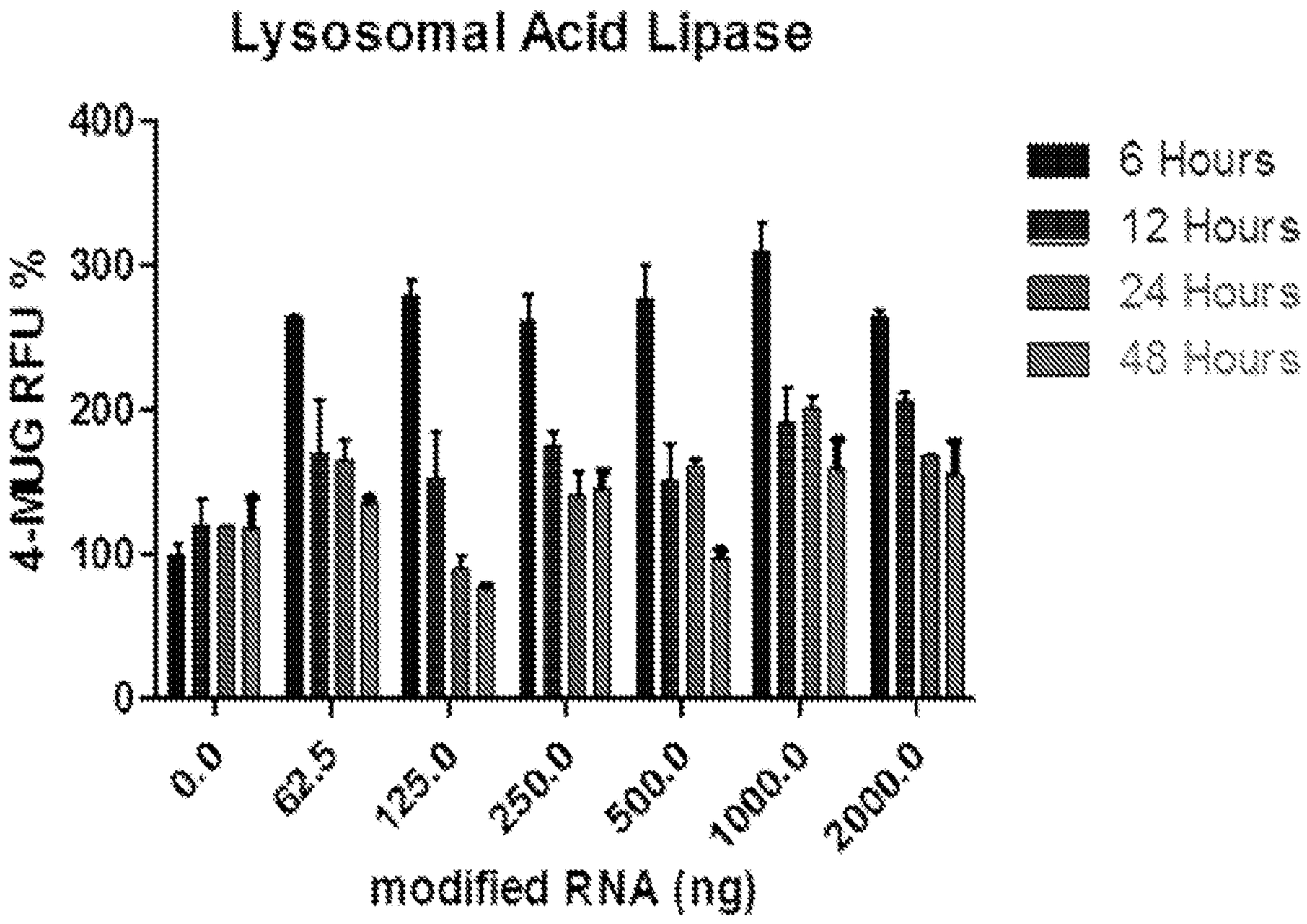

Lysosomal Acid Lipase
400
300
200
100
0
4-MUG RFU %
0.0
62.5
125.0
250.0
500.0
1000.0
2000.0
modified RNA (ng)
6 Hours
12 Hours
24 Hours
48 Hours

FIGURE 64

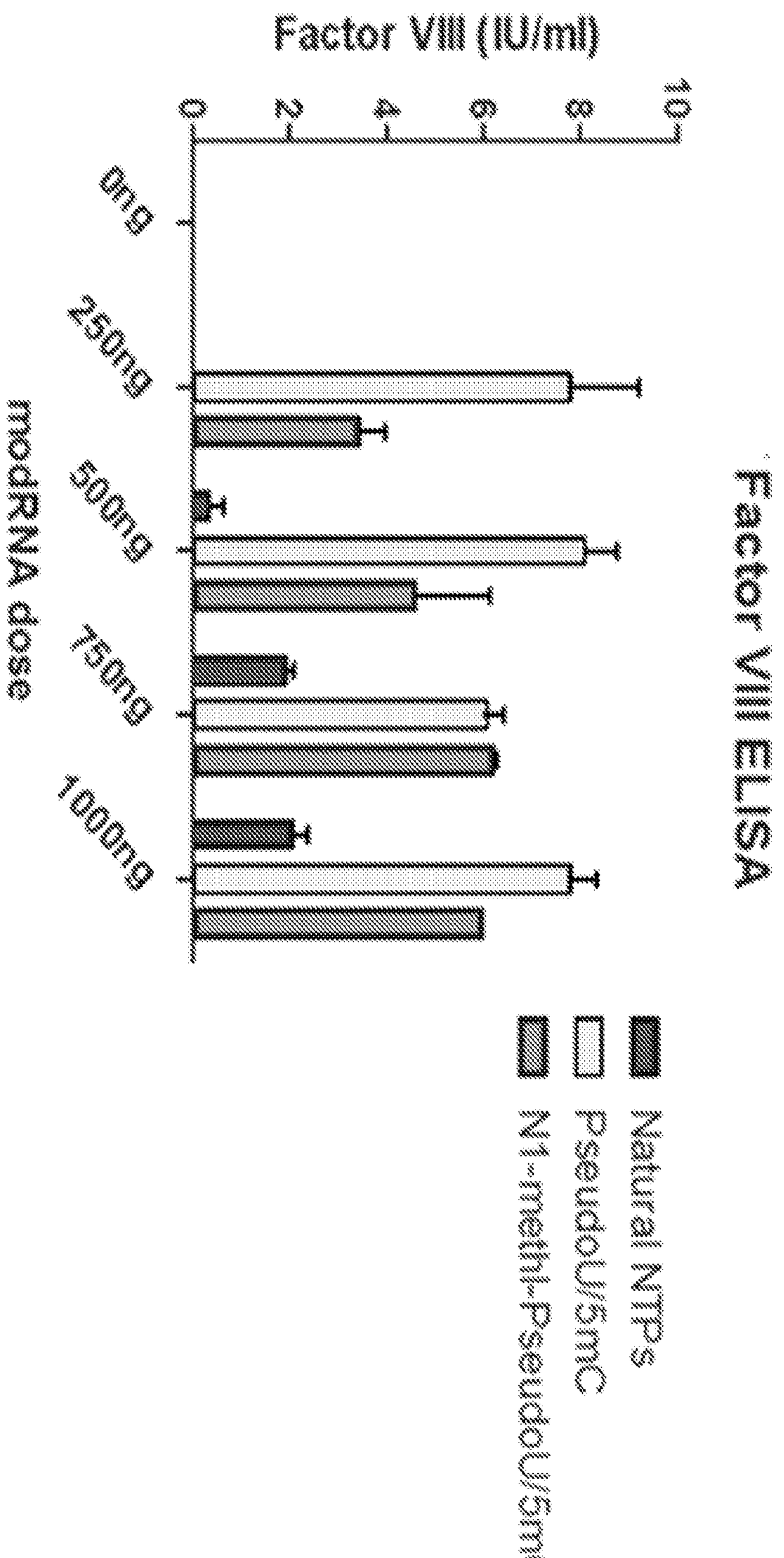

Factor VIII (IU/ml)
0
2
4
6
8
10
Vehicle
25mg
50mg
75mg
100mg
mRNA dose
Factor VIII ELISA
Natural Nab
Pseudouridine
N1-methyl-Pseudouridine

FIGURE 65

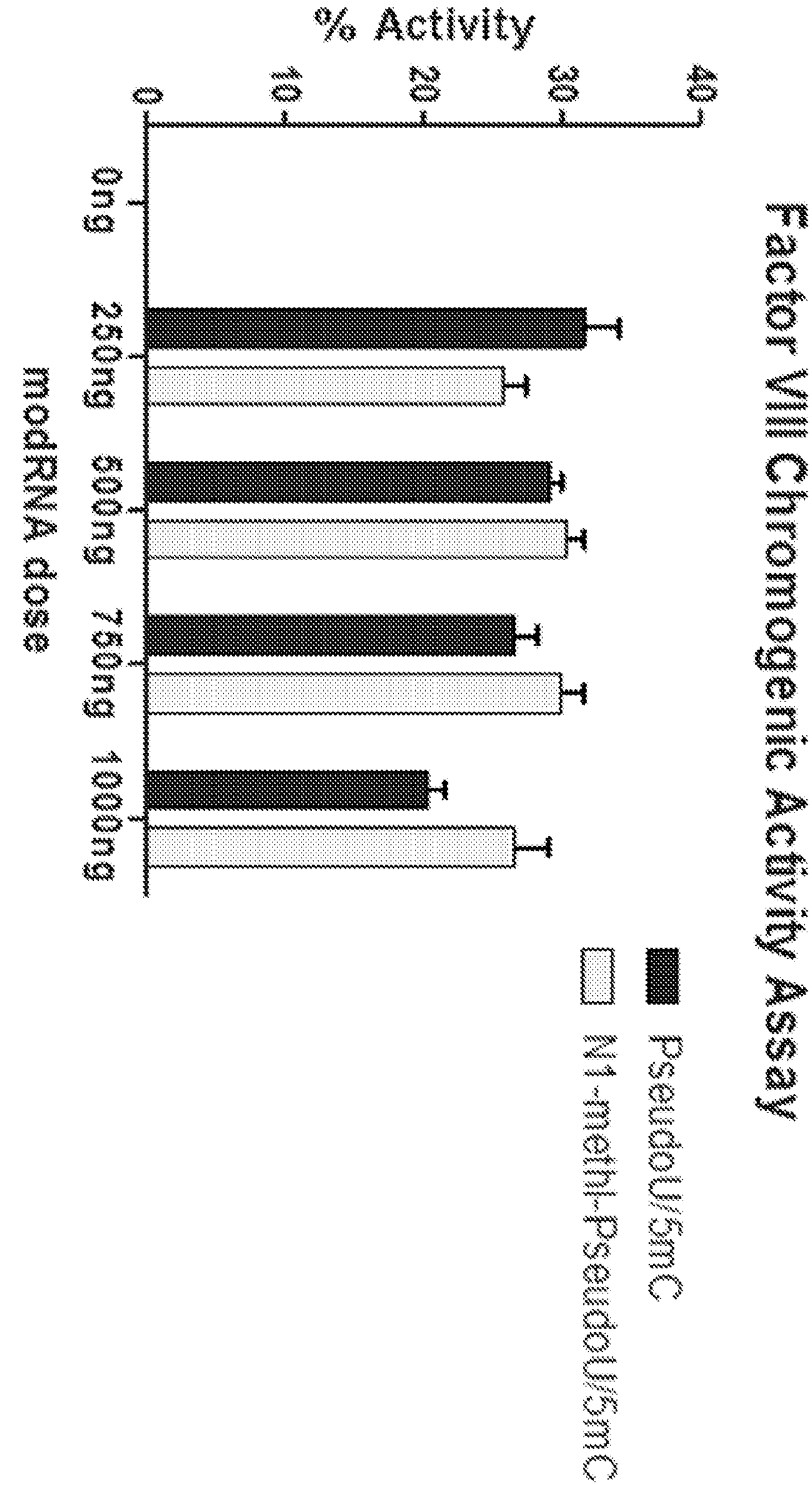

% Activity
0
6uo
6uo2
6uo4
6uo8
6uo16
6uo32
6uo64
6uo128
modRNA dose
Pseudouridine/5mC
N1-methyl-pseudouridine/5mC
Human Vim Chromogenic Activity Assay
Factor VIII

FIGURE 66

A.

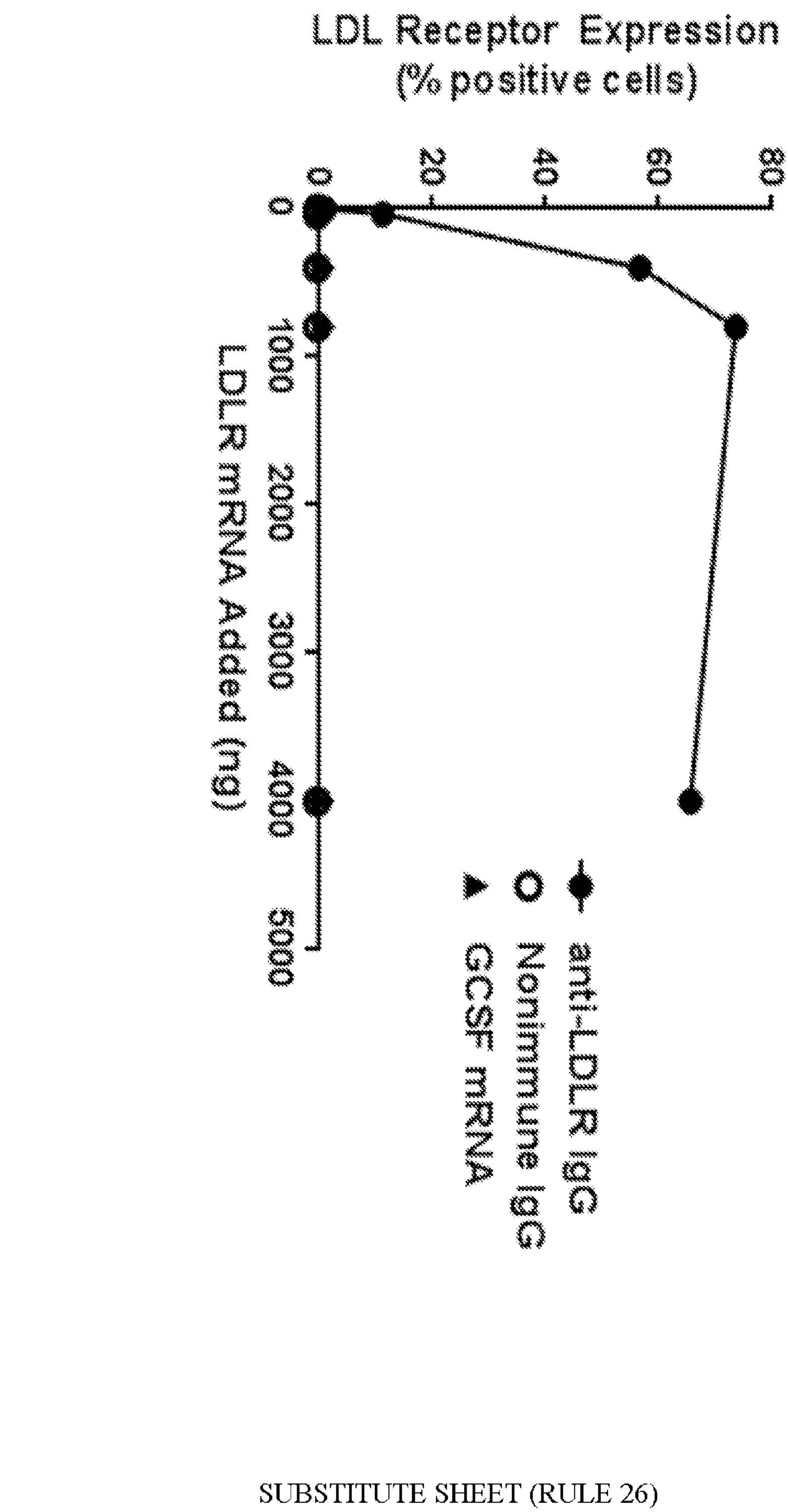

LDL Receptor Expression
(% positive cells)
0
20
40
60
80
0
1000
2000
3000
4000
5000
LDLR mRNA Added (ng)
anti-LDLR IgG
Non-immune IgG
SCS mRNA

FIGURE 66 CONT.

B.

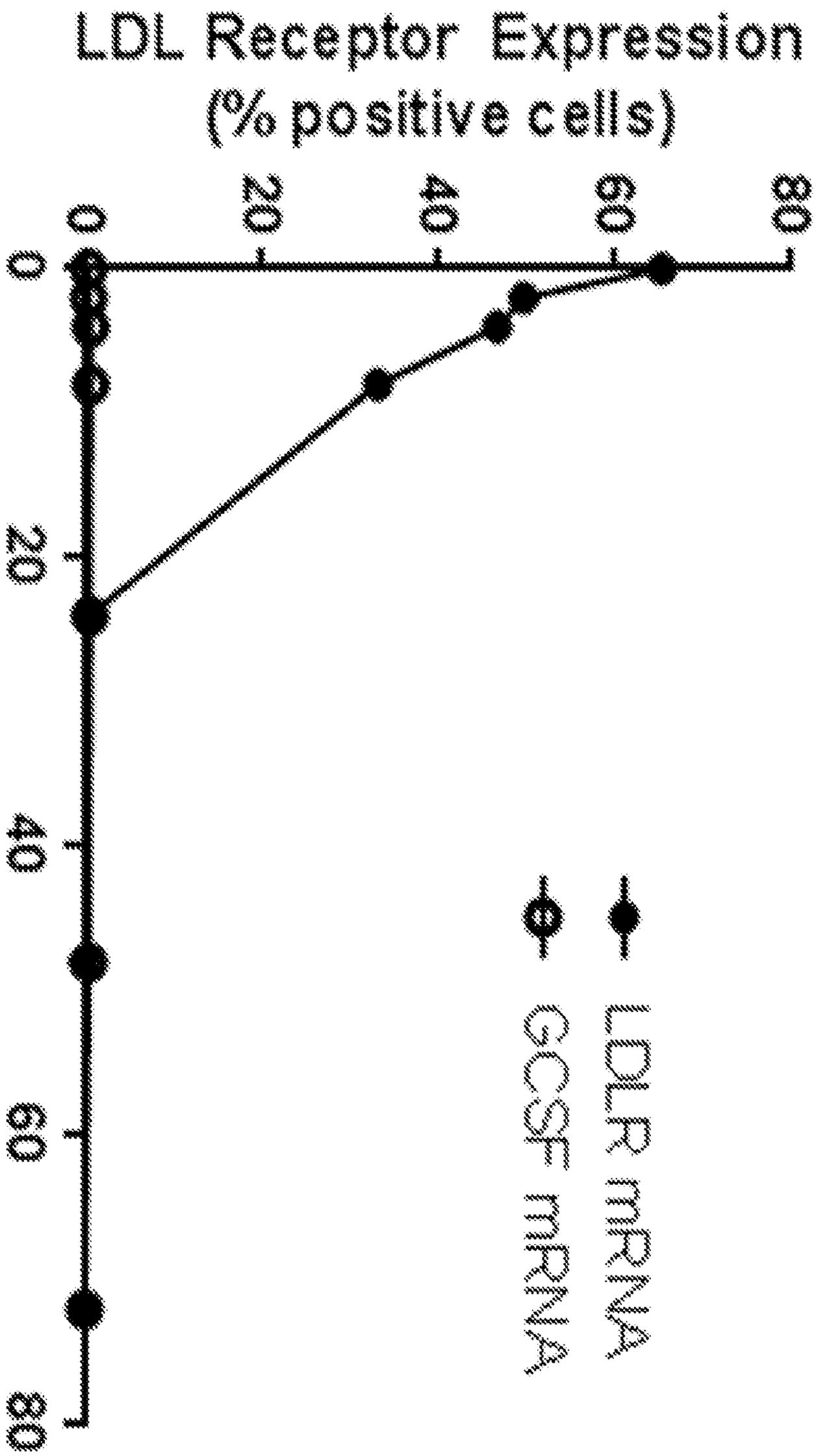


FIGURE 66 CONT.

C.

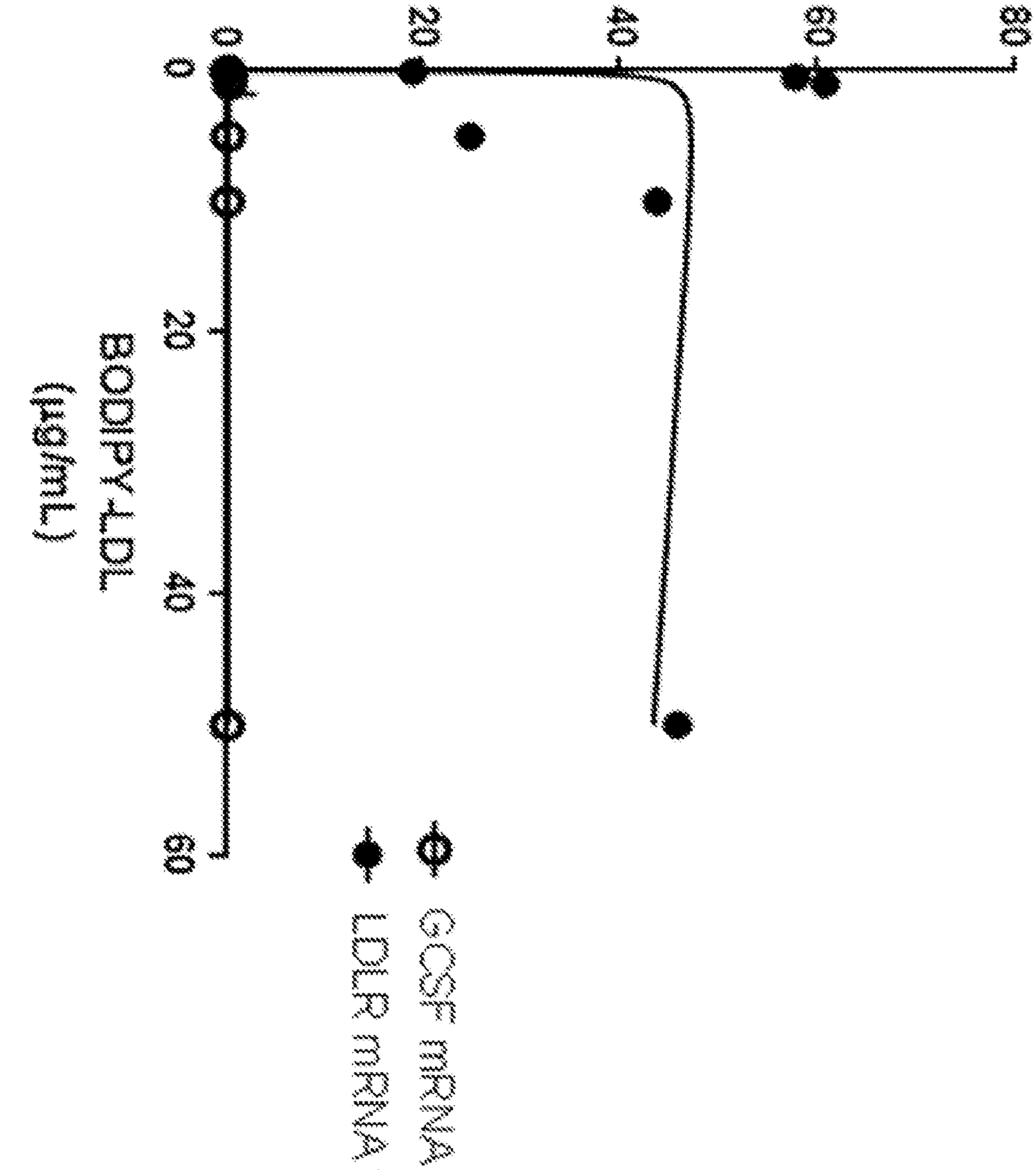


FIGURE 66 CONT.

D.

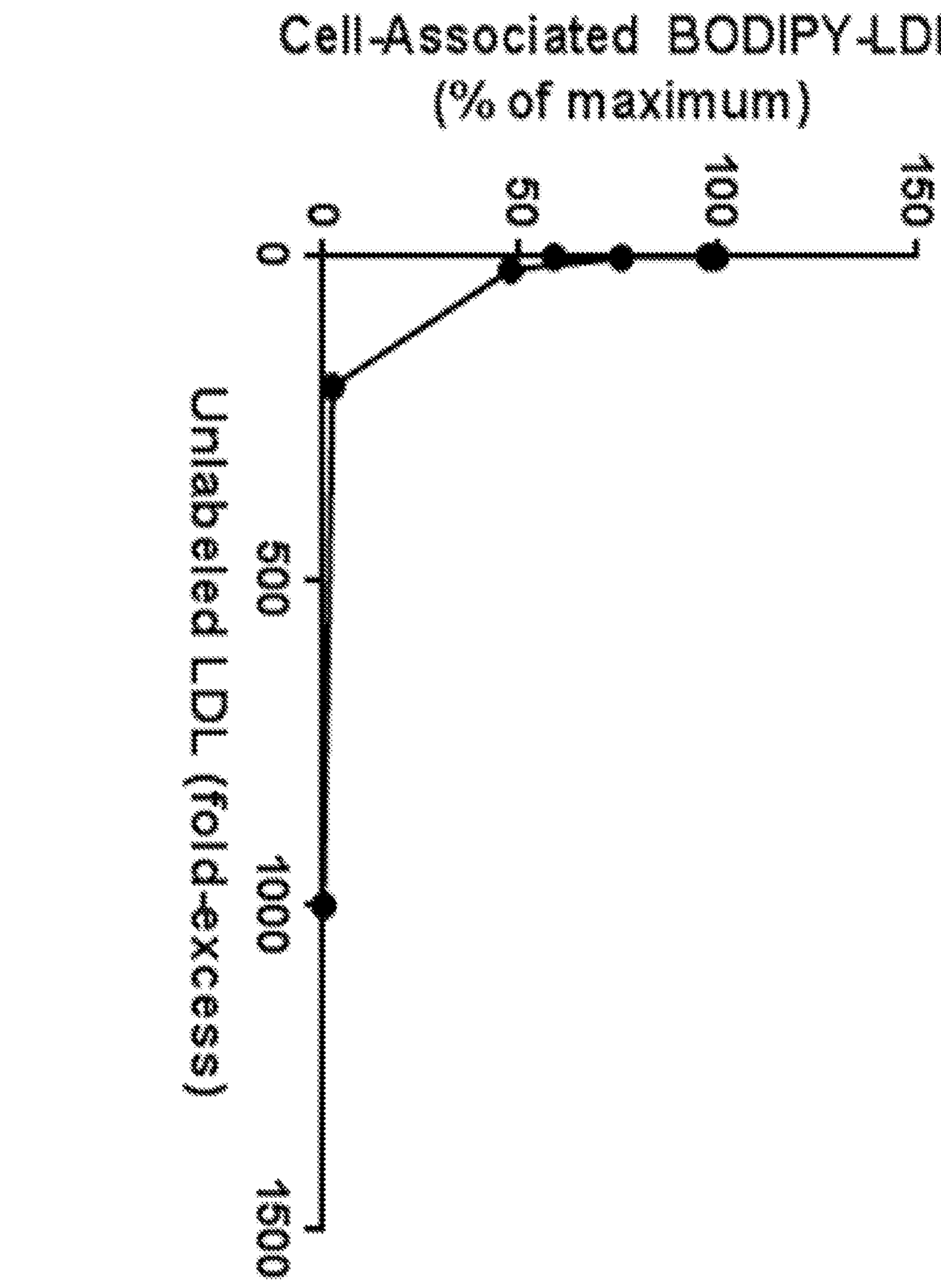


FIGURE 67

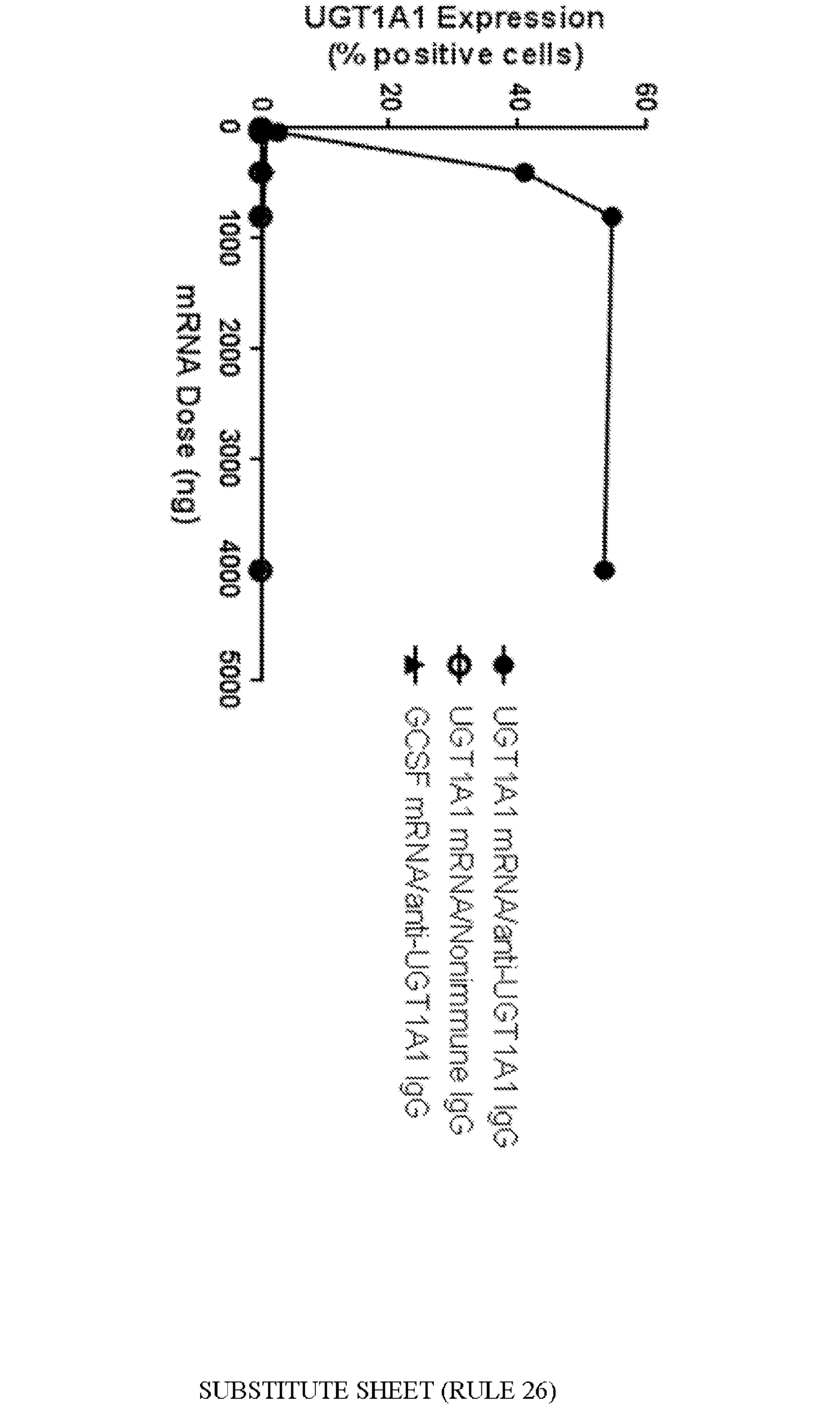

UGT1A1 Expression
(% positive cells)
0
20
40
60
mRNA Dose (ng)
0
1000
2000
3000
4000
5000
UGT1A1 mRNA/ampli-ug
UGT1A1 mRNA/Normalization gene
GCSF mRNA/ampli-ug
log UGT1A1 ng/g
log Normalization gene
log UGT1A1 ng/g

FIGURE 68

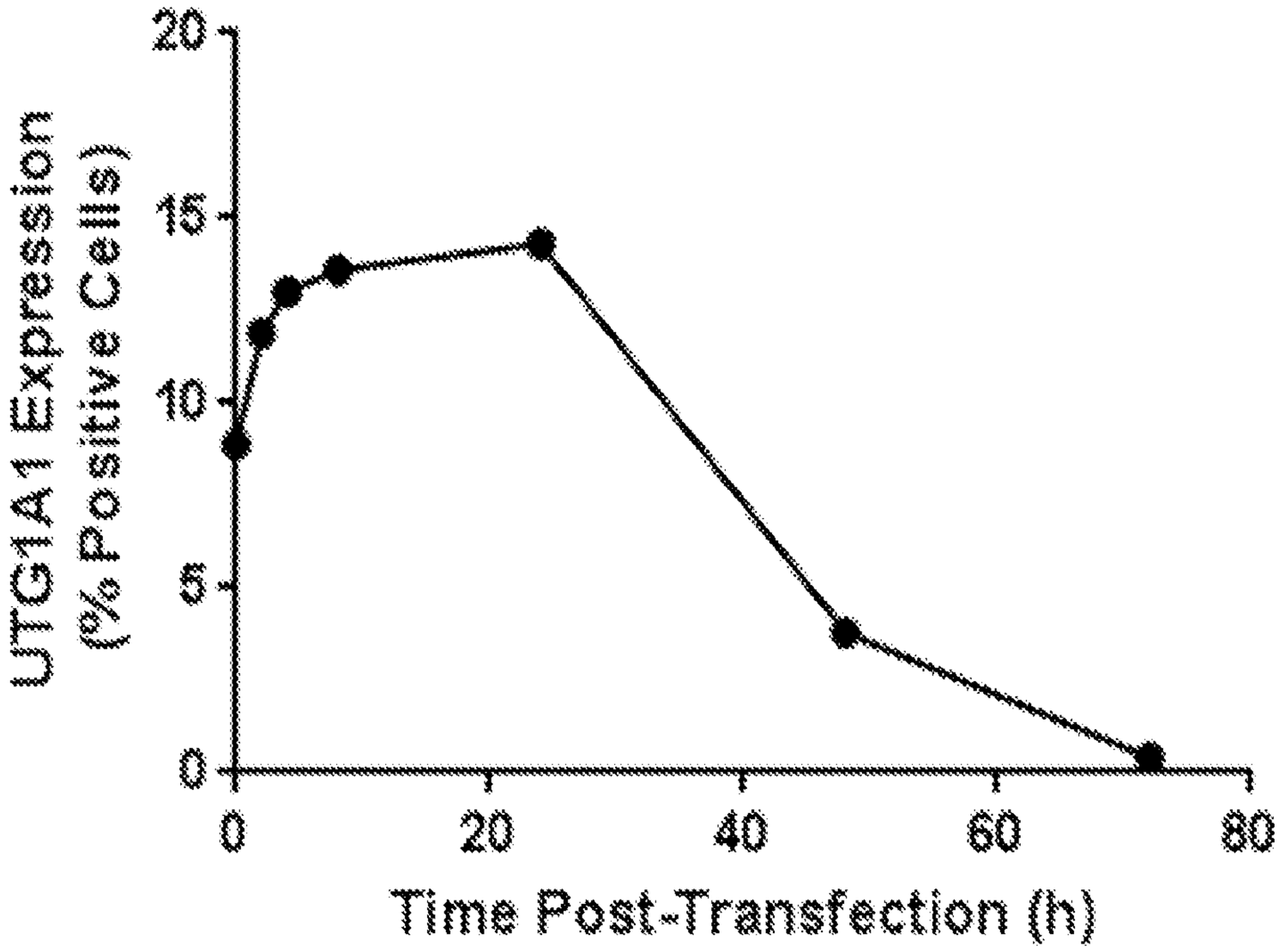

UTG1A1 Expression
(% Positive Cells)
0
5
10
15
20
0
20
40
60
80
Time Post-Transfection (h)

FIGURE 69

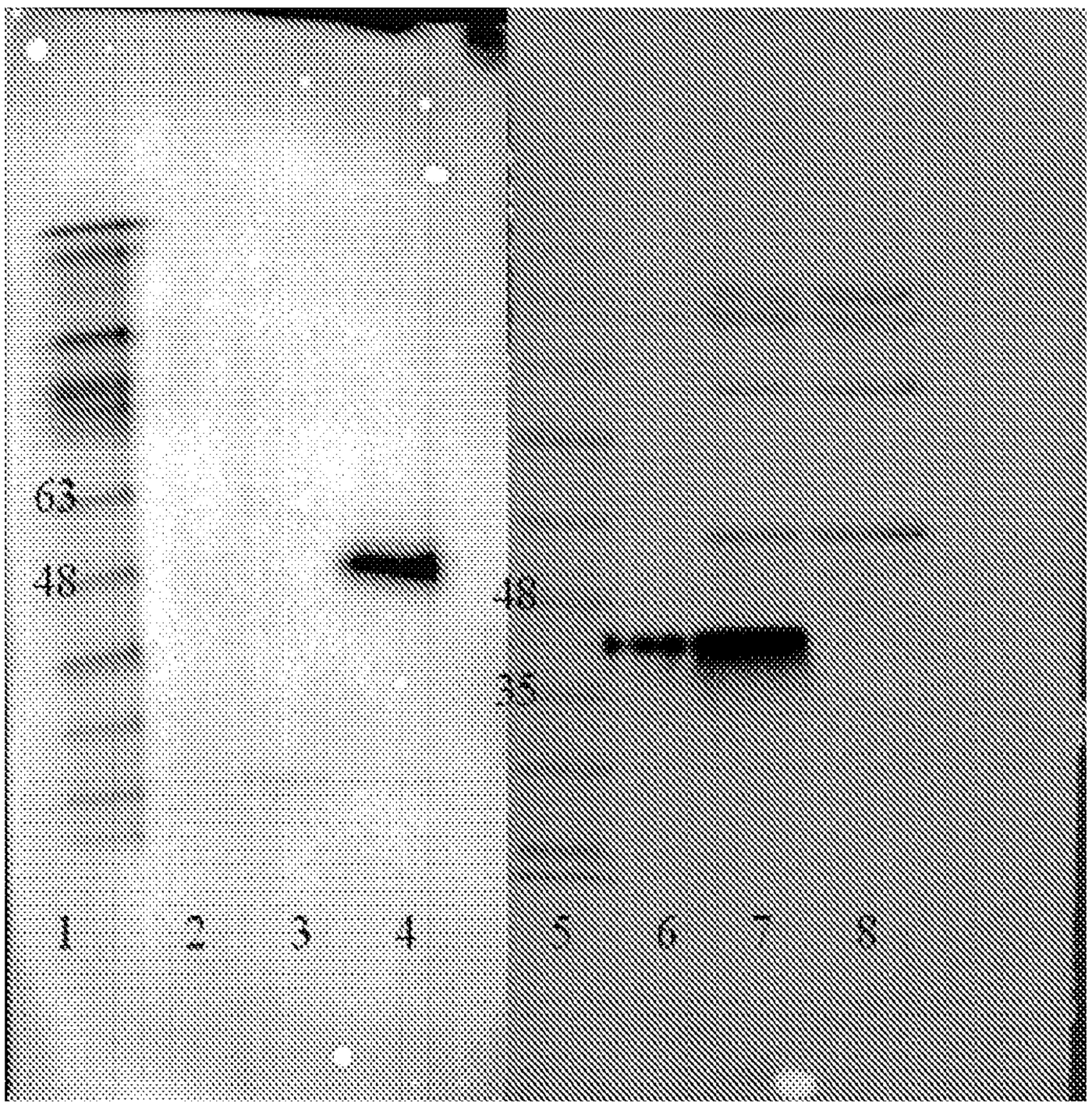


FIGURE 70

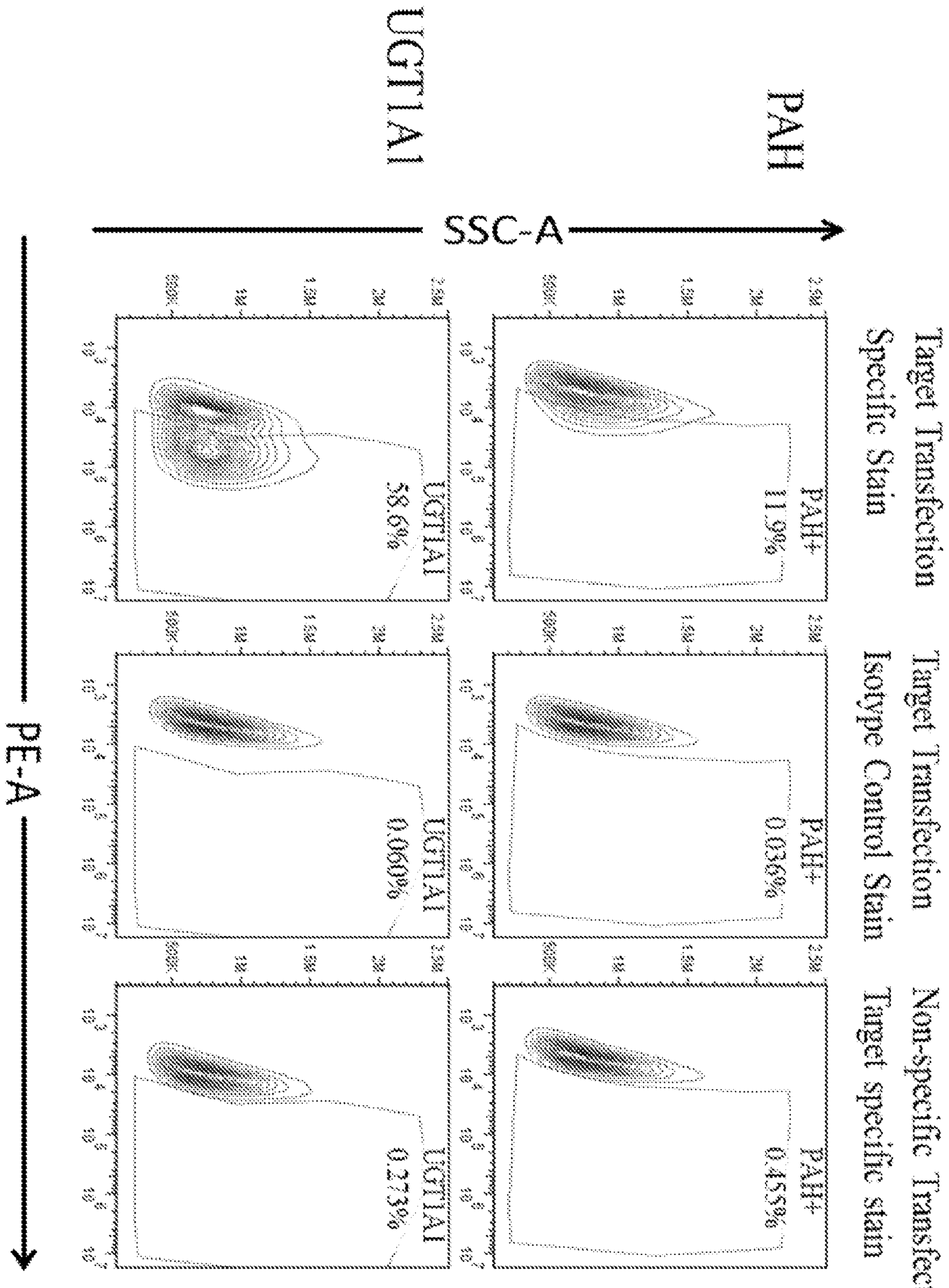

UGT1A1
PAH
SSC-A
Target Transfection
Specific Stain
Non-specific Transfection
Target specific stain
Isotype Control Stain
PAH+
38.6%
UGT1A1
PAH+
11.9%
UGT1A1
PAH+
0.036%
UGT1A1
0.060%
PAH+
0.455%
UGT1A1
0.273%
PE-A

FIGURE 71

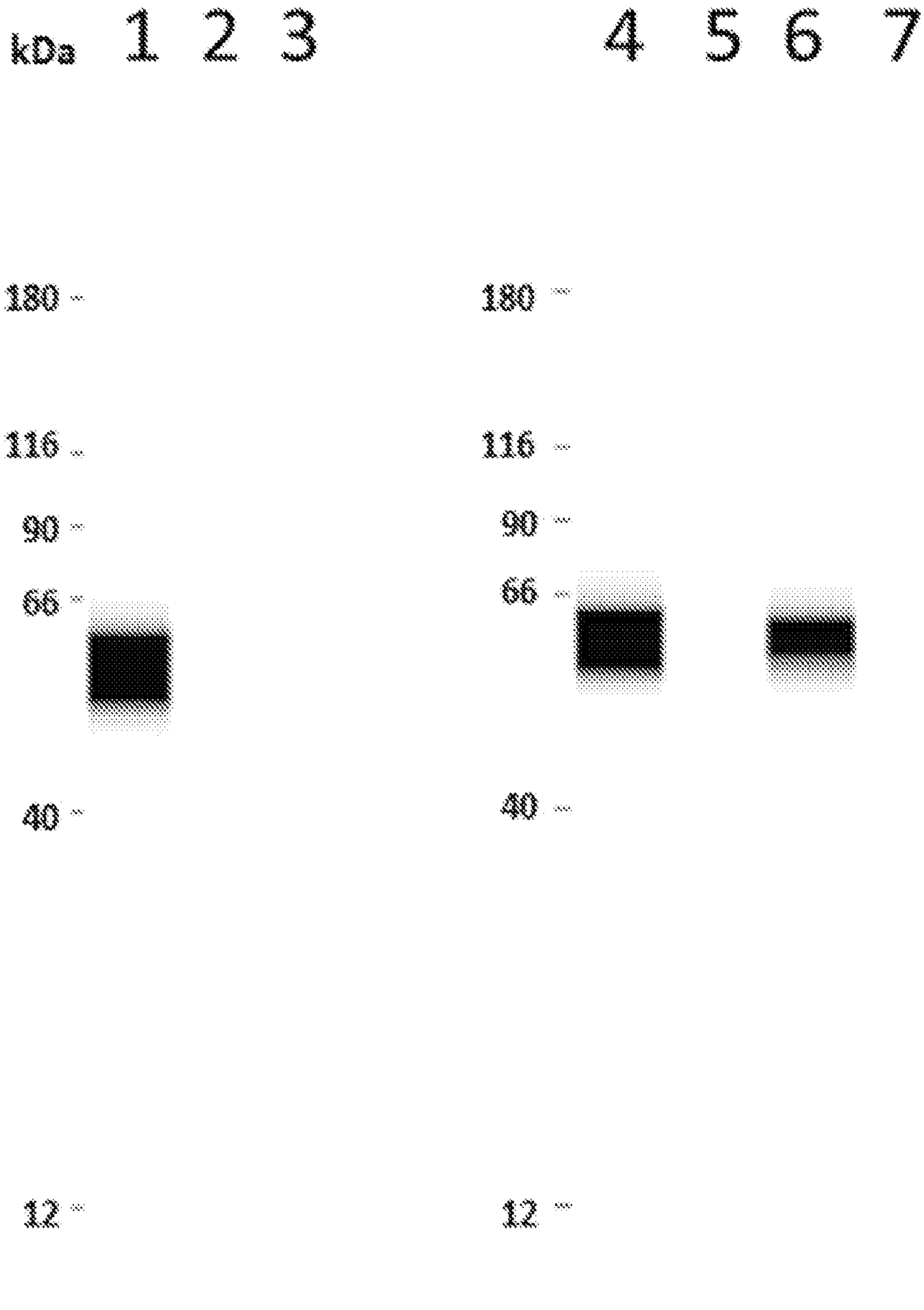
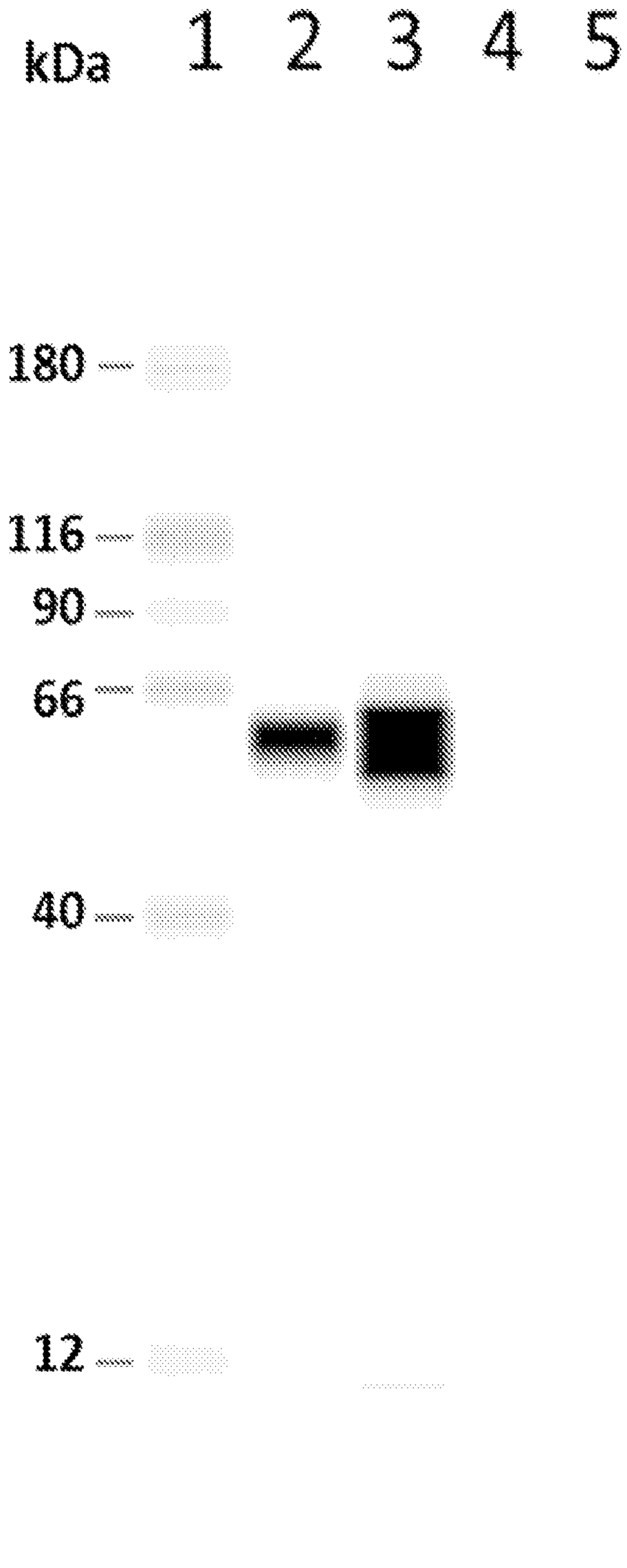


FIGURE 72



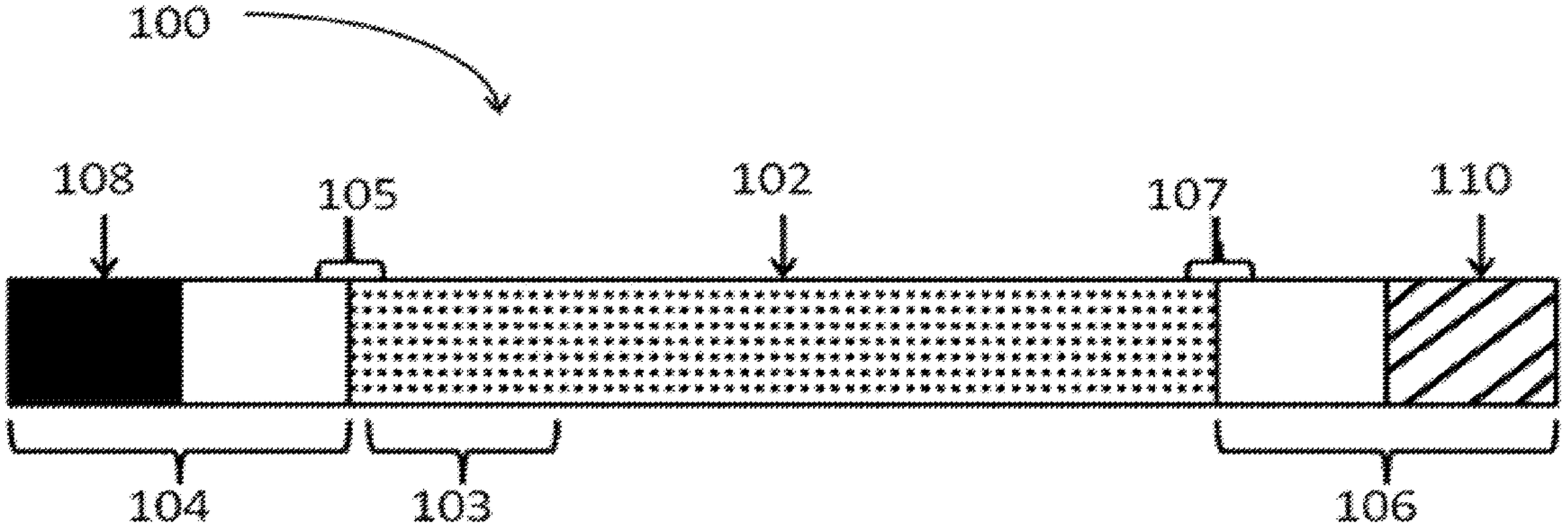


FIGURE 1