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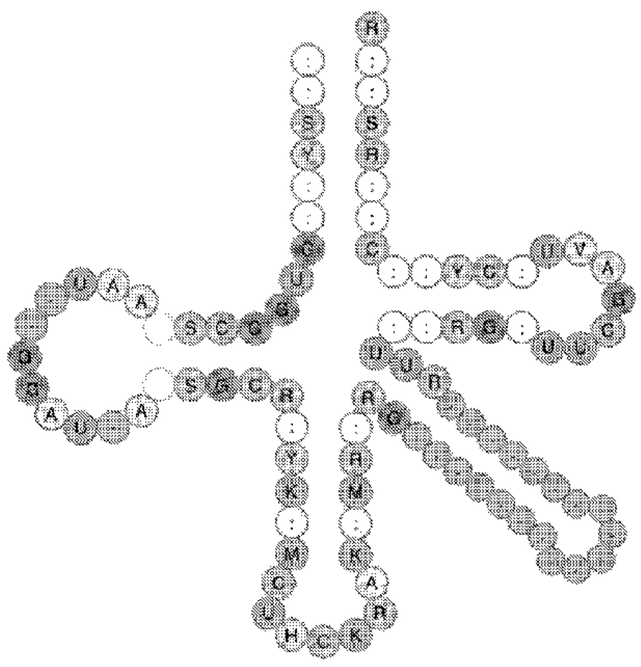
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(54) Title: TARGETED TRANSFER RNAS FOR TREATMENT OF DISEASES



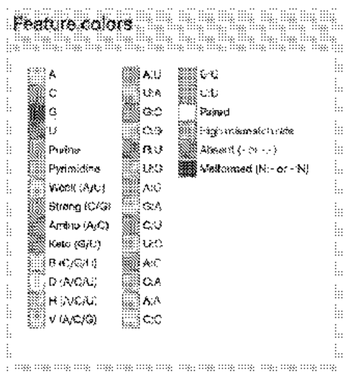


FIG. 2A

(57) Abstract: Provided are engineered tRNA molecules and vectors encoding engineered suppressor tRNA molecules that recognize and readthrough of disease-causing premature stop codons. Pharmaceutical formulations of the engineered tRNA or vectors encoding the engineered tRNA are provided for use in treating diseases associated with truncation of a protein caused by premature stop codons present in the nucleic acid sequence encoding the protein.

[Continued on next page]

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Declarations under Rule 4.17:

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TARGETED TRANSFER RNAS FOR TREATMENT OF DISEASES

CROSS-REFERENCE

[1] This application claims the benefit of U.S. Provisional Application Nos. 62/942690 filed December 2, 2019, 62/942667 filed December 2, 2019, 63/010856 filed April 16, 2020, and 63/111856 filed November 10, 2020, which applications are all incorporated herein by reference in their entireties.

INCORPORATION BY REFERENCE OF SEQUENCE LISTING

[2] The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing is provided as a file entitled 54761-709_601_SL.txt, created November 30, 2020, which is 86,501 bytes in size. The information in the electronic format of the Sequence Listing is incorporated by reference in its entirety.

SUMMARY

[3] Disclosed herein, in some embodiments, are compositions comprising: an engineered tRNA variant comprising a mutation in a T-loop, a T-stem, a D-loop, a D-stem, a variable loop, an anticodon stem, or an anticodon loop relative to the nucleic acid sequence of any one of SEQ ID NOs: 3-22 or 103-122, wherein upon administration to a subject, the engineered tRNA variant is capable of restoring production of at least 10% of a substantially full-length polypeptide by suppression of a premature stop codon in a target mRNA encoding for the substantially full-length polypeptide, relative to a comparable polypeptide produced using a comparable mRNA that lacks the premature stop codon, as optionally determined by: transfecting a first vector encoding the engineered tRNA or variant thereof and a second vector encoding a screening mRNA encoding a first green fluorescent protein into a first human cell, wherein the screening mRNA encoding the first green fluorescent protein comprises the premature stop codon; transfecting a third vector encoding a comparable screening mRNA encoding a second green fluorescent protein into a second human cell, wherein the comparable screening mRNA does not comprise the premature stop codon; and comparing an amount of fluorescence emitted from the first human cell and the second human cell. In some embodiments, the engineered tRNA variant can comprise at least 70% sequence identity to any one of SEQ ID NOs: 23-48 or 123-148. In some embodiments, the engineered tRNA variant can comprise a sequence that is at least 70% identical to SEQ ID NO: 6 or 106, and can have a substitution at position 2, 4, 6, 12, 23, 27, 28,

31, 39, 40, 42, 43, 44, 46, 49, 50, 64, 65, 67, 69, or 71, of SEQ ID NO: 6 or 106. In some embodiments, the substitution at position 2 can be to a C, the substitution at position 4 can be to a C, the substitution at position 6 can be to a T, the substitution at position 6 can be to an A, the substitution at position 12 can be to a C, the substitution at position 23 can be to a G, the substitution at position 27 can be to a C, the substitution at position 28 can be to a C, the substitution at position 31 can be to a C, the substitution at position 39 can be to a G, the substitution at position 40 can be to a C, the substitution at position 42 can be to a G, the substitution at position 43 can be to a G, the substitution at position 44 can be to a G, the substitution at position 46 can be to an A, the substitution at position 49 can be to a G, the substitution at position 50 can be to a T, the substitution at position 64 can be to an A, the substitution at position 65 can be to a C, the substitution at position 67 can be to an A, the substitution at position 67 can be to a T, the substitution at position 69 can be to a G, the substitution at position 71 can be to a C, or the substitution at position 71 can be to a G. In some embodiments, the sequence of the engineered tRNA variant can be identical to SEQ ID NO: 6 or 106, except for the substitution. In some embodiments, the engineered tRNA variant can exhibit an increased stability in vivo, as compared with a comparable tRNA comprising the sequence provided in SEQ ID NO: 6 or 106, as determined by a proxy measurement, a half-life measurement, an amino acid charging efficiency measurement, or a measurement of binding to a synthetase or ribosomal machinery. In some embodiments, the engineered tRNA variant can comprise the sequence of SEQ ID NO: 45 or 145. In some embodiments, the engineered tRNA variant can comprise a sequence that is at least 70% identical to any one of SEQ ID NO: 3 or 103, and can comprise a substitution at position 2, 6, 13, 15, 22, 28, 31, 37, 39, 42, 44, 50, 64, 67, 71, or 72, of SEQ ID NO: 3 or 103. In some embodiments, the substitution at position 2 can be to a G, the substitution at position 6 can be to a G, the substitution at position 13 can be to a C, the substitution at position 15 can be to a G, the substitution at position 22 can be to a G, the substitution at position 28 can be to a C, the substitution at position 31 can be to an A, the substitution at position 37 can be to a G, the substitution at position 39 can be to a T, the substitution at position 42 can be to a G, the substitution at position 44 can be to an A, the substitution at position 50 can be to a C, the substitution at position 64 can be to a G, the substitution at position 67 can be to a C, the substitution at position 71 can be to a C, or the substitution at position 72 can be to a C. In some embodiments, the sequence of the engineered

