(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
20 January 2022 (20.01.2022)

WIPO | PCT

(10) International Publication Number
WO 2022/015956 A1

(51) International Patent Classification:
*C12N 15/90* (2006.01) *C12N 5/0783* (2010.01)
*C12N 15/11* (2006.01) *A61K 39/00* (2006.01)
*C12N 5/10* (2006.01)

(21) International Application Number:
PCT/US2021/041790

(22) International Filing Date:
15 July 2021 (15.07.2021)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
63/052,319 15 July 2020 (15.07.2020) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, IT, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

**Declarations under Rule 4.17:**
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*
— *of inventorship (Rule 4.17(iv))*

**Published:**
— *with international search report (Art. 21(3))*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

WO 2022/015956 A1

(54) Title: IMPROVED PROCESS FOR DNA INTEGRATION USING RNA-GUIDED ENDONUCLEASES

(57) Abstract: There is disclosed an improved, safer and commercially efficient process for developing genetically engineered cells. More specifically, there is disclosed a process comprises introducing a donor DNA construct, a guide RNA, and an RNA-guided nuclease with the host cells to be transfected; and introducing the three components into the host cell. There is further disclosed donor DNA constructs designed for inserting genetic constructs into a defined genomic site of a host cell, such as a target site of the TRAC gene. Further, the present disclosure provides host cells transfected with genetic constructs that lacks viral vectors that can present a safety concern. The disclosure provides for more efficient and more cost-effective process for engineering T ceils to express CAR or DAR constructs.

# IMPROVED PROCESS FOR DNA INTEGRATION USING RNA-GUIDED ENDONUCLEASES

## RELATED APPLICATIONS

This application claims priority to U.S. Provisional Patent Application No. 63/052,319, filed July 15, 2020 which is incorporated by reference in its entirety.

## TECHNICAL FIELD

The present disclosure provides methods and compositions for efficiently integrating a DNA sequence of interest into a target DNA molecule, such as a host genome using an RNA-guided endonuclease such as a cas protein. The disclosure also provides cells such as T cells modified to include a genetic construct integrated into a genetic locus such as the T cell receptor locus.

## BACKGROUND

Targeted integration of an exogenous DNA sequence into a genomic locus has been highly desired. CRISPR-Cas genome engineering is a fast and relatively simple way to knockout gene function or precisely knock-in a DNA sequence for gene correction or gene tagging. Targeted gene knockout is achieved through generation of a double-strand break (DSB) in the DNA using Cas9 nuclease and guide RNA (gRNA). The DSB is then repaired, often imperfectly, by random insertions or deletions (indels), through the endogenous non-homologous end joining (NHEJ) repair pathway. For knock-in experiments, in addition to the Cas9 nuclease and gRNA, a DNA donor template is required and the DSB is repaired with the donor template, typically through the homology-directed repair (HDR) pathway.

Knock-in using a donor template, either a single-stranded DNA (ssDNA) donor oligo or donor plasmid (dsDNA), has a relatively low efficiency, often in the 1-10% range. Therefore, successful HDR-mediated knock-in experiments require important design considerations and experimental optimization. Using single-stranded oligodeoxynucleotides (ssODNs) with short homology arms, several groups have achieved precise DNA editing such as SNP correction or epitope tag addition. A donor plasmid (dsDNA) is able to integrate much longer exogenous DNA, however efficiency is very low. Several groups used an AAV (viral) vector to provide HDR donor ssDNA and combined with CRISPR/Cas9 to achieve 40-60% gene knock-in efficiency. However, these methods still need to produce high titer AAV vectors which is time-consuming and needs to be compatible with cGMP production for clinical application.

A genome engineering tool has been developed based on the components of the type II prokaryotic CRISPR (Clustered Regularly Interspaced Short palindromic Repeats) adaptive immune system of some bacteria such as *S. pyogenes*. This multi-component system referred to as RNA-guided Cas nuclease system or more simply as CRISPR, involves a Cas endonuclease, coupled with a guide RNA molecule, that have the ability to create double-stranded breaks in genomic DNA at specific sequences that are targeted by the guide RNA. The RNA-guided Cas endonuclease has the ability to cleave the DNA where the RNA guide hybridizes to the genome sequence. Additionally, the Cas9 nuclease cuts the DNA only if a specific sequence known as protospacer adjacent motif (PAM) is present immediately downstream of the target sequence in the genome. The canonical PAM sequence in *S. pyogenes* is 5′-NGG-3′, where N refers to any nucleotide.

It has been demonstrated that the expression of a single chimeric crRNA:tracrRNA transcript, which normally is expressed as two different RNAs in the native type II CRISPR system, is sufficient to direct the Cas9 nuclease to sequence-specifically cleave target DNA sequences. In addition, several mutant forms of Cas9 nuclease have been developed. For instance, one mutant form of Cas9 nuclease functions as a nickase, generating a break in complementary strand of DNA rather than both strands as with the wild-type Cas9. This allows repair of the DNA template using a high-fidelity pathway rather than NHEJ, which prevents formation of indels at the targeted locus, and possibly other locations in the genome to reduce possible off-target/toxicity effects while maintaining ability to undergo homologous recombination. Paired nicking can reduce off-target activity by 50- to 1,500-fold in cell lines and to facilitate gene knockout in mouse zygote without losing on-target cleavage efficiency.

In addition, cas proteins have been isolated from a variety of bacteria and have been found to use different PAM sequences than S. pyogenes cas9. In addition, some cas proteins such as cas12a naturally use a single RNA guide – that is, they use a crRNA that hybridizes to a target sequence but do not use a tracrRNA.

Adoptive immunotherapy involves transfer of autologous antigen-specific cells generated *ex vivo*, is a promising strategy to treat viral infections and cancer. The cells used for adoptive immunotherapy can be generated either by expansion of antigen-specific cells or redirection of cells through genetic engineering.

CARs are synthetic receptors consisting of a targeting moiety that is associated with one or more signaling domains in a single fusion molecule. In general, the binding moiety of a CAR consists of an antigen-binding domain of a single-chain antibody (scFv), comprising the light and variable fragments of a monoclonal antibody joined by a flexible linker. Binding moieties based

on receptor or ligand domains have also been used successfully. The signaling domains for first generation CARs are derived from the cytoplasmic region of the CD3zeta or the Fc receptor gamma chains. First generation CARs have been shown to successfully redirect T cell cytotoxicity, however, they failed to provide prolonged expansion and anti-tumor activity *in vivo*. Signaling domains from co-stimulatory molecules including CD28, OX-40 (CD134), and 4-1BB (CD137) have been added alone (second generation) or in combination (third generation) to enhance survival and increase proliferation of CAR modified T cell. CARs have successfully allowed T cells to be redirected against antigens expressed at the surface of tumor cells from various malignancies including lymphomas and solid tumors.

CAR (chimeric antigen receptor) cell immunotherapy, which involves removing T-cells from a patient's blood, adding a CAR through gene transfer, and infusing the genetically engineered cells back into the body, is one of the most promising methods in treating cancer. Currently, the gene transfer techniques include viral-based gene transfer methods using gamma-retroviral vectors or lentiviral vectors. To make GMP (FDA's required good manufacturing practice regulations) level viral-vector, the viral vector has to comply with clinical safety standards such as replication incompetence, low genotoxicity, and low immunogenicity. These conventional approaches have ease of use and reasonable expression, however they can give rise to secondary transformation events, e.g., unwanted blood cancers and other events resulting from viral genome integration into the T cells.

A review article (Ren and Zhao, *Protein Cell* 8(9):634-643, 2017) indicates that any use of CRISPR/Cas9 still involves the use of viral vector for a knocking in process to insert a CAR (chimeric antigen receptor) construct into a T cell genome. "Gene editing with CRISPR encoded by non-integrating virus, such as adenovirus and adenovirus-associated virus (AAV), has also been reported." In addition, Ren et al., *Clin. Cancer Res.* 16:1300, published online 04 November 2016 used a CD19 CAR construct and found that gene disruption in T cells is not very efficient with lentiviral and adenoviral CRISPR.

Although RNA-guided endonucleases, such as the Cas9/CRISPR system, appear to be an attractive approach for genetically engineering some mammalian cells, the use of Cas9/CRISPR in primary cells, in particular in T cells, is significantly more difficult because: (1) T-cells are adversely affected by the introduction of DNA in their cytoplasm: high rate of apoptosis is observed when transforming cells with DNA vectors; (2) the CRISPR system requires stable expression of Cas9 in the cells, however, prolonged expression of Cas9 in living cells may lead to chromosomal defects; and (3) the specificity of current RNA-guided endonuclease is determined only by sequences comprising 11 nucleotides (N12-20NGG, where NGG represents

the PAM), which makes it very difficult to identify target sequences in desired loci that are unique in the genome. Other nucleases, in addition to CAS9, are zinc finger nucleases (ZFN) or TAL effector nucleases (TALEN).

The present disclosure aims to provide solutions to these limitations in order to efficiently implement RNA-guided endonuclease engineering in host cells such as T cells. There is a need in the art for safer techniques for introducing genetic constucts, such as Chimeric Antigen Receptor constructs, into cells that do not include transduction with viral vectors but instead can use transfection techniques, where the risk of off-target mutations is minimized.

## SUMMARY

According to a first aspect, a method for site-specific integration of a donor DNA into a target DNA molecule is described. The method includes introducing into a cell: an RNA-guided endonuclease or a nucleic acid molecule encoding an RNA-guided endonuclease; at least one engineered guide RNA or at least one nucleic acid molecule encoding an engineered guide RNA; and a donor DNA molecule comprising at least two nucleic acid modifications. The guide RNA includes a target sequence designed to hybridize with a target site in the target DNA and the donor DNA is inserted into the target DNA molecule at the target site.

In any of the disclosed implementations, the method may further include any of the following details, which may be combined with one another in any combinations unless clearly mutually exclusive:

(i) the at least two nucleic acid modifications may be on a single strand of the donor DNA molecule;

(ii) one or more nucleic acid modifications may be a modification of one or more nucleotides or nucleotide linkages within 10 nucleotides of the 5' end of the modified strand of the donor DNA molecule;

(iii) one or more nucleic acid modifications may be a backbone modification;

(iv) one or more nucleic acid modifications may be a phosphorothioate modification or a phosphoramidite modification, or a combination thereof;

(v) one or more nucleic acid modifications may be a modification or substitution of a nucleobase;

(vi) one or more nucleic acid modifications may be a modification or substitution of a sugar;

(vii) one or more nucleic acid modifications may be a 2'-O-methyl group modification of deoxyribose;

(viii) the donor DNA molecule may be a double stranded DNA molecule;

(ix) the donor DNA molecule may have a 5' terminal phosphate on the strand opposite to the modified strand;

(x) the donor molecule may have between one and three phosphorothiorate modifications on the backbone within ten nucleotides of the 5' terminus of one strand of the donor molecule and between one and three 2'-O-methyl nucleotide modifications within ten nucleotides of the 5' terminus of one strand of the donor molecule;

(xi) the donor molecule may have between one and three phosphorothiorate modifications on the backbone within five nucleotides of the 5' terminus of one strand of the donor molecule and between one and three 2'-O-methyl nucleotide modifications within five nucleotides of the 5' terminus of one strand of the donor molecule;

(xii) the donor DNA molecule may include homology arms flanking a sequence for integration into the genome;

(xiii) the guide RNA may be a crRNA;

(xiv) the method may further include introducing a tracr RNA into the cell;

(xv) the guide RNA may be a chimeric guide RNA;

(xvi) the RNA-guided endonuclease may be Cas9, Cas12a, Cas12b, Cas13, Cas14, or CasX;

(xvii) at least one guide RNA may be introduced into the cell;

(xviii) an RNA-guided endonuclease may be introduced into the cell;

(xix) the RNA-guided endonuclease may be introduced into the cell as a ribonucleoprotein;

(xx) the RNA-guided endonuclease or the nucleic acid molecule encoding an RNA-guided endonuclease, the at least one engineered guide RNA or the at least one nucleic acid molecule encoding an engineered guide RNA, and the donor DNA molecule may be introduced into the cell simultaneously;

(xxi) the cell may be a prokaryotic cell or a eukaryotic cell;

(xxii) the cell may be a mammalian cell;

(xxiii) the cell may be a human cell;

(xxiv) the cell may be a hematopoietic cell;

(xxv) the cell may be a T cell;

(xxvi) the homology arms may be from 50 to 2000 nucleotides in length;

(xxvii) the homology arms may be from 100 and 1000 nucleotides in length;

(xxviii) the homology arms may be from 150 to 650 nucleotides in length;

(xxix) the homology arms may be from 150 and 350 nucleotides in length;

(xxx) the homology arms may be from 150 to 200 nucleotides in length;

(xxxi) the donor DNA molecule may include a modified strand and an opposite strand, wherein the modified strand may include two or more nucleic acid modifications, and the opposite strand may include a terminal phosphate;

(xxxii) the RNA-guided endonuclease and the guide RNA are introduced into the cell as a ribonucleoprotein complex (RNP);

(xxxiii) the RNP may include a tracr RNA;

(xxxiv) the RNP may be introduced into the cell by electroporation or liposome transfer;

(xxxv) the donor DNA and the RNP may be introduced into the cell simultaneously or separately;

(xxxvi) the donor DNA may include a gene construct;

(xxxvii) the donor DNA may include a gene construct that is at least 1.0 kb, at least 1.2 kb, at least 1.4 kb, at least 1.6 kb, at least 1.8 kb, at least 2.0 kb, at least 2.2 kb, at least 2.4 kb, or at least 2.5 kb in size;

(xxxviii) the donor DNA may include a chimeric antigen receptor (CAR) or a dimeric antigen receptor (DAR) construct;

(xxxix) the target site may be selected from a T cell receptor gene, a PD-1 gene, or a TIM3 gene;

(xxxx) the efficiency of insertion of the donor DNA into the target site may be at least 15%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, or at least 80%;

(xxxxi) the frequency of total off-target mutations occurring at a frequency of at least 0.2% may be less than 25%, 20%, or 15% of the frequency of mutation at the target site;

(xxxxii) the frequency of mutation at the highest frequency non-target mutation site may be less than 25%, 20%, or 15% of the frequency of mutation at the target site; and

(xxxxiii) there may be no detectable nontarget mutations in protein encoding regions of the genome.

In some embodiments of the method the RNA-guided endonuclease is Cas9 and the method includes introducing into a cell: a Cas9 endonuclease or a nucleic acid molecule encoding a Cas9 endonuclease; at least one engineered guide RNA or at least one nucleic acid molecule encoding an engineered guide RNA, where the guide RNA includes the target sequence of SEQ ID NO:1; and a donor DNA molecule comprising at least two nucleic acid modifications.

The guide RNA target sequence is designed to hybridize with a target site in the TCRα subunit constant (TRAC) gene and the donor DNA is inserted into the target DNA molecule at the target site. The donor DNA can include a gene that may be expressed in the cell. In various examples the donor DNA includes a gene or genetic construct the genetic construct that is at least 1.0 kb, at least 1.2 kb, at least 1.4 kb, at least 1.6 kb, at least 1.8 kb, at least 2.0 kb, at least 2.2 kb, at least 2.4 kb, or at least 2.5 kb in size. In various embodiments the gene or genetic construct can encode an immunological molecule, an immunomodulatory protein, or a receptor. For example, the donor can encode a CAR or DAR, or can encode a T Cell Receptor Fusion Construct (TFP; US20210079057, incorporated herein by reference), or an antibody-TCR chimeric molecule (abTCR; US 10, 098,451, incorporated herein by reference).

The methods herein can be used to introduce an RNA-guided endonuclease, e.g., Cas9, a guide RNA, and a modified donor DNA as disclosed herein for integration into the genetic locus targeted by the guide RNA, into a population of cells for integration of the donor DNA into the genome of the cells of the population. The efficiency of insertion of the genetic construct of the donor DNA can be, in various examples, at least 15%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, or at least 80%. In various embodiments at least 20%, at least 30%, at least 40%, or at least 50% of the cells transfected with the RNA-guided endonuclease in combination with the guide and a donor DNA modified as disclosed herein that includes a genetic construct may express the genetic construct while not expressing the T cell receptor. Using the methods provided herein the Cas9 endonuclease in combination with the guide comprising the target sequence of SEQ ID NO:1 and a donor DNA may result in cells of the population having a frequency of indels at the highest-frequency off-target site of less than 10%. Using the methods provided herein the Cas9 endonuclease in combination with the guide comprising the target sequence of SEQ ID NO:1 may result in cells of the population having no detectable off-target mutations in the coding regions of genes.

In various embodiments, the RNA-guided endonuclease, e.g., Cas9, is delivered to the cells as a protein complexed with a guide RNA, which may be an sgRNA (e.g., a chimeric guide), or the guide RNA may be a crRNA and the RNP that is introduced into the cell may further include a tracrRNA. A donor DNA, such as a ds donor DNA fragment having one or more chemical modifications at the 5' terminus of one strand as described herein, and optionally having a 5' terminal phosphate at the 5' terminus of the opposite strand, can be introduced into the cell along with the RNP, i.e., in the same transfection, or can be separately transfected into the cell. Introduction of the RNP can be, for example, by electroporation, or may be by chemical transfection, or for example, by lipofection, liposome delivery, or nanoparticle delivery. In

certain embodiments, the donor DNA can include an expression construct. For example, the donor DNA can include a construct that encodes an immunological molecule, i.e., a molecule comprising a binding domain of an antibody and/or an immunomodulatory molecule, such as, for example, a cytokine, a receptor domain, a checkpoint molecule or checkpoint inhibitor, etc. Delivery of an RNA-guided endonuclease (or a nucleic acid molecule encoding an RNA-guided endonuclease), a guide RNA (or a nucleic acid molecule encoding a guide RNA), and a donor DNA as disclosed herein into a cell results in integration of the donor DNA into the target site recognized by the guide RNA. The methods can result in a low level of off-target mutations, for example, less than a 10% frequency of off-target mutations at the highest-frequency off-target site.

In further embodiments, the RNA-guided endonuclease, e.g., Cas9, is delivered to a population of cells as a protein complexed with a guide RNA that includes the target site of SEQ ID NO:1, which may be an sgRNA (e.g., a chimeric guide), or the guide RNA may be a crRNA and the RNP that is introduced into the cells may further include a tracrRNA. A donor DNA, such as a double stranded (ds) donor DNA fragment having one or more chemical modifications at the 5' terminus of one strand as described herein, and optionally having a 5' terminal phosphate at the 5' terminus of the opposite strand, can be introduced into the cell along with the RNP, i.e., in the same transfection, or can be separately transfected into the cell. Introduction of the RNP can be, for example, by electroporation, or may be by chemical transfection, for example, by lipofection. In certain embodiments, the guide RNA may target the T cell receptor. In certain embodiments, the donor DNA can include an expression construct. For example, the donor DNA can include a construct that encodes an immunological molecule, i.e., a molecule comprising a binding domain of an antibody or an immunomodulatory molecule. The population of cells may be a population of T cells, e.g., a population of primary T cells. Delivery of an RNA-guided endonuclease (or a nucleic acid molecule encoding an RNA-guided endonuclease), a guide RNA (or a nucleic acid molecule encoding a guide RNA), and a donor DNA as disclosed herein into a cell population results in integration of the donor DNA into the genome of the cells in at least 20%, at least 30%, at least 40%, or at least 50% of the cells. Delivery of an RNA-guided endonuclease (or a nucleic acid molecule encoding an RNA-guided endonuclease), a guide RNA (or a nucleic acid molecule encoding a guide RNA), and a donor DNA as disclosed herein into a cell population results in expression of a gene encoded by the donor DNA and the absence of T cell receptor expression in at least 20%, at least 30%, at least 40%, or at least 50% of the cells. The frequency of mutation of the highest frequency nontarget site by Cas9 complexed with a guide RNA that include the target sequence of SEQ ID NO:1 in the cell

population transfected with the Cas9 nuclease, guide sequence that includes SEQ ID NO:1, and donor DNA can be less than 10%, less than 5%, less than 2%, less than 1%, or less than 0.5%. The frequency of insertion of the donor DNA into a coding region of a gene other than the targeted TRAC gene in some embodiments can be undetectable in the cell population.

According to a second aspect, a system for targeted integration of a donor DNA into a target locus is described. The system includes: an RNA-guided endonuclease or a nucleic acid molecule encoding an RNA guided endonuclease; a guide RNA or a nucleic acid molecule encoding a guide RNA; and a double-stranded donor DNA molecule. The donor DNA molecule includes one or more phosphorothioate bonds on a single strand of the double stranded DNA molecule within ten or within five nucleotides of the 5' terminus of the modified strand of the donor DNA molecule, and may further include one or more base or sugar modifications on one or more nucleotides within ten or within five nucleotides of the 5' terminus of the modified strand of the donor DNA molecule. The donor can be, for example, any of the donor DNA molecules having any of the modifications provided herein.

In any of the disclosed implementations, the system may further include any of the following details, which may be combined with one another in any combinations unless clearly mutually exclusive:

(i) the system may include an RNA-guided endonuclease;

(ii) the system may include a guide RNA;

(iii) the donor DNA molecule may further include at least one modification of a sugar moiety or nucleobase of the modified strand within ten nucleotides of the 5' terminus of the modified strand of the nucleic acid molecule;

(iv) the donor DNA may have homology arms flanking a sequence of interest for integration into the genome;

(v) the donor DNA may include a genetic construct flanked by homology arms where the genetic construct is at least 1.0 kb, at least 1.2 kb, at least 1.4 kb, at least 1.6 kb, at least 1.8 kb, at least 2.0 kb, at least 2.2 kb, at least 2.4 kb, or at least 2.5 kb in size;

(v) the donor DNA may include a chimeric antigen receptor (CAR) or a dimeric antigen receptor (DAR) construct;

(v) the one or more phosphorothioate bonds on the single strand of the double stranded DNA molecule may be within five nucleotides of the 5' terminus of the modified strand of the nucleic acid molecule;

(vi) the at least one modification of a sugar moiety or nucleobase of the modified strand may be within five nucleotides of the 5' terminus of the modified strand of the nucleic acid molecule;

(vii) the at least one modification of a sugar moiety may include a 2'-O methylation;

(viii) the sequence of interest may include an expression cassette;

(ix) the expression cassette may include a construct including one or more antibody or receptor domains;

(x) the homology arms may be between 50 and 5000 nucleotides in length;

(xi) the homology arms may be between 100 and 1000 nucleotides in length;

(xii) the homology arms are between 150 and 800 nucleotides in length;

(xiii) the nuclease may be selected from the group consisting of Cas9, Cas12a, Cas12b, CasX, and combinations thereof;

(xiii) the guide RNA may be a chimeric guide having sequences of both a crRNA and a tracrRNA;

(xiv) the guide RNA may be a crRNA;

(xv) the guide RNA may include one or more phosphorothioate (PS) oligonucleotides;

(xvi) the guide RNA may be a crRNA and the system may further include a tracrRNA;

(xvii) the guide RNA may be a single guide RNA;

(xviii) the system may include a ribonucleoprotein complex including the RNA-guided endonuclease and the guide RNA.

In some embodiments, a system for targeted integration of a donor DNA into a target locus as provided herein includes: an Cas9 endonuclease or a nucleic acid molecule encoding a Cas9 endonuclease; a guide RNA or a nucleic acid molecule encoding a guide RNA, where the guide RNA includes the target sequence of SEQ ID NO:1; and a double-stranded donor DNA molecule. The donor DNA molecule includes one or more phosphorothioate bonds and one or more O-methylated modified nucleotides on a single strand of the double stranded DNA molecule within ten nucleotides of the 5' terminus of the modified strand of the nucleic acid molecule.

According to a third aspect, a composition for generating a donor DNA molecule is described. The composition includes: a first primer (single stranded deoxyoligonucleotide) having one or more phosphorothioate bonds and one or more modified nucleotides within five nucleotides of the 5' terminus of the oligonucleotide; and a second primer (single stranded deoxyoligonucleotide) having a 5' terminal phosphate. The first and second primers may be

homologous to sequences on opposite sides of a target site for an RNA-guided endonuclease in a target genome. The first primer can include one or more phosphorothioate bonds and one or more 2'-O-methylated nucleotides within five nucleotides of the 5' terminus of the modified strand of the oligonucleotide, for example, the first primer can have three phosphorothioate linkages and three 2'-O-methylated nucleotides. The primers can be, for example, from 17 to 100 nucleotides in length, for example, from 17 to 30 oligonucleotides in length. The primers are designed to hybridize to opposite strands of a genomic sequence on either side of cas target site in a genome, such as in a human gene, so that donor fragments based on constructs having flanking sequences homologous to sequences surrounding a target site of interest can be produced with the desired nucleotide or backbone modifications.

According to a fourth aspect, a double-stranded donor DNA molecule configured to integrate a sequence of interest into a target site of a host genome is described. The double-stranded donor DNA molecule includes one or more modifications to nucleotides of one donor DNA strand; homology arms flanking the sequence of interest, where the homology arms include sequences homologous to sequences occurring in the host genome on either side of the target site; and from one to ten modified nucleotides that occur within ten nucleotides of the 5' end of one strand of the donor DNA.

In any of the disclosed implementations, the double-stranded donor DNA molecule may further include any of the following details, which may be combined with one another in any combinations unless clearly mutually exclusive:

(i) the double-stranded donor DNA molecule may include from one to five modified nucleotides that be within five nucleotides of the 5' end of one strand of the donor DNA;

(ii) the modified nucleotides may include from 1 to 4 phosphorothioate (PS) linkages, or from 1 to 4 2'-O-methylation modifications, or a combination thereof;

(iii) one strand of the double-stranded donor DNA molecule may have two or more modifications on any of the first ten or first five nucleotides from the 5' end and the other strand has a terminal 5' phosphate.

(iv) the sequence of interest may include a CAR or DAR construct;

(v) the sequence of interest may be is at least 1.0 kb, at least 1.2 kb, at least 1.4 kb, at least 1.6 kb, at least 1.8 kb, at least 2.0 kb, at least 2.2 kb, at least 2.4 kb, or at least 2.5 kb in size;

(v) the target site may be selected from a T cell receptor gene, a PD-1 gene, or a TIM3 gene.

(vi) the target site may be exon 1 of the T cell receptor gene, and may be the target site of SEQ ID NO:1.

According to a fifth aspect, a host cell is described. The host cell includes a donor DNA as described herein integrated into a target DNA molecule as described herein, wherein the host cell may be produced by any of the methods described herein. Also provided is a population of cells provided in which at least 20%, at least 30%, or at least 40% of the cells of the population express a genetic construct of at least 1.0, at least 1.2 kb, at least 1.4 kb, at least 1.6 kb, at least 1.8 kb, at least 2.0 kb, at least 2.2 kb, at least 2.4 kb, or at least 2.5 kb in size that is integrated into a targeted site of the genome), where none of the cells include a viral vector or any sequences derived therefrom. In some examples, the none of the cells of the population include any vector sequences, any viral sequences, or any bacterial sequences. In some examples, a population of T cells provided herein that include a genetic construct integrated into a targeted site of the genome does not have any retroviral or adenoviral sequences detectable in the population.

In a further aspect a population of primary T cells is provided in which at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, or at least 90% of the cells of the population express a genetic construct that is integrated into the genome and do not express a gene that is knocked out by the targeted integration (e.g., a TRAC gene or another targeted gene), where none of the cells include a viral vector or any sequences derived therefrom. In some examples, none of the cells of the population include any vector sequences, any viral sequences, or any bacterial sequences. In some examples, a population of T cells provided herein that include a genetic construct integrated into a targeted site of the genome does not have any retroviral or adenoviral sequences detectable in the population. The integrated genetic construct can be at least 1.5 kb, at least 1.7 kb, at least 1.9 kb, at least 2 kb, at least 2.1 kb, at least 2.2 kb, at least 2.3 kb, or at least 2.4 kb in length. In nonlimiting example, the genetic construct can be a construct encoding an immunological molecule (e.g., a protein having at least one binding domain derived from an antibody) or engineered receptor, such as for example a CAR or DAR construct.

In some embodiments of the primary T cell population provided herein having a genetic construct integrated into the genome, the construct is integrated into a Cas9 site, i.e., a site

adjacent to a Cas9 PAM. In various embodiments, the population of primary T cells includes a genetic construct integrated into exon 1 of the TRAC gene, and in some embodiments, the genetic construct is integrated into the target site of SEQ ID NO:1. In some embodiments, the frequency of mutation of the highest frequency mutated nontarget site in the population can be less than 20%, less than 15%, less than 10%, less than 5%, less than 2%, less than 1%, or less than 0.5%. In some embodiments the frequency of insertion of the donor DNA into a coding region of a gene other than the targeted TRAC gene can be undetectable, for example, using a nonbiased method such as iGUIDE or by amplicon sequencing.

In another aspect, provided herein is a population of cells in which at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% of the cells of the population have a protein-encoding exogenous genetic construct integrated into the TRAC locus, where the cells express the encoded protein and do not express the T cell receptor. In various embodiments the protein encoded by the construct may be a receptor, such as an engineered receptor, and in particular embodiments can be a DAR. In various embodiments at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% of the cells of the population have an exogenous genetic construct integrated into the TRAC locus and the frequency of mutation at the highest frequency off-target site is less than 25%, less than 20%, less than 15%, or less than 10% of the frequency of mutation at the target site. The frequency of off-target mutations that occur in a protein encoding region of the genome may be undetectable using an unbiased off-target detection method such as iGUIDE. The exogenous genetic construct may be integrated into the TRAC gene target site of SEQ ID NO:1. In some embodiments the exogenous genetic construct can be a DAR and in some embodiments is a CD38 DAR. For example, the cells of the population can include a CD38 DAR genetic construct that encodes the precursor polypeptide of SEQ ID NO:52. In some embodiments the CD38 DAR construct comprises SEQ ID NO:51 or a nucleotide sequence having at least 95% identity thereto. The population of cells can be a population of T cells, for example primary human T cells. The population of cells may be a population of human primary T cells enriched for cells that express the CD38DAR and do not express the T cell receptor for use in allogeneic cell-based therapies.

## BRIEF DESCRIPTION OF THE FIGURES

**FIG. 1A** provides chemical drawings that show, in the right structure, a phosphorothioate (PS) modification of the bond between nucleotides as they might occur in a primer. The nucleotides shown in the oligonucleotide on the left are attached via a (nonmodified) phosphodiester bond. **FIG. 1B** provides a chemical drawing of an oligonucleotide having two PS bonds that join the 5'-most nucleotide to the next nucleotide "downstream" in the oligonucleotide, which in turn is attached to the following downstream nucleotide of the oligonucleotide by a PS bond. The 5'-most nucleotide of the oligonucleotide includes a 2' O-methyl modification.

**FIG. 2A** is a diagram showing structure of a donor DNA construct, such as a CAR donor DNA construct that includes an open reading frame having a sequence encoding a single chain variable fragment (scFv), followed by the CD8a leader peptide which is then followed by a CD28 hinge-CD28 transmembrane-intracellular regions and then a CD3 zeta intracellular domain. The coding sequence is preceded by a JeT promoter (SEQ ID NO:3) and the construct includes homology arms (HA), in this case matching sequences of the human TRAC locus, flanking the promoter plus coding sequences. This provides a diagram of the template DNA used for generating donor DNA. The anti-CD38A2 contains a CD38 CAR transgene with expression driven by the JeT promoter and flanked by homology arms on the 5' and 3' sides to enable targeted integration. **FIG. 2B** shows primer design for confirming knock in, showing the same diagram as in FIG. 2A and also indicating the positions of PCR primers used to confirm CAR integration by amplification with one primer located within the CAR and one primer in TRAC outside of the homology arms at both the 5' and 3' ends to generate 1371-bp and 1591-bp products, respectively, when integration is at the targeted integration site.

**FIG. 3A** provides flow cytometry plots of PBMCs 8 days after transformation with a donor DNA that included a construct for expressing an anti-CD38 CAR and an RNP comprising a guide RNA targeting the TRAC locus. The CAR cassette was flanked by homology arms having homology to TRAC locus sequences flanking the integration target site in exon 1 of the TRAC gene. The Y axis reports cell size. Anti-CD38 construct expression is along the x axis. Negative control: no donor DNA was transformed into the target cells; No modification- the donor DNA had no chemical modifications; PS modification: three phosphorothioate bonds

occurred within the 5'-most five nucleotide backbone positions; PS + 2'-OMe: in addition to phosphorothioate bonds, the three nucleotides within the 5'-most five nucleotides of the donor included 2'-OMe in addition to PS modifications; TCR KO/retroviral construct: the cells were transfected with the RNP in the absence of donor DNA to knock out the TCR gene and transduced with a retrovirus to express the anti-CD38 CAR. **FIG. 3B** provides the results of flow cytometry performed on the same cultures as in A) ten days after transfection. **FIG. 3C** provides the results of flow cytometry performed on the culture that received the doubly-modified donor DNA and control (TRAC knockout only and TRAC knockout with retroviral transduction) twenty days after transfection.

**FIG. 4** shows a gel of PCR products showing integration of the donor DNA at the targeted TRAC (Exon1) site. Primary human T cells were electroporated with TRAC RNP only or together with ssDNA. PCR was used to confirm the presence of the anti-CD38A2 CAR transgene integrated in the TRAC locus two weeks post-electroporation (lanes 3 and 6, depicting products from 5' and 3' integration regions). No bands were observed in non-transformed ATCs (lanes 1 and 4) or T cells that were transformed with the TRAC exon 1 targeting RNP but did not receive the donor DNA (lanes 2 and 5).

**FIG. 5** is a graph showing cytotoxicity assay results with Activated T cells (ATCs, stars) as a control, TCR knock out ATC, anti-CD38A2 retrovirus transduced CART cells RV CART, black line), TRAC knock out retrovirus transduced CART cells (dots), TRAC knock out together with phosphorothioate modified ss donor DNA knock in (dashes), TRAC knock out together with phosphorothioate and 2' O-Methyl modified ssDNA knock in (dashes and dots).

**FIGS. 6A-6C** provide graphs of the results of cytokine secretion assays using anti-CD38 CART cells and controls co-cultured with K52 or RPM18226 cells. The T cell cultures tested are as provided in FIG. 5.

**FIG. 7** provides the results of testing donor DNAs having homology arms (HAs) of different lengths. Cultures were assessed by flow cytometry for loss of TCR expression (Y axis) and anti-CD38 expression (X axis).

**FIG. 8** provides the results of testing double stranded donor DNAs modified by the addition of three PS bonds and three 2'O methyl nucleotides proximal to the 5' end of one strand

of the donor DNA molecule. Cultures were assessed by flow cytometry for loss of TCR expression (Y axis) and anti-CD38 expression (X axis).

**FIG. 9** provides the results of flow cytometry on cells transfected with a ds PS and 2'-OMe- modified donor DNA that included a cassette for expressing an anti-CD19 CAR. The donor was directed to the TRAC exon 1 locus by cotransfection with an RNP. TCR expression is determined on the Y axis and anti-CD19 CAR expression on the Y axis.

**FIG. 10** provides the results of flow cytometry on cells 22 days post-transfection with a ds PS and 2'-OMe- modified donor DNA that included a cassette for expressing an anti-BCMA CAR. The donor was directed to the TRAC exon 1 locus by cotransfection with an RNP. TCR expression is determined on the Y axis and anti-BCMA CAR expression on the Y axis.

**FIG. 11** provides the results of flow cytometry on cells 10 days post-transfection with a ds PS and 2'-OMe- modified donor DNA that included a cassette for expressing an anti-CD19 CAR. In one culture, the donor had homology arms derived from TRAC exon 3 was directed to the TRAC exon 3 locus by cotransfection with an RNP having an exon 3 guide RNA ($2^{nd}$ panel). In another culture, the donor had homology arms derived from TRAC exon 1 was directed to the TRAC exon 1 locus by cotransfection with an RNP having an exon 1 guide RNA ($2^{nd}$ panel). TCR expression is determined on the Y axis and anti-CD19 CAR expression on the Y axis.

**FIG. 12** provides the results of flow cytometry on cells transfected with a ds PS and 2'-OMe- modified donor DNA that included a cassette for expressing an anti-C38 CAR and homology arms derived from the TRAC gene or the PD-1 gene. In one culture, the donor had homology arms derived from TRAC exon 1 was directed to the TRAC exon 1 locus by cotransfection with an RNP having an exon 1 guide RNA (3rd panel). In another culture, the donor had homology arms derived from the PD-1 locus and was directed to the PD-1 gene by cotransfection with an RNP having a PD-I gene guide RNA (4th panel). TCR expression is determined on the Y axis and anti-CD38 or anti-PD-1 CAR expression on the Y axis.

**FIG. 13** provides the results of flow cytometry on cells transfected with a ds PS and 2'-OMe- modified donor DNA that included a cassette for expressing a CD38 DAR. TCR expression is determined on the Y axis and anti-CD19 CAR expression on the Y axis.

**FIG. 14** is a graph showing cytotoxicity assay results with TCR knock out ATCs and T cells produced using the methods provided herein that express a CD38DAR.

## DETAILED DESCRIPTION

The present disclosure provides an improved, safer, and commercially efficient process for developing genetically engineered and transduced cells for immunotherapy. More specifically, the disclosed process comprises introducing an RNA-guided endonuclease, a guide RNA, and a donor DNA construct into host cells, where the guide RNA is engineered to direct the cas protein with which it is complexed to a targeted site of the host genome. Cleavage of the genomic DNA at the target site by the RNA-guided endonuclease and subsequent repair of the double stranded break using the donor fragment that includes homology arms by homology-directed repair (HDR) results in integration of sequences of the donor DNA molecule positioned between the homology arms. The method can be used to simultaneously knock out a gene at the target locus and insert or "knock in" at the disrupted locus a transgene that is provided in the donor DNA molecule. The method can be used on any host cells, including prokaryotic and eukaryotic cells, and can be used with mammalian cells, such as human cells. The method has advantages in ease of use, efficiency, and the ability to generate genome modifications that do not entail the use of selectable markers or viral vectors that are undesirable in many applications, including clinical applications. In some embodiments, the host cells are hematopoietic cells, such as, for example, T cells.

The present disclosure also provides donor DNA compositions, where the donor DNA molecule includes one or more modifications to nucleotides of one donor DNA strand. The donor DNA can include homology arms flanking a sequence of interest whose integration into the host genome is desired, where the homology arms have sequences homologous to sequences occurring in the host genome on either side of the target sequence. The donor DNA in some embodiments is double-stranded. In various embodiments the donor DNA includes from one to ten modified nucleotides that are proximal to the 5' end of one strand of the donor DNA, for example, that occur within ten nucleotides or within five nucleotides of the 5' terminus of one strand of the donor DNA. In some embodiments the donor DNA has at least two types of nucleic acid modification of from one to ten nucleotides at the 5' end of one strand of the donor DNA. In some embodiments the donor DNA has two types of nucleic acid modification of from one to ten nucleotides at the 5' end of one strand of the donor DNA. The modification may be, for example, phosphorothioate (PS) linkages between nucleotides, or may be 2'-O-methylation of the deoxyribose of one or more nucleotides of the donor DNA molecule. For example, a donor DNA molecule can have one, two, three or four PS bonds within the first five, first six, or first seven nucleotides from the 5' end of the modified strand and can also have one, two, three or four 2'-

O-methyl modified nucleotides within the first five, first six, or first seven nucleotides from the 5' end of the modified strand. In some embodiments the donor DNA molecule is double-stranded and one strand comprises the modifications at the 5' end. In some embodiments the donor DNA molecule is double-stranded and one strand has two or more modifications on any of the first ten or first five nucleotides from the 5' end and the opposite strand has a terminal 5' phosphate. In various embodiments, the donor DNA molecule is double-stranded and has at least two PS bonds and at least two 2'O-methyl-modified nucleotides on one strand of the donor DNA, where the PS and 2'-O methyl modifications occur within the first five nucleotides from the 5' end of the modified strand. In various embodiments, the donor DNA molecule is double-stranded and has three PS bonds and three 2'O-methyl-modified nucleotides on one strand of the donor DNA, where the PS and 2'-O methyl modifications occur within the first five nucleotides from the 5' end of the modified strand. In some examples of these embodiments, the opposite strand includes a terminal 5' phosphate. The donor DNA is introduced into the cell as a double-stranded molecule.

The present disclosure further provides a donor DNA construct designed for inserting a CAR (chimeric antigen receptor) into a host cell. Further, the present disclosure provides a host cell transduced with a CAR that lacks viral vectors. The disclosure provides for more efficient and more cost-effective process for engineering T cells to express CAR constructs. The CAR construct can include homology arms that target the construct to a T cell receptor gene, PD-1 gene, or TIM3 gene, as nonlimiting examples, for simultaneous knock-in of the CAR construct and knock out of the TCR, PD-1, or TIM3 gene.

In a further aspect, provided herein is a system for genome modification that comprises: an RNA-guide endonuclease or a nucleic acid molecule encoding an RNA-guide endonuclease; a guide RNA or a nucleic acid molecule encoding a guide RNA; and a donor DNA molecule, where the donor DNA molecule includes at least one nucleotide modification within ten or within five nucleotides of the 5' terminus. In some embodiments the donor DNA is double-stranded and includes at least one, at least two, or at least three modifications on at least one, at least two, or at least three nucleotides occurring within ten or within five nucleotides of one strand of the double stranded donor molecule. The modifications can be, for example, phosphorothioate bonds and/or 2'-O methylation of nucleotides. The donor DNA can have homology arms flanking a sequence of interest to be integrated into the genome. The sequence of interest can be an expression cassette, for example, for expression of a construct that includes one or more antibody or receptor domains. Homology arms can be between about 50 and about

5000 nucleotides in length, or between about 100 and 1000 nucleotides in length, for example between about 150 and about 800 nucleotides in length.

In some embodiments, the nuclease is selected from the group consisting of Cas9, Cas12a, Cas12b, CasX, and combinations thereof. The guide RNA can be a chimeric guide, having sequences of both crRNA and tracrRNA, or can be a crRNA, and can optionally include one or more phosphorothioate (PS) oligonucleotides. Where the guide is a crRNA, and the RNA-guided endonuclease uses a tracrRNA, the system can also include a tracrRNA. For example, Cas9 can be used with a crRNA and a tracrRNA or can be used with a chimeric guide RNA (sometimes called a single guide or "sgRNA") that combines structural features of the crRNA and tracrRNA. Cas12a on the other hand naturally uses only a crRNA and has no associated tracrRNA. In various embodiments, the RNA-guide endonuclease, guide RNA (that can be a crRNA or a chimeric guide RNA), and, when included, tracr RNA, can be complexed as a ribonucleoprotein complex that is introduced to the cell. The donor DNA can be introduced into the target cell together with the RNP, or separately, for example, in a separate electroporation or transfection.

Also provided herein is a method for site-specific integration of a donor DNA into a target DNA molecule, where the method includes introducing into a cell: an RNA-guided endonuclease or a nucleic acid molecule encoding an RNA-guided endonuclease; at least one engineered guide RNA or at least one nucleic acid molecule encoding an engineered guide RNA; and a donor DNA molecule comprising at least one nucleic acid modification; where the guide RNA comprises a target sequence designed to hybridize with a target site in the target DNA and the donor DNA is inserted into the target DNA molecule at the target site. In various embodiments the donor DNA includes at least two modified nucleotides, which can have the same or different modifications, and preferably occur within ten or within five nucleotides of the 5' terminus of one strand of the donor DNA. In some embodiments, the donor DNA is double-stranded and the one or more nucleotide modifications occur on a single strand of the donor DNA molecule. In some embodiments, the donor DNA is double-stranded and the one or more nucleotide modifications occur on a single strand of the donor DNA molecule within ten or within five nucleotides of the 5' terminus of the modified strand. In some embodiments, the donor DNA includes a backbone modification such as a phosphoramidite or phoshorothioate modification. In some embodiments, the donor DNA includes a modification of a sugar moiety of a nucleotide. In some embodiments, the donor DNA is double stranded and includes at least one, at least two, or at least three phosphorothioate modifications within five nucleotides of the 5' end of a single strand of the donor DNA molecule and further includes at least one, at least

two, or at least three 2'-O-methylated nucleotides within five nucleotides of the 5' end of a single strand of the donor DNA molecule. In various embodiments the donor DNA includes homology arms flanking a DNA sequence of interest, such as, for example, an expression cassette, where the homology arms have homology to sites in the target genome on either side of the target site of the RNA-guide endonuclease. Homology arms can be from about 50 to about 2000 nt in length, and may be, for example between 100 and 1000 nt in length, or between 150 and 650 nt in length, for example, between 150 and 350 nt in length, or 150 to 200 nt in length. In various embodiments a donor DNA molecule has two or more nucleotide modifications on the modified strand and the opposite strand includes a terminal phosphate.

The RNA-guided endonuclease can be a cas protein and can be, as nonlimiting example, a cas9, cas12a, or casX protein. In various embodiments of the method, the RNA-guided endonuclease and an RNA guide are introduced into the cell as a ribonucleoprotein complex (RNP). The RNP can in some embodiments further include a tracr RNA. An RNP can be introduced into a target cell by any feasible means, including electroporation or liposome transfer, for example. The donor DNA can be delivered to the cell simultaneously with the RNP, or separately.

Also included herein are methods of producing a donor DNA molecule, where the method includes amplifying a template DNA that includes homology arms flanking a sequence of interest using a first primer that includes at least two nucleotide modifications within the first five nucleotides of the 5' terminus of the primer, and a second primer that includes a 5' terminal phosphate. In various embodiments the first primer can include at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, or at least ten modifications, and can include more than one type of modification. For example, a primer for producing a donor DNA molecule can include at least one phosphorothioate modification and at least one 2'O-methyl modification of a nucleotide within five nucleotides of the 5' terminus of the primer.

Further included is a composition that comprises primers for amplification of a donor DNA fragment for insertion into a mammalian genome. The composition can include a first DNA oligonucleotide primer and a second DNA oligonucleotide primer, where each of the first and second oligonucleotide primers include at least one 2'O-methyl modification of a nucleotide within five nucleotides of the 5' terminus of the primer and at least one phosphorothioate modification within five nucleotides of the 5' terminus of the primer. The first and second oligonucleotide primers are a primer pair, where the first and second primers are able to amplify a sequence that includes a target site of comprises a sequence of at least 18 nucleotides that is on

one side of a target site for a cas nuclease and the second primer comprises a sequence of at least 18 nucleotides that is on the opposite strand side of a target site for a cas nuclease

Definitions

Unless specifically indicated otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art. In addition, any method or material similar or equivalent to a method or material described herein can be used in the practice of the present disclosure.

The terms "a," "an," or "the" as used herein not only include aspects with one member, but also include aspects with more than one member. For instance, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a cell" includes a plurality of such cells and reference to "the agent" includes reference to one or more agents known to those skilled in the art, and so forth.

Where the term "comprising" is used, embodiments that "consist essentially of" and "consist of" the item(s) or steps are also encompassed.

The term "primary cell" refers to a cell isolated directly from a multicellular organism. Primary cells typically have undergone very few population doublings and are therefore more representative of the main functional component of the tissue from which they are derived in comparison to continuous (tumor or artificially immortalized) cell lines. In some cases, primary cells are cells that have been isolated and then used immediately. In other cases, primary cells cannot divide indefinitely and thus cannot be cultured for long periods of time *in vitro*.

The term "genome editing" refers to a type of genetic engineering in which DNA is inserted, replaced, or removed from a target DNA, e.g., the genome of a cell, using one or more nucleases. The nucleases create specific double-strand breaks (DSBs) at desired locations in a genome and harness a cell's endogenous mechanisms to repair the induced break by homology-directed repair (HDR) (*e.g.*, homologous recombination) or by nonhomologous end joining (NHEJ). Any suitable nuclease can be introduced into a cell to induce genome editing of a target DNA sequence including, but not limited to, CRISPR-associated protein (Cas) nucleases, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), meganucleases, other endo- or exo-nucleases, variants thereof, fragments thereof, and combinations thereof. Nuclease-mediated genome editing of a target DNA sequence can be "induced" or "modulated" (*e.g.*, enhanced) using the modified single guide RNAs (sgRNAs) described herein in combination with Cas nucleases (e.g., Cas9 polypeptides or Cas9 mRNA), to improve the efficiency of precise genome editing via homology-directed repair (HDR).

The term "homology-directed repair" or "HDR" refers to a mechanism in cells to accurately and precisely repair double-strand DNA breaks using a homologous template to guide repair. The most common form of HDR is homologous recombination (HR), a type of genetic recombination in which nucleotide sequences are exchanged between two similar or identical molecules of DNA.

The term "nonhomologous end joining" or "NHEJ" refers to a pathway that repairs double-strand DNA breaks in which the break ends are directly ligated without the need for a homologous template.

The term "nucleic acid," "nucleotide," or "polynucleotide" refers to deoxyribonucleic acids (DNA), ribonucleic acids (RNA) and polymers thereof in either single-, double- or multi-stranded form. The term includes, but is not limited to, single-, double- or multi- stranded DNA or RNA, genomic DNA, cDNA, DNA-RNA hybrids, or a polymer comprising purine and/or pyrimidine bases or other natural, chemically modified, biochemically modified, non-natural, synthetic or derivatized nucleotide bases. In some embodiments, a nucleic acid can comprise a mixture of DNA, RNA and analogs thereof. The term also encompasses nucleic acids containing known analogs of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. A particular nucleic acid sequence also encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions), alleles, orthologs, single nucleotide polymorphisms (SNPs), and complementary sequences as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer et al, *Nucleic Acid Res.* 19:5081 (1991); Ohtsuka et al, *J. Biol. Chem.* 260:2605-2608 (1985); and Rossolini et al, *Mol. Cell. Probes* 8:91-98 (1994)). The term nucleic acid is used interchangeably with gene, cDNA, and mRNA encoded by a gene.

This application discloses targeted integration of nucleic acid constructs into a host genome directed by an RNA-guided endonuclease. As is known in the art, the use of the term "target site" refers to a site proximal to a PAM sequence of a CRISPR system where double-stranded cutting of the RNA-guided endonuclease (and, where applicable, nucleic acid construct integration) occurs, although due to cellular repair mechanisms, modifications that occur due to a Cas enzyme may be proximal to, but outside, the defined target site, for example, may be observed within 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 120, 150, or 200 nucleotides of the defined target site. As disclosed herein, a guide RNA that is designed for modification at a referenced target site or that "targets" a referenced sequence (target site) in the

genome, or that has, includes, or comprises a referenced target site sequence, or has, includes, or comprises a sequence corresponding to a referenced target site sequence, or similar language, is understood to include a sequence of ribonucleotides (i.e., a ribonucleotide sequence) or a sequence that comprises both deoxynucleotides plus ribonucleotides, where the sequence is equivalent in base sequence to the referenced target sequence, with uracil substituting for thymine in the ribonucleotides of the guide sequence. For example, where the target sequence is SEQ ID NO:1 (CAGGGTTCTGGATATCTGT), the sequence in a guide RNA (for example, crRNA) that includes the target site sequence is understood to comprise ribonucleotides rather than deoxyribonucleotides and to substitute uracil ribonucleotides for thymine deoxynucleotides, e.g., the guide will include the sequence: CAGGGUUCUGGAUAUCUGU.

The term "nucleotide analog" or "modified nucleotide" refers to a nucleotide that contains one or more chemical modifications (e.g., substitutions), in or on the nitrogenous base of the nucleoside (e.g., cytosine (C), thymine (T) or uracil (U), adenine (A) or guanine (G)), in or on the sugar moiety of the nucleoside (e.g., ribose, deoxyribose, modified ribose, modified deoxyribose, six-membered sugar analog, or open-chain sugar analog), or the phosphate.

The term "gene" or "nucleotide sequence encoding a polypeptide" means the segment of DNA involved in producing a polypeptide chain. The DNA segment may include regions preceding and following the coding region (leader and trailer) involved in the transcription/translation of the gene product and the regulation of the transcription/translation, as well as intervening sequences (introns) between individual coding segments (exons).

The terms "polypeptide," "peptide," and "protein" are used interchangeably to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymers. The terms encompass amino acid chains of any length, including full-length proteins, wherein the amino acid residues are linked by covalent peptide bonds.

The term "variant" refers to a form of an organism, strain, gene, polynucleotide, polypeptide, or characteristic that deviates from what occurs in nature.

The term "complementarity" refers to the ability of a nucleic acid to form hydrogen bond(s) with another nucleic acid sequence by either traditional Watson-Crick or other non-traditional types. A percent complementarity indicates the percentage of residues in a nucleic acid molecule which can form hydrogen bonds (e.g., Watson-Crick base pairing) with a second nucleic acid sequence (e.g., 5, 6, 7, 8, 9, 10 out of 10 being 50%, 60%, 70%, 80%, 90%, and 100% complementary). "Perfectly complementary" means that all the contiguous residues of a

nucleic acid sequence will hydrogen bond with the same number of contiguous residues in a second nucleic acid sequence. "Substantially complementary" as used herein refers to a degree of complementarity that is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%. 97%, 98%, 99%, or 100% over a region of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, or more nucleotides, or refers to two nucleic acids that hybridize under stringent conditions.

The term "stringent conditions" for hybridization refers to conditions under which a nucleic acid having complementarity to a target sequence predominantly hybridizes with the target sequence, and substantially does not hybridize to non-target sequences. Stringent conditions are generally sequence-dependent and vary depending on a number of factors. In general, the longer the sequence, the higher the temperature at which the sequence specifically hybridizes to its target sequence. Non-limiting examples of stringent conditions are described in detail in Tijssen (1993), Laboratory Techniques In Biochemistry And Molecular Biology - Hybridization With Nucleic Acid Probes Part 1, Second Chapter "Overview of principles of hybridization and the strategy of nucleic acid probe assay", Elsevier, N.Y.

The term "hybridization" refers to a reaction in which one or more polynucleotides react to form a complex that is stabilized via hydrogen bonding between the bases of the nucleotide residues. The hydrogen bonding may occur by Watson Crick base pairing, Hoogstein binding, or in any other sequence specific manner. The complex may comprise two strands forming a duplex structure, three or more strands forming a multi stranded complex, a single self-hybridizing strand, or any combination of these.

A "recombinant expression vector" is a nucleic acid construct, generated recombinantly or synthetically, with a series of specified nucleic acid elements that permit transcription of a particular polynucleotide sequence in a host cell. An expression vector may be part of a plasmid, viral genome, or nucleic acid fragment. Typically, an expression vector includes a polynucleotide to be transcribed, operably linked to a promoter.

"Operably linked" means two or more genetic elements, such as a polynucleotide coding sequence and a promoter, placed in relative positions that permit the proper biological functioning of the elements, such as the promoter directing transcription of the coding sequence.

The term "promoter" refers to an array of nucleic acid control sequences that direct transcription of a nucleic acid. As used herein, a promoter includes necessary nucleic acid sequences near the start site of transcription, such as, in the case of a polymerase II type promoter, a TATA element. A promoter also optionally includes distal enhancer or repressor elements, which can be located as much as several thousand base pairs from the start site of

transcription. Other elements that may be present in an expression vector include those that enhance transcription (*e.g.*, enhancers) and terminate transcription (*e.g.*, terminators), as well as those that confer certain binding affinity or antigenicity to the recombinant protein produced from the expression vector.

"Recombinant" refers to a genetically modified polynucleotide, polypeptide, cell, tissue, or organism. For example, a recombinant polynucleotide (or a copy or complement of a recombinant polynucleotide) is one that has been manipulated using well known methods. A recombinant expression cassette comprising a promoter operably linked to a second polynucleotide (e.g., a coding sequence) can include a promoter that is heterologous to the second polynucleotide as the result of human manipulation (e.g., by methods described in Sambrook et al, Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, (1989) or Current Protocols in Molecular Biology Volumes 1-3, John Wiley & Sons, Inc. (1994-1998)). A recombinant expression cassette (or expression vector) typically comprises polynucleotides in combinations that are not found in nature. For instance, human manipulated restriction sites or plasmid vector sequences can flank or separate the promoter from other sequences. A recombinant protein is one that is expressed from a recombinant polynucleotide, and recombinant cells, tissues, and organisms are those that comprise recombinant sequences (polynucleotide and/or polypeptide).

The term “genetic construct” indicates a gene or combination of genes that has been assembled and, potentially, modified using molecular cloning techniques, including but not limited to, endonuclease digestion, ligation, in vitro recombination, PCR, gene synthesis using primers and polymerases, etc. In the descriptions herein, a cell produced by the disclosed methods that includes an integrated donor fragment encoding a gene is a transgenic cell.

The term "single nucleotide polymorphism" or "SNP" refers to a change of a single nucleotide with a polynucleotide, including within an allele. This can include the replacement of one nucleotide by another, as well as deletion or insertion of a single nucleotide. Most typically, SNPs are biallelic markers although tri- and tetra-allelic markers can also exist. By way of non-limiting example, a nucleic acid molecule comprising SNP A\C may include a C or A at the polymorphic position.

The terms "culture," "culturing," "grow," "growing," "maintain," "maintaining," "expand," "expanding," etc., when referring to cell culture itself or the process of culturing, can be used interchangeably to mean that a cell (e.g., primary cell) is maintained outside its normal environment under controlled conditions, e.g., under conditions suitable for survival. Cultured cells are allowed to survive, and culturing can result in cell growth, stasis, differentiation or

division. The term does not imply that all cells in the culture survive, grow, or divide, as some may naturally die or senesce. Cells are typically cultured in media, which can be changed during the course of the culture.

The terms "subject," "patient," and "individual" are used herein interchangeably to include a human or animal. For example, the animal subject may be a mammal, a primate (e.g., a monkey), a livestock animal (e.g., a horse, a cow, a sheep, a pig, or a goat), a companion animal (e.g., a dog, a cat), a laboratory test animal (e.g., a mouse, a rat, a guinea pig, a bird), an animal of veterinary significance, or an animal of economic significance.

The term "administering" includes oral administration, topical contact, administration as a suppository, intravenous, intraperitoneal, intramuscular, intralesional, intrathecal, intranasal, or subcutaneous administration to a subject. Administration is by any route, including parenteral and transmucosal (e.g., buccal, sublingual, palatal, gingival, nasal, vaginal, rectal, or transdermal). Parenteral administration includes, e.g., intravenous, intramuscular, intra-arteriole, intradermal, subcutaneous, intraperitoneal, intraventricular, and intracranial. Other modes of delivery include, but are not limited to, the use of liposomal formulations, intravenous infusion, transdermal patches, etc.

The term "treating" refers to an approach for obtaining beneficial or desired results including but not limited to a therapeutic benefit and/or a prophylactic benefit. By therapeutic benefit is meant any therapeutically relevant improvement in or effect on one or more diseases, conditions, or symptoms under treatment. For prophylactic benefit, the compositions may be administered to a subject at risk of developing a particular disease, condition, or symptom, or to a subject reporting one or more of the physiological symptoms of a disease, even though the disease, condition, or symptom may not have yet been manifested.

The term "effective amount" or "sufficient amount" refers to the amount of an agent (e.g., Cas nuclease, modified single guide RNA, etc.) that is sufficient to effect beneficial or desired results. The therapeutically effective amount may vary depending upon one or more of: the subject and disease condition being treated, the weight and age of the subject, the severity of the disease condition, the manner of administration and the like, which can readily be determined by one of ordinary skill in the art. The specific amount may vary depending on one or more of: the particular agent chosen, the target cell type, the location of the target cell in the subject, the dosing regimen to be followed, whether it is administered in combination with other agents, timing of administration, and the physical delivery system in which it is carried.

The term "pharmaceutically acceptable carrier" refers to a substance that aids the administration of an agent (e.g., Cas nuclease, modified single guide RNA, etc.) to a cell, an

organism, or a subject. "Pharmaceutically acceptable carrier" refers to a carrier or excipient that can be included in a composition or formulation and that causes no significant adverse toxicological effect on the patient. Non-limiting examples of pharmaceutically acceptable carrier include water, NaCl, normal saline solutions, lactated Ringer's, normal sucrose, normal glucose, binders, fillers, disintegrants, lubricants, coatings, sweeteners, flavors and colors, and the like. One of skill in the art will recognize that other pharmaceutical carriers are useful in the present invention.

The term "increasing stability," with respect to components of the CRISPR system, refers to modifications that stabilize the structure of any molecular component of the CRISPR system. The term includes modifications that decrease, inhibit, diminish, or reduce the degradation of any molecular component of the CRISPR system.

The term "increasing specificity," with respect to components of the CRISPR system, refers to modifications that increase the specific activity (e.g., the on-target activity) of any molecular component of the CRISPR system. The term includes modifications that decrease, inhibit, diminish, or reduce the non-specific activity (e.g., the off-target activity) of any molecular component of the CRISPR system.

The term "decreasing toxicity," with respect to components of the CRISPR system, refers to modifications that decrease, inhibit, diminish, or reduce the toxic effect of any molecular component of the CRISPR system on a cell, organism, subject, and the like.

The term "enhanced activity," with respect to components of the CRISPR system and in the context of gene regulation, refers to an increase or improvement in the efficiency and/or the frequency of inducing, modulating, regulating, or controlling genome editing and/or gene expression.

The term "about" in relation to a reference numerical value can include a range of values plus or minus 10% from that value. For example, the amount "about 10" includes amounts from 9 to 11, including the reference numbers of 9, 10, and 11. The term "about" in relation to a reference numerical value can also include a range of values plus or minus 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1% from that value. IV.

All references cited herein are incorporated by reference in their entireties.

Non-Viral Transfection Process

Disclosed herein is a process that provides a high efficiency targeted gene integration approach. The methods can be used for genome engineering of any cell type, and can be used, for example, in applications where engineered cells are introduced into a patient. The following

patent applications are incorporated by reference herein in their entireties: US Patent Application Publication No. US 2020/0224160; WO 2020/176740; WO 2020/185867; and US Patent Application Publication No. US 2020/02399393.

In some embodiments, the methods provided herein can be used for installing a cancer treating construct, e.g. a CAR or a DAR, for example against any of CD38, CD19, CD20, CD123, BCMA and the like into T cells. The efficiency of gene transfer can be for example, at least 10%, at least 20%, at least 30% or at least 40%, or at least 50%, for example, can reach 40-80%. The efficiency of gene transfer and disruption of a targeted endogenous gene can be for example, at least 10%, at least 20%, at least 30% or at least 40%, or at least 50%. In some examples, the gene targeted for integration is the TRAC gene encoding the alpha subunit of the T cell receptor, and the efficiency of targeted integration of the construct into the genome at the TRAC locus can be assessed by expression of the construct and the absence of expression of the T cell receptor. As provided herein, the efficiency of gene transfer can be assessed using flow cytometry, where cells can be stained with antibodies or other reagents (e.g., proteins or portions thereof that bind, for example, a recombinant receptor expressed by a transfected cell. Flow cytometry to assess gene transfer (and gene knockout) efficiency can be performed for example, from five to fifteen days after transfection with a donor construct as provided herein and CRISPR/cas components. Efficiency is assessed on cell populations following transfection but in the absence of cell sorting or any enrichment of construct-expressing cells (for example using beads or cells expressing an antigen recognized by the engineered receptor of by using bead to subtract cells expressing the T cell receptor.

This approach, employing a targeted gene integration, can be used for both autologous and allogenic approaches, and importantly, does not carry a risk of secondary and unwanted cell transformation when engineered cells are introduced into a patient and is therefore safer than current conventional approaches. Additional advantages include a modified guide strand, reliable gene integration, integration of large genes, gene integration of a CAR or DAR, for example, and gene integration of a CAR or DAR with high expression.

For preparation of knock-out/knock in ("KOKI") cells for allogeneic treatments, the percentage of construct expressing cells (e.g., CAR or DAR expressing cells) can be increased by removal of cells expressing the T cell receptor for the population, as such cells do not have integration of a transgene at the TRAC site. Such subtraction methods simultaneously reduces the number of cells of the population expressing a T cell receptor that has the potential to react against patient antigens.

The examples disclose making CAR-T cells via RNA-guided endonuclease-mediated genome editing that uses phosphorothioate and 2' O-methyl modified single-stranded or double-stranded donor DNA synthesized by PCR. Preferably, the modified single-stranded (ss) or double-stranded (ds) DNA is produced by adding three PS bonds to the nucleotides within 10 nucleotides or five nucleotides of the 5'-end of one primer. Without limiting the invention to any particular mechanism, it is believed the PS modification inhibits exonuclease degradation of the modified strand of the donor DNA. Nucleotides within ten or within five nucleotides of the 5' end of the primer were also modified with 2' O-methyl to avoid the non-specific binding which is caused by phosphorothioate bonds. The phosphorothioate and 2' O-methyl modified ds donor DNA and ss donor DNA can be made through PCR, asymmetric PCR or reverse transcription. In the alternative, the final ds DNA product of a synthesis can be modified with phosphorothioate and 2' O-methyl and dsDNA can be produced with modification on one strand only.

There is further disclosed a donor DNA construct, such as a donor DNA construct having chemical modifications such as phosphorothioate and 2' O-methyl that include a CAR construct, i.e., are designed for inserting a CAR (chimeric antigen receptor) into a defined genomic site of a host cell. Further, the present disclosure provides a host cell transfected with a CAR that lacks viral vectors that can present a safety concern.

This process – using a donor DNA with modifications on one strand - can increase knock-in efficiency at least two-fold, which is comparable with viral vector methods and has advantages for site specificity of integration and very stable for CAR expression in T cells compared to conventional retrovirus or lentivirus approaches. At least double modification of one donor chain with phosphorothioate and/or 2' O-methyl can increase knock-in efficiency. This one step knock-out/knock-in method provides a faster and cheaper CAR-T production process for multiple cancer therapy. The ability to use double stranded DNA and avoid nuclease treatment of the donor construct and recovery of the single strand which is laborious and reduces yields is another benefit of the method.

In this application, we present a simple and robust method for knock in long dsDNA or ssDNA (e.g. ~3kb anti-CD38 CAR and CD19 CAR) by modified dsDNA or ssDNA donor with phosphorothioate and 2' O-methyl modification. We show that modified long dsDNA and ssDNA sequences are highly efficient HDR templates for the integration of CAR into primary T cells. Further we demonstrate that this method has advantages for site specificity of integration and very stable for CAR expression in T cells compared to conventional retrovirus or lenti-virus approaches.

Introduction of a CAR construct into a T cell using the methods provided herein for targeted integration into the genome avoids the use of viral vectors, for example, retroviral or adenoviral vectors entirely. Thus, the present disclosure provides a population of primary human T cells in which at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, or at least 80% of the cells of the population express a CAR construct that is integrated into the genome, where none of the cells of the population include a viral vector or any sequences derived therefrom. For example, a population of T cells provided herein that include a CAR construct integrated into the genome does not have any retroviral or adenoviral sequences detectable in the population. The population can be a population that was transfected with a donor DNA encoding a CAR construct using the methods and/or systems provided herein. The CAR construct is integrated into a site in the genome of the T cells targeted by a guide RNA. The CAR construct is integrated at a cas target site, i.e., a site adjacent to a PAM specific for a cas nuclease. For example, a CAR construct is integrated at a target site in the genome that is adjacent to a cas9 PAM. As nonlimiting examples, the CAR construct can be integrated into a T cell receptor gene (e.g., a TRAC gene) or a PD-1 gene, such as but not limited to, SEQ ID NO:1, SEQ ID NO:26, and SEQ ID NO:32. In various embodiments, the insertion of the CAR construct into a targeted site in the genome, such as a site in a TRAC gene or a site in a PD-1 gene results in knockout (disrupted expression) of the TRAC gene or PD-1 gene. Thus, the present disclosure provides a population of primary T cells in which at least 20%, at least 30%, or at least 40% of the cells of the population express a CAR construct that is integrated into the genome and do not express a gene that is knocked out by the targeted integration (e.g., a TRAC or PD-1 gene), where none of the cells include a viral vector or any sequences derived therefrom. For example, a population of T cells provided herein that include a CAR construct integrated into the genome, does not have any retroviral or adenoviral sequences detectable in the population. The integrated CAR construct can be at least 1.5 kb, at least 1.7 kb, at least 1.9 kb, at least 2 kb, or at least 2.1 kb in length. In nonlimiting example, the CAR construct can be a CAR construct that encodes a CAR that binds any of CD38, CD19, CD20, CD123, or BCMA. In some exemplary embodiments a CAR construct can be an anti-CD38 CAR, an anti-CD19 CAR, or an anti-BCMA CAR.

The present disclosure provides methods for expressing a CAR gene in a primary cell, the method comprising introducing into the primary cell:

(a) a single guide RNA (sgRNA) comprising a first nucleotide sequence that is complementary to the selected knockout nucleic acid and a second nucleotide sequence that interacts with a

CRISPR-associated protein (Cas) polypeptide, wherein one or more of the nucleotides of the sgRNA sequence are optionally modified nucleotides; and

(b) a Cas polypeptide, an mRNA encoding a Cas polypeptide, and/or a recombinant expression vector comprising a nucleotide sequence encoding a Cas polypeptide, or Cas polypeptide wherein the modified sgRNA guides the Cas polypeptide to the site of knockout nucleic acid, and (c) a donor target DNA comprising a 5' HA sequences, a promoter sequence, a CAR construct, and 3' HA sequence, wherein the donor target DNA is preferably double-stranded and has both or preferably one strand modified with at least one phosphothioate bond within five nucleotides of the 5'-end of the donor for reducing 5'exonuclease cleavage, and optionally includes one, two three, or four 2'-O-methyl-modified nucleotides within 5 nucleotides of the 5' end. Preferably the opposite strand to the modified strand has a 5' terminal phosphate.

The present disclosure provides a method for inducing gene expression of a CAR gene in a primary cell, the method comprising introducing into the primary cell:

(a) a tracrRNA and a crRNA comprising a first nucleotide sequence that is complementary to the selected target knockout nucleic acid, wherein one or more of the nucleotides in the tracrRNA and a crRNA are optionally modified nucleotides; and

(b) a Cas polypeptide, an mRNA encoding a Cas polypeptide, and/or a recombinant expression vector comprising a nucleotide sequence encoding a Cas polypeptide, or a Cas polypeptide; wherein the crRNA guides the Cas polypeptide to the site of knockout nucleic acid; and (c) a donor target DNA comprising a 5' HA sequences, a promoter sequence, a CAR construct, and 3'HA sequence, wherein the donor target DNA is preferably double-stranded and has both or preferably one strand modified with at least one phosphothioate bond within five nucleotides of the 5'-end of the donor for reducing 5'exonuclease cleavage, and optionally includes one, two three, or four 2'-O-methyl-modified nucleotides within 5 nucleotides of the 5' end. Preferably the opposite strand to the modified strand has a 5' terminal phosphate.

RNA-guided endonucleases that may be used in the methods disclosed herein and to generate the recombinant cells disclosed herein can be those described in the literature and patent applications as well as RNA-guided endonucleases of the same type but isolated from or whose sequence is found in species other than currently identified or characterized RNA-guided endonucleases. Variants, including variants engineered to have higher specificity for the target site (reduced off-target effects) and/or increased activity with respect to the naturally-occurring RNA-guided nuclease on which they are based. Further included are chimeric RNA-guided nucleases.

In some embodiments, the endonuclease is a Cas9 (CRISPR associated protein 9). In some embodiments, the Cas9 endonuclease is from *Streptococcus pyogenes*, although other Cas9 homologs may be used, e.g., *S. aureus* Cas9, N. meningitidis Cas9, *S. thermophilus* CRISPR1 Cas9, *S. thermophilus* CRISPR 3 Cas9, or *T denticola* Cas9. In other instance s, the CRISPR endonuclease is Cpf1, e.g., *L. bacterium* ND2006 Cpf1 or *Acidaminococcus* sp. BV3L6 Cpf1. In some embodiments, the endonuclease is Cas1,Cas1B, Cas2, Cas3, Cas4, Cas5, Cas6, Cas7, Cas8, Cas9 (also known as Csn1 and Csx12), Cas100, Csy1, Csy2, Csy3, Cse1, Cse2, Csc1, Csc2, Csa5, Csn2, Csm2, Csm3, Csm4, Csm5, Csm6, Cmr1, Cmr3, Cmr4, Cmr5, Cmr6, Csb1, Csb2, Csb3, Csx17, Csx14, Csx10, Csx16, CsaX, Csx3, Csx1, Csx15, Csf1, Csf2, Csf3, Csf4, or Cpf1 endonuclease. In some embodiments, wild-type variants may be used. In some embodiments, modified versions (e.g., a homolog thereof, a recombination of the naturally occurring molecule thereof, codon-optimized thereof, or modified versions thereof) of the preceding endonucleases may be used.

[0102] A "guide RNA" is a nucleic acid molecule that promotes the specific association (or "targeting") of an RNA-guided nuclease such as a Cas9 or a Cpf1 to a target sequence such as a genomic sequence in a cell. As used herein, a guide RNA can be a crRNA that associates with a tracr RNA in an RNP complex or may be a chimeric RNA molecule with both crRNA and tracrRNA sequences in a single molecule, as may be used with a Cas9 endonuclease. (Cpf1 uses only a crRNA.) An RNA-guided nuclease protein such as Cas9 includes a CRISPR RNA (crRNA) that includes a 5′ region that is complementary to a target sequence and a trans-activating crRNA (tracrRNA) that includes a 5′ region that is complementary to, and forms a duplex with, a 3′ region of the crRNA. A crRNA and a tracrRNA can be joined into a single chimeric guide RNA, in one non-limiting example, by means of a four nucleotide (e.g., GAAA) "tetraloop" or "linker" sequence bridging complementary regions of the crRNA (at its 3′ end) and the tracrRNA (at its 5′ end). (Mali et al. Science. 2013 Feb. 15; 339(6121): 823-826; Jiang et al. Nat Biotechnol. 2013 March; 31(3): 233-239; and Jinek et al., 2012 Science August 17; 337(6096): 816-821, all of which are incorporated by reference herein.)

Guide RNAs include a "target sequence" or "targeting domain" that is fully or partially complementary to a target domain within a target sequence, such as a DNA sequence in the genome of a cell where editing is desired. Targeting domains are referred to by various names in the literature, including without limitation "guide sequences" (Hsu et al., Nat Biotechnol. 2013 September; 31(9): 827-832), "complementarity regions", "spacers", and generically as

"crRNAs". Targeting domains (target sequences of a guide RNA) are typically 12-30 nucleotides in length, and in certain embodiments are 16-24 nucleotides in length (for instance, 16, 17, 18, 19, 20, 21, 22, 23 or 24 nucleotides in length), and are at or near the 5′ terminus of in the case of a Cas9 gRNA, and at or near the 3′ terminus in the case of a Cpf1 gRNA. Guide RNAs and their component parts are described throughout the literature, for instance in Briner et al. (Molecular Cell 56(2), 333-339, Oct. 23, 2014).

## EXAMPLES

The examples show the advantages of the disclosed process to provide high transfection efficiency without the use of viral vectors for knocking in donor DNA and knocking out a targeted endogenous gene such as a T cell receptor (TCR) or PD-1 gene.

Buffy coats from healthy volunteer donors were obtained from the San Diego blood bank. Some fresh whole blood or leukapheresis products were obtained from StemCell Techologies. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation. PBMCs were activated with CD3 antibody (BioLegend, San Diego, CA) 100 ng/mL for two days in AIM-V medium (ThermoFisher Scientific, Waltham, MA) supplemented with 5% fetal bovine serum (Sigma, St. Louis, MO) with 300 U/mL IL-2 (Proleukin) at a density of $10^6$ cells per mL. The medium was changed every two to three days, and cells were re-plated at $10^6$ per mL. This treatment selectively amplifies T cells in the culture. In some experiments, cells were cultured in CTS™ OpTmizer™ T Cell Expansion SFM (ThermoFisher) supplemented with 5% CTS™ Immune Cell SR (Thermofisher scientific) with 300U/mL IL-2 (Proleukin) at a density of $10^6$ cells per mL. In some experiments T cells were isolated from PBMCs using magnetic negative selection using EasySep™ Human T Cell Isolation Kit or CD3 positive selective kit (Stemcell Technology Inc.) according to the manufacturer's instructions.

For use in cytotoxicity assays, RPMI-8226 multiple myeloma cell line) cells, which express CD38, were transduced to express green fluorescent protein (GFP). K562 (human immortalized myelogenous leukemia) cells, which do not express CD38, were transduced to express R-phycoerythrin (RPE). Both cell lines were cultured in RPMI1640 medium (ATCC) supplemented with 10% fetal bovine serum (Sigma). CAR plasmids were generated with an In-

Fusion® HD Cloning Kit (Takara Bio USA, Inc, Mountain View, CA). Backbone plasmid pAAV-MCS was purchased from Cell Biolabs (San Diego, CA).

In some experiments, retrovirus-transduced T cells were compared with cas-mediated knock-in cells. Transduction of T cells with the retroviral construct was performed essentially as described in Ma et al., 2004 *The Prostate* 61:12-25; and Ma et al., *The Prostate* 74(3):286-296, 2014 (the disclosures of which are incorporated by reference herein in their entireties). In brief, the anti-CD38 CAR MFG retroviral vector plasmid DNA was transfected into Phoenix-Eco cell line (ATCC) using FuGene reagent (Promega, Madison, WI) to produce Ecotropic retrovirus, then harvested transient viral supernatant (Ecotropic virus) was used to transduce PG13 packaging cells with Gal-V envelope to produce retrovirus to infect human cells. Viral supernatant from PG13 cells was then used to transduce activated T cells (or PBMCs) two to three days after CD3 or CD3/CD28 activation. Activated human T cells were prepared by activating normal healthy donor peripheral blood mononuclear cells (PBMC) with 100 ng/ml mouse anti-human CD3 antibody OKT3 (Orth Biotech, Rartian, NJ) or anti-CD3,anti-CD28 TransAct (Miltenly Biotech, German) as manufacturer's manual and 300-1000 U/ml IL-2 in AIM-V growth medium (GIBCO-Thermo Fisher scientific, Waltham, MA) supplemented with 5% FBS for two days. $5\times10^6$ activated human T cells were transduced in a 10 μg/ml retronectin (Takara Bio USA) pre-coated 6-well plate with 3 ml viral supernatant and were centrifuged at 1000 g for 1 hour at 32 °C. After transduction, the transduced T cells were expanded in AIM-V growth medium supplemented with 5% FBS and 300-1000 U/ml IL-2.

**Table 1. Primers used for generating double-stranded donor DNAs:**
an asterisk indicates a phosphorothioate (PS) linkage; Am, 2'-O-methylated deoxyadenosine; Cm, 2'-O-methylated deoxycytosine; Gm, 2'-O-methylated deoxyguanosine

| Primer | Sequence | SEQ ID NO |
|---|---|---|
| Forward primer for generating anti-CD38 donor DNA having 660 and 650 nt HAs from TRAC gene exon 1 | 5′-T*Gm*Gm*AmGCTAGGGCACCATATT-3′ | 8 |
| Reverse primer for generating anti-CD38 donor DNA having 660 and 650 nt HAs from TRAC gene exon 1 | p-5′-CAACTTGGAGAAGGGGCTT-3′ | 9 |
| Forward primer for generating anti-CD38 donor DNA having 375 and 321 nt HAs from TRAC gene exon 1 | 5′-C*Cm*Am*TGmCCTGCCTTTACTCTG-3′ | 14 |
| Reverse primer for generating anti-CD38 donor DNA having 375 and 321 nt HAs from TRAC gene exon 1 | p-5′-TCCTGAAGCAAGGAAACAGC-3′ | 15 |

| | | |
|---|---|---|
| Forward primer for generating anti-CD38 donor DNA having 171 and 161 nt HAs from TRAC gene exon 1 | 5′-A*TCm*Am*CmGAGCAGCTGGTTTCT-3′ | 18 |
| Reverse primer for generating anti-CD38 donor DNA having 171 and 161 nt HAs from TRAC gene exon 1 | p-5′-GACCTCATGTCTAGCACAGTTTTG-3′ | 19 |
| Forward primer for generating anti-CD38 donor DNA having 171 and 161 nt HAs from TRAC gene exon 1 - unmodified | 5′-ATCACGAGCAGCTGGTTTCT-3′ | 20 |
| Reverse primer for generating anti-CD38 donor DNA having 171 and 161 nt HAs from TRAC gene exon 1 - unmodified | 5′-GACCTCATGTCTAGCACAGTTTTG-3′ | 21 |
| Forward primer for generating anti-CD38 donor DNA having 183 and 140 nt HAs from TRAC gene exon 3 | 5′-T*Am*T*GmCmACAGAAGCTGCAAGG-3′ | 28 |
| Reverse primer for generating anti-CD38 donor DNA having 183 and 140 nt HAs from TRAC gene exon 3 | p-5′-TTAGGATGCACCCAGAGACC-3′ | 29 |
| Forward primer for generating anti-CD38 donor DNA having 326 and 380 nt HAs from PD-1 locus | p-5′-CTCCCCATCTCCTCTGTCTC-3′ | 34 |
| Reverse primer for generating anti-CD38 donor DNA having 326 and 380 nt HAs from PD-1 locus | 5′-Cm*Cm*T*GmACCCGTCATTCTACAG-3′ | 35 |
| Forward primer for generating anti-CD38 donor DNA having 660 and 650 nt HAs from TRAC gene exon 1 - unmodified | 5′-TGGAGCTAGGGCACCATATT-3′ | 36 |
| Forward primer for generating anti-CD38 donor DNA having 171 and 161 nt HAs from TRAC gene exon 1 | 5′-ATCACGAGCAGCTGGTTTCT-3′ | 37 |

**Example 1. Simultaneous knockout of the T-cell receptor gene and knock-in of anti-CD38 CAR in human T cells.**

In this example, the T cell receptor alpha constant (TRAC) gene was targeted with an anti-CD38 CAR construct as the donor DNA. The pAAV-TRAC-anti-CD38 construct was designed with approximately 1.3kb of genomic DNA sequence of the T cell receptor alpha constant (TRAC) that flanks the target sequence (CAGGGTTCTGGATATCTGT (SEQ ID NO:1)) in the genome. The target sequence was identified as a site upstream of a Cas9 PAM in exon 1 of the TRAC gene for Cas9-mediated gene disruption and insertion of the donor construct. The anti-CD38 CAR gene construct (SEQ ID NO:2) comprised a sequence encoding a single chain variable fragment (scFv) specific for human CD38, followed by CD8 and CD28 hinge-CD28 transmembrane-CD28 intracellular regions and a CD3 zeta intracellular domain. An exogenous JeT promoter (US Patent No. 6,555, 674; SEQ ID NO:3) was used to initiate transcription of the anti-CD38 CAR.

To construct the pAAV-anti-CD38A2 donor plasmid which was used as a PCR template for generating donor fragments for genome editing, the anti-CD38A2 CAR construct with 650-660 bp homology arms (SEQ ID NO:4) was synthesized by Integrated DNA Technologies (IDT, Coralville, IA). An in-fusion cloning reaction was performed at room temperature, containing pAAV-MCS vector double digested with MluI and BstEII (50 ng), the anti-CD38A2 CAR fragment with flanking homology arms (SEQ ID NO:4) (50ng), 1ul 5X In-Fusion HD Enzyme Premix (Takara Bio), and nuclease-free water. The reaction was briefly vortexed and centrifuged prior to incubation at 50 °C for 30 min. Stellar™ Competent Cells (Takara Bio USA) were then transformed with the in-fusion product and plated on ampicillin-treated agar plates. Multiple colonies were chosen for Sanger sequencing (Genewiz, South Plainfield, NJ) to identify the correct clones using the primers CTTAGGCTGGGCATTAGCAG (SEQ ID NO:5), CATGGAATGGTCATGGGTCT (SEQ ID NO:6), and GGCTACGTATTCGGTTCAGG (SEQ ID NO:7). Correct clones were cultured and the DNA plasmids from these clones were purified.

For RNA guide-directed targeting of the TCR alpha (TRAC) gene, the tracr RNA (ALT-R® CRISPR-Cas9 tracrRNA) and crispr RNA (ALT-R® CRISPR-Cas9 crRNA) were purchased from IDT (Coralville, IA), where the crRNA was designed to include the target sequence CAGGGTTCTGGATATCTGT (SEQ ID NO:1) that occurs directly upstream of a cas9 PAM sequence (NGG) in first exon of the TRAC gene.

To make donor fragment DNA, PrimeSTAR Max Premix (Takara Bio USA) was used for PCR reactions. The AAV donor plasmid pAAV-anti-CD38A2 described above was used as a template. To generate a donor fragment with homology arms of 660 nt and 650 nt, the forward primer had the sequence: TGGAGCTAGGGCACCATATT (SEQ ID NO:36), and the reverse primer had the sequence: CAACTTGGAGAAGGGGCTTA (SEQ ID NO:9). In various experiments to test the effectiveness of different homology arm lengths, primers having sequences hybridizing to specific positions within the homology arms of the pAAV-anti-CD38A2 construct were used to produce donor fragments with homology arms of desired lengths by PCR. Phosphorothioate bonds (**FIG. 2A**) were introduced into the terminal three nucleotides at the 5'-end of the forward primer (SEQ ID NO:36) to inhibit exonuclease degradation (that is, between the first and second, second and third, and third and fourth nucleotides from the 5' terminus). The nucleotides at the second, third and fourth positions from

the 5'-end of the forward oligonucleotide primer were also 2'-O-methyl modified to avoid non-specific binding, potentially caused by the phosphorothioate (PS) backbone of the terminal 3 nucleotides (SEQ ID NO:8, **FIG. 2B**). The reverse primer (SEQ ID NO:9) was modified by 5'-end phosphorylation so that the strand could be digested by a strandase provided by the Guide-it™ Long ssDNA Production System kit (Takara Bio USA). To produce the donor DNA fragment, the thermocycler settings were: one cycle of 98 °C for 30s, 35 cycles of 98 °C for 10s, 66 °C for 5s, 72 °C for 30s and one cycle of 72 °C for 10 min. Digestion with the strandase was done according to the manufacturer's instructions (Takara Bio USA), and ssDNA was purified using the NucleoSpin Gel and PCR Clean-Up kits (Takara Bio USA). The concentration of ssDNA was determined by NanoDrop (Denovix, Wilmington, DE). As controls, donor fragments were produced with unmodified primers, such that the resulting donor fragment had no chemical modifications (no PS or 2'-O-methyl groups) or had the PS modification only (no 2'-O-methyl groups).

To generate TCR knockouts / anti-CD38 CAR knock-ins, T cells were activated by adding CD3 to the cultures. About 48 to 72 hours after initiating T-cell activation with CD3, the PBMC cultures including activated T cells were electroporated with SpCas9 protein plus crRNA (containing guide sequence SEQ ID NO:1) and tracrRNA using a Neon® Transfection System (ThermoFisher Scientific) and 10-µl tip or 100-µl tips. Briefly, Alt-R CRISPR-Cas9 crRNA and Alt-R tracrRNA (IDT) were first mixed and heated at 95 °C for 5 min. The mixture was then removed from heat and allow to cool to room temperature (15–25 °C) on the bench top for about 20 min. For each transfection, 10 µg SpCas9 protein (IDT) was mixed with 200 pmol crRNA:tracrRNA duplex to form RNPs. 1 x $10^6$ cells were mixed with the RNP and electroporated with 1700 V, 20 ms pulse width, 1 pulse. One to two hours later, 10ug single-stranded donor DNA was electroporated into the cells with 1600 V, 20ms pulse width, 1 pulse. In some cases, T cells were mixed with the RNP and donor DNA and RNP and donor were electroporated at the same time. Following electroporation cells were diluted into culture medium and incubated at 37 °C, 5% $CO_2$.

As controls for the cas-mediated knock-in methods, CAR-expressing PBMCs were generated by transduction of T cells with a retrovirus that included the same anti-CD38A2

expression cassette (SEQ ID NO:2) in the retroviral vector that was used to make the donor fragment employed in CRISPR targeting.

To determine knock-in efficiency by detecting CAR expression of transformed cells by FACS, transfected or transduced PBMCs were washed with DPBS/5% human serum albumin, then stained with anti-CD3-BV421 antibody SK7 (BioLegend) and PE conjugated anti-CD38-Fc protein (Chimerigen Laboratories, Allston, MA) for 30-60 min at 4 °C. CD3 and anti-CD38 CAR expression were analyzed using iQue Screener Plus (Intellicyte Co.) Negative controls were cells that had been transfected with an RNP that included cas9 protein complexed with a hybridized tracrRNA and crRNA targeting the first exon of the TRAC gene, but were not transfected with the anti-CD38 CAR donor DNA. PBMCs that had been transfected with the RNP that included the guide targeting the TRAC locus were subsequently transduced with a retrovirus that included the anti-CD38 CAR construct as described above and analyzed for expression of the anti-CD38 CAR as well. **FIG. 3A** shows that 8 days after transfection no expression of an anti-CD38 construct was detected in cells transformed with the RNP (for knocking out the TRAC gene) in the absence of a donor fragment for expression of the anti-CD38 CAR (leftmost panel). On the other hand, PBMCs that had a TRAC knockout and were subsequently transduced with a retrovirus that included a construct for expressing the anti-CD38 CAR did show expression of the anti-CD38 CAR in about 70% of the cells 8 days after transfection (rightmost panel of **FIG. 3A**). For cultures transformed with anti-CD38 CAR ss donor DNA in addition to an RNP targeting exon 1 of the TRAC gene, approximately 12% of the population that received the ss donor DNA having no chemical modifications and approximately 13% of cultures that were transduced with ss donor DNA having only PS backbone modifications on nucleotides near the 5'-end of the donor DNA (introduced by using a PCR primer having PS bonds between nucleotides 1 and 2, 2 and 3, and 3 and 4, numbering from the 5' end) demonstrated expression of the anti-CD38 construct. Adding methyl groups to the 2' oxygen of the three nucleotides at the second, third, and fourth nucleotides from the 5'-end of the donor fragment strand that also included PS modifications (by using the primer of SEQ ID NO:8 that included these modifications to generate the donor DNA by PCR) resulted in significantly higher expression of the anti-CD38 CAR in the transfected population, where expression of the anti-CD38 CAR was seen in approximately 20% of the cells that received the 'double modified'

(2'-O-methyl and PS) single-stranded donor fragment at 8 days. Notably, chemical modifications of the donor DNA did not affect viability of the transfected cultures.

Increased expression of the anti-CD38 CAR was observed over time in cultures that had been transfected with anti-CD38 CAR donor fragments plus RNPs targeting the TRAC gene. At 10 days post-transfection, flow cytometry of PBMC cultures transfected with unmodified single-stranded donor or single-stranded donor modified to include PS linkages on the 5'-most three nucleotides demonstrated that among all cultures that were transfected with the TRAC-targeting RNP, at least 80% of the cells did not express the TCR. Moreover, in cultures transfected with the anti-CD38 CAR donor in addition to the TRAC-targeting RNP, at least 42% of the cells that did not express the TCR expressed the anti-CD38 construct (**FIG. 3B**, panels 2-4). For cultures transfected with an anti-CD38 CAR donor fragment with both PS and 2'-O-methyl groups on 5'-proximal nucleotides, 57% of the cells were expressing the anti-CD38 construct by day ten. At the same time, the expression of the anti-CD38 CAR in cultures that had been transduced with the retrovirus dropped to about half of what had been seen at 8 days, to approximately 34% of the cells on day ten post-transfection or transduction. Analysis of the culture transfected with doubly modified ss donor and the retrovirus-transduced culture at day 20 (**FIG. 3C**) showed that expression of the anti-CD38 construct in the cultures had stabilized, with the cas9-modified culture that had been transfected with a ss donor having both PS and 2'-O-methyl modifications at the 5' end demonstrating 54% of the TCR-negative cells were expressing the construct and the culture that had been transduced with a retrovirus demonstrating 31% of the TCR-negative cells were expressing the construct.

To confirm the occurrence of homology directed repair (HDR) at the targeted locus in Exon 1 of the TRAC gene, PCR was performed on DNA isolated from cultures to verify that the donor fragment had inserted into the TRAC site targeted by the guide RNA. Genomic DNA was amplified from non-transfected activated T cells (ATCs), TRAC knockout cells that were transformed with the RNP that included the TRAC Exon 1 guide RNA, and from T cells transfected with the RNP plus phosphorothioate and 2' O-Methyl modified donor DNA to detect targeted insertion of an anti-CD38 CAR transgene into the TRAC locus. To confirm the position of the donor DNA in the genome, oligonucleotide primers were targeted to sequences outside of the TRAC homology arms but adjacent to the homology arm sequences in the genome. A total of

1 x $10^5$ cells were resuspended in 30 µL of Quick Extraction solution (Epicenter) to extract the genomic DNA. The cell lysate was incubated at 65 °C for 5 min and then at 95 °C for 2 min and stored at -20 °C. The concentration of genomic DNA was determined by NanoDrop (Denovix). Genomic regions containing the TRAC target sites were PCR-amplified using the following primer sets: 5' PCR forward primer on TRAC: CTGCTTTCTGAGGGTGAAG (SEQ ID NO:10), 5' PCR Reverse primer on CAR: CTTTCGACCAACTGGACCTG (SEQ ID NO:11); 3' Forward primer on CAR: CGTTCTGGGTACTCGTGGTT (SEQ ID NO:12), 3' Reverse primer on TRAC: GAGAGCCCTTCCCTGACTTT (SEQ ID NO:13) (see **FIG. 1B**). Both primer sets were designed to avoid amplifying the HDR templates by annealing outside of the homology arms.

The concentration of genomic DNA was determined by NanoDrop (Denovix). Both primer sets were designed such that one primer of the pair annealed to a site in the genome outside of the homology arm, and the other primer of the pair annealed to a site within the coding region of the construct (*i.e.*, not in a homology arm). The PCR contained 400 ng of genomic DNA and Q5 high fidelity 2X mix (New England Biolabs). The thermocycler setting consisted of one cycle of 98 °C for 2 min, 35 cycles of 98 °C for 10s, 65 °C for 15s, 72 °C for 45s and one cycle of 72 °C for 10 min. The PCR products were purified on 1% agarose gel containing SYBR Safe (Life Technologies). The PCR products were then eluted from the agarose gel and isolated using NucleoSpin® Gel and PCR Clean-up kit (MACHEREY-NAGEL GmbH & Co. KG). The PCR products were submitted for Sanger sequencing (Genewiz). **FIG. 4** provides a photograph of the gel separating PCR products. The positive bands corresponding to the anti-CD38 construct adjacent to genomic sequences adjacent to the homology arms in the genome at the 5' and 3' ends of the construct were only seen in cells transfected with donor DNA (lanes 3 and 6) and not in non-transfected ATCs (lanes 1 and 4) or TRAC knock out cells (lanes 2 and 5). Sequencing of these PCR products confirmed that they included the anti-CD38 CAR sequence. SEQ ID NO:

To test for function of transfected cells, three weeks after electroporation, the activated T cells that had been transfected with the anti-CD38 CAR targeted to the TRAC locus were starved with IL-2 overnight and tested in specific killing assays (**FIG. 5**). The activated T cells were co-cultured with a target cell mixture of CD38 positive RPMI-8226/GFP cells and CD38 negative K562/RPE cells. The incubation effector-to-target cell ratio ranged from 10:1 to 0.08:1. After

overnight incubation, the cells were analyzed by flow cytometry to measure the GFP-positive and RPE-positive cell populations to determine the specific target cell killing by anti-CD38A2 CART cells. **FIG. 5** shows that while non-transfected ATC cells showed some toxicity at the highest effector to target ratios, TRAC knockout cells showed virtually no killing regardless of effector-to-target cell ratio. The anti-CD38A2 CART cells however exhibited potent killing activity of CD38 positive cells- RPMI8226 but not CD38 negative cells – K562 (**FIG. 5**). T cells that had integrated the chemically modified donor that included the anti-CD38 CAR cassette demonstrated cytotoxicity toward target cells similarly to that of cells transduced with retrovirus that included the anti-CD38 CAR construct.

The transfected activated T cells (ATCs) were also tested for cytokine secretion (**FIGS. 6A-6C**). T cells were starved in IL-2 free medium overnight. Anti-CD38 CAR-T cells or ATC controls were then co-cultured with CD38 negative K562 or CD38 positive RPMI8226 cells. The incubation effector to target cell ratio was 2:1. After overnight incubation, the cells were centrifuged to collect the supernatants for quantitating cytokine IL-2, IFN-gamma and TNF alpha (Affymetrix eBioscience) according to the manufacturer's instructions. The gene-edited TCR knockout anti-CD38A2 CART cells also released similar amount of IFN-γ and other pro-inflammatory cytokines when co-cultured with CD38 positive tumor cells (RPMI8226) but not CD38 negative cells (K562).

In summary, *in vitro* cellular functional studies did not reveal any notable differences between TRAC-site-specific integrated anti-CD38A2 CAR achieved by this novel and efficient process and virus-mediated randomly integrated anti-CD38A2 CAR, in terms of both specific killing assay (**FIG. 5**) and cytokine secretion assay (**FIGS. 6A-6C**).

**Example 2. Reducing length of homology arms of donor DNAs**

When synthesizing donor DNA by PCR, the nuclease reaction and resulting purification of the single stranded donor fragment is time consuming, typically results in losses in the yield of donor fragment for transfections, and can be difficult to control the length of homology arms (homology can be over-chewed). In further experiments testing the efficiency of directed gene knockouts and antibody construct knock-ins, double-stranded donor DNAs were tested to eliminate the nuclease digestion of the PCR-synthesized donor.

For knock-in of the anti-CD38 CAR construct, donor fragments having homology arms (HAs) of different lengths were produced. The pAAV-TRAC-anti-CD38 construct described in Example 1 that included the anti-CD38 cassette plus TRAC exon 1 homology arms of 660 and 650 nts (SEQ ID NO:4) was used as the template. A first set of primers, SEQ ID NO:8 and SEQ ID NO:9, was used to generate a donor fragment having homology arms of 660 nt and 650 nt from this template. A second set of primers, SEQ ID NO:14 and SEQ ID NO:15, was used to generate a donor fragment having homology arms of approximately 350 nt (375 and 321 nucleotides), where the primer of SEQ ID NO:14 had PS linkages between the between first and second, second and third, and third and fourth nucleotides from the 5' terminus and had 2'-O-methyl-modified nucleotides at positions 2, 3, and 5. A third set of primers, SEQ ID NO:18 and SEQ ID NO:19, was used to generate a donor fragment having homology arms of approximately 165 nt (171 and 161 nts), where the primer of SEQ ID NO:18 had PS linkages between the between first and second, third and fourth, and fourth and fifth nucleosides from the 5' terminus and had 2'-O-methyl-modified nucleotides at positions 3, 4, and 5. In each case, the forward primer (SEQ ID Nos: 8, 14, and 18) was designed to have three PS linkages within the 5'terminal-most five nucleotides (for example, between any of the first and second, second and third, third and fourth, and fourth and fifth nucleosides from the 5' terminus of the primer, and three 2'-O-methyl groups occurring in any of the five 5'terminal-most nucleotides. In each case, the reverse primer (SEQ ID Nos: 9, 15, and 17) had a 5' terminal phosphate (see **Table 1**).

Each of the primer sets was used to generate a donor DNA molecule having multiple PS and 2'-O methyl modifications proximal to the 5'end of one strand of the donor and a 5' phosphate at the 5' terminus of the opposite strand of the donor. RNPs were assembled to include tracr and crRNAs as described in Example 1, where the crRNA included the target sequence of SEQ ID NO:1, a sequence found in exon 1 of the TRAC gene. The donor molecules, having homology arms of approximately 665, 350, and 165 base pairs in length, were independently transfected into activated T cells as described in Example 1 except that donor fragments and RNPs were transfected in the same electroporation under conditions for electroporating the RNP (using a Neon® Transfection System (ThermoFisher Scientific) 1700 V, 20 ms pulse width, 1 pulse). As a control, activated T cells were transfected with the RNP in the absence of a donor fragment, which should result in knockout of the targeted TRAC locus, but

without donor DNA insertion. To test for expression of the T cell receptor and the anti-CD38 CAR construct, flow cytometry was performed as provided in Example 1. **FIG. 7** shows that, as expected, the T cell culture transfected with the RNP only had low levels of expression of the T cell receptor and also demonstrates no expression of the anti-CD38 CAR. T cells transfected with the RNP plus donor DNAs having homology arms of different sizes however show low levels of T cell receptor expression and good expression of anti-CD38 CAR in the cultures, demonstrating that transfection of a double-stranded DNA in highly effective for targeted knock-ins. Further, the shortest HA lengths tested, 161/171 nt, worked at least as well as longer lengths, with the percentages of knockout cells expressing the introduced construct being approximately 24% for approximately 665 nt arms, approximately 30% for approximately 350 nt arms, and approximately 38% for approximately 165 nt arms. The short homology arms are thus found to be very effective in targeted knock in genome modification using double-stranded DNA donors, which has the benefit of allowing for smaller constructs and/or allowing for more capacity in a construct to allow inclusion of additional or lengthier sequences to be included in the donor DNA.

**Example 3. Modified versus non-modified double-stranded donor DNA**

Donor DNAs that included anti-CD38 CAR and having the approximately 165 nt TRAC exon 1 homology arms as set forth in Example 2, above, were synthesized using primers with and without nucleotide modifications to test their relative effectiveness in promoting HDR. In the first case, primer SEQ ID NO:18 had three PS linkages, occurring between first and second, third and fourth, and fourth and fifth nucleosides and three 2'-O-methyl-modified nucleotides within the first five nucleotides of the 5' terminus of the primer (at nucleotide positions 2, 3, and 5) and primer SEQ ID NO:19 had a 5' terminal phosphate (**Table 1**). These primers were used to generate a donor DNA with the corresponding nucleotide modifications (i.e., three PS linkages and three 2'-O-methyl groups within five nucleotides of the 5' terminus of the first strand of the donor DNA product, and a phosphate on the 5' end of the second strand of the donor DNA product). In the second case, primer SEQ ID NO:37 was identical to primer SEQ ID NO:18 except that primer SEQ ID NO:37 lacked chemical modifications see **Table 1**). The SEQ ID NO:37 primer and the SEQ ID NO:19 primer lacking a 5' terminal phosphate were used to generate a donor DNA with no nucleotide modifications having the anti-CD38 CAR cassette.

These donor DNAs were transfected as double-stranded DNA molecules (with no denaturation or nuclease digestion of either strand) along with RNPs that included a tracr RNA and a crRNA that included the target sequence of SEQ ID NO:1 (within exon 1 of the TRAC gene) into activated T cells. In the electroporations with double stranded DNA as donor, 5ug dsDNA was used to transfect one million activated T cells.

As in Example 2, control activated T cells were transfected with the RNP in the absence of a donor fragment, which should result in knockout of the targeted TRAC locus without construct insertion. To test for expression of the T cell receptor and the anti-CD3 CAR construct, flow cytometry was performed essentially as provided in Example 1. The results, shown in **FIG. 8**, show that transfection with the RNP and a modified double stranded donor resulted in at least twice the expression of the anti-CD38 construct across the culture as compared with transfection with the RNP and the unmodified double-stranded donor, resulting in over 50% of the cells of the culture expressing the anti-CD38 CAR transgene and not expressing the TCR (CD3 negative).

Sequencing of PCR products produced using primers to diagnose the insertion locus (see **FIG. 2B**) provided sequences demonstrating the anti-CD38 CAR donor fragment integrated into exon 1 of the TRAC gene. The PCR product sequences (SEQ ID NO:39 and SEQ ID NO:40) included sequences adjacent to the homology arm in the genome, the homology arm present in the donor fragment, and portions of the anti-CD38 CAR in a single PCR product, demonstrating the expected insertion.

**Example 4. HDR-mediated knock-in of anti-CD19 and anti-BCMA CAR constructs with simultaneous TCR knockout**

Additional donor DNAs that included anti-CD19 CAR and anti-BCMA CAR expression constructs were also tested for insertion into the TRAC locus.

An anti-CD19 CAR construct that included an anti-CD19 CAR cassette (SEQ ID NO:22) that included the Jet promoter (SEQ ID NO:3), and intron, an anti-CD19 CAR construct, and an SV40 polyA sequence was made essentially as described for the anti-CD38 CAR pAAV construct described in Example 1 and was cloned in a vector flanked by the TRAC gene exon 1 homology arms (HAs) of SEQ ID NO:20 and SEQ ID NO:21. The anti-CD19 CAR with HAs pAAV construct was used as a template in PCR reactions as provided in Example 1 using the

primers provided as SEQ ID NO:18 and SEQ ID NO:19 that result in the production of modified donor DNA having HAs of approximately 170 and 160 nucleotides (see **Table 1**). The forward primer (SEQ ID NO:18) had three PS bonds between the first and second, third and fourth, and fourth and fifth nucleosides and three 2'-O-methyl modifications at nucleotides 3, 4, and 5 when numbering from the 5'-terminus of the primer. The reverse primer (SEQ ID NO:19) had a 5'-terminal phosphate. The resulting double-stranded donor DNA was therefore synthesized to have the corresponding modifications, a first strand with three PS and three 2'-O-methyl modifications within five nucleotides of the 5'-terminus, and a second strand with a 5'-terminal phosphate.

The double-stranded chemically modified donor fragment having the sequence of SEQ ID NO:38 with the nucleotide modifications of primers SEQ ID NO:18 and SEQ ID NO:19 described above incorporated was used to transfect cells along with an RNP that was produced according to the methods provided in Example 1, where the crRNA of the RNP included the target sequence of SEQ ID NO:1, targeting exon 1 of the TRAC gene. As a control, activated T cells were transfected with the RNP in the absence of a donor fragment, which should result in knockout of the targeted TRAC locus without construct insertion. Flow cytometry was performed on cells 10 days after transfection essentially as described in Example 1 to evaluate the efficiency of introducing a different construct into the TRAC locus, except that anti-CD19 CAR expression was detected by CD19-Fc (Speed Biosystem) followed by APC anti-human IgG Fcγ (Jackson Immunoresearch). The results are shown in **FIG. 9**, where it can be seen that the anti-CD19 CAR was expressed in the absence of T cell receptor expression in approximately 42% of the cells in the culture.

An anti-BCMA CAR construct was made through replacing the CD38 CAR with BCMA CAR based on the anti-CD38 CAR pAAV construct described in Example 1. The BCMA CAR fragment was synthesized by IDT. The sequence of the insert is provided as SEQ ID NO:23. The anti-BCMA CAR construct was used as a template in PCR reactions as set forth in Example 1 using the primers provided as SEQ ID NO:18 and SEQ ID NO:19 that result in the production of donor DNA having HAs of approximately 160-170 nucleotides (see **Table 1**). The forward primer (SEQ ID NO:18) had three PS and three 2'-O-methyl modifications within five nucleotides of the 5'-terminus of the primer. The reverse primer (SEQ ID NO:19) had a 5'-

terminal phosphate. The resulting double-stranded donor DNA was therefore synthesized to have a first strand with three PS and three 2'-O-methyl modifications within five nucleotides of the 5'-terminus, and a second strand with a 5'-terminal phosphate.

The double-stranded donor fragment having the sequence of SEQ ID NO:37, having modified nucleotides by incorporation of chemically modified primers as provided above, was used to transfect cells along with an RNP that was produced according to the methods provided in Example 1, where the crRNA of the RNP included the target sequence of SEQ ID NO:1, targeting exon 1 of the TRAC gene. As a control, activated T cells were transfected with the RNP in the absence of a donor fragment, which should result in knockout of the targeted TRAC locus without construct insertion. Flow cytometry was performed on cells 22 days after transfection essentially as described in Example 1 to evaluate the efficiency of introducing a different construct into the TRAC locus, except that anti-BCMA CAR expression was detected by PE or APC conjugated BCMA-Fc (R&D). The results are shown in **FIG. 10**, where it can be seen that the anti-BCMA CAR was expressed in the absence of T cell receptor expression in approximately 66% of the cells in the culture. In other experiments, transfection of cells with an RNP targeting exon 1 of the TRAC locus with a different anti-BCMA CAR construct resulted in an efficiency of 23% of the cell population expressing the anti-BCMA CAR in the absence of T cell receptor expression as assessed by flow cytometry 14 days after transfection.

**Example 5. HDR mediated knock-in targeting TRAC Exon 3**

To test the efficiency of inserting donor DNAs into loci other than exon 1 of the TRAC gene using the methods for donor insertion provided herein, an anti-CD38 CAR construct was made for producing a donor DNA having HAs from Exon 3 of the TRAC gene. In this case, the construct was produced essentially as described in Example 1 for the TRAC exon 1 targeting construct, except that the HAs (5' HA SEQ ID NO:24 (183 nt) and 3' HA SEQ ID NO:25 (140 nt)) were sequences surrounding the exon3 target site (SEQ ID NO:26). The sequence of the insert of the pAAV construct that was then produced as a donor DNA with TRAC gene exon 3 homology arms is provided as SEQ ID NO:27. To generate the donor fragment, the forward primer (SEQ ID NO:28) included PS linkages between first and second, second and third, and third and fourth nucleosides and 2'-O-methyl modifications on the second, fourth, and fifth positions from the 5'-terminus, and the reverse primer (SEQ ID NO:29) had a 5'-terminal

phosphate. The resulting double-stranded donor DNA that incorporated the primers had a first strand with corresponding PS and 2'-O-methyl modifications on the 5'-terminal most nucleotides, and a second strand having a 5'-terminal phosphate.

The double-stranded donor fragment having modified nucleotides by incorporation of the primers above and having the sequence of SEQ ID NO:27 was used to transfect cells along with an RNP that was produced according to the methods provided in Example 1, where the crRNA included the target sequence of SEQ ID NO:26, targeting exon 3 of the TRAC gene. As a control, activated T cells were transfected with the RNP in the absence of a donor fragment, which should result in knockout of the targeted TRAC locus without construct insertion. A further control was non-transfected activated T cells (ATCs). Flow cytometry was performed essentially as described in Example 1. The results are shown in **FIG. 11**, where it can be seen that transfection with the RNP or the RNP plus donor DNA result in greater than 80% of cells across the culture losing TCR expression. Further, anti-CD38 CAR was expressed in the absence of T cell receptor expression in approximately 42% of the cells in the culture that was transfected with the targeting RNP plus the donor DNA with HAs derived from the TRAC gene exon 3.

Sequencing of PCR products produced using primers to diagnose the insertion locus (see **FIG. 2B**) provided sequences demonstrating the anti-CD38 CAR donor fragment integrated into exon 3 of the TRAC gene. The PCR product sequences (SEQ ID NO:41 and SEQ ID NO:42) included sequences adjacent to the homology arm in the genome, the homology arm present in the donor fragment, and portions of the anti-CD38 CAR in a single PCR product, demonstrating the expected insertion.

**FIG. 11** compares targeting of the anti-CD19 CAR to exon 3 and exon 1 of the TRAC gene. The anti-CD19 CAR donor DNA directed to exon 3 is synthesized to include the anti-CD19 CAR cassette (SEQ ID NO:22) as set forth in the Examples above, where the anti-CD19 expression cassette is flanked by sequences from the exon 3 locus (SEQ ID NO:24 and SEQ ID NO:25) as set forth above. The anti-CD19 CAR donor directed to exon 1 (having the sequence of SEQ ID NO:38) is provided in Example 4. Each of these constructs – one having the anti-CD19 CAR cassette (SEQ ID NO:22) flanked by TRAC exon 1 HAs (SEQ ID NO:18 and SEQ ID NO:19), and the other having the anti-CD19 CAR cassette (SEQ ID NO:22) flanked by TRAC exon 3 HAs (SEQ ID NO:24 and SEQ ID NO:25), was used to produce donor fragment using

modified forward primers having PS and 2'-O-methyl modifications on the three 5'-terminal most nucleotides. The reverse primers had 5'-terminal phosphates. The primers for producing the anti-CD19 CAR donor flanked by exon 1 HAs were SEQ ID NO:18 and SEQ ID NO:19, where the SEQ ID NO:18 primer included PS linkages between first and second, third and fourth, and fourth and fifth nucleosides and 2'-O methyl groups at position 3, position 4, and position 5 from the 5' end. The primers for producing the anti-CD19 CAR donor flanked by exon 3 HAs were SEQ ID NO:28 and SEQ ID NO:29, where the SEQ ID NO:28 primer had PS linkages between the first and second, second and third, and third and fourth nucleosides from the 5' end and 2'-O-methyl groups at position 2, position 4, and position 5 from the 5' end. The resulting double-stranded donor DNAs thus had a first strand with corresponding PS and 2'-O-methyl modifications on the 5'-terminal end nucleotides, and a second strand having a 5'-terminal phosphate.

The donor fragments were independently transfected into activated T cells with RNPs. RNPs were produced as described in Example 1, except that for targeting TRAC gene exon 1, the target sequence of the crRNA was SEQ ID NO:1, and for targeting TRAC gene exon 3, the target sequence of the crRNA was SEQ ID NO:26. As can be seen in **FIG. 11**, approximately 41% of the culture that was transfected with an RNP targeting exon 3 of the TRAC gene and a donor fragment for expressing the anti-CD19 CAR were both TCR negative and positive for anti-CD19 CAR, while approximately 20% of the culture that was transfected with an RNP targeting exon 1 of the TRAC gene and a donor fragment for expressing the anti-CD19 CAR were both TCR negative and positive for anti-CD19 CAR. T cell cultures transduced with a retrovirus including the anti-CD19 CAR expression cassette demonstrated a higher percentage of anti-CD19 CAR expressing cells, but these cells did not have a TCR knockout.

**Example 6. Targeted insertion of an anti-CD38 dimeric antibody receptor (DAR) construct into the TRAC exon 1 locus with Cas9.**

In further experiments, further configurations of synthetic antibody-receptors were expressed in T cells. Constructs were made for the expression of dimeric antibody receptors (DARs, see, for example, US Patent Application Publication No. US 2020/0399393 and WO 2019/173837, both of which are incorporated herein by reference in their entireties), where the DAR constructs included a nucleic acid sequence encoding two polypeptides linked by a "self-

cleaving" 2A sequence that was used to generate two polypeptides from a single open reading frame. Various DAR constructs were generated using sequences of the 'A2' CD38 (US Patent No. 10,059,774, incorporated herein by reference). The first encoded polypeptide was a heavy chain polypeptide that included a signal peptide, the heavy chain variable region and the first heavy chain constant region (CH1) of CD38 monoclonal antibody 'A2' , a hinge region from CD28, the transmembrane domain of CD28, and cytoplasmic signaling domains of 4-1BB and CDζ. This was followed by the *Thosea asigna* virus T2A peptide-encoding sequence (SEQ ID NO:44**)** and then by the sequence encoding the second polypeptide, where the second polypeptide included, proceeding from the N-terminus to the C-terminus, a signal peptide, and the immunoglobulin light chain variable region (VL) plus constant region (lambda) of CD38 monoclonal antibody 'A2'. The precursor polypeptide that included the first and second polypeptides, each preceded by a signal peptide and joined by a 2A sequence, is provided as SEQ ID NO:52. The mature first and second polypeptides of the CD38 DAR, as synthesized by a cell, are provided as SEQ ID NO:53 and SEQ ID NO:54 respectively.

The nucleic acid sequence encoding the heavy chain polypeptide sequence, 2A peptide, and light chain polypeptide sequence (SEQ ID NO:51) were operably linked to the JeT promoter (SEQ ID NO:3) at the 5' end of the DAR-encoding sequence and an SV40 polyA addition sequence (SEQ ID NO:45) at the 3'end of the DAR-encoding sequence. The entire anti-CD38 DAR construct with promoter and polyadenylation sequences (SEQ ID NO:46), was cloned between homology arms of 660 bp and 650 bp in a vector. The homology arms included sequences of the TRAC exon 1 locus on either side of the target sequence (SEQ ID NO:1). Donor fragments for use in transfection experiments were synthesized by PCR using a forward primer that included three PS bonds between the first and second, third and fourth, and fourth and fifth nucleotides and three 2'-O-methyl modifications at nucleotides 3, 4, and 5 when numbering from the 5'-terminus of the primer (SEQ ID NO:18), and a reverse primer that included a 5' terminal phosphate (SEQ ID NO:19) (**Table 1**). The resulting PCR product (SEQ ID NO:49) included the homology arms (SEQ ID NO:20 and SEQ ID NO:21) flanking the anti-CD38 DAR-encoding construct (SEQ ID NO:46) and had the primer modifications of SEQ ID NO:18 incorporated into the first strand and a 5' terminal phosphate but no introduced chemical modifications added to the opposite, or second, strand.

Isolation of primary human T cells from PBMCs, preparation of the RNP that included Cas9 and hybridized tracrRNA and crRNA that included the target site sequence (SEQ ID NO:1), and transformation of isolated T cells with the RNP targeting exon 1 of the TRAC gene and the donor fragment of SEQ ID NO:49 that included the CD38 DAR construct (SEQ ID NO:46) was performed essentially as set forth in Example 1.

**Fourteen days after transfection**, the T cell population transfected with Cas9 RNP plus the donor DNA having homology arms for targeting the Cas9 target site (SEQ ID NO:47) was analyzed by flow cytometry alongside the knockout controls as described in Example 1 (**Figure 13**). Only about 32% of the cell population that was transfected with a Cas9 RNP targeting the TRAC gene in the absence of a donor fragment ("TRAC KO", middle panel) expressed the TCR. By comparison, nearly all (approximately 98%) nonmodified activated T cells (activated T cells "ATC"), shown in the leftmost panel of **Figure 13**, expressed the TCR. As expected, cells that did not receive donor DNA showed no expression of the anti-CD38 constructs (**Figure 13**, middle panel). On the other hand, a majority (5.5%) of the cell population transfected with an anti-CD38 DAR construct donor DNA in addition to a Cas9 RNP demonstrated expression of the CD38 DAR construct (**Figure 13**, rightmost panel) and did not express the T cell receptor.

The insertion of the anti-CD38 DAR construct into the Cas9 target site of exon 1 of the TRAC gene (SEQ ID NO:1) was confirmed by PCR performed on genomic DNA isolated from both transfected cell populations and sequencing of the junction fragments. For Cas9-mediated insertion, PCR of the 5' homology arm region used SEQ ID NO:48 as the forward primer and SEQ ID NO:49 as the reverse primer. PCR of the 3' homology arm region used SEQ ID NO:50 as the forward primer and SEQ ID NO:51 as the reverse primer. Sequencing of the resulting PCR fragments demonstrated that the anti-CD38 DAR construct had inserted in the targeted Cas9 target site.

The results of cytotoxicity assays with the transfected populations co-cultured with RPMI8226 cells demonstrated that T cells transfected with the DAR construct by using a Cas9 system had the expected physiological behavior (**Figure 14**). Cells transfected with the Cas9 RNP plus anti-CD38 DAR construct donor DNA (SEQ ID NO:47) showed specific killing of RPMI88226 cells that was dramatically higher that of the control population having a knocked out TCR gene but not transfected with an anti-CD38 DAR construct donor DNA.

**Example 7. *In silico* prediction of Double-Stranded Breaks (DSBs)**

The target sequence of SEQ ID NO:1 was selected for construct integration because its modification would disrupt the reading frame of the TRAC gene and because there is no identical sequence elsewhere in the human genome. The Benchling (San Francisco, CA) CRISPR Guide RNA Design software (benching.com) was used to identify potential off-target sites in the human genome when using the guide RNA that targeted exon 1 of the TRAC locus (having the targeting sequence of SEQ ID NO:1). Gene function annotation was done using ANNOVAR software (Wang et al. (2010) *Nucl Acids Res.* 38:e164). Fifty *in silico* predicted DSBs of the crRNA that included the target site of SEQ ID NO:1 are listed in **Table 2** including genome coordinates, (off-)target sequence in the genome, predicted cleavage score, and gene function annotation. The predicted DSBs were ranked by score from highest to lowest cleavage score. The predicted DSB site with the highest score is in the TRAC locus which is the on-target cleavage, and the remaining entries are potential off-target DSBs having some degree of sequence homology to the target sequence of SEQ ID NO:1.

**Table 2. *In-silico* predicted DSBs of crRNA against TRAC locus.**

| Chr | Position | Sequence | Score | Func | Gene |
|---|---|---|---|---|---|
| chr14 | 22547505 | CAGGGTTCTGGATATCTGT | 100 | Exon 1 junction | TRAC |
| chr4 | 57114018 | CA**A**G**T**TTCT**T**GATATCTGT | 2.5 | ncRNA_intronic | RP11-12A1.1 |
| chr16 | 19151786 | CA**TT**GTTCT**A**GATATCTGT | 2.5 | intronic | CTD-2349B8.1 |
| chr17 | 12425260 | **G**A**T**GGTTC**C**GGATATCTGT | 1.7 | intergenic | RP11-471L13.2;LINC00670 |
| chr2 | 225701288 | **T**AG**T**GTTCTGG**C**TATCTGT | 1.6 | ncRNA_exonic | RP11-314B1.2 |
| chr11 | 33910945 | **TG**GGG**G**TCTGGATATCTGT | 1.6 | intergenic | LMO2;CAPRIN1 |
| chr10 | 29620188 | CA**T**GG**C**T**A**TGGATATCTGT | 1.5 | intronic | SVIL |
| chr16 | 3810642 | **A**AG**TA**TTCT**T**GATATCTGT | 1.4 | exonic | CREBBP |
| chr12 | 83242253 | **G**AGG**T**TTCTGGATATCTG**G** | 1.4 | intergenic | RP11-87P13.2;RP11-384P14.1 |
| chr1 | 163589101 | **A**A**TCA**TTCTGGATATCTGT | 1.4 | intergenic | RNA5SP63;RF00012 |
| chr11 | 76117929 | CA**ATT**TTCT**T**GATATCTGT | 1.3 | intronic | UVRAG |
| chr7 | 123204620 | CAG**T**GTTCT**A**GATATCTG**A** | 1.1 | intergenic | SLC13A1;IQUB |

| | | | | | |
|---|---|---|---|---|---|
| chr7 | 47512568 | CAGAGTGCTGTATATCTGT | 1 | intronic | TNS3 |
| chr11 | 72679292 | CAGGTTCCAGGATATCTGT | 1 | intergenic | PDE2A;ARAP1 |
| chr15 | 99175578 | GAGGGGTCTGTATATCTGT | 1 | intronic | TTC23 |
| chr2 | 48154612 | ATGGGTCCTTGATATCTGT | 1 | ncRNA_intronic | AC079807.4 |
| chr8 | 104075440 | CATTCTGCTGGATATCTGT | 0.9 | intronic | RIMS2 |
| chr15 | 86891532 | CATTCTACTGGATATCTGT | 0.9 | intronic | AGBL1 |
| chr5 | 140082170 | CATGCTACTAGATATCTGT | 0.9 | intergenic | NRG2;CTB-131B5.4 |
| chr2 | 205415665 | CAAGGTAATTGATATCTGT | 0.9 | intronic | PARD3B |
| chrX | 84161433 | GAGAGTTTTGCATATCTGT | 0.9 | intronic | RPS6KA6 |
| chr3 | 89713738 | AAAGGTTTTGCATATCTGT | 0.9 | intergenic | EPHA3;RF00012 |
| chrX | 71572437 | AATGGTTGTGCATATCTGT | 0.9 | intronic | OGT |
| chr2 | 241712463 | TAGATTTCTGTATATCTGT | 0.9 | intronic | ING5 |
| chr16 | 4419087 | CTGAGCTGTGGATATCTGT | 0.9 | intergenic | CORO7;DNAJA3 |
| chr21 | 41988729 | ATAGGTTCTGCATATCTGT | 0.9 | UTR3 | ZBTB21 |
| chr14 | 21406177 | CAGATTTTAGGATATCTGT | 0.9 | intronic | CHD8 |
| chr5 | 93337288 | CTGGTGTTTGGATATCTGT | 0.9 | intergenic | CTD-2091N23.1;RP11-65F13.2 |
| chr19 | 30371678 | AGGGTATCTGGATATCTGT | 0.9 | upstream | ZNF536 |
| chr6 | 142672594 | CTCGCTTCTGAATATCTGT | 0.9 | intergenic | RP11-440G9.1;RP1-67K17.3 |
| chr13 | 90486292 | CTGAGTTCTTTATATCTGT | 0.8 | intergenic | MIR622;RP11-158A8.1 |
| chrX | 49750504 | CATTTTTCTGAATATCTGT | 0.8 | intergenic | PAGE1;PAGE4 |
| chr4 | 158960302 | CATGGGTTTTGATATCTGT | 0.8 | exonic | C4orf45 |
| chr4 | 13938348 | CATGGTTCTTGATACCTGT | 0.8 | ncRNA_intronic | RP11-341G5.1 |
| chr5 | 15599009 | AAAGATTCTGGTTATCTGT | 0.8 | intronic | FBXL7 |
| chr5 | 146441246 | CAAATCTCTGGATATCTGT | 0.8 | intergenic | CTB-1H10.1;TCERG1 |
| chr5 | 99820618 | CATTGTTCTAAATATCTGT | 0.8 | intergenic | CTD-2151A2.1;RF00019 |
| chr2 | 209202308 | CAGGCTTTCAGATATCTGT | 0.8 | intergenic | RNA5SP117;MAP2 |
| chr12 | 124473886 | CAGAGTCCTGGACATCTGT | 0.8 | intronic | NCOR2 |
| chr11 | 112561353 | AAGGGCTCTGGACATCTGT | 0.8 | ncRNA_intronic | RP11-65M17.3 |

| | | | | | |
|---|---|---|---|---|---|
| chrX | 18700855 | GAGGTTTCTAGCTATCTGT | 0.8 | intronic | PPEF1 |
| chr2 | 1486193 | CAAGGTTTTTAATATCTGT | 0.8 | intronic | TPO |
| chr10 | 78376800 | CTGGGGTCTGGAGATCTGT | 0.8 | ncRNA_intronic | LINC00595;RP11-90J7.3 |
| chr7 | 132290688 | CATGATTCTGGATATCTCT | 0.7 | intronic | PLXNA4 |
| chr4 | 6221527 | CAGGGTTCGCCATATCTGT | 0.7 | intergenic | RP11-586D19.2;WFS1 |
| chr17 | 46650565 | TTGGGTTGTGGAAATCTGT | 0.7 | intronic | NSF |
| chr17 | 46432993 | TTGGGTTGTGGAAATCTGT | 0.7 | intergenic | ARL17B;LRRC37A2 |
| chr13 | 35346557 | AAAGGTTATGGAAATCTGT | 0.7 | intronic | NBEA |
| chr6 | 94570974 | AAGCTTTCTGGAGATCTGT | 0.7 | intergenic | RP3-399J4.3;RP13-44L19.2 |
| chrX | 111474546 | CTAGGTTTTGGACATCTGT | 0.7 | intergenic | RNU6-496P;RP6-170F5.2 |

**Example 8. Efficiency of DAR construct integration at Targeted TRAC Locus Using Cas9**

T cells of three different donors (donors #301, #302, and #303) were separately isolated from PBMCs by thawing frozen PBMCs in a 37° C water bath the cells were washed once with CTS OpTmizer T Cell Expansion SFM basal medium and resupended in CTS™ Dynabeads™ CD3/CD28 and beads (1.25 mL per 1 x $10^9$ PBMC cells) were transferred to a new tube. The beads were washed once with CTS™ OpTmizer™ T-Cell Expansion SFM basal medium, and then the resuspended cells and beads were combined and cell concentration was adjusted to 3 x $10^7$ cells/mL. The tubes were rotated on a sample mixer at 10 rpm for 30 min at room temperature and then placed on a magnet (Easy 50 EasySep™ Magnet) for 3 minutes, after which the supernatant was removed. The pellet was washed once with CTS™ OpTmizer™ T-Cell Expansion SFM basal medium and resuspended in T cell complete medium (CTS™ OpTmizer™ T-Cell Expansion SFM supplemented with 5% CTS™ Immune Cell SR, GlutaMAX™ Supplement and 300 IU/mL Human IL-2). Cell concentration was adjusted to 5 x $10^5$ cells/mL with T cell complete medium and cells were incubated at 37 °C / 5% $CO_2$. After 3 days of incubation the cells were collected by centrifugation and resuspended in T cell complete medium. The cells plus beads were pipeted up and down and then the tube containing the cells plus beads was placed on magnet for 3 minutes, and the supernatant containing T cells was transferred to a new tube. Medium was added to the beads remaining in the original tube, the mixture was pipetted up and down, and the beads were again captured with the supernatant being

removed and added to the first supernatant. The T cells in the combined supernatants were counted.

For transfection of T cells with a Cas9 RNP and donor oligonucleotides, the Cas9 crRNA and tracrRNA were first annealed. For each sample with CRISPR components, 5 µL 500 µM Alt-R CRISPR crRNA (having a sequence targeting the sequence of SEQ ID NO:1) and 5 µL 500 µM Alt-R CRISPR-CAS9 tracrRNA were mixed in a tube. Alt-R CRISPR crRNA and Alt-R CRISPR-CAS9 tracrRNA were synthesized by Integrated DNA Technologies (IDT; Coralville, IA). The crRNA and tracrRNA were incubated in a 95 °C heat block for 5 minutes and then incubated at room temperature for 20 minutes, after which 100 µg sNLS-SpCas9-sNLS nuclease was added, and the annealed RNAs and Cas9 enzyme were mixed and incubated at 4 °C for 30 minutes to form Cas9 RNP. 500 pmol dsODNs were added to the RNP and the donor oligonucleotides and RNP were mixed. Cells were washed once with Opti-MEM™ I Reduced Serum Medium and resuspended in Opti-MEM™ I Reduced Serum Medium to obtain a cell concentration of 2 x $10^7$ cells per 200 µL. 200 µL of cell suspension were transferred to the RNP plus donor oligonucleotides mixture in a 200 µL electroporation tube. As controls, T cells from each donor were mock electroporated, where the cells were mixed with dsODN donor oligonucleotides but no CRISPR components (Cas protein or guide RNA, e.g., no RNP) were added to the electroporation mixture. Electroporation was performed using Celetrix electroporator at 1325 V, 20 ms, 1 pulse. After electroporation cells were transferred into T cell complete medium at a concentration of 1 x $10^6$ cells/mL and the cells were incubated in a 37 °C $CO_2$ incubator. Cell concentration was adjusted every other day to 0.5 – 1 x $10^6$ cells/mL using T cell complete medium.

At 7 days post transfection cells were counted and gene editing efficiency (knockout of the T cell receptor) was assessed by flow cytometry using an antibody against CD3. For each sample 5 x $10^6$ cells were transferred into a tube, washed once with DPBS and cell pellets were stored at -80 °C. These sample were used for library generation and NGS as described in Example 9.

The results of gene editing efficiency assessment on day 7 post transfection are summarized in **Table 3**. Because disruption of the TRAC gene eliminates CD3 expression on T cells, we evaluated gene editing efficiency by flow cytometry using an antibody against CD3.

Our control samples without CRISPR components had 95.4% – 95.8% CD3 positive cells and this percentage decreased to 6.1% - 9.7% in the samples with CRISPR components.

**Table 3. Assessment of gene editing efficiency using commercial guide crRNA/tracrRNA.**

| Sample ID | Donor ID | CRISPR components | CD3+ % |
|---|---|---|---|
| STGG-201 | #301 | Yes | 9.7 |
| STGG-204 | #301 | No | 95.8 |
| STGG-205 | #302 | Yes | 6.7 |
| STGG-208 | #302 | No | 93.6 |
| STGG-209 | #303 | Yes | 6.1 |
| STGG-212 | #303 | No | 95.4 |

Another series of transfections was performed essentially identically to those described immediately above with the exception that instead of using a tracrRNA and crRNA from a commercial source, a tracrRNA and a crRNA that included a ribonucleotide sequence corresponding to the target sequence of SEQ ID NO:1 were produced in-house. In this case isolated T cells were from donors 301, 304, and 305. **Table 4** provides the gene editing/targeted donor fragment integration efficiency using the in-house guides.

**Table 4. Assessment of gene editing efficiency using in-house produced guide crRNA/tracrRNA.**

| Sample ID | Donor ID | CRISPR components | CD3+ % |
|---|---|---|---|
| STGG-201 | #301 | Yes | 4.7 |
| STGG-204 | #301 | No | 84.2 |
| STGG-205 | #304 | Yes | 4.9 |
| STGG-208 | #304 | No | 91.4 |
| STGG-209 | #305 | Yes | 3.9 |
| STGG-212 | #305 | No | 91.3 |

In this case the control samples without CRISPR components had 84.2% – 91.4% CD3 positive cells and this percentage decreased to 3.9% - 4.9% in the samples with CRISPR components.

These results demonstrate that robust gene editing was achieved in our iGUIDE-Seq samples consistently, which ensures the detection of potential off-target DSBs.

**Example 9. Off-Target Mutation Analysis Using Improved Genome-Wide Unbiased Identification of Double-Stranded Breaks Enabled by Sequencing (iGuide).**

To find off-target mutations and their frequency using a nonbiased method, the methodology known as Improved Genome-Wide Unbiased Identification of Double-Stranded Breaks Enabled by Sequencing (iGuide; see for example Nobles *et al.* (2019) *Genome Biology* 20:14; https://doi.org/10.1186/s13059-019-1625-3) was used to analyze the T cells transfected in Example 8. Library preparation and next-generation sequencing for iGUIDE-Seq were performed on the transfected T cell samples of Example 8 by Genenius Genetics (San Diego, CA).

The double-stranded oligodeoxynucleotides (dsODNs) (46 nucleotides in length) that were included in the transfections were used for detection of off-target editing sites. Insertions of the dsODNs at off-target sites after the creation of double-stranded breaks by Cas9 allowed for sequencing of the off-target loci using sequencing primers that hybridized to the ODN sequences.

Libraries were made from DNA isolated from the cell populations shown in **Tables 3** and **4** and next generation sequencing was performed according to established methods by Genenius Genetics (San Diego, CA). The regions containing DSBs determined by iGUIDE-Seq from cells of three different donors transfected with dsODNs and an RNP that included the Alt-R crRNA/tracrRNA (IDT, Coralville, IA) (**Table 3**) resulting from the sequence analysis are listed below in **Tables 5**–7. **Tables 5**–7 also include the genome coordinates, sequencing depth, and functional annotation of the gene at or proximal to the observed DSB sites.

Fourteen, thirteen, and seventeen DSB regions were identified from the transfections of T cells of donors #301, #302, and #303 respectively using the Alt-R RNAs. The most abundant DSB regions identified in the three donor genomes are all located in TRAC locus, which is the on-target DSB region. The percentage of on-target DSBs at the TRAC locus out of all DSBs

identified by iGUIDE-Seq ranges from 88% to 91% in the three different transfections. Six off-target DSB regions were found in more than two samples that included CRISPR components, which are highlighted in bold type in the tables below. Combining experiments from the three donors, a total of 30 potential off-target DSBs regions were identified. Importantly none of potential off-target DSB regions was found to occur in a protein coding region, indicating that any off-target DSBs of the CRISPR components used are unlikely to disrupt critical gene functions.

The sequencing depth at the peaks of on-target DSBs region (TRAC locus) ranged from 7704 to 44951 and the threshold of analysis was set to 10. Based on these parameters, we calculated the detection sensitivity of the iGUIDE-Seq method used to detect off-target DSBs was about 0.02% - 0.12% (10/44951 – 10/7704) of on-target DSBs.

**Table 5. DSBs regions identified by iGUIDE-seq for Donor #301 transfected with RNP including Alt-R crRNA/tracrRNA**

| Chr | Start | End | Depth | % of reads relative to on-target site | Func | Gene |
|---|---|---|---|---|---|---|
| chr14 | 22547436 | 22547586 | 22339 | 100.00% | exonic | TRAC |
| **chr20** | **24956161** | **24956287** | **1358** | **6.08%** | **intronic** | **CST7** |
| **chr4** | **49709342** | **49709546** | **407** | **1.82%** | **intergenic** | **RP11-1281K21.8; NONE** |
| **chr19** | **1512270** | **1512295** | **262** | **1.17%** | **intronic** | **ADAMTSL5** |
| chr13 | 18211906 | 18212104 | 176 | 0.79% | ncRNA_ intronic | RP11-341D18.3 |
| **chr5** | **128810747** | **128810772** | **156** | **0.70%** | **intergenic** | **CTC-573M9.1; SLC27A6** |
| **chr18** | **28607975** | **28607985** | **131** | **0.59%** | **intergenic** | **CDH2; RP11-510D21.1** |
| **chr20** | **32791392** | **32791407** | **98** | **0.44%** | **intronic** | **DNMT3B** |
| chr16 | 34588067 | 34588254 | 72 | 0.32% | intergenic | RNA5-8SP2; CTD-2144E22.9 |
| chrY | 11328342 | 11328458 | 68 | 0.30% | intergenic | RNA5-8SP6; RN7SL702P |
| chr1 | 28725622 | 28725626 | 67 | 0.30% | intergenic | GMEB1; YTHDF2 |

| | | | | | | |
|---|---|---|---|---|---|---|
| chr1 | 125179221 | 125179402 | 67 | 0.30% | intergenic | NONE; NONE |
| chr10 | 5820171 | 5820177 | 63 | 0.28% | intergenic | RP11-318E3.9; ANKRD16 |
| chr4 | 178180900 | 178180902 | 57 | 0.26% | intergenic | RNU1-45P; RNA5SP173 |

**Table 6. DSBs regions identified by iGUIDE-seq for Donor #302 transfected with RNP including Alt-R crRNA/tracrRNA**

| Chr | Start | End | Depth | % of reads relative to on-target site | Func | Gene |
|---|---|---|---|---|---|---|
| chr14 | 22547426 | 22547594 | 68252 | 100.00% | exonic | TRAC |
| **chr20** | **24956165** | **24956255** | **3858** | **5.65%** | **intronic** | **CST7** |
| **chr19** | **1512210** | **1512321** | **889** | **1.30%** | **intronic** | **ADAMTSL5** |
| **chr18** | **28607960** | **28608028** | **584** | **0.86%** | **intergenic** | **CDH2; RP11-510D21.1** |
| **chr5** | **128810717** | **128810777** | **476** | **0.70%** | **intergenic** | **CTC-573M9.1; SLC27A6** |
| chr3 | 145805989 | 145805990 | 223 | 0.33% | intergenic | RP11-42F12.1; RP11-88H10.2 |
| **chr4** | **49709486** | **49709698** | **171** | **0.25%** | **intergenic** | **RP11-1281K21.8; NONE** |
| chr8 | 24484213 | 24484222 | 158 | 0.23% | ncRNA_ intronic | RP11-624C23.1 |
| chr3 | 52940234 | 52940237 | 150 | 0.22% | intronic | SFMBT1 |
| **chr20** | **32791399** | **32791407** | **121** | **0.18%** | **intronic** | **DNMT3B** |
| chr16 | 34571787 | 34571860 | 107 | 0.16% | intergenic | RNA5-8SP2; CTD-2144E22.9 |
| chr4 | 85007083 | 85007104 | 96 | 0.14% | ncRNA_ exonic | WDFY3-AS2 |
| chr10 | 104352184 | 104352193 | 76 | 0.11% | ncRNA_ intronic | RP11-127L20.6 |

**Table 7. DSBs regions identified by iGUIDE-seq for Donor #303 transfected with RNP including Alt-R crRNA/tracrRNA**

| Chr | Start | End | Depth | % of reads relative to on-target site | Func | Gene |
|---|---|---|---|---|---|---|
| chr14 | 22547422 | 22547602 | 55881 | 100.00% | exonic | TRAC |
| **chr20** | **24956149** | **24956236** | **4324** | **7.74%** | **intronic** | **CST7** |
| **chr19** | **1512270** | **1512291** | **831** | **1.49%** | **intronic** | **ADAMTSL5** |
| **chr20** | **32791391** | **32791429** | **354** | **0.63%** | **intronic** | **DNMT3B** |
| **chr18** | **28607976** | **28607989** | **280** | **0.50%** | **intergenic** | **CDH2; RP11-510D21.1** |
| **chr5** | **128810713** | **128810778** | **274** | 0.49% | **intergenic** | **CTC-573M9.1; SLC27A6** |
| chr6 | 90769404 | 90769431 | 208 | **0.37%** | intergenic | MAP3K7; RP1-177I10.1 |
| chr3 | 133343722 | 133343768 | 164 | 0.29% | intronic | TMEM108 |
| chr17 | 54299505 | 54299507 | 160 | 0.29% | intergenic | KIF2B; RN7SKP14 |
| chr1 | 143262680 | 143262897 | 144 | **0.26%** | intergenic | NONE; RNA5SP533 |
| chr1 | 31254953 | 31254987 | 142 | 0.25% | intergenic | NKAIN1; SNRNP40 |
| chr1 | 143232982 | 143233147 | 123 | 0.22% | intergenic | NONE; RNA5SP533 |
| chr6 | 67751230 | 67751237 | 111 | 0.20% | intergenic | RF00019; RP11-301G19.1 |
| chr6 | 18439213 | 18439219 | 82 | 0.15% | intronic | RNF144B |
| chr18 | 108091 | 108251 | 67 | 0.12% | intergenic | RP11-683L23.7; MIR8078 |
| chr11 | 73623119 | 73623124 | 59 | 0.11% | intergenic | FAM168A; PLEKHB1 |
| chrX | 17972943 | 17972948 | 56 | 0.10% | ncRNA_ intronic | RP3-410B11.1 |

The regions containing DSBs determined by iGUIDE-Seq from cells of three different donors transfected with dsODNs and an RNP that included in-house produced crRNA/tracrRNA (IDT, Coralville, IA) (**Table 4**) resulting from the sequence analysis are listed below in **Tables 8–10. Tables 8-10** also include genome coordinates, sequencing depth, and functional annotation of the gene at or proximal to the observed DSB sites.

Fifteen, sixteen, and sixteen DSB regions were identified from the transfections of T cells of donors #301, #304, and #305 respectively using the Alt-R RNAs. The most abundant DSB regions identified in the three donor genomes are all located in TRAC locus, which is the on-target DSB region. The percentage of on-target DSBs at the TRAC locus out of all DSBs identified by iGUIDE-Seq ranges from 78% to 80% in the three different transfections. Nine off-target DSB regions were found in more than two samples that included CRISPR components, which are highlighted in bold type in the tables below. Combining experiments from the three donors, a total of 30 potential off-target DSBs regions were identified. Importantly none of potential off-target DSB regions was found to occur in a protein coding region, indicating that any off-target DSBs of the CRISPR components used are unlikely to disrupt critical gene functions.

The sequencing depth at the peaks of on-target DSBs region (TRAC locus) ranged from 105336 to 328583 and the threshold of analysis was set to 10. Based on these parameters, we calculated the detection sensitivity of the iGUIDE-Seq method used to detect off-target DSBs was about 0.01% - 0.12% (10/105336) of on-target DSBs.

The off-target DSBs identified by iGUIDE-Seq using IDT Alt-R crRNA/tracrRNA and in-house produced crRNA/tracrRNA are highly similar, especially those with higher reads percentage relative to the on-target site.

**Table 8. DSBs regions identified by iGUIDE-seq for Donor #301.**

| Chr | Start | End | Reads | Reads % relative to on-target site | Func | Gene |
|---|---|---|---|---|---|---|
| chr14 | 22547420 | 22547595 | 778976 | 100.00% | exonic | TRAC |
| **chr20** | **24956133** | **24956259** | **107213** | **13.76%** | **intronic** | **CST7** |
| **chr19** | **1512247** | **1512314** | **28331** | **3.64%** | **intronic** | **ADAMTSL5** |
| **chr18** | **28607925** | **28608057** | **18547** | **2.38%** | **intergenic** | **CDH2; RP11-510D21.1** |
| **chr20** | **32791355** | **32791448** | **9922** | **1.27%** | **intronic** | **DNMT3B** |
| **chr5** | **128810713** | **128810798** | **9076** | **1.17%** | **intergenic** | **CTC-573M9.1; SLC27A6** |
| **chr3** | **15513813** | **15513821** | **5317** | **0.68%** | **intronic** | **COLQ** |
| chrX | 17972933 | 17972976 | 4330 | 0.56% | ncRNA_intronic | RP3-410B11.1 |
| chr19 | 42923112 | 42923122 | 3784 | 0.49% | intergenic | PSG6;PSG7 |
| **chr3** | **133343757** | **133343809** | **3052** | **0.39%** | **intronic** | **TMEM108** |
| chr20 | 34298705 | 34298719 | 2523 | 0.32% | intronic | AHCY |
| **chr17** | **45045074** | **45045110** | **1536** | **0.20%** | **intronic** | **DCAKD** |

| | | | | | | |
|---|---|---|---|---|---|---|
| **chr2** | **105366661** | **105366669** | **1095** | **0.14%** | **ncRNA_ intronic** | **AC012360.6** |
| chr15 | 54767271 | 54767286 | 538 | 0.07% | intergenic | UNC13C;RP11-548M13.1 |
| chr2 | 74203685 | 74203691 | 523 | 0.07% | intronic | MTHFD2 |
| chr19 | 30371673 | 30371678 | 509 | 0.07% | upstream | ZNF536 |

**Table 9. DSBs regions identified by iGUIDE-seq for Donor #304.**

| Chr | Start | End | Reads | Reads % relative to on-target site | Func | Gene |
|---|---|---|---|---|---|---|
| chr14 | 22547420 | 22547599 | 709884 | 100.00% | exonic | TRAC |
| **chr20** | **24956138** | **24956286** | **122535** | **17.26%** | **intronic** | **CST7** |
| **chr19** | **1512243** | **1512324** | **24067** | **3.39%** | **intronic** | **ADAMTSL5** |
| **chr18** | **28607945** | **28608029** | **12443** | **1.75%** | **intergenic** | **CDH2; RP11-510D21.1** |
| **chr5** | **128810739** | **128810798** | **8921** | **1.26%** | **intergenic** | **CTC-573M9.1; SLC27A6** |
| **chr20** | **32791360** | **32791444** | **7309** | **1.03%** | **intronic** | **DNMT3B** |
| **chr17** | **45045070** | **45045112** | **6319** | **0.89%** | **intronic** | **DCAKD** |
| **chr2** | **105366649** | **105366668** | **3930** | **0.55%** | **ncRNA_ intronic** | **AC012360.6** |
| chr20 | 34298703 | 34298731 | 3493 | 0.49% | intronic | AHCY |
| chr6 | 67751217 | 67751242 | 3325 | 0.47% | intergenic | RF00019; RP11-301G19.1 |
| chr3 | 145805982 | 145805996 | 2815 | 0.40% | intergenic | RP11-42F12.1; RP11-88H10.2 |
| chr15 | 54767274 | 54767289 | 2621 | 0.37% | intergenic | UNC13C; RP11-548M13.1 |
| **chr3** | **133343752** | **133343804** | **2566** | **0.36%** | **intronic** | **TMEM108** |
| **chr3** | **15513809** | **15513819** | **1984** | **0.28%** | **intronic** | **COLQ** |
| chr19 | 30371674 | 30371683 | 1065 | 0.15% | upstream | ZNF536 |
| chrX | 17972939 | 17972976 | 1056 | 0.15% | ncRNA_ intronic | RP3-410B11.1 |
| chr6 | 88864446 | 88864453 | 109 | 0.02% | intronic | RNGTT |

**Table 10. DSBs regions identified by iGUIDE-seq for Donor #305.**

| Chr | Start | End | Reads | Reads % relative to on-target site | Func | Gene |
|---|---|---|---|---|---|---|
| chr14 | 22547416 | 22547595 | 342542 | 100.00% | exonic | TRAC |
| **chr20** | **24956120** | **24956298** | **59846** | **17.47%** | **intronic** | **CST7** |
| **chr19** | **1512232** | **1512345** | **9121** | **2.66%** | **intronic** | **ADAMTSL5** |
| **chr20** | **32791359** | **32791408** | **7975** | **2.33%** | **intronic** | **DNMT3B** |
| **chr5** | **128810739** | **128810809** | **4133** | **1.21%** | **intergenic** | **CTC-573M9.1; SLC27A6** |
| **chr18** | **28607963** | **28608026** | **4048** | **1.18%** | **intergenic** | **CDH2; RP11-510D21.1** |
| chr3 | 145805966 | 145805999 | 1908 | 0.56% | intergenic | RP11-42F12.1; RP11-88H10.2 |
| **chr17** | **45045067** | **45045122** | **1755** | **0.51%** | **intronic** | **DCAKD** |
| **chr3** | **133343758** | **133343804** | **1504** | **0.44%** | **intronic** | **TMEM108** |
| **chr2** | **105366659** | **105366689** | **1110** | **0.32%** | **ncRNA_ intronic** | **AC012360.6** |
| chr3 | 93470613 | 93470727 | 1006 | 0.29% | intergenic | NONE; RNU6-488P |
| chr6 | 67751215 | 67751245 | 939 | 0.27% | intergenic | RF00019; RP11-301G19.1 |
| chr9 | 75844622 | 75844629 | 780 | 0.23% | intergenic | RP11-470F18.1; PCSK5 |
| **chr3** | **15513809** | **15513824** | **429** | **0.13%** | **intronic** | **COLQ** |
| chr19 | 42923100 | 42923116 | 376 | 0.11% | intergenic | PSG6; PSG7 |
| chr4 | 73671341 | 73671346 | 300 | 0.09% | intergenic | RASSF6; AC112518.3 |
| chr21 | 7926402 | 7926435 | 176 | 0.05% | intergenic | KCNE1B; CH507-338C24.3 |

The sequencing depth at the peaks of the on-target DSB region (the TRAC locus, top row of all of **Tables 5-10**) ranged from 7,704 to 346,110, and the threshold of analysis was set to 10.. the sensitivity in detecting off-target DSBs is calculated to be about 0.12% (10/7,704) of on-target DSBs.

In summary, **Tables 5-10**, which include the DSB regions identified by the unbiased iGUIDE method ranked by the abundance of reads, show that the potential off-target sites are similar from sample to sample, with five of the high-confidence off target DSB regions (highlighted in bold type in Tables 6-11) detected in all experiments. Importantly, none of the off-target sites identified by iGUIDE-Seq were found to be in protein coding regions.

**Example 10. Next Generation Sequencing of Identified and Predicted Off-Target Sites for Cells Engineered to Express an anti-CD38 DAR Construct.**

Based on the iGUIDE-Seq results and in-silico prediction results (Example 7), we selected 79 potential editing sites to be evaluated by amplicon sequencing. To evaluate the off-target effect of CRISPR mediated gene editing in CD38A2 DAR-T cells produced by the methods for gene editing described in Example 6 using in-house produced tracrRNA and crRNA for targeting exon 1 of the TRAC locus, a panel of amplicon sequencing primers was designed and optimized. The panel contained 155 amplicons designed for the on-target TRAC site and 78 potential off-target sites determined by biased in-silico prediction or unbiased iGUIDE-Seq. The targeted regions contained the on-target region, confirmed off-target regions, in silico predicted off-target sites in exonic regions (although no indel was detected in those regions) and as controls some potential off-target sites in which there was not a consistent indel detected. Out of 79 targeted genome regions, 1 on-target site and 5 off-target sites were confirmed with indel variants in more than one CD38A2 KOKI DAR-T cell sample (**Table 11**).

Amplicon sequencing was performed in Anti-CD38A2 KOKI DAR-T cells produced using three independent donors (#306, #307, #308). T cells that were not subjected to gene editing from the three donors were used as controls to remove native genome variants in the donor samples. Post-transfection and prior to amplicon analysis, subtraction of T cell receptor-expressing T cells were performed using magnetic beads that bound CD3 to use the process that

would be used for preparing transfected cells for allogeneic therapy, resulting in T cell populations with very high levels of CD38A2 DAR expression (**Table 11**).

With a variant calling out threshold at 0.5% AF (allele frequency), out of 79 targeted genome regions, 1 on-target site and 5 off-target sites were detected with indel variants in more than one gene edited CD38A2 KOKI DAR-T cell sample. On-target and off-target sites confirmed by targeted amplicon sequencing are listed in **Table 11** including genome coordinates, gene functional annotation, and detected frequency of variants (including deletion, insertion and substitution) in each gene-edited CD38A2 KOKI DAR-T cell sample. No confirmed off-target sites were in exonic regions. For intergenic and intronic off-target gene editing, there was no evidence found to support their relationship to gene regulatory elements that could relate to activation of oncogenes, inhibition of tumor suppressors, and abnormal activity of gene regulation.

To assess the assay performance of amplicon sequencing for gene editing evaluation, two gBlocks (IDT double stranded DNA fragment) per targeted region were designed and synthesized as standards to create a calibration curve. One gBlock contained a single 40 bp deletion and the other contained one 2 bp deletion, one 1 bp insertion, and two single-nucleotide polymorphisms. Calibration curves were created by spiking synthetic gBlocks at 0, 0.25%, 0.5%, 1%, 5% and 10% allele frequency in genomic DNA derived from 12 cell lines from Coriell Institute separately. Amplicon sequencing data confirmed that all designed variants can be detected at the lowest spike-in allele frequency with detection rate > 90% and coefficient of variation $\leq 0.6$. Determined LOD and LOQ were 0.25% allele frequency for majority of targets except 0.31% allele frequency for target STGG-29 (0.31% allele frequency was the lowest spike-in level created for the long deletion variant of this target). Assay accuracy (sensitivity and specificity) were analyzed at 1% allele frequency. Both calculated sensitivity and specificity for all targets were 100% with a 95% confident interval 75.75% - 100%. To evaluate the assay precision (Repeatability and Reproducibility), three intra-run replicates and three inter-run replicates of amplicon sequencing were performed for 12 Coriell Institute cell lines genomic DNA with synthetic gBlocks spike-in at 1% allele frequency individually. Overall, the intra-run repeatability of detecting all variants at average 1% allele frequency were verified to be 100% while the inter-run reproducibility of detecting vast majority variants at average 1% allele

frequency were verified to be 100%, except 97% for STGG-29 (in one inter-run replicate the long deletion variant for STGG-29 was not detected at frequency ≥ 60% of expected allele frequency).

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**Table 11. On/Off-target sites confirmed by targeted amplicon sequencing.**

| Target | Chr | Start | end | Func | Gene | Donor 306 | Donor 307 | Donor 308 |
|---|---|---|---|---|---|---|---|---|
| STGG-22 | chr14 | 22547421 | 22547602 | exonic | TRAC | 94.86% | 96.89% | 95.63% |
| STGG-28 | chr19 | 1512209 | 1512321 | intronic | ADAMTSL5 | 7.58% | 5.79% | 8.24% |
| STGG-13 | chr5 | 128810712 | 128810778 | intergenic | CTC-573M9.1;SLC27A6 | 4.18% | 4.12% | 4.25% |
| STGG-29 | chr20 | 24956148 | 24956287 | intronic | CST7 | 1.75% | 4.65% | 3.47% |
| STGG-27 | chr18 | 28607959 | 28608028 | intergenic | CDH2;RP11-510D21.1 | 1.13% | 1.18% | 1.26% |
| STP-48 | chrX | 84161392 | 84161473 | intronic | RPS6KA6 | 0.51% | 0.50% | 0.00% |

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**Table 12. On/Off-target editing efficiency determined by amplicon sequencing.**

| Target | Chr | start | end | Func | Gene | Editing efficiency | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Sample 1 | Sample 2 | Sample 3 |
| STGG-07 | chr3 | 133343721 | 133343768 | intronic (non-coding) | TMEM108 | 0.00% | 0.00% | 0.00% |
| STGG-08 | chr3 | 145805969 | 145806009 | intergenic (non-coding) | RP11-42F12.1;RP11-88H10.2 | 0.00% | 0.00% | 0.00% |
| STGG-13 | chr5 | 128810712 | 128810778 | intergenic (non-coding) | CTC-573M9.1;SLC27A6 | 4.18% | 4.12% | 4.25% |
| STGG-27 | chr18 | 28607959 | 28608028 | intergenic (non-coding) | CDH2;RP11-510D21.1 | 1.13% | 1.18% | 1.26% |
| STGG-28 | chr19 | 1512209 | 1512321 | intronic (non-coding) | ADAMTSL5 | 7.58% | 5.79% | 8.24% |
| STGG-29 | chr20 | 24956148 | 24956287 | intronic (non-coding) | CST7 | 1.75% | 4.65% | 3.47% |
| STGG-30 | chr20 | 32791389 | 32791430 | intronic (non-coding) | DNMT3B | 0.00% | 0.00% | 0.00% |
| STP-09 | chr4 | 6221486 | 6221567 | intergenic (non-coding) | RP11-586D19.2;WFS1 | 0.00% | 0.00% | 0.00% |
| STP-12 | chr4 | 158960261 | 158960342 | exonic (coding) | C4orf45 | 0.00% | 0.00% | 0.00% |
| STP-27 | chr11 | 72679251 | 72679332 | intergenic (non-coding) | PDE2A;ARAP1 | 0.00% | 0.00% | 0.00% |
| STP-29 | chr11 | 112561332 | 112561373 | ncRNA_intronic (non-coding) | RP11-65M17.3 | 0.00% | 0.00% | 0.00% |
| STP-37 | chr16 | 3810601 | 3810682 | exonic (coding) | CREBBP | 0.00% | 0.00% | 0.00% |
| STP-48 | chrX | 84161392 | 84161473 | intronic (non-coding) | RPS6KA6 | 0.51% | 0.50% | 0.00% |
| STGG-22 | chr14 | 22547421 | 22547602 | exonic (coding) | TRAC | 94.86% | 96.89% | 95.63% |

## CD38 DAR SEQUENCES

SEQ ID NO:46
DNA
Artificial
CD38 DAR insert 2652 nt

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SEQ ID NO:47
DNA
Artificial
CD38 DAR insert 2652 nt flanked by 5' 171 nt and 3' 161 nt TRAC exon 1 homology arms

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SEQ ID NO:51
DNA
encodes the 2 polypeptide CD38 DAR precursor joined by 2A sequence

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CGAAATGGGCGGCAAACCGAGACGCAAAAATCCTCAGGAGGGGCTCTACAATGAACTTCAAAAAGACAAA
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AAGCGGAGAGGGCAGAGGAAGTCTGCTAACATGCGGTGACGTCGAGGAGAATCCTGGACCTATGTCCGTC
CCTACCCAGGTGCTGGGCCTGCTGCTGCTGTGGCTGACCGATGCTAGATGCCAGTCCGTTCTGACCCAGC
CTCCTTCCGCCTCTGGCACATCCGGCCAGAGAGTGACCATCTCCTGCAGCGGCTCCAGCTCTAACATCGG
CATCAATTTCGTGTACTGGTATCAGCACCTGCCAGGCACAGCTCCCAAGCTGCTGATCTACAAGAACAAT
CAGAGGCCTTCCGGCGTGCCAGACCGGTTCTCTGGCTCCAAGAGCGGCAACTCTGCCTCCCTGGCTATCT
CCGGCCTGCGCAGCGAGGACGAGGCTGATTACTATTGCGCCGCTTGGGACGATAGCCTGTCTGGCTACGT
GTTCGGCAGCGGCACAAAGGTGACCGTGCTGGGACAGCCAAAGGCTGCTCCTTCTGTGACACTGTTTCCC
CCTTCCAGCGAGGAGCTGCAGGCCAATAAGGCCACCCTGGTGTGCCTGATCAGCGACTTCTATCCTGGAG
CTGTGACCGTGGCTTGGAAGGCTGATTCTTCCCCAGTGAAGGCTGGCGTGGAGACAACAACCCCCAGCAA
GCAGTCTAACAATAAGTACGCCGCTAGCTCTTATCTGTCTCTGACCCCAGAGCAGTGGAAGTCCCATAGG
TCCTATAGCTGTCAGGTCACCCACGAAGGGAGCACAGTCGAAAAAACCGTCGCACCAACCGAGTGTTCC

SEQ ID NO:52
Protein
2 polypeptide CD38 DAR precursor joined by 2A sequence

MEWSWVFLFFLSVTTGVHSQVQLVESGGGLVKPGGSLRLSCAASGFTFSDDYMSWIRQAPGKGLEWVASV
SNGRPTTYYADSVRGRFTISRDNAKNSLYLQMNSLRAEDTAVYYCAREDWGGEFTDWGRGTLVTVSSAST
KGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPS

SSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTKIEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFP
GPSKPFWVLVVVGGVLACYSLLVTVAFIIFWVKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEG
GCELRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDK
MAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPRGSGEGRGSLLTCGDVEENPGPMSV
PTQVLGLLLLWLTDARCQSVLTQPPSASGTSGQRVTISCSGSSSNIGINFVYWYQHLPGTAPKLLIYKNN
QRPSGVPDRFSGSKSGNSASLAISGLRSEDEADYYCAAWDDSLSGYVFGSGTKVTVLGQPKAAPSVTLFP
PSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASSYLSLTPEQWKSHR
SYSCQVTHEGSTVEKTVAPTECS

SEQ ID NO:53
Protein
Amino acid sequence of first CD38 DAR polypeptide (transmembrane polypeptide)

QVQLVESGGGLVKPGGSLRLSCAASGFTFSDDYMSWIRQAPGKGLEWVASVSNGRPTTYYADSVRGRFTI
SRDNAKNSLYLQMNSLRAEDTAVYYCAREDWGGEFTDWGRGTLVTVSSASTKGPSVFPLAPSSKSTSGGT
AALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNT
KVDKRVEPKSCDKTHTKIEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFPGPSKPFWVLVVVGGVLACY
SLLVTVAFIIFWVKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYQQ
GQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKG
HDGLYQGLSTATKDTYDALHMQALPPR

SEQ ID NO:54
Protein
Amino acid sequence of second CD38 DAR polypeptide

QSVLTQPPSASGTSGQRVTISCSGSSSNIGINFVYWYQHLPGTAPKLLIYKNNQRPSGVPDRFSGSKSGN
SASLAISGLRSEDEADYYCAAWDDSLSGYVFGSGTKVTVLGQPKAAPSVTLFPPSSEELQANKATLVCLI
SDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTV
APTECS

## CLAIMS

What is claimed is:

1. A population of cells comprising a plurality of T cells comprising a DNA construct encoding at least one polypeptide, wherein the DNA construct is inserted into the TRAC gene, wherein at least 10% of the cells of the population express the polypeptide encoded by the DNA construct and do not express the T cell receptor, further wherein the frequency of mutation at the highest frequency non-target site in the cells of the population is less than 20% of the frequency of mutation at the target site.

2. The cell population of claim 1, wherein the frequency of mutation at the highest frequency non-target site in the cells of the population is less than 15% of the frequency of mutation at the target site.

3. The cell population of claim 1, wherein the frequency of mutation at the highest frequency non-target site in the cells of the population is less than 10% of the frequency of mutation at the target site.

4. The cell population of claim 1, wherein the sum of the frequency of mutation at all non-target sites occurring at a frequency of 0.1% or greater relative to the frequency of mutation at the target site is less than 25%.

5. The cell population of claim 1, wherein the frequency of mutation at all non-target sites is less than 20%.

6. The cell population of claim 1, wherein none of the detected non-target sites is in the protein-encoding portion of a gene.

7. The cell population of claim 1, wherein the cells are human T cells.

8. The cell population of claim 1, wherein the cells are primary T cells.

9. The cell population of claim 1, wherein the target site comprises SEQ ID NO:1.

10. The cell population of claim 1, wherein the DNA construct includes a promoter operably linked to the protein-encoding sequence of the construct.

11. The cell population of claim 1, wherein the DNA construct encodes an immunological molecule or a receptor.

12. The cell population of claim 1, wherein the DNA construct encodes an engineered receptor.

13. The cell population of claim 1, wherein the DNA construct encodes a chimeric antigen receptor (CAR), a dimeric antigen receptor (DAR), a T Cell Receptor Fusion Construct (TFP), or an antibody-TCR chimeric molecule (abTCR).

14. The cell population of claim 12, wherein the DNA construct encodes a CD38 DAR.

15. The cell population of claim 13, wherein the DNA construct encodes a CD38 DAR precursor comprising a sequence having at least 95% identity to SEQ ID NO:52.

16. The cell population of claim 14, wherein the DNA construct encodes a CD38 DAR precursor comprising SEQ ID NO:52.

17. The cell population of any of claims 1-16, wherein at least 95% of the population does not express a T cell receptor.

18. A method of producing a population of T cells for the treatment of a disease, wherein the cells are transfected with a Cas9 protein or a nucleic acid molecule encoding a Cas9 protein, a guide RNA that targets the target site of SEQ ID NO:1, and a double-stranded donor DNA fragment the encodes a recombinant receptor.

19. A method according to claim 18, wherein the double-stranded donor DNA fragment is chemically modified.

20. A method according to claim 18, wherein the guide RNA is a crRNA and the cells are transfected with a Cas9 protein or a nucleic acid molecule encoding a Cas9 protein, a

crRNA that targets the target site of SEQ ID NO:1, a tracrRNA, and a donor fragment the encodes a recombinant receptor.

21. A method according to any of claims 18-20, wherein the cells are transfected with a Cas 9 protein.

22. A method according to claim 21, wherein the cells are transfected with an RNP comprising the Cas 9 protein.

23. A method according to any of claims 18-22, further comprising removing CD3 positive cells from the population.

24. A method of treating a patient having cancer, comprising delivering to the patient a composition comprising any of the cell populations of claims 1-17.

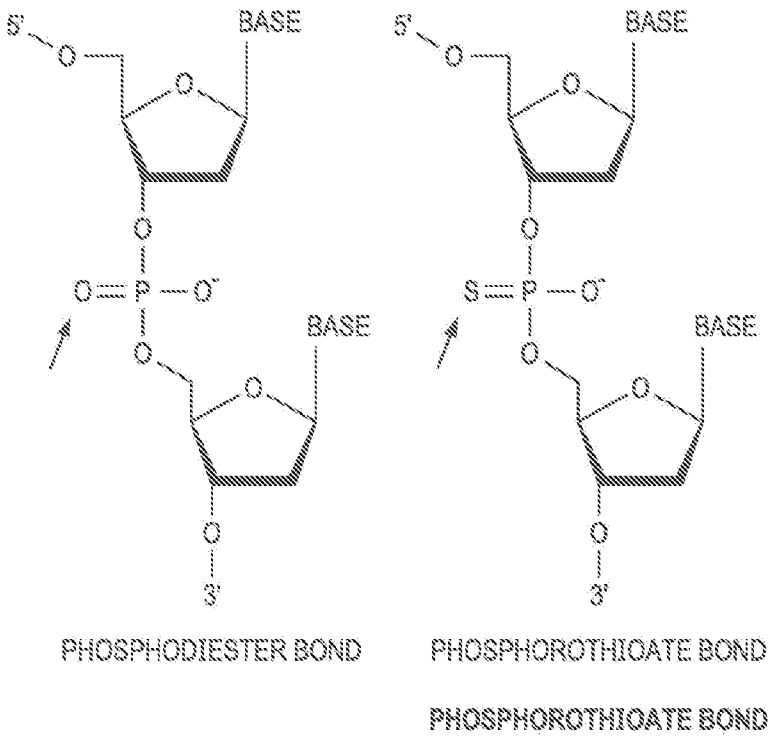
5'
BASE
O
O
O
O
P
O⁻
BASE
O
O
3'
PHOSPHODIESTER BOND
5'
BASE
O
O
O
S
P
O⁻
BASE
O
O
3'
PHOSPHOROTHIOATE BOND
PHOSPHOROTHIOATE BOND

FIG. 1A

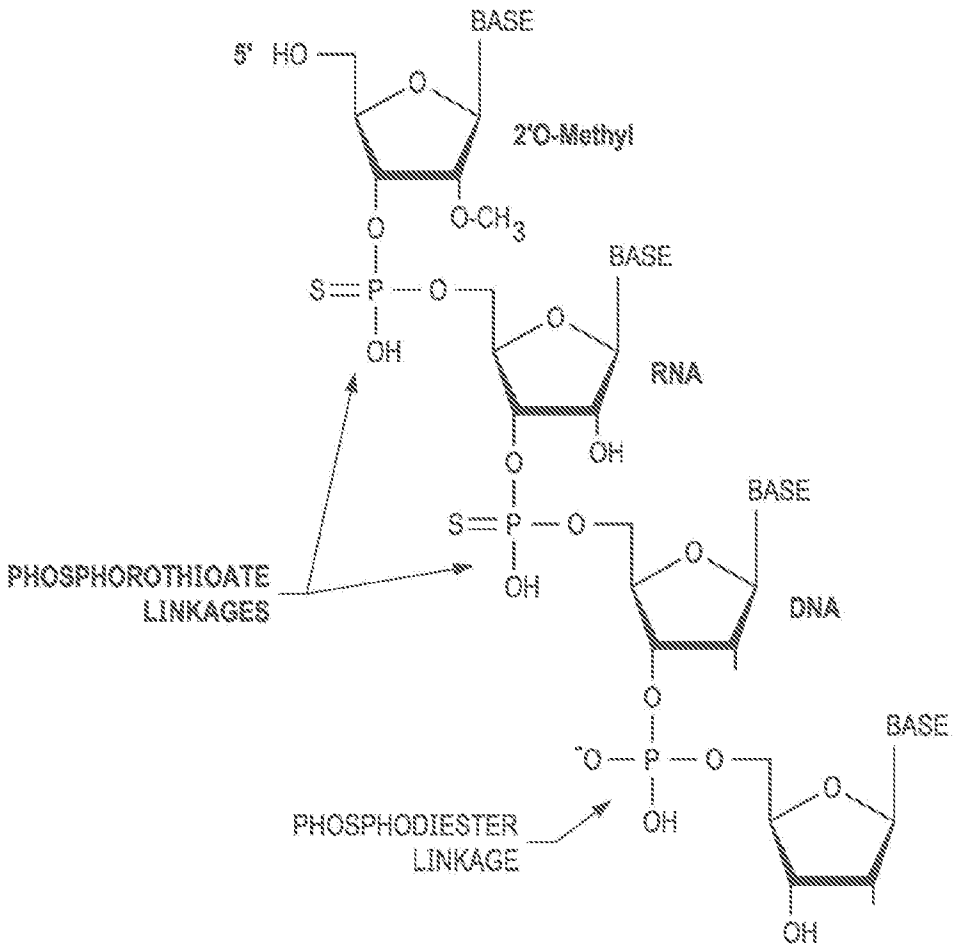
5' HO
BASE
O
2'O-Methyl
O-CH3
O
S=P—O
OH
BASE
O
RNA
OH
O
S=P—O
OH
BASE
O
DNA
O
-O—P—O
OH
BASE
O
OH
PHOSPHOROTHIOATE LINKAGES
PHOSPHODIESTER LINKAGE

FIG. 1B

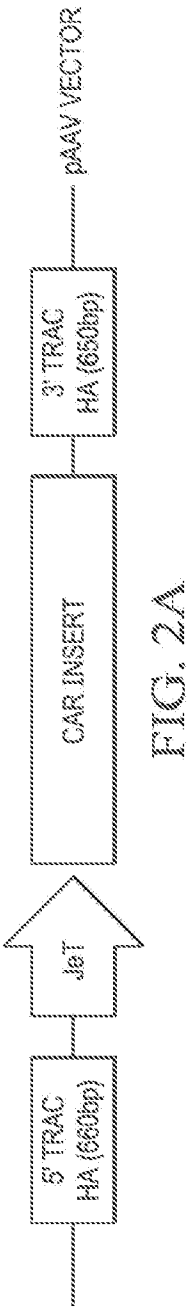
pAAV VECTOR
3' TRAC HA (650bp)
CAR INSERT
JeT
5' TRAC HA (650bp)

FIG. 2A

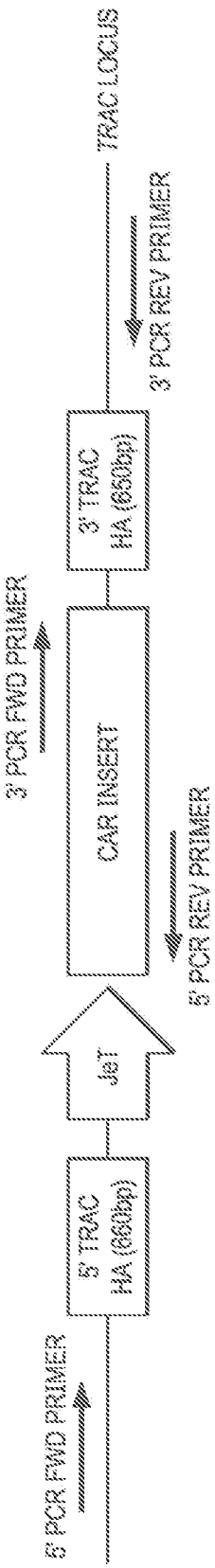
TRAC LOCUS
3' PCR REV PRIMER
3' TRAC HA (650bp)
3' PCR FWD PRIMER
CAR INSERT
5' PCR REV PRIMER
JeT
5' TRAC HA (650bp)
5' PCR FWD PRIMER

FIG. 2B

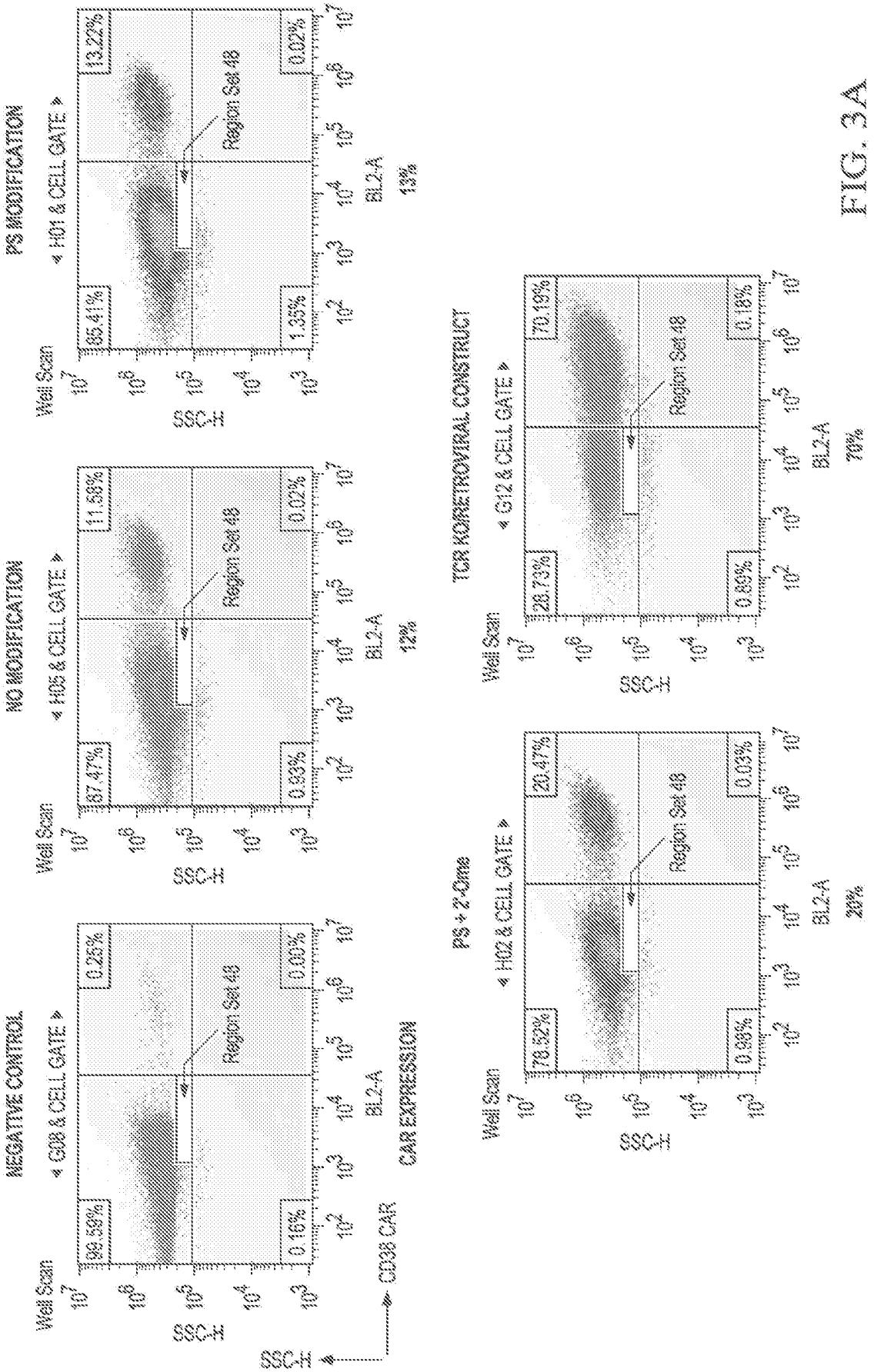
NEGATIVE CONTROL
NO MODIFICATION
PS MODIFICATION
TCR KORETROVIRAL CONSTRUCT
PS + 2'-Ome
Well Scan
G08 & CELL GATE
H05 & CELL GATE
H01 & CELL GATE
G12 & CELL GATE
H02 & CELL GATE
Region Set 48
99.59%
0.25%
0.16%
0.00%
87.47%
11.68%
0.93%
0.02%
65.41%
13.22%
1.35%
0.02%
28.73%
70.19%
0.89%
0.18%
78.52%
20.47%
0.98%
0.03%
SSC-H
BL2-A
CD38 CAR
CAR EXPRESSION
12%
13%
19%
20%

FIG. 3A

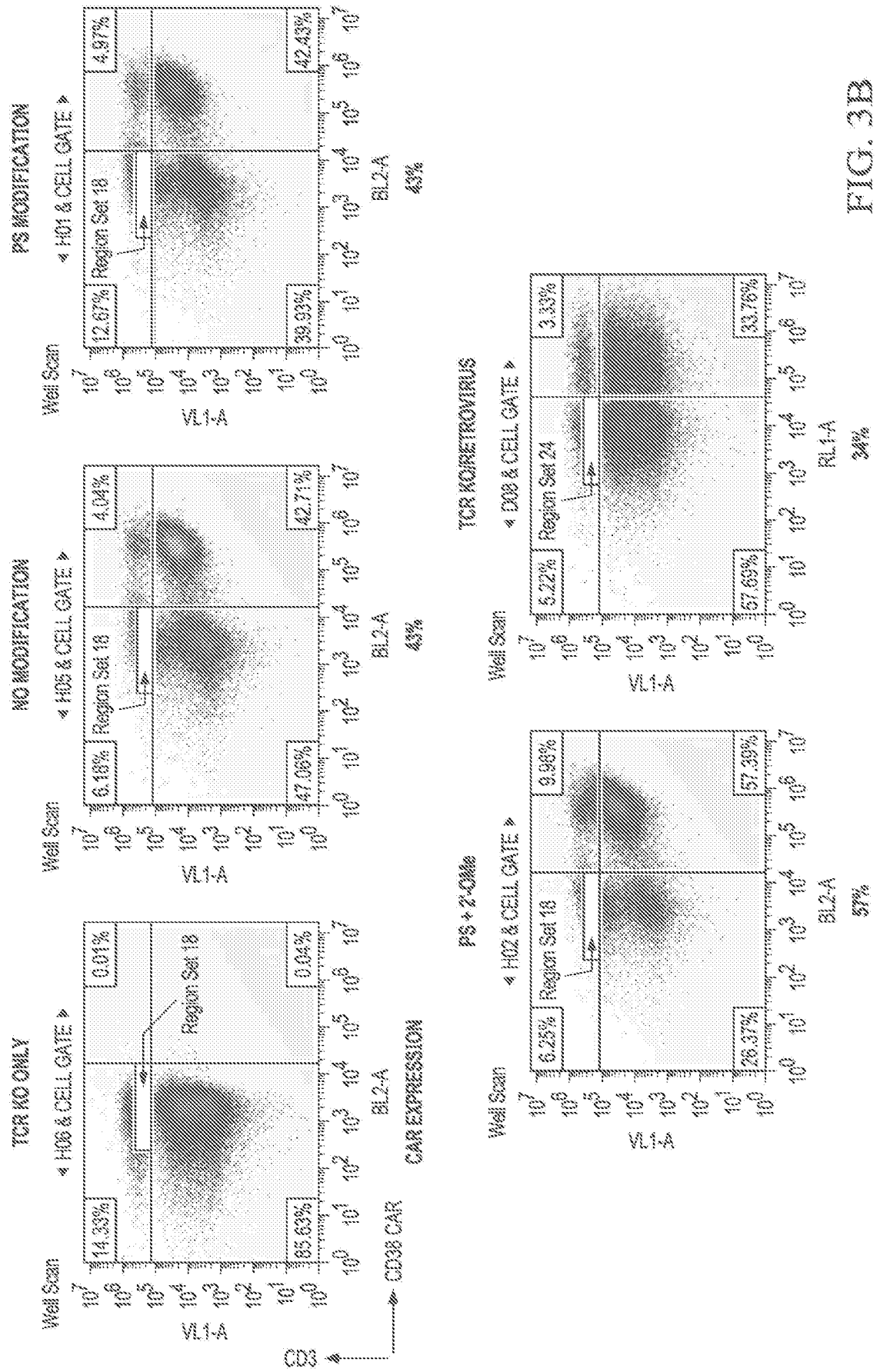
TCR KO ONLY
NO MODIFICATION
PS MODIFICATION
TCR KO/RETROVIRUS
PS + 2'-OMe
Well Scan
H06 & CELL GATE
H05 & CELL GATE
H01 & CELL GATE
H02 & CELL GATE
D08 & CELL GATE
Region Set 18
Region Set 24
14.33%
0.01%
85.63%
0.04%
6.18%
4.04%
47.06%
42.71%
12.67%
4.97%
39.93%
42.43%
6.25%
9.98%
26.37%
57.39%
5.22%
3.33%
67.69%
33.76%
VL1-A
BL2-A
RL1-A
CD3
CD38 CAR
CAR EXPRESSION
43%
43%
57%
34%

FIG. 3B

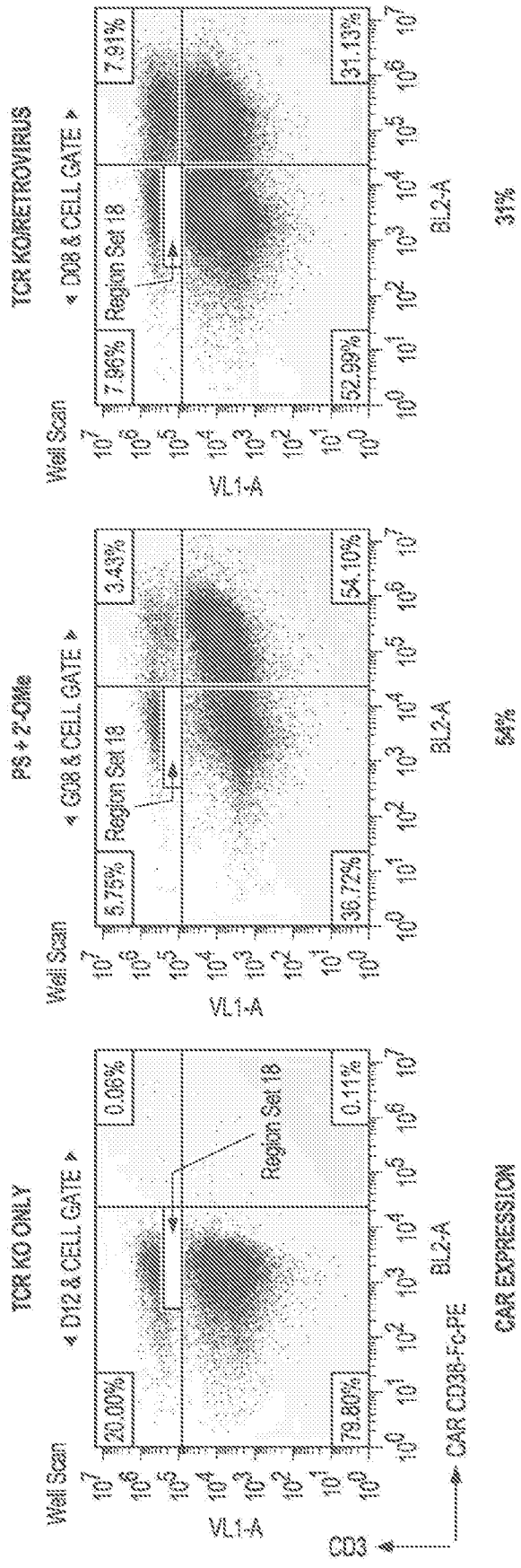
TCR KO ONLY
D12 & CELL GATE
Well Scan
20.00%
0.06%
Region Set 18
79.80%
0.11%
VL1-A
BL2-A
CD3
CAR CD38-Fc-PE
CAR EXPRESSION
PS + 2'-OMe
G08 & CELL GATE
Well Scan
8.75%
Region Set 18
3.43%
36.72%
54.10%
VL1-A
BL2-A
54%
TCR KO/RETROVIRUS
D08 & CELL GATE
Well Scan
7.86%
Region Set 18
7.91%
52.99%
31.13%
VL1-A
BL2-A
31%

FIG. 3C

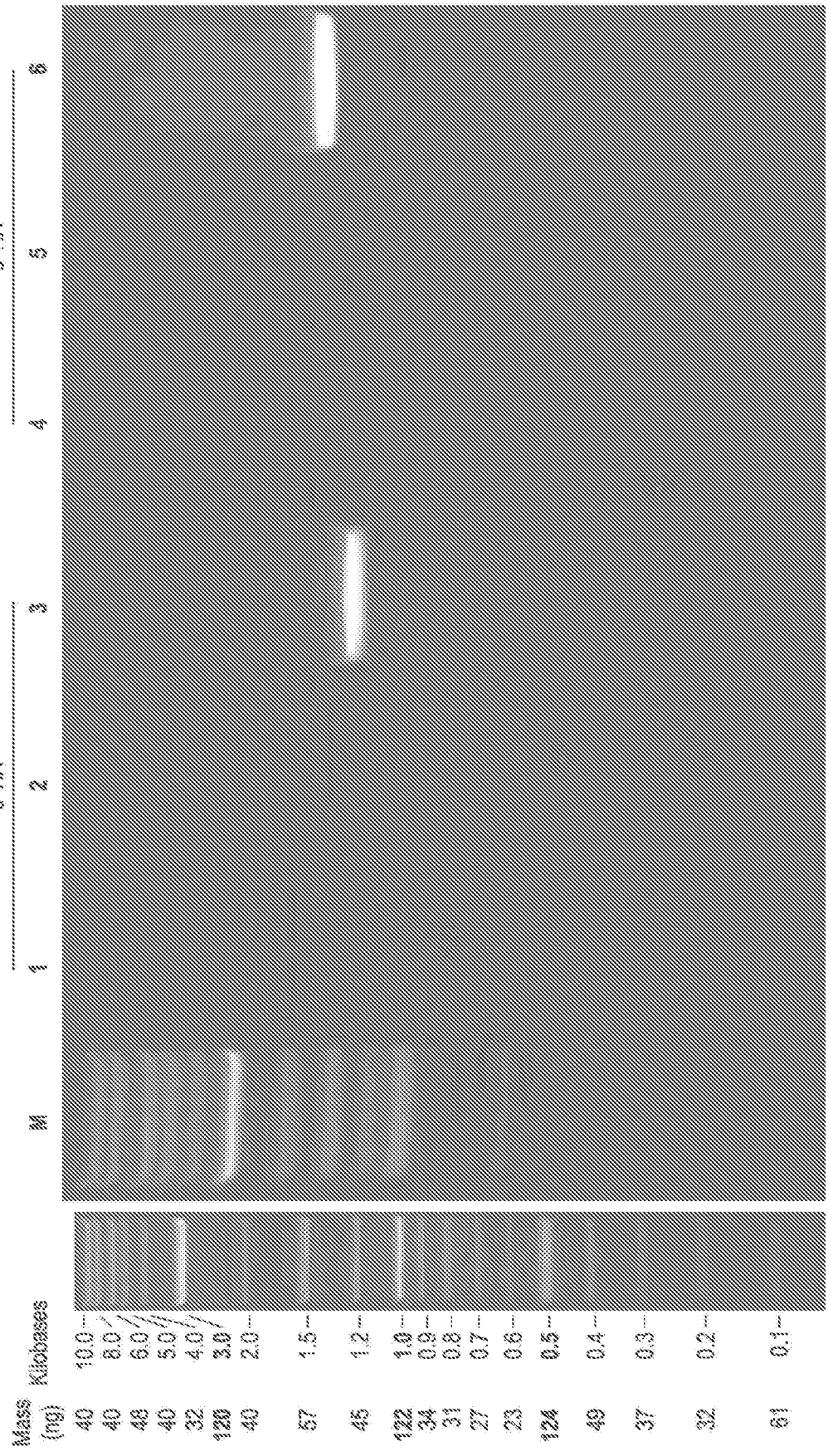
5' HA
3' HA
M
1
2
3
4
5
6
Kilobases
Mass (ng)
10.0
8.0
6.0
5.0
4.0
3.0
2.0
1.5
1.2
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
40
40
48
40
32
120
40
57
45
122
34
31
27
23
124
49
37
32
61

FIG. 4

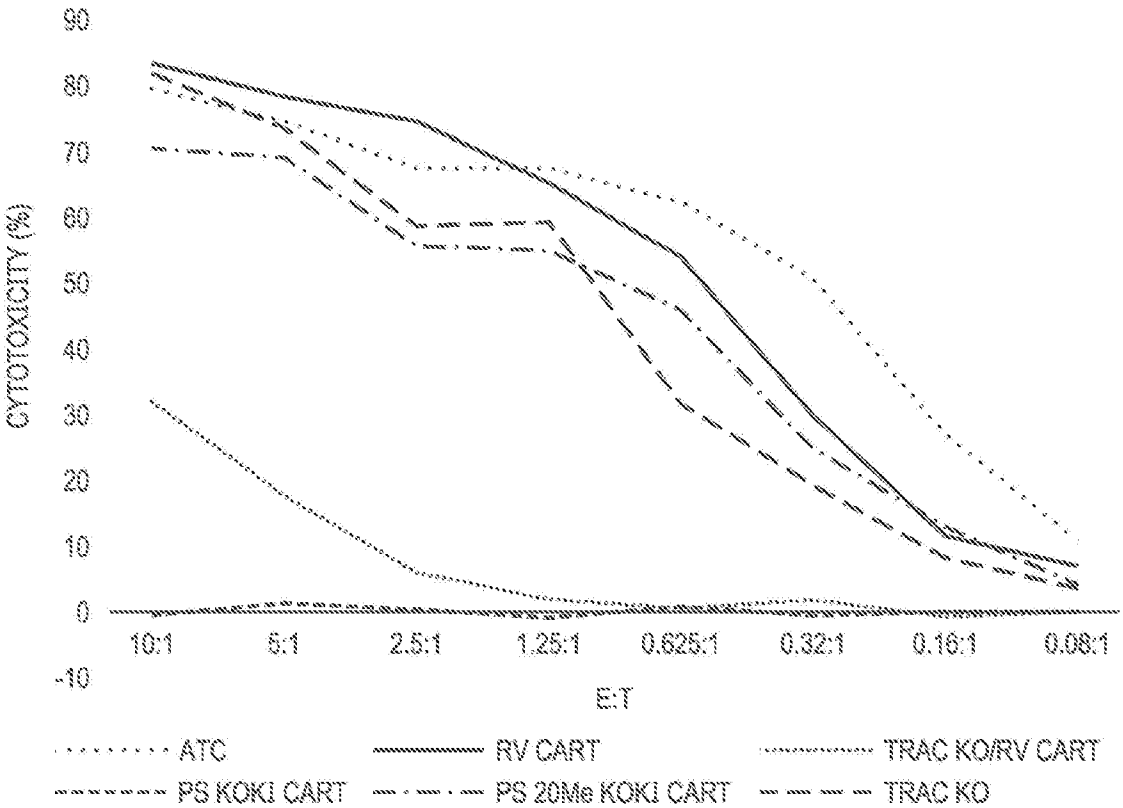
90
80
70
60
50
40
30
20
10
0
-10
CYTOTOXICITY (%)
10:1
5:1
2.5:1
1.25:1
0.625:1
0.32:1
0.16:1
0.08:1
E:T
ATC
RV CART
TRAC KO/RV CART
PS KOKI CART
PS 20Me KOKI CART
TRAC KO

FIG. 5

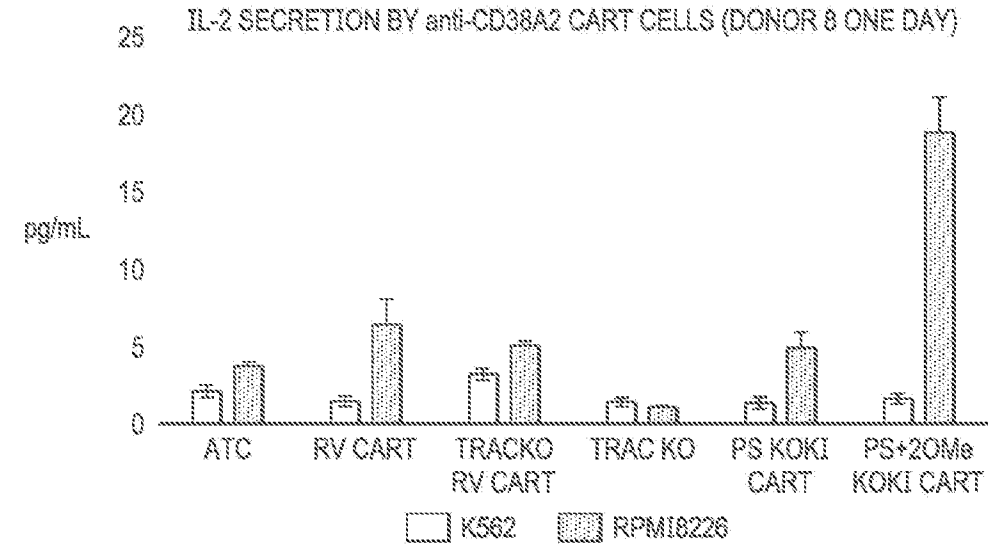


FIG. 6A

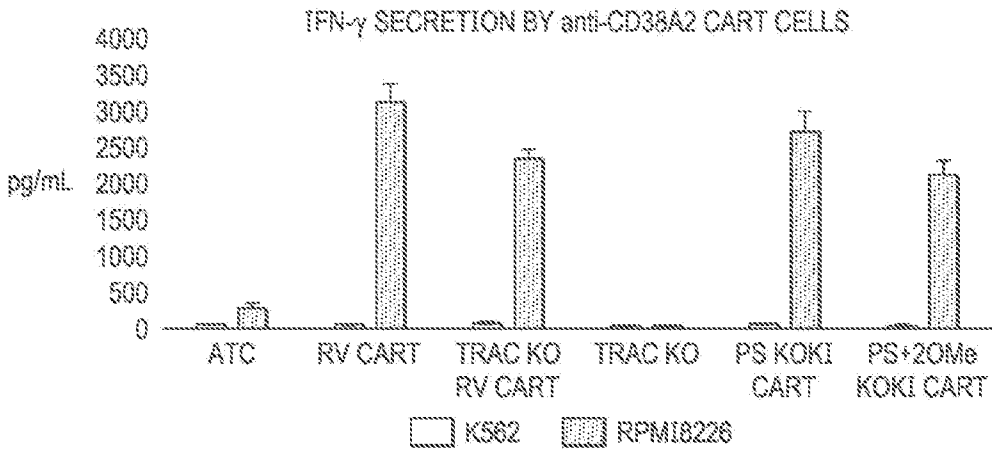


FIG. 6B

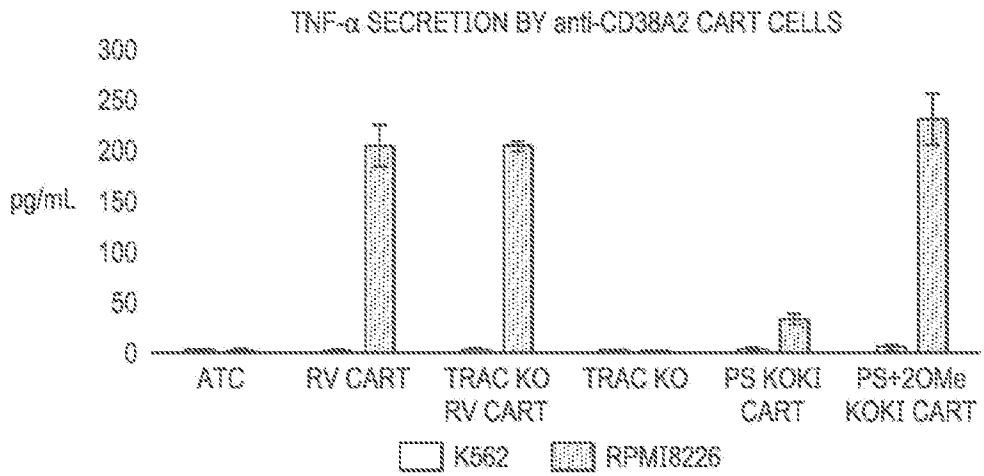


FIG. 6C

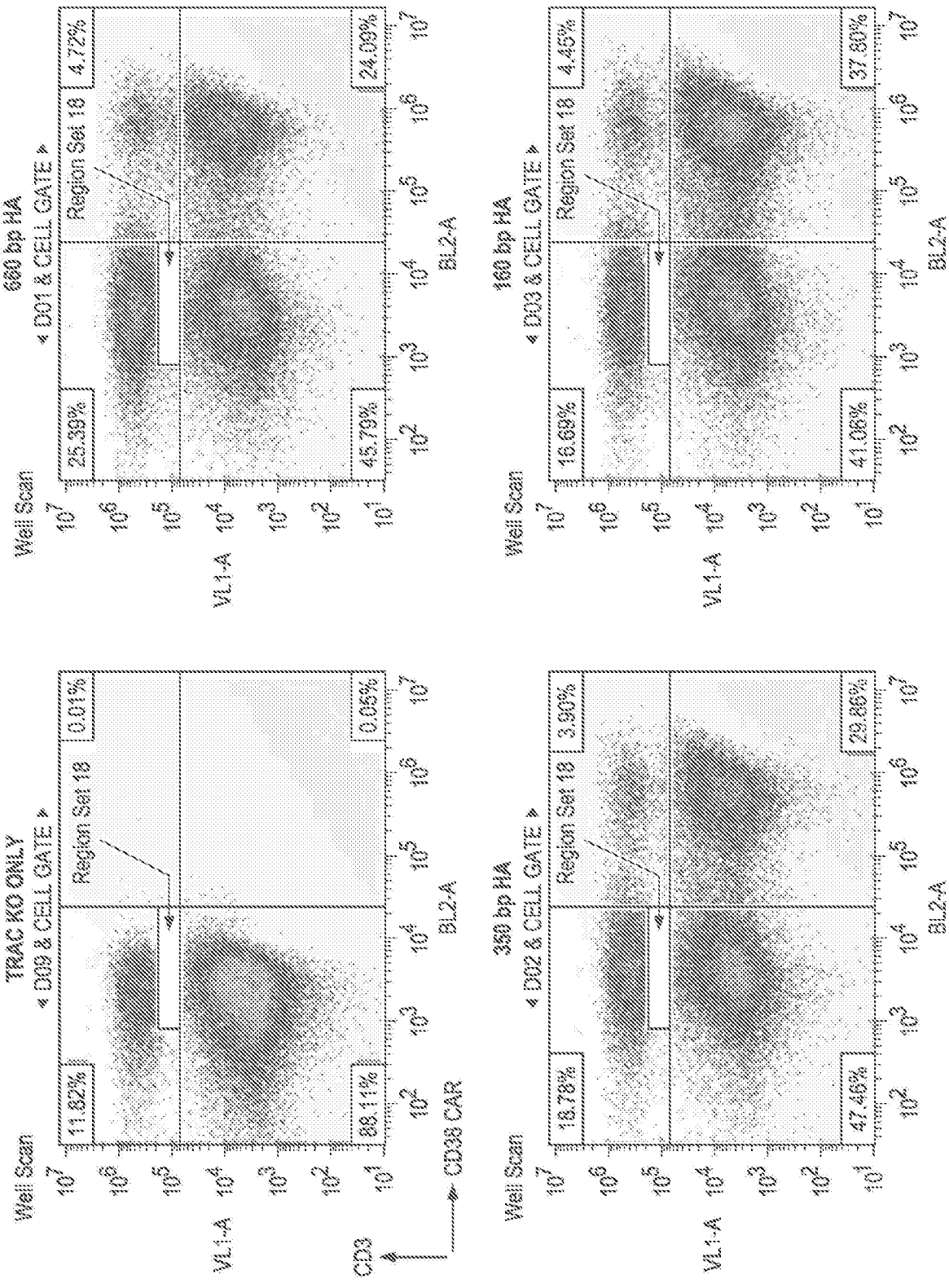
TRAC KO ONLY
D09 & CELL GATE
Well Scan
Region Set 18
11.82%
0.01%
88.11%
0.06%
VL1-A
CD3
BL2-A
CD38 CAR
660 bp HA
D01 & CELL GATE
25.39%
4.72%
45.79%
24.09%
160 bp HA
D03 & CELL GATE
16.69%
4.45%
41.06%
37.80%
350 bp HA
D02 & CELL GATE
18.78%
3.90%
47.46%
29.86%

FIG. 7

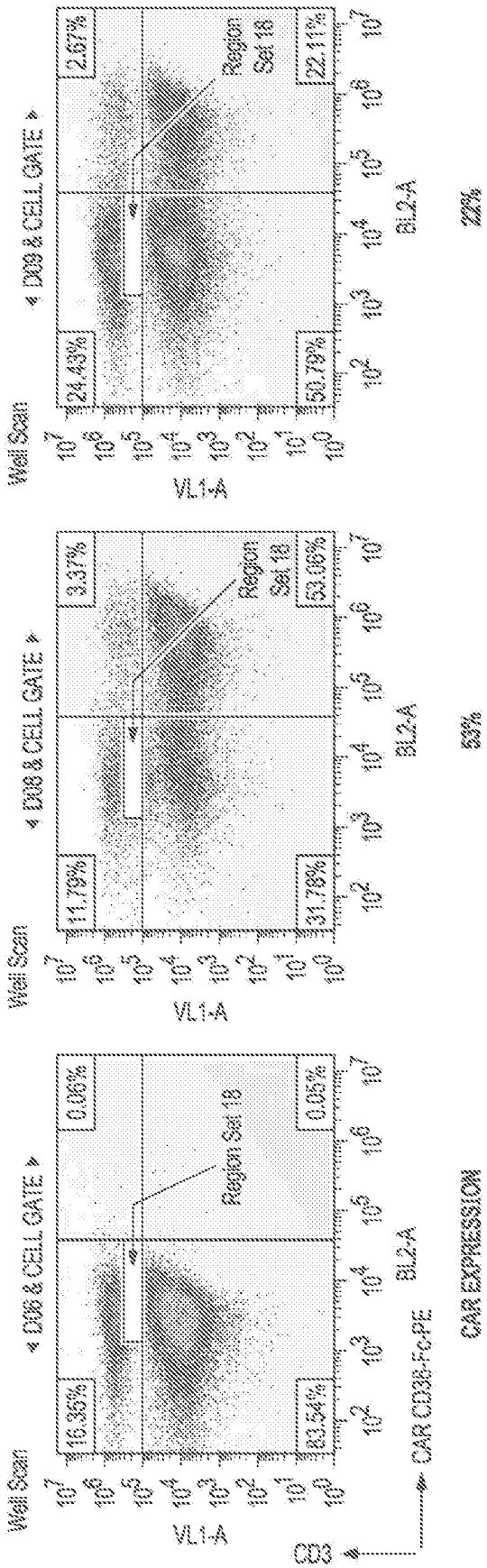
TRAC KO ONLY
D06 & CELL GATE
Well Scan
VL1-A
CD3
CAR CD38-Fc-PE
BL2-A
16.35%
0.06%
83.54%
0.05%
Region Set 18
53%
PS+ZOMe MODIFIED DONOR CART
D08 & CELL GATE
11.79%
3.37%
31.78%
53.06%
Region Set 18
NON-MODIFIED DONOR CART
D09 & CELL GATE
24.43%
2.67%
50.79%
22.11%
Region Set 18
22%
CAR EXPRESSION

FIG. 8

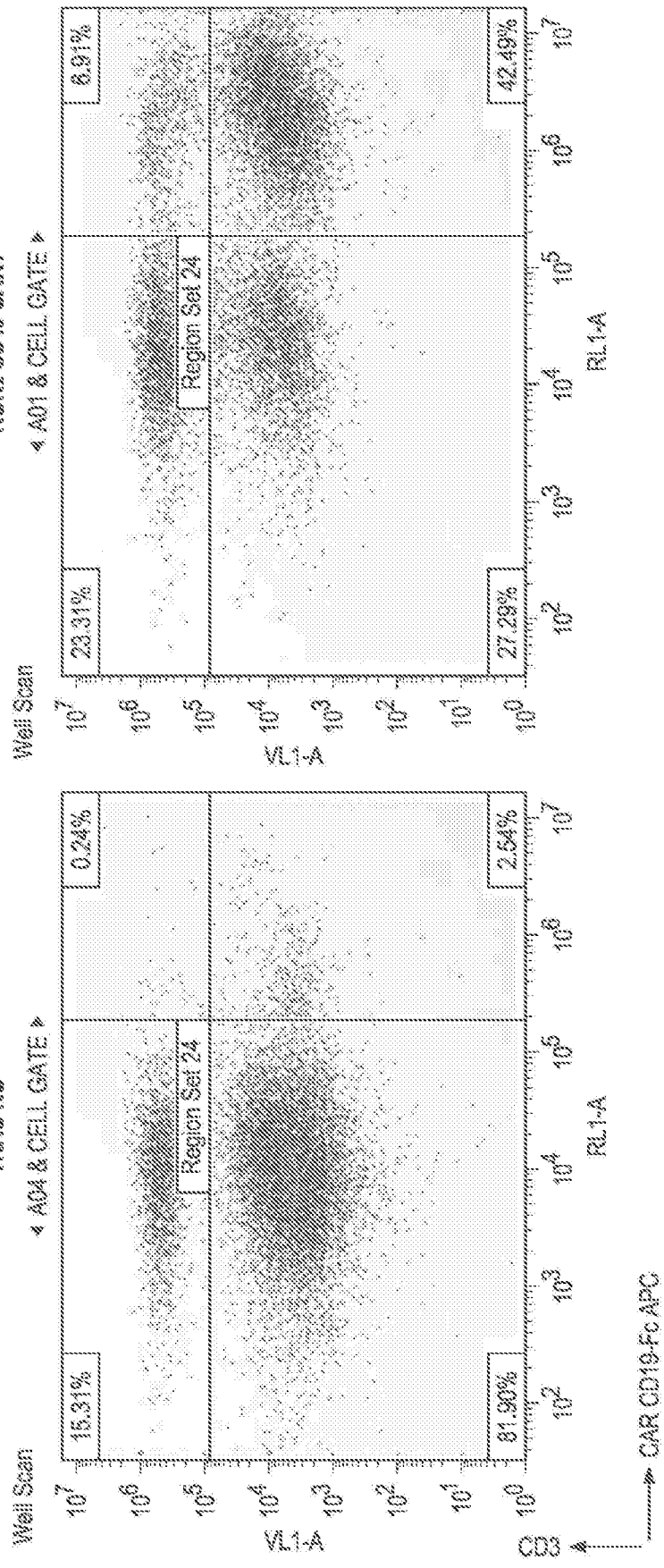
KOK1 CD19 CART
A01 & CELL GATE
Well Scan
Region Set 24
23.31%
6.91%
27.29%
42.49%
VL1-A
RL1-A
TRAC KO
A04 & CELL GATE
Well Scan
Region Set 24
15.31%
0.24%
81.90%
2.54%
VL1-A
RL1-A
CD3
CAR CD19-Fc APC

FIG. 9

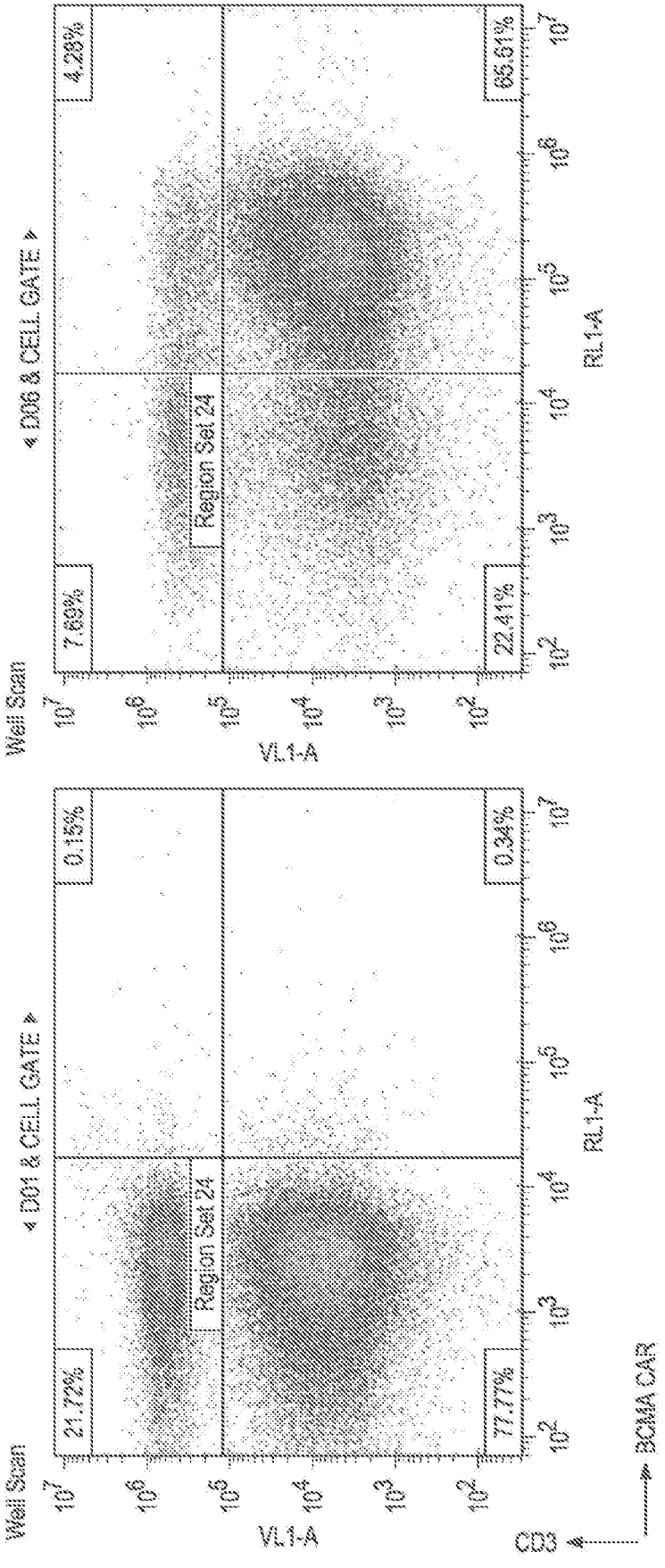
TRAC KO
D01 & CELL GATE
Well Scan
21.72%
0.15%
77.77%
0.34%
Region Set 24
VL1-A
RL1-A
CD3
BCMA CAR
BCMA CART
D06 & CELL GATE
Well Scan
7.69%
4.28%
22.41%
65.61%
Region Set 24
VL1-A
RL1-A

FIG. 10

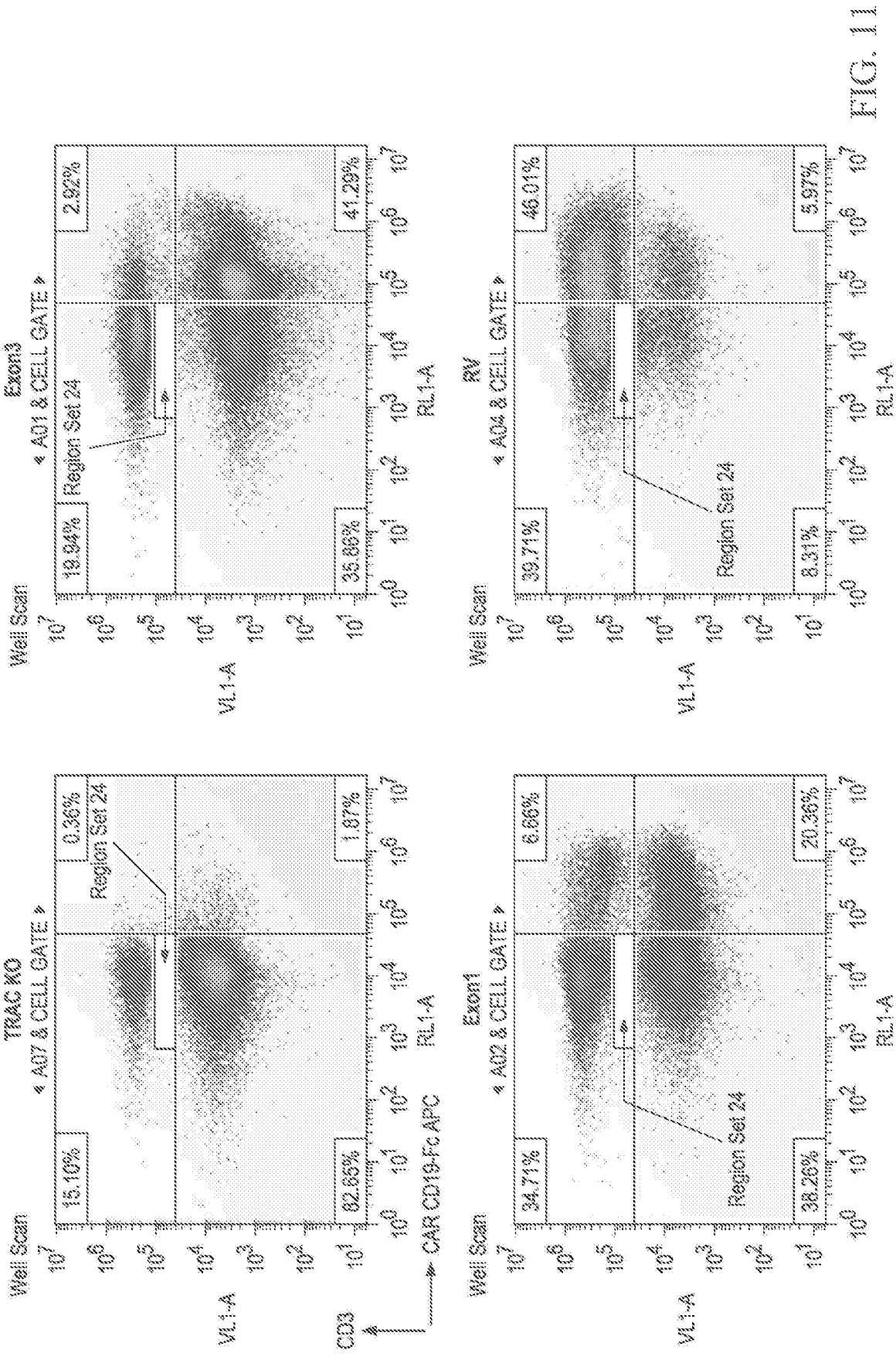
TRAC KO
A07 & CELL GATE
Well Scan
15.10%
0.36%
Region Set 24
82.65%
1.87%
VL1-A
CD3
CAR CD19-Fc APC
RL1-A
Exon3
A01 & CELL GATE
19.94%
Region Set 24
2.92%
35.86%
41.29%
RV
A04 & CELL GATE
39.71%
46.01%
8.31%
5.97%
Exon1
A02 & CELL GATE
34.71%
6.66%
38.26%
20.36%

FIG. 11

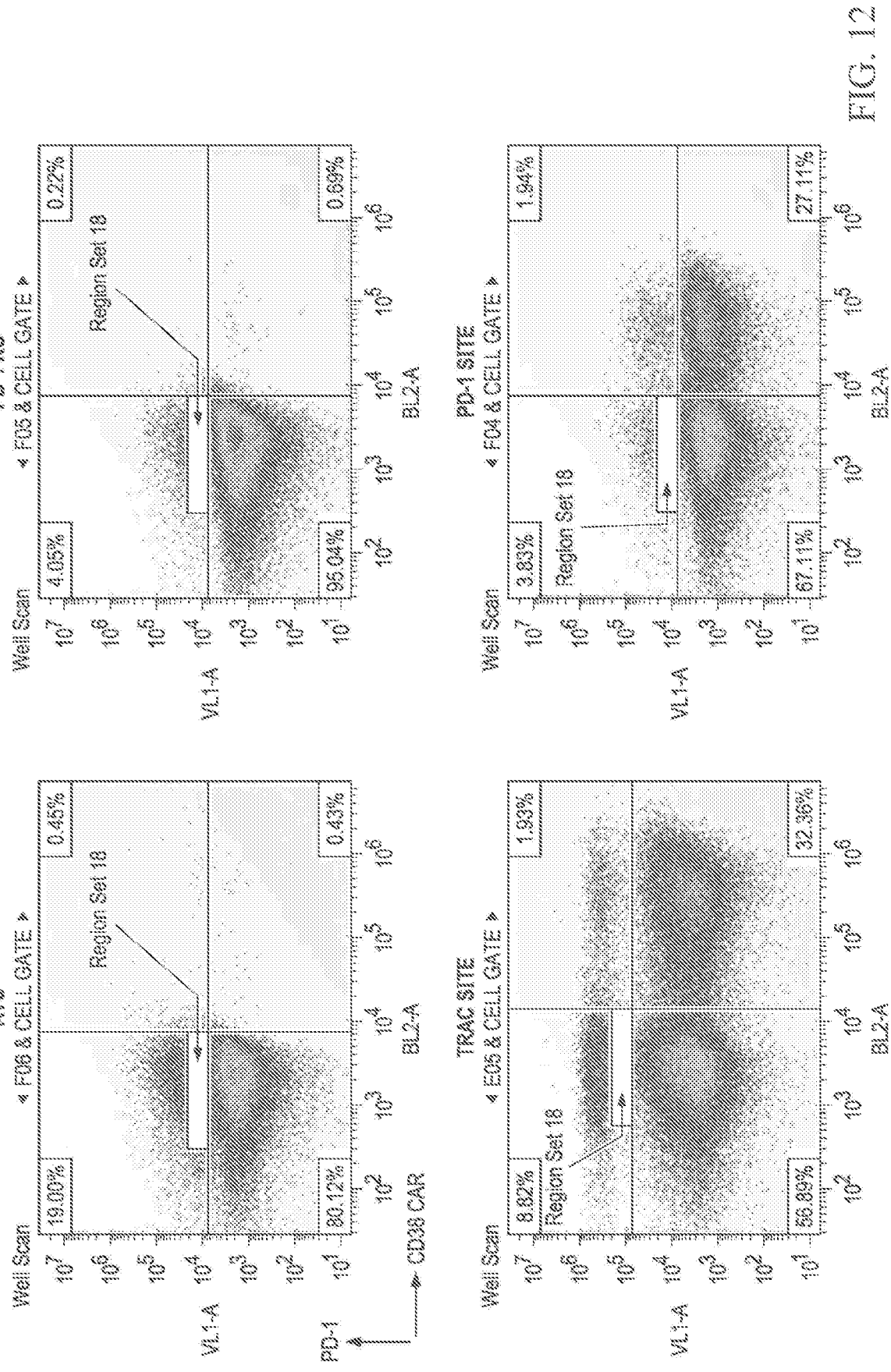
ATC
F06 & CELL GATE
Well Scan
Region Set 18
19.00%
0.45%
80.12%
0.43%
VL1-A
PD-1
CD38 CAR
BL2-A
PD-1 KO
F05 & CELL GATE
Well Scan
Region Set 18
4.05%
0.22%
95.04%
0.69%
VL1-A
BL2-A
TRAC SITE
E05 & CELL GATE
Well Scan
Region Set 18
8.82%
1.93%
56.89%
32.36%
VL1-A
BL2-A
PD-1 SITE
F04 & CELL GATE
Well Scan
Region Set 18
3.83%
1.94%
67.11%
27.11%
VL1-A
BL2-A

FIG. 12

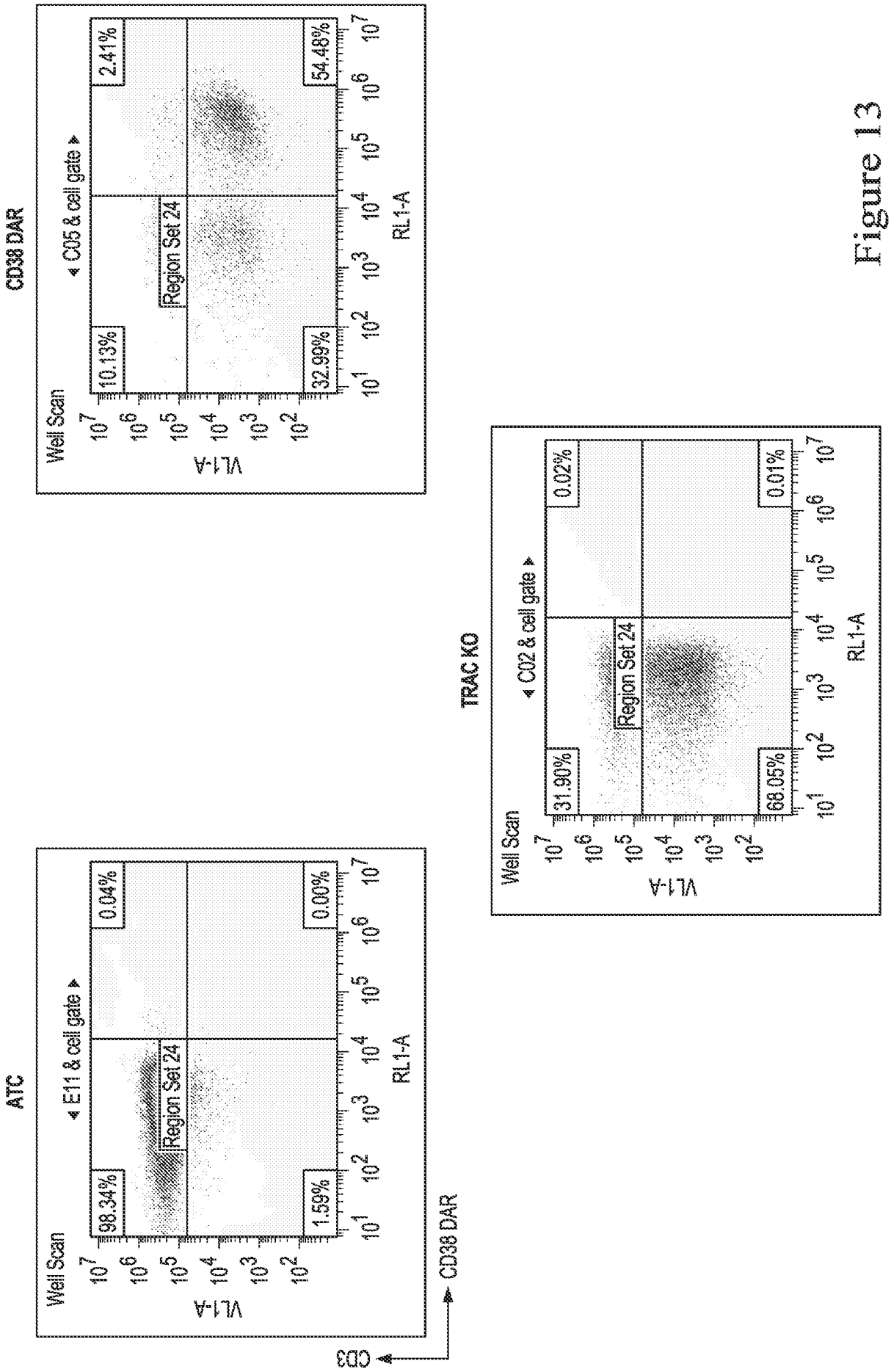
ATC
CD38 DAR
TRAC KO
Well Scan
E11 & cell gate
C05 & cell gate
C02 & cell gate
Region Set 24
98.34%
1.59%
0.04%
0.00%
10.13%
32.99%
2.41%
54.48%
31.90%
68.05%
0.02%
0.01%
VL1-A
RL1-A
CD3
CD38 DAR

Figure 13

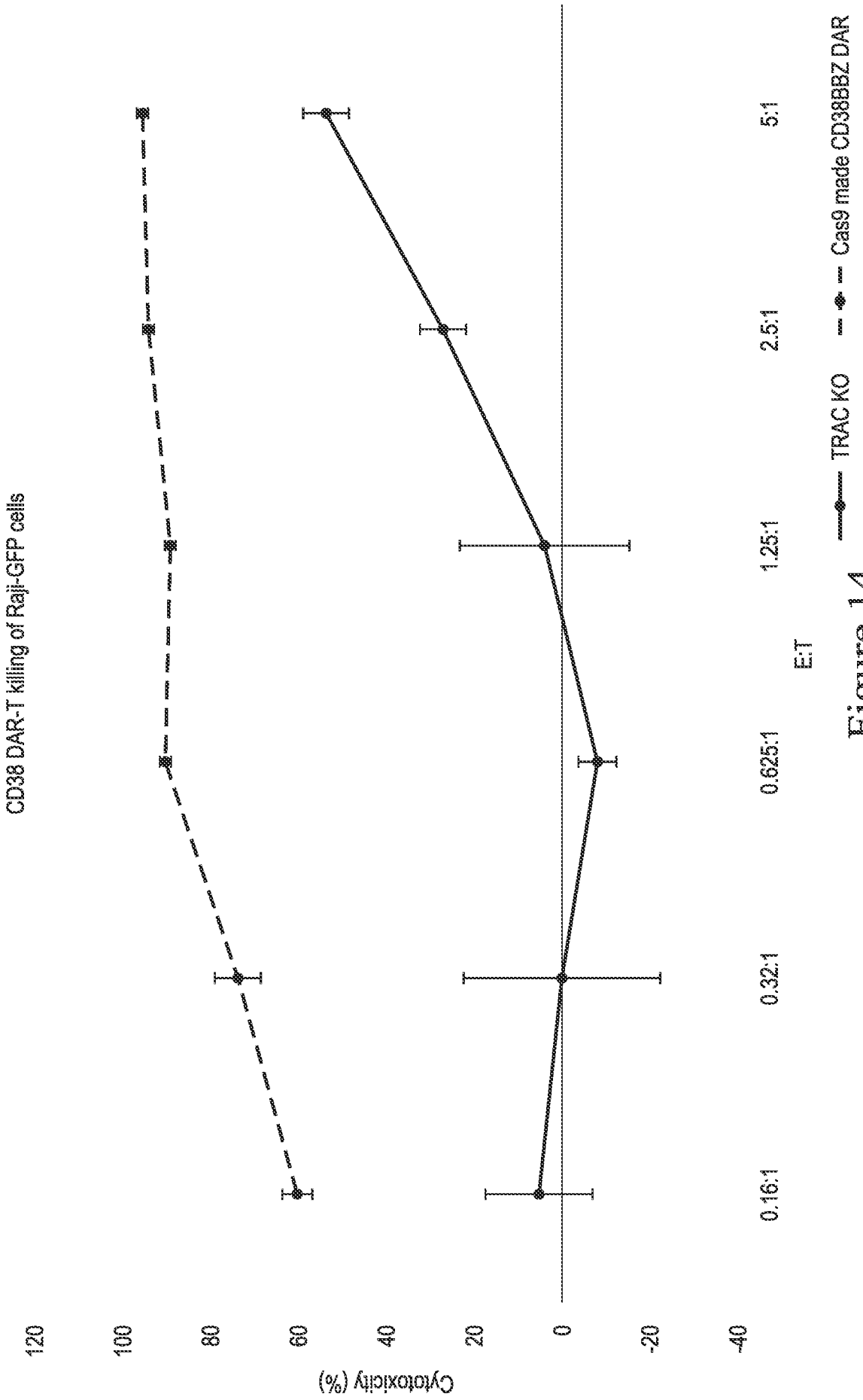
CD38 DAR-T killing of Raji-GFP cells
Cytotoxicity (%)
120
100
80
60
40
20
0
-20
-40
0.16:1
0.32:1
0.625:1
1.25:1
2.5:1
5:1
E:T
TRAC KO
Cas9 made CD38BBZ DAR

Figure 14

# INTERNATIONAL SEARCH REPORT

International application No
PCT/US2021/041790

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/90 C12N15/11 C12N5/10 C12N5/0783 A61K39/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 2019/097305 A2 (CRISPR THERAPEUTICS AG [CH]) 23 May 2019 (2019-05-23) | 1-8, 10-13, 17,24 |
| Y | claims 1-21, 32, 66; examples 1, 9-13 | 9,14-16, 18-23 |
| | ----- | |
| X | WO 2020/041387 A1 (BRIGHAM & WOMENS HOSPITAL INC [US]) 27 February 2020 (2020-02-27) | 1-8, 10-13, 17,24 |
| Y | paragraph [0235] - paragraph [0239] | 9,14-16, 18-23 |
| | ----- | |
| | -/-- | |

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 15 November 2021 | 25/11/2021 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040,<br>Fax: (+31-70) 340-3016 | Deleu, Laurent |

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International application No
PCT/US2021/041790

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International application No.
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**Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [ ] forming part of the international application as filed:

      [ ] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

   b. [ ] furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

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2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

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