tRNA variant can be identical to SEQ ID NO: 3 or 103, except for the substitution. In some embodiments, the engineered tRNA variant can exhibit an increased stability in vivo, as compared with a comparable tRNA comprising the sequence provided in SEQ ID NO: 3 or 103, as determined by a proxy measurement, a half-life measurement, an amino acid charging efficiency measurement, or a measurement of binding to a synthetase or ribosomal machinery. In some embodiments, the engineered tRNA variant can comprise the sequence of SEQ ID NO: 32 or 132. In some embodiments, the engineered tRNA variant can comprise a sequence that is at least 70% identical to SEQ ID NO: 5 or 105 and can have a substitution at position 73 of SEQ ID NO: 5 or 105. In some embodiments, the substitution at position 73 can be to a G. In some embodiments, the sequence of the engineered tRNA variant can be identical to SEQ ID NO: 5 or 105, except for the substitution. In some embodiments, the engineered tRNA variant can exhibit an increased stability in vivo, as compared with a comparable tRNA comprising the sequence provided in SEQ ID NO: 5 or 105, as determined by a proxy measurement, a half-life measurement, an amino acid charging efficiency measurement, or a measurement of binding to a synthetase or ribosomal machinery. In some embodiments, the engineered tRNA variant can be acylated with an amino acid comprising lysine, arginine, histidine, glycine, alanine, valine, leucine, isoleucine, methionine, phenylalanine, tryptophan, proline, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartate, glutamate, pyrolysine, or selenocysteine, or can be acylated with a non-canonical amino acid. In some embodiments, the premature stop codon can be an opal stop codon, an ochre stop codon, or an amber stop codon. In some embodiments, the polypeptide can comprise an MeCP2 polypeptide, a FoxG1 polypeptide, a CDKL5 polypeptide, a MYH9 polypeptide, a COL11A2 polypeptide, or a MYO7A polypeptide. In some embodiments, the engineered tRNA variant is capable of restoring production of at least 20% of the substantially full-length polypeptide. In some embodiments, the engineered tRNA variant is capable of restoring production of at least 40% of the substantially full-length polypeptide. In some embodiments, the engineered tRNA variant is capable of restoring production of at least 60% of the substantially full-length polypeptide. In some embodiments, the engineered tRNA variant is capable of restoring production of at least 80% of the substantially full-length polypeptide. In some embodiments, the composition can comprise a polynucleotide encoding the engineered tRNA variant. In some embodiments, the polynucleotide can be in a viral vector. In some embodiments, the viral vector can be an adenoviral vector, an adeno-associated viral

(AAV) vector, or a lentiviral vector. In some embodiments, the AAV vector comprises an AAV 2/5 vector, an AAV 2/6 vector, an AAV 2/7 vector, an AAV2/8 vector, or an AAV 2/9 vector. In some embodiments, the AAV can comprise a capsid from AAV5. In some embodiments, the polynucleotide can comprise an inverted terminal repeat (ITR) sequence from AAV2. In some embodiments, the polynucleotide can comprise an inverted terminal repeat with a mutated terminal resolution site and lacking terminal nucleotides. In some embodiments, the terminal nucleotides can comprise at least a portion of the A region, at least a portion of the D region, or both. In some embodiments, the polynucleotide can be self-complementary. In some embodiments, the polynucleotide can encode two or more, three or more, four or more, five or more, or six or more copies of the same engineered tRNA variant. In some embodiments, the polynucleotide can encode two or more, three or more, four or more, five or more, or six or more copies of different engineered tRNA variants. In some embodiments, the polynucleotide can comprise a stuffer sequence comprising at least about 50 nucleotides, at least about 100 nucleotides, at least about 150 nucleotides, or at least about 200 nucleotides. In some embodiments, the stuffer sequence can be 3' or 5' of one or more copies of the engineered tRNA variant within the polynucleotide. In some embodiments, the stuffer sequence can separate two or more copies of the engineered tRNA variant within the polynucleotide. In some embodiments, the engineered tRNA variant or the polynucleotide encoding the engineered tRNA can be present in a delivery system. In some embodiments, the delivery system can comprise a liposome, a charged polymer, an uncharged polymer, a nanoparticle, a surfactant, a penetrating enhancer, a gene transfer agent, a phospholipid, a micelle, a synthetic vector, a macromolecule, a dendrimer, a biopolymer, a viral particle, or any combination thereof. In some embodiments, the polypeptide can comprise an MeCP2 polypeptide. In some embodiments, the premature stop codon can result from a mutation encoding for an R at amino acid position 168, 255, 270, 294, 198, 186, 453, 8 (e.g., in isoform 2), 9 (e.g., in isoform 1), 84, 85, 89, 91, 106, 111, 115, 133, 162, 167, 188, 190, 211, 250, 253, 268, 306, 309, 344, 354, 420, 458, 468, 471, 478, or 484, of the MeCP2 polypeptide. Disclosed herein, in some embodiments, are pharmaceutical compositions comprising the composition and a pharmaceutically acceptable excipient, carrier or diluent. In some embodiments, the pharmaceutical composition can comprise a dose unit form. Disclosed herein, in some embodiments, are kits comprising the composition or pharmaceutical composition, and a packaging or container. Disclosed herein, in some embodiments, are methods

of making the kit comprising contacting the composition or pharmaceutical composition with the packaging or container. Disclosed herein, in some embodiments, are methods of treatment or prevention of a disease or disorder, comprising: administering to a subject in need thereof the composition.

[4] Disclosed herein, in some embodiments, are methods of treating or preventing a disease or condition in a subject in need thereof, comprising: administering to the subject an engineered tRNA or variant thereof or a polynucleotide encoding the engineered tRNA or variant thereof; and producing a substantially full-length polypeptide in vivo at an efficiency of at least about 10%, relative to a comparable polypeptide produced using an mRNA that lacks a premature stop codon, wherein the engineered tRNA or the variant thereof is capable of reading through the premature stop codon in a target mRNA encoding for the substantially full-length polypeptide. Disclosed herein, in some embodiments, are methods of treating or preventing a disease or condition in a subject in need thereof, comprising: administering to the subject an engineered tRNA or variant thereof or a polynucleotide encoding the engineered tRNA or variant thereof, thereby at least partially treating the disease or condition in the subject; wherein the engineered tRNA or variant thereof recognizes a premature stop codon in a target mRNA encoding a polypeptide, wherein the engineered tRNA or variant thereof during translation of the target mRNA can at least partially transform interpretation of the premature stop codon into a sense codon and produce a substantially full-length polypeptide in vivo at an efficiency of at least about 10%, relative to a comparable polypeptide produced using a comparable mRNA that lacks the premature stop codon, as optionally determined by: transfecting a first vector encoding the engineered tRNA or variant thereof and a second vector encoding a screening mRNA encoding a first marker into a first human cell, wherein the screening mRNA encoding the first marker can comprise the premature stop codon; transfecting a third vector encoding a comparable screening mRNA encoding a second marker into a second human cell, wherein the comparable screening mRNA may not comprise the premature stop codon; and comparing an amount of a detectable signal emitted from the first human cell and the second human cell. In some embodiments, the engineered tRNA or variant thereof can comprise at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% sequence identity to any one of SEQ ID NOs: 003-48 or 103-148. In some embodiments, the engineered tRNA or variant thereof can comprise any one of SEQ ID NOS: 3-48 or 103-148. In some embodiments, the engineered tRNA or variant thereof

can comprise any one of SEQ ID NOS: 3, 6, 7, 32, 45, 103, 106, 107, 132, or 145. In some embodiments, administering to the subject an engineered tRNA or variant thereof can comprise administering a virus comprising a polynucleotide encoding the engineered tRNA or variant thereof. In some embodiments, the virus can comprise an adenovirus, an adeno-associated virus (AAV), or lentivirus. In some embodiments, the virus can comprise AAV 2/5, AAV 2/6, AAV 2/7, AAV2/8, or AAV 2/9. In some embodiments, the virus can comprise an AAV5 capsid. In some embodiments, the polynucleotide encoding the engineered tRNA or variant thereof can comprise an AAV2 inverted terminal repeat (ITR) sequence. In some embodiments, the polynucleotide encoding the engineered tRNA or variant thereof can comprise an inverted terminal repeat with a mutated terminal resolution site and lacking terminal nucleotides. In some embodiments, the terminal nucleotides comprise at least a portion of the A region, at least a portion of the D region, or both. In some embodiments, the polynucleotide encoding the engineered tRNA or variant thereof is self-complementary. In some embodiments, the polynucleotide encoding the engineered tRNA or variant thereof can encode an additional one or more, two or more, three or more, four or more, five or more, or six or more copies of the engineered tRNA or variant thereof. In some embodiments, the polynucleotide encoding the engineered tRNA or variant thereof can encode two or more, three or more, four or more, five or more, or six or more copies of a second engineered tRNA or variant thereof that is different from the engineered tRNA or variant thereof. In some embodiments, the polynucleotide encoding the engineered tRNA or variant thereof can comprise a stuffer sequence comprising at least about 50 nucleotides, at least about 100 nucleotides, at least about 150 nucleotides, or at least about 200 nucleotides. In some embodiments, the stuffer sequence can be 3' or 5' of one or more copies of the engineered tRNA or variant thereof within the polynucleotide. In some embodiments, the stuffer sequence can separate two or more copies of the engineered tRNA or variant thereof within the polynucleotide. In some embodiments, the engineered tRNA or variant thereof or the polynucleotide encoding the engineered tRNA or variant thereof, can be in a delivery system. In some embodiments, the delivery system can comprise a liposome, a charged polymer, an uncharged polymer, a nanoparticle, a surfactant, a penetrating enhancer, a gene transfer agent, a phospholipid, a micelle, a synthetic vector, a macromolecule, a dendrimer, a biopolymer, a viral particle, or any combination thereof. In some embodiments, the engineered tRNA or variant thereof or the polynucleotide encoding the engineered tRNA or variant thereof, is in a

pharmaceutical composition comprising a pharmaceutically acceptable excipient, carrier or diluent. In some embodiments, the pharmaceutical composition can be in a dose unit form. In some embodiments, the subject can have a disease or condition related to the premature stop codon in the target mRNA. In some embodiments, the polypeptide can be produced at an efficiency of at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, or at least about 90%, relative to a comparable polypeptide produced using a comparable mRNA that lacks the premature stop codon, thereby at least partially treating the disease or condition in the subject. In some embodiments, the disease or condition can comprise Rett Syndrome, cystic fibrosis, retinitis pigmentosa, or deafness. In some embodiments, the deafness can comprise autosomal dominant 17 deafness, autosomal dominant 13 deafness, or autosomal dominant 11 deafness. In some embodiments, the subject is a human. In some embodiments, the subject can be a non-human animal. In some embodiments, the administration can be oral, rectal, parenteral, intravenous, intra-arterial, intrathecal, intraocular, otic, intracerebroventricular, intra cisterna magna, intracerebroventricular, or intraperitoneal. In some embodiments, the disease or condition can be Rett Syndrome and at least one symptom of Rett Syndrome is alleviated. In some embodiments, the at least one symptom of Rett Syndrome can comprise slowed growth, slowed brain growth, microcephaly, a decrease or loss of movement or coordination, reduced hand control, decreased walking ability, rigid or spastic movement, a decreased ability to speak, decreased eye, disinterestedness, repetitive hand movement, unusual eye movements, difficulty breathing, irritability, fear, anxiety, a cognitive defect, seizures, an abnormal electroencephalogram, scoliosis, irregular heartbeat, or an abnormal sleep pattern. In some embodiments, from 1 x 1012 to 1 x 1015 viral genomes can be administered. In some embodiments, the subject can be around 10 to 30 years of age. In some embodiments, the composition can decrease or inhibit nonsense-mediated mRNA decay (NMD) of the target mRNA. In some embodiments, the composition can increase an amount of the target mRNA in the subject, relative to a baseline target mRNA measurement. In some embodiments, the polypeptide can comprise an MeCP2 polypeptide. In some embodiments, the premature stop codon can result from a mutation encoding for an R at amino acid position 168, 255, 270, 294, 198, 186, 453, 8 (e.g., in isoform 2), 9 (e.g., in isoform 1), 84, 85, 89, 91, 106, 111, 115, 133,

162, 167, 188, 190, 211, 250, 253, 268, 306, 309, 344, 354, 420, 458, 468, 471, 478, or 484, of the MeCP2 polypeptide.

[5] An aspect of the present disclosure can provide a method of treating a disease or condition in a subject thereof. The method can comprise: administering to the subject an engineered tRNA or variant thereof or a polynucleotide encoding the engineered tRNA or variant thereof; and producing a substantially full-length polypeptide in vivo at an efficiency of at least about 10%, relative to a comparable polypeptide produced using a comparable mRNA that lacks the premature stop codon, where the engineered tRNA or the variant thereof can be capable of reading through a premature stop codon in an mRNA encoding for the substantially full-length polypeptide. Another aspect of the present disclosure can provide a method of treating a disease or condition in a subject in need thereof. The method can comprise: administering to the subject an engineered tRNA or variant thereof or a polynucleotide encoding the engineered tRNA or variant thereof, thereby at least partially treating the disease or condition in the subject; where the engineered tRNA or variant thereof recognizes a premature stop codon in an mRNA encoding a polypeptide, where the engineered tRNA or variant thereof during translation of the mRNA can at least partially transform interpretation of the premature stop codon into a sense codon and produce a substantially full-length polypeptide in vivo at an efficiency of at least about 10%, relative to a comparable polypeptide produced using a comparable mRNA that lacks the premature stop codon, as determined by: (a) transfecting a first vector encoding the engineered tRNA or variant thereof and a second vector encoding a screening mRNA encoding a first marker into a first human cell, where the screening mRNA encoding the first marker can comprise the premature stop codon; (b) transfecting a third vector encoding a comparable screening mRNA encoding a second marker into a second human cell, where the comparable screening mRNA may not comprise the premature stop codon; and (c) comparing an amount of a detectable signal emitted from the first human cell and the second human cell. In some embodiments, the first human cell or the second human cell can be a HEK293 cell. In some embodiments, the method can further comprise restoring at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 92%, at least about 95%, at least about 97%, at least about 99%, or 100% expression of the substantially full-length polypeptide. In some embodiments, the engineered tRNA or variant thereof can produce the substantially full-length

polypeptide at an efficiency of at least about 35%, relative to the comparable polypeptide produced using the comparable mRNA that lacks the premature stop codon. In some embodiments, the engineered tRNA or variant thereof can be acylated with an amino acid selected from the group consisting of: lysine, arginine, histidine, glycine, alanine, valine, leucine, isoleucine, methionine, phenylalanine, tryptophan, proline, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartate, glutamate, pyrolysine, and selenocysteine. In some embodiments, the engineered tRNA or variant thereof can be acylated with arginine. In some embodiments, the engineered tRNA or variant thereof can be acylated with a non-canonical amino acid. In some embodiments, the mRNA encoding the polypeptide corresponds to MeCP2. In some embodiments, the engineered tRNA or variant thereof, during translation of the mRNA, can insert an amino acid into a nascent MeCP2 polypeptide chain encoded by the mRNA in vivo in response to the premature stop codon, where the amino acid when inserted can be sufficient to produce an at least partially functional MeCP2 polypeptide, as compared to a comparable MeCP2 polypeptide produced using the comparable mRNA that lacks the premature stop codon. In some embodiments, the mRNA can comprise at least two premature stop codons. In some embodiments, the at least two premature stop codons can be the same stop codon. In some embodiments, the at least two premature stop codons can be different stop codons. In some embodiments, the premature stop codon can result from an R to an X at amino acid position 168, 255, 270, 294, 198, 186, 453, 8 (e.g., in isoform 2), 9 (e.g., in isoform 1), 84, 85, 89, 91, 106, 111, 115, 133, 162, 167, 188, 190, 211, 250, 253, 268, 306, 309, 344, 354, 420, 458, 468, 471, 478, or 484, or any combination thereof, in a sequence corresponding to a MeCP2 polypeptide, where X can be a premature stop codon. In some embodiments, the premature stop codon can be an opal stop codon. In some embodiments, the premature stop codon can be an amber stop codon. In some embodiments, the premature stop codon can be an ochre stop codon. In some embodiments, the engineered tRNA or variant thereof can be a lysyl-tRNA, an arginyl-tRNA, a histidyl-tRNA, a glycyl-tRNA, an alanyl-tRNA, a valyl-tRNA, a leucyl-tRNA, an isoleucyl-tRNA, methionyl-tRNA, a phenylalanyl-tRNA, a tryptophanyl-tRNA, a prolyl-tRNA, a seryl-tRNA, a threonyl-tRNA, a cysteinyl-tRNA, a tyrosyl-tRNA, an asparaginyl tRNA, a glutaminyl-tRNA, an aspartyl-tRNA, a pyrrolysyl tRNA, a selenocytstyl tRNA or a glutamyl-tRNA. In some embodiments, the engineered tRNA or variant thereof can be an engineered pre-tRNA. In some embodiments, the engineered tRNA or variant thereof can comprise an intronic sequence. In

some embodiments, the intronic sequence can be spliced within a cell containing the engineered tRNA or variant thereof, thereby producing a mature engineered tRNA or variant thereof. In some embodiments, the subject can be a human. In some embodiments, the subject can be a non-human animal. In some embodiments, the human can be aged from about birth to about 40 years old. In some embodiments, the human can be aged about 6 months to about 15 years old. In some embodiments, the human can be an embryo or a fetus. In some embodiments, the disease or condition can comprise Rett Syndrome. In some embodiments, the disease or condition can comprise cystic fibrosis. In some embodiments, the disease or condition can comprise retinitis pigmentosa. In some embodiments, the disease or condition can comprise deafness. In some embodiments, the deafness can comprise autosomal dominant 17 deafness, autosomal dominant 13 deafness, or autosomal dominant 11 deafness. In some embodiments, the administering can be oral administering, rectal administering, or parenteral administering. In some embodiments, the administering can be the parenteral administering, and where the parenteral administering can be an intravenous administering, an intra-arterial administering, an intrathecal administering, an intraocular administering, an otic administering, an intracerebroventricular administering, or an intraperitoneal administering. In some embodiments, the administering can be intra-cisternal magna (ICM). In some embodiments, the administering can be intra-cerebroventricular (ICV). In some embodiments, the polypeptide can comprise an MeCP2 polypeptide. In some embodiments, the polypeptide can comprise a FoxG1 polypeptide. In some embodiments, the polypeptide can comprise a CDKL5 polypeptide. In some embodiments, the polypeptide can comprise a MYH9 polypeptide. In some embodiments, the polypeptide can comprise a COL11A2 polypeptide. In some embodiments, the polypeptide can comprise a MYO7A polypeptide. In some embodiments, the administering can be performed at least twice during a time period. In some embodiments, the time period can be about 24 hours. In some embodiments, the method can be a method of preventing the disease or condition, and where the preventing can comprise a prophylactic administration of the engineered tRNA or variant thereof or the polynucleotide encoding the engineered tRNA or variant thereof to the subject. In some embodiments, the polynucleotide encoding the engineered tRNA or variant thereof can be administered to the subject. In some embodiments, the polynucleotide encoding the engineered tRNA or variant thereof can be comprised in a vector. In some embodiments, the vector can be a viral vector. In some embodiments, the vector can comprise a plasmid, adenoviral vector, an adeno-associated viral

(AAV) vector, a lentiviral (LV) vector. In some embodiments, the AAV vector can be from an AAV having: (a) a serotype comprising AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11 or AAV12, or (b) a pseudotype comprising AAV-DJ, AAV-DJ/8, AAV-Rh10, AAV-Rh74, AAV-retro, AAV-PHP.B, AAV8-PHP.eB, AAV-PHP.S or AAV-2i8. In some embodiments, the AAV vector can comprise a genome comprising a replication gene and inverted terminal repeats from a first AAV serotype and a capsid protein from a second AAV serotype. In some embodiments, the AAV vector can comprise an AAV 2/5 vector, an AAV 2/6 vector, an AAV 2/7 vector, an AAV2/8 vector, or an AAV 2/9 vector. In some embodiments, the AAV vector can be an AAV 2/5 vector. In some embodiments, the engineered tRNA or variant thereof, or the polynucleotide encoding the engineered tRNA or variant thereof can be present in a delivery system. In some embodiments, the delivery system can comprise a liposome, a charged polymer, an uncharged polymer, a nanoparticle, a surfactant, a penetrating enhancer, a gene transfer agent, a phospholipid, a micelle, a synthetic vector, a macromolecule, a dendrimer, a biopolymer, a viral particle, or any combination thereof. In some embodiments, the method further comprising administering a composition comprising a polynucleotide sequence comprising or encoding for: (i) a recruiting region and (ii) a targeting region, where the polynucleotide sequence recruits an RNA editing entity, and where the editing entity when contacted with the polynucleotide sequence and the mRNA performs a chemical modification on a base of a nucleotide of the premature stop codon of the mRNA, thereby converting the premature stop codon into a sense codon. In some embodiments, the RNA editing entity can comprise: (a) an ADAR polypeptide; (b) an APOBEC polypeptide; (c) a biologically active fragment of (a) or (b); or (d) a fusion protein comprising the biological active fragment of (c). In some embodiments, the disease or condition can comprise Rett Syndrome, where the mRNA encodes an MeCP2 polypeptide, where the premature stop codon can be an opal stop codon, where the engineered tRNA or variant thereof can be an arginyl-tRNA, where the subject can be a human aged 0-6 years old, and where when the engineered tRNA or variant thereof can be administered to the subject as an AAV vector, the engineered tRNA or variant thereof during translation of the mRNA encoding the MeCP2 polypeptide at least partially transforms interpretation of the premature stop codon into an arginine sense codon and produces a substantially full-length MeCP2 polypeptide, thereby at least partially treating the Rett Syndrome. In some embodiments, the engineered tRNA or variant thereof can comprise a

chemical modification comprising a methyl group, a fluoro group, a methoxyethyl group, an ethyl group, a phosphate group, an amide group, an ester group, or any combination thereof. In some embodiments, the subject can be given a diagnosis with the disease or the condition prior to administration. In some embodiments, the diagnosis can be determined by an in vitro diagnostic test.

[6] Another aspect of the present disclosure provides a composition. The composition can comprise: an engineered tRNA or variant thereof described herein; and a pharmaceutically acceptable excipient, diluent, or carrier. Another aspect of the present disclosure provides a kit comprising the composition mentioned herein in a container. Another aspect of the present disclosure provides a method of treating a disease or condition in a subject in need thereof, comprising: administering to the subject an engineered tRNA variant or a polynucleotide encoding the engineered tRNA variant, where the engineered tRNA variant can comprise one or more mutations in a sequence of a reference tRNA provided in any one of SEQ ID NOS: 3-22, and where the engineered tRNA variant recognizes a premature stop codon in an mRNA encoding a polypeptide and at least partially transforms interpretation of the premature stop codon into a sense codon during translation of the mRNA to produce a substantially full-length polypeptide in vivo. In some embodiments, the one or more mutations can comprise a substitution of at least two nucleotides in an acceptor stem or an anticodon stem of the engineered tRNA variant that do not Watson-Crick base pair to each other with at least two nucleotides that do Watson-Crick base pair. In some embodiments, the one or more mutations can comprise a substitution of a thymine with a cytosine at nucleotide position 72 with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 23. In some embodiments, the one or more mutations can comprise a substitution of a uracil with a cytosine at nucleotide position 72 with reference to SEQ ID NO: 103 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 123. In some embodiments, the engineered tRNA variant can comprise a cytosine at nucleotide position 2 and a guanine at nucleotide position 71 with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 24. In some embodiments, the engineered tRNA variant can comprise a cytosine at nucleotide position 2 and a guanine at nucleotide position 71 with reference to SEQ ID NO: 103 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of

SEQ ID NO: 124. In some embodiments, the one or more mutations can comprise an adenine substituted with a guanine at nucleotide position 6 and a thymine substituted with a cytosine at nucleotide position 67, where the nucleotide position 6 and the nucleotide position 67 can be with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 25. In some embodiments, the one or more mutations can comprise a an adenine substituted with a guanine at nucleotide position 6 and a uracil substituted with a cytosine at nucleotide position 67, where the nucleotide position 6 and the nucleotide position 67 can be with reference to SEQ ID NO: 103 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 125. In some embodiments, the one or more mutations can comprise a thymine substituted with a cytosine at nucleotide position 13 and an adenine substituted with a guanine at nucleotide position 22, where the nucleotide position 13 and the nucleotide position 22 can be with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 26. In some embodiments, the one or more mutations can comprise a uracil substituted with a cytosine at nucleotide position 13 and an adenine substituted with a guanine at nucleotide position 22, where the nucleotide position 13 and the nucleotide position 22 can be with reference to SEQ ID NO: 103 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 126. In some embodiments, the one or more mutations can comprise an adenine substituted with to a guanine at nucleotide position 15 with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 27. In some embodiments, the one or more mutations can comprise an adenine substituted with to a guanine at nucleotide position 15 with reference to SEQ ID NO: 103 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 127. In some embodiments, the one or more mutations can comprise a thymine substituted with a cytosine at nucleotide position 28 and an adenine substituted with a guanine at nucleotide position 42, where the nucleotide position 28 and the nucleotide position 42 can be with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 28. In some embodiments, the one or more mutations can comprise a uracil substituted with a cytosine at nucleotide position 28 and an adenine substituted with a guanine at nucleotide position 42, where the nucleotide position 28 and the nucleotide position 42 can be with reference to SEQ ID NO: 103 or a 5' end of the reference

tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 128. In some embodiments, the one or more mutations can comprise a cytosine substituted with an adenine at nucleotide position 31 and a guanine substituted with a thymine at nucleotide position 39, where the nucleotide position 31 and the nucleotide position 39 can be with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 29. In some embodiments, the one or more mutations can comprise a cytosine substituted with an adenine at nucleotide position 31 and a guanine substituted with a uracil at nucleotide position 39, where the nucleotide position 31 and the nucleotide position 39 can be with reference to SEQ ID NO: 103 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 129. In some embodiments, the one or more mutations can comprise an adenine substituted with a guanine at nucleotide position 37 with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 30. In some embodiments, the one or more mutations can comprise an adenine substituted with a guanine at nucleotide position 37 with reference to SEQ ID NO: 103 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 130. In some embodiments, the one or more mutations can comprise a guanine substituted with an adenine at nucleotide position 44 with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 31. In some embodiments, the one or more mutations can comprise a guanine substituted with an adenine at nucleotide position 44 with reference to SEQ ID NO: 103 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 131. In some embodiments, the one or more mutations can comprise a thymine substituted with a cytosine at nucleotide position 50 and an adenine substituted with a guanine at nucleotide position 64, where the nucleotide position 50 and the nucleotide position 64 can be with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 32. In some embodiments, the one or more mutations can comprise a uracil substituted with a cytosine at nucleotide position 50 and an adenine substituted with a guanine at nucleotide position 64, where the nucleotide position 50 and the nucleotide position 64 can be with reference to SEQ ID NO: 103 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 132. In some embodiments, the one or more mutations can comprise a cytosine substituted with a thymine at nucleotide

position 6 and a guanine substituted with an adenine at nucleotide position 67, where the nucleotide position 6 and the nucleotide position 67 can be with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 35. In some embodiments, the one or more mutations can comprise a cytosine substituted with a uracil at nucleotide position 6 and a guanine substituted with an adenine at nucleotide position 67, where the nucleotide position 6 and the nucleotide position 67 can be with reference to SEQ ID NO: 106 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 135. In some embodiments, the one or more mutations can comprise a cytosine substituted with a guanine at nucleotide position 49 and a guanine substituted with a cytosine at nucleotide position 65, where the nucleotide position 49 and the nucleotide position 65 can be with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 36. In some embodiments, the one or more mutations can comprise a cytosine substituted with a guanine at nucleotide position 49 and a guanine substituted with a cytosine at nucleotide position 65, where the nucleotide position 49 and the nucleotide position 65 can be with reference to SEQ ID NO: 106 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 136. In some embodiments, the one or more mutations can comprise a cytosine substituted with a thymine at nucleotide position 50 and a guanine substituted with an adenine at nucleotide position 64, where the nucleotide position 50 and the nucleotide position 64 can be with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 37. In some embodiments, the one or more mutations can comprise a cytosine substituted with a uracil at nucleotide position 50 and a guanine substituted with an adenine at nucleotide position 64, where the nucleotide position 50 and the nucleotide position 64 can be with reference to SEQ ID NO: 106 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 137. In some embodiments, the one or more mutations can comprise a thymine substituted with a cytosine at nucleotide position 71 with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 38. In some embodiments, the one or more mutations can comprise a uracil substituted with a cytosine at nucleotide position 71 with reference to SEQ ID NO: 106 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 138. In some embodiments, the one or more

mutations can comprise a guanine substituted with a cytosine at nucleotide position 2 and a thymine substituted with a guanine at nucleotide position 71, where the nucleotide position 2 and the nucleotide position 72 can be with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 39. In some embodiments, the one or more mutations can comprise a guanine substituted with a cytosine at nucleotide position 2 and a uracil substituted with a guanine at nucleotide position 71, where the nucleotide position 2 and the nucleotide position 72 can be with reference to SEQ ID NO: 106 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 139. In some embodiments, the one or more mutations can comprise a thymine substituted with a cytosine at nucleotide position 4 and an adenine substituted with a guanine at nucleotide position 69, where the nucleotide position 4 and the nucleotide position 69 can be with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 40. In some embodiments, the one or more mutations can comprise a uracil substituted with a cytosine at nucleotide position 4 and an adenine substituted with a guanine at nucleotide position 69, where the nucleotide position 4 and the nucleotide position 69 can be with reference to SEQ ID NO: 106 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 140. In some embodiments, the one or more mutations can comprise a cytosine substituted with an adenine at nucleotide position 6 and a guanine substituted with a thymine at nucleotide position 67, where the nucleotide position 6 and the nucleotide position 67 can be with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 41. In some embodiments, the one or more mutations can comprise a cytosine substituted with an adenine at nucleotide position 6 and a guanine substituted with a uracil at nucleotide position 67, where the nucleotide position 6 and the nucleotide position 67 can be with reference to SEQ ID NO: 106 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 141. In some embodiments, the one or more mutations can comprise a guanine substituted with a cytosine at nucleotide position 12 and a cytosine substituted with a guanine at nucleotide position 23, where the nucleotide position 12 and the nucleotide position 23 can be with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 42. In some embodiments, the one or more mutations can comprise a guanine substituted with a cytosine at

nucleotide position 12 and a cytosine substituted with a guanine at nucleotide position 23, where the nucleotide position 12 and the nucleotide position 23 can be with reference to SEQ ID NO: 106 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 142. In some embodiments, the one or more mutations can comprise a thymine substituted with a cytosine at nucleotide position 27 and an adenine substituted with a guanine at nucleotide position 43, where the nucleotide position 27 and the nucleotide position 43 can be with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 43. In some embodiments, the one or more mutations can comprise a uracil substituted with a cytosine at nucleotide position 27 and an adenine substituted with a guanine at nucleotide position 43, where the nucleotide position 27 and the nucleotide position 43 can be with reference to SEQ ID NO: 106 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 143. In some embodiments, the one or more mutations can comprise a thymine substituted with a cytosine at nucleotide position 28 and an adenine substituted with a guanine at nucleotide position 42, where the nucleotide position 28 and the nucleotide position 42 can be with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 44. In some embodiments, the one or more mutations can comprise a uracil substituted with a cytosine at nucleotide position 28 and an adenine substituted with a guanine at nucleotide position 42, where the nucleotide position 28 and the nucleotide position 42 can be with reference to SEQ ID NO: 106 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 144. In some embodiments, the one or more mutations can comprise a thymine substituted with a cytosine at nucleotide position 40 with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 45. In some embodiments, the one or more mutations can comprise a uracil substituted with a cytosine at nucleotide position 40 with reference to SEQ ID NO: 106 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 145. In some embodiments, the one or more mutations can comprise an adenine substituted with a cytosine at nucleotide position 31 and a thymine substituted with a guanine at nucleotide position 39, where the nucleotide position 31 and the nucleotide position 39 can be with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 46. In some embodiments, the one or

more mutations can comprise an adenine substituted with a cytosine at nucleotide position 31 and a uracil substituted with a guanine at nucleotide position 39, where the nucleotide position 31 and the nucleotide position 39 can be with reference to SEQ ID NO: 106 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 146. In some embodiments, the one or more mutations can comprise an adenine substituted with a guanine at nucleotide position 44 with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 47. In some embodiments, the one or more mutations can comprise an adenine substituted with a guanine at nucleotide position 44 with reference to SEQ ID NO: 106 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 147. In some embodiments, the one or more mutations can comprise a guanine substituted with an adenine at nucleotide position 46 with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 48. In some embodiments, the one or more mutations can comprise a guanine substituted with an adenine at nucleotide position 46 with reference to SEQ ID NO: 106 or a 5' end of the reference tRNA. The engineered tRNA variant can include the sequence of SEQ ID NO: 148. In some embodiments, the polypeptide can be produced at an efficiency of at least about 10%, relative to a comparable polypeptide produced using a comparable mRNA that can lack the premature stop codon, as determined by: (a) transfecting a first vector encoding the engineered tRNA variant and a second vector encoding a screening mRNA encoding a first green fluorescent protein into a first human cell, where the screening mRNA encoding the first green fluorescent protein can comprise the premature stop codon; (b) transfecting a third vector encoding a comparable screening mRNA encoding a second green fluorescent protein into a second human cell, where the comparable screening mRNA does not comprise the premature stop codon; and (c) comparing an amount of fluorescence emitted from the first human cell and the second human cell. In some embodiments, the polypeptide can be produced at an efficiency of at least about 80%, relative to a comparable polypeptide produced using a comparable mRNA that lacks the premature stop codon, thereby at least partially treating the disease or condition in the subject. In some embodiments, the engineered tRNA variant can be acylated with an amino acid selected from the group consisting of: lysine, arginine, histidine, glycine, alanine, valine, leucine, isoleucine, methionine, phenylalanine, tryptophan, proline, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartate, glutamate, pyrolysine, and

selenocysteine. In some embodiments, the engineered tRNA variant can be acylated with a non-canonical amino acid. In some embodiments, the engineered tRNA variant during translation of the mRNA inserts an amino acid into a nascent MeCP2 polypeptide chain encoded by the mRNA in vivo in response to the premature stop codon, where the amino acid when inserted can be sufficient to produce an at least partially functional MeCP2 polypeptide, as compared to a comparable MeCP2 polypeptide produced using the comparable mRNA that lacks the premature stop codon. In some embodiments, the mRNA can comprise at least two premature stop codons. In some embodiments, the at least two premature stop codons can be the same stop codon. In some embodiments, the at least two premature stop codons can be different stop codons. In some embodiments, the engineered tRNA variant can be a lysyl-tRNA, an arginyl-tRNA, a histidyl-tRNA, a glycyl-tRNA, an alanyl-tRNA, a valyl-tRNA, a leucyl-tRNA, an isoleucyl-tRNA, methionyl-tRNA, a phenylalanyl-tRNA, a tryptophanyl-tRNA, a prolyl-tRNA, a seryl-tRNA, a threonyl-tRNA, a cysteinyl-tRNA, a tyrosyl-tRNA, an asparaginyl tRNA, a glutaminyl-tRNA, an aspartyl-tRNA, a pyrrolysyl tRNA, a selenocytstyl tRNA, or a glutamyl-tRNA. In some embodiments, the engineered tRNA variant can be an engineered pre-tRNA. In some embodiments, the engineered tRNA variant can comprise an intronic sequence. In some embodiments, the intronic sequence can be spliced within a cell containing the engineered tRNA variant, thereby producing a mature engineered tRNA variant. In some embodiments, the subject can be a human. In some embodiments, the human can be aged from about birth to about 40 years old. In some embodiments, the human can be aged about 6 months to about 15 years old. In some embodiments, the human can be an embryo or a fetus. In some embodiments, the disease or condition can comprise Rett Syndrome. In some embodiments, the disease or condition can comprise cystic fibrosis. In some embodiments, the disease or condition can comprise retinitis pigmentosa. In some embodiments, the disease or condition can comprise deafness. In some embodiments, the deafness can comprise autosomal dominant 17 deafness, autosomal dominant 13 deafness, or autosomal dominant 11 deafness. In some embodiments, the premature stop codon can be an opal stop codon.

[7] In some embodiments, the administering can be oral administering, rectal administering, or parenteral administering. In some embodiments, the administering can be the parenteral administering, and where the parenteral administering can be an intravenous administering, an intra-arterial administering, an intrathecal administering, an intraocular

administering, an otic administering, an intracerebroventricular administering, intracranial, intracranial into the parenchyma, or an intraperitoneal administering. In some embodiments, the polypeptide can comprise an MeCP2 polypeptide. In some embodiments, the polypeptide can comprise a FoxG1 polypeptide. In some embodiments, the polypeptide can comprise a CDKL5 polypeptide. In some embodiments, the polypeptide can comprise a MYH9 polypeptide. In some embodiments, the polypeptide can comprise a COL11A2 polypeptide. In some embodiments, the polypeptide can comprise a MYO7A polypeptide. In some embodiments, the administering can be performed at least twice during a time period. In some embodiments, the time period can be about 24 hours. In some embodiments, the method can be a method of preventing the disease or condition, and where the preventing can comprise a prophylactic administering of the engineered tRNA variant or the polynucleotide encoding the engineered tRNA variant to the subject. In some embodiments, the polynucleotide encoding the engineered tRNA variant can be administered to the subject. In some embodiments, the vector can be a viral vector. In some embodiments, the viral vector can be an adenoviral vector, an adeno-associated viral (AAV) vector, or a lentiviral vector. In some embodiments, the AAV vector can comprise a genome comprising a replication gene and inverted terminal repeats from a first AAV serotype and a capsid protein from a second AAV serotype. In some embodiments, the AAV vector can comprise an AAV 2/5 vector, an AAV 2/6 vector, an AAV 2/7 vector, an AAV2/8 vector, or an AAV 2/9 vector. In some embodiments, the AAV vector can be an AAV 2/5 vector. In some embodiments, the engineered tRNA variant or the polynucleotide encoding the engineered tRNA can be present in a delivery system. In some embodiments, the delivery system can comprise a liposome, a charged polymer, an uncharged polymer, a nanoparticle, a surfactant, a penetrating enhancer, a gene transfer agent, a phospholipid, a micelle, a synthetic vector, a macromolecule, a dendrimer, a biopolymer, a viral particle, or any combination thereof. In some embodiments, the method can further comprise administering a composition comprising a polynucleotide sequence comprising or encoding an RNA editing polynucleotide comprising: (i) a recruiting region and (ii) a targeting region, where the polynucleotide sequence recruits an editing entity via at least a portion of the recruiting region, and where the editing entity when contacted with the RNA editing polynucleotide sequence and the mRNA performs a chemical modification on a base of a nucleotide of the premature stop codon of the mRNA, thereby converting the premature stop codon into a sense codon. In some embodiments, the editing entity can comprise: (a) an ADAR

polypeptide; (b) an APOBEC polypeptide; (c) a biologically active fragment of (a) or (b); or (d) a fusion protein comprising the biological active fragment of (c). In some embodiments, the disease or condition can comprise Rett Syndrome, where the mRNA encodes an MeCP2 polypeptide, where the premature stop codon can be an opal stop codon, where the engineered tRNA can be an arginyl-tRNA, where the subject can be a human aged 0-6 years old, and where when the engineered tRNA variant can be administered to the subject as an AAV vector, the engineered tRNA variant during translation of the mRNA encoding the MeCP2 polypeptide at least partially transforms interpretation of the premature stop codon into an arginine sense codon and produces a substantially full-length MeCP2 polypeptide, thereby at least partially treating the Rett Syndrome. In some embodiments, the engineered tRNA variant can comprise a chemical modification can comprise a methyl group, a fluoro group, a methoxyethyl group, an ethyl group, a phosphate group, an amide group, an ester group, or any combination thereof. In some embodiments, the engineered tRNA variant or the polynucleotide encoding the engineered tRNA variant can be comprised in a pharmaceutical unit dose form further comprising a pharmaceutically acceptable carrier excipient, a diluent, or a carrier. In some embodiments, the subject was given a diagnosis with the disease or the condition. In some embodiments, the diagnosis was determined by an in vitro diagnostic test.

[8] Another aspect of the present disclosure provides a composition. The composition can comprise: an engineered tRNA variant comprising one or more mutations with reference to a sequence provided in any of SEQ ID NOS: 3-22. In some embodiments, the engineered tRNA variant can comprise at least 70%, at least 80%, at least 85%, at least 90%, at least 92%, at least 95%, at least 97%, at least 99%, or 100% sequence identity to any one of SEQ ID NO: 23 - SEQ ID NO: 48. In some embodiments, the engineered tRNA variant can comprise at least 70%, at least 80%, at least 85%, at least 90%, at least 92%, at least 95%, at least 97%, at least 99%, or 100% sequence identity to SEQ ID NO: 1. In some embodiments, the engineered tRNA variant can comprise at least 70%, at least 80%, at least 85%, at least 90%, at least 92%, at least 95%, at least 97%, at least 99%, or 100% sequence identity to SEQ ID NO: 2. Another aspect of the present disclosure provides a composition; the composition can comprise: an engineered tRNA variant or a polynucleotide encoding the engineered tRNA variant, where the engineered tRNA variant can comprise one or more mutations in a sequence of the engineered tRNA as compared with a reference tRNA comprising a sequence provided in any of SEQ ID NOS: 3-22, and where

the engineered tRNA variant recognizes a premature stop codon in an mRNA encoding a polypeptide and at least partially transforms interpretation of the premature stop codon into a sense codon during translation of the mRNA to produce a substantially full-length polypeptide. In some embodiments, the engineered tRNA variant can comprise a sequence that can be at least 70% identical to SEQ ID NO: 6, and can comprise a substitution at position 2, 4, 6, 12, 23, 27, 28, 31, 39, 40, 42, 43, 44, 46, 49, 50, 64, 65, 67, 69, or 71, of the sequence of SEQ ID NO: 6. In some embodiments, the substitution at position 2 can be to a C, the substitution at position 4 can be to a C, the substitution at position 6 can be to a T, the substitution at position 6 can be to an A, the substitution at position 12 can be to a C, the substitution at position 23 can be to a G, the substitution at position 27 can be to a C, the substitution at position 28 can be to a C, the substitution at position 31 can be to a C, the substitution at position 39 can be to a G, the substitution at position 40 can be to a C, the substitution at position 42 can be to a G, the substitution at position 43 can be to a G, the substitution at position 44 can be to a G, the substitution at position 46 can be to an A, the substitution at position 49 can be to a G, the substitution at position 50 can be to a T, the substitution at position 64 can be to an A, the substitution at position 65 can be to a C, the substitution at position 67 can be to an A, the substitution at position 67 can be to a T, the substitution at position 69 can be to a G, the substitution at position 71 can be to a C, or the substitution at position 71 can be to a G. In some embodiments, the sequence of the engineered tRNA variant can include multiple substitutions. In some embodiments, the sequence of the engineered tRNA variant can be identical to the sequence of SEQ ID NO: 6, except for the substitution(s). In some embodiments, the engineered tRNA variant exhibits an increased stability in vivo, as compared with a comparable tRNA comprising the sequence provided in SEQ ID NO: 6, as determined by a proxy measurement, a half-life measurement, an amino acid charging efficiency measurement, or a measurement of binding to a synthetase or ribosomal machinery. In some embodiments, the engineered tRNA variant can comprise a sequence that can be at least 70% identical to SEQ ID NO: 3, and can comprise a substitution at position 2, 6, 13, 15, 22, 28, 31, 37, 39, 42, 44, 50, 64, 67, 71, or 72, of SEQ ID NO: 3. In some embodiments, the substitution at position 2 can be to a G, the substitution at position 6 can be to a G, the substitution at position 13 can be to a C, the substitution at position 15 can be to a G, the substitution at position 22 can be to a G, the substitution at position 28 can be to a C, the substitution at position 31 can be to an A, the

substitution at position 37 can be to a G, the substitution at position 39 can be to a T, the substitution at position 42 can be to a G, the substitution at position 44 can be to an A, the substitution at position 50 can be to a C, the substitution at position 64 can be to a G, the substitution at position 67 can be to a C, the substitution at position 71 can be to a C, or the substitution at position 72 can be to a C. In some embodiments, the sequence of the engineered tRNA variant can include multiple substitutions. In some embodiments, the sequence of the engineered tRNA variant can be identical to SEQ ID NO: 3, except for the substitution(s). In some embodiments, the engineered tRNA variant exhibits an increased stability in vivo, as compared with a comparable tRNA comprising the sequence provided in SEQ ID NO: 3, as determined by a proxy measurement, a half-life measurement, an amino acid charging efficiency measurement, or a measurement of binding to a synthetase or ribosomal machinery. In some embodiments, the engineered tRNA variant can comprise a sequence that can be at least 70% identical to SEQ ID NO: 5, and can comprise a substitution at position 73 of SEQ ID NO: 5. In some embodiments, the substitution at position 73 can be to a G. In some embodiments, the sequence of the engineered tRNA variant can include multiple substitutions. In some embodiments, the sequence of the engineered tRNA variant can be identical to SEQ ID NO: 5, except for the substitutions. In some embodiments, the engineered tRNA variant exhibits an increased stability in vivo, as compared with a comparable tRNA comprising the sequence provided in SEQ ID NO: 5, as determined by a proxy measurement, a half-life measurement, an amino acid charging efficiency measurement, or a measurement of binding to a synthetase or ribosomal machinery. In some embodiments, the one or more mutations can comprise a substitution at least two nucleotides in an acceptor stem or an anticodon stem of the engineered tRNA variant that do not Watson-Crick base pair to each other with at least two nucleotides that do Watson-Crick base pair. In some embodiments, the engineered tRNA variant can comprise a cytosine at nucleotide position 2 and a guanine at nucleotide position 71 with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA. In some embodiments, the one or more mutations can comprise a substitution of a thymine with a cytosine at nucleotide position 72 with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA. In some embodiments, the one or more mutations can comprise a cytosine substituted with a guanine at nucleotide position 22 and a guanine substituted with a cytosine at nucleotide position 71, where the nucleotide position 22 and nucleotide position 71 can be with reference to SEQ ID NO: 3 or a 5' end of the reference

tRNA. In some embodiments, the one or more mutations can comprise an adenine substituted with a guanine at nucleotide position 6 and a thymine substituted with a cytosine at nucleotide position 67, where the nucleotide position 6 and the nucleotide position 67 can be with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA. In some embodiments, the one or more mutations can comprise a thymine substituted with a cytosine at nucleotide position 13 and an adenine substituted with a guanine at nucleotide position 22, where the nucleotide position 13 and the nucleotide position 22 can be with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA. In some embodiments, the one or more mutations can comprise an adenine substituted with to a guanine at nucleotide position 15 with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA. In some embodiments, the one or more mutations can comprise a thymine substituted with a cytosine at nucleotide position 28 and an adenine substituted with a guanine at nucleotide position 42, where the nucleotide position 28 and the nucleotide position 42 can be with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA. In some embodiments, the one or more mutations can comprise a cytosine substituted with an adenine at nucleotide position 31 and a guanine substituted with a thymine at nucleotide position 39, where the nucleotide position 31 and the nucleotide position 39 can be with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA. In some embodiments, the one or more mutations can comprise an adenine substituted with a guanine at nucleotide position 37 with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA. In some embodiments, the one or more mutations can comprise a guanine substituted with an adenine at nucleotide position 44 with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA. In some embodiments, the one or more mutations can comprise an adenine substituted with a guanine at nucleotide position 73 with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA. In some embodiments, the one or more mutations can comprise a cytosine substituted with a thymine at nucleotide position 50 and a guanine substituted with an adenine at nucleotide position 64, where the nucleotide position 50 and the nucleotide position 64 can be with reference to SEQ ID NO: 4 or a 5' end of the reference tRNA. In some embodiments, the one or more mutations can comprise a cytosine substituted with a uracil at nucleotide position 50 and a guanine substituted with an adenine at nucleotide position 64, where the nucleotide position 50 and the nucleotide position 64 can be with reference to SEQ ID NO: 104 or a 5' end of the reference tRNA. In some embodiments, the one or more mutations can comprise a cytosine substituted with a thymine at nucleotide position

6 and a guanine substituted with an adenine at nucleotide position 67, where the nucleotide position 6 and the nucleotide position 67 can be with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA. In some embodiments, the one or more mutations can comprise a cytosine substituted with a guanine at nucleotide position 49 and a guanine substituted with a cytosine at nucleotide position 65, where the nucleotide position 49 and the nucleotide position 65 can be with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA. In some embodiments, the one or more mutations can comprise a cytosine substituted with a thymine at nucleotide position 50 and a guanine substituted with an adenine at nucleotide position 64, where the nucleotide position 50 and the nucleotide position 64 can be with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA. In some embodiments, the one or more mutations can comprise a cytosine substituted with a uracil at nucleotide position 50 and a guanine substituted with an adenine at nucleotide position 64, where the nucleotide position 50 and the nucleotide position 64 can be with reference to SEQ ID NO: 106 or a 5' end of the reference tRNA. In some embodiments, the one or more mutations can comprise a thymine substituted with a guanine at nucleotide position 71 with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA. In some embodiments, the one or more mutations can comprise a thymine substituted with a cytosine at nucleotide position 4 and an adenine substituted with a guanine at nucleotide position 69, where the nucleotide position 4 and the nucleotide position 69 can be with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA. In some embodiments, the one or more mutations can comprise a cytosine substituted with an adenine at nucleotide position 6 and a guanine substituted with a thymine at nucleotide position 67, where the nucleotide position 6 and the nucleotide position 67 can be with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA. In some embodiments, the one or more mutations can comprise a guanine substituted with a cytosine at nucleotide position 12 and a cytosine substituted with a guanine at nucleotide position 23, where the nucleotide position 12 and the nucleotide position 23 can be with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA. In some embodiments, the one or more mutations can comprise a thymine substituted with a cytosine at nucleotide position 27 and an adenine substituted with a guanine at nucleotide position 43, where the nucleotide position 27 and the nucleotide position 43 can be with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA. In some embodiments, the one or more mutations can comprise a thymine substituted with a cytosine at nucleotide position 28 and an adenine substituted with a guanine at nucleotide position 42, where the nucleotide

position 28 and the nucleotide position 42 can be with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA. In some embodiments, the one or more mutations can comprise a thymine substituted with a cytosine at nucleotide position 40 with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA. In some embodiments, the one or more mutations can comprise an adenine substituted with a cytosine at nucleotide position 31 and a thymine substituted with a guanine at nucleotide position 39, where the nucleotide position 31 and the nucleotide position 39 can be with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA. In some embodiments, the one or more mutations can comprise an adenine substituted with a guanine at nucleotide position 44 with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA. In some embodiments, the one or more mutations can comprise a guanine substituted with an adenine at nucleotide position 46 with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA. In some embodiments, the polypeptide can be produced at an efficiency of at least about 10%, relative to a comparable polypeptide produced using a comparable mRNA that lacks the premature stop codon, as determined by: (a) transfecting a first vector encoding the engineered tRNA or variant thereof and a second vector encoding a screening mRNA encoding a first green fluorescent protein into a first human cell, where the screening mRNA encoding the first green fluorescent protein can comprise the premature stop codon; (b) transfecting a third vector encoding a comparable screening mRNA encoding a second green fluorescent protein into a second human cell, where the comparable screening mRNA does not comprise the premature stop codon; and (c) comparing an amount of fluorescence emitted from the first human cell and the second human cell. In some embodiments, the polypeptide can be produced at an efficiency of at least about 80%, relative to a comparable polypeptide produced using a comparable mRNA that lacks the premature stop codon, thereby at least partially treating the disease or condition in the subject. In some embodiments, the engineered tRNA variant can be acylated with an amino acid selected from the group consisting of: lysine, arginine, histidine, glycine, alanine, valine, leucine, isoleucine, methionine, phenylalanine, tryptophan, proline, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartate, glutamate, pyrolysine, and selenocysteine. In some embodiments, the engineered tRNA variant can be acylated with a non-canonical amino acid. In some embodiments, the engineered tRNA variant during translation of the mRNA inserts an amino acid into a nascent MeCP2 polypeptide chain encoded by the mRNA in vivo in response to the premature stop codon, where the amino acid when inserted can be sufficient to produce an

at least partially functional MeCP2 polypeptide, as compared to a comparable MeCP2 polypeptide produced using the comparable mRNA that lacks the premature stop codon. In some embodiments, the mRNA can comprise at least two premature stop codons. In some embodiments, the at least two premature stop codons can be the same stop codon. In some embodiments, the at least two premature stop codons can be different stop codons. In some embodiments, the engineered tRNA variant can be a lysyl-tRNA, an arginyl-tRNA, a histidyl-tRNA, a glycyl-tRNA, an alanyl-tRNA, a valyl-tRNA, a leucyl-tRNA, an isoleucyl-tRNA, methionyl-tRNA, a phenylalanyl-tRNA, a tryptophanyl-tRNA, a prolyl-tRNA, a seryl-tRNA, a threonyl-tRNA, a cysteinyl-tRNA, a tyrosyl-tRNA, an asparaginyl tRNA, a glutaminyl-tRNA, an aspartyl-tRNA, a pyrrolysyl tRNA, a selenocytstyl tRNA, or a glutamyl-tRNA. In some embodiments, the engineered tRNA variant can be an engineered pre-tRNA. In some embodiments, the engineered tRNA variant can comprise an intronic sequence. In some embodiments, the intronic sequence can be spliced within a cell containing the engineered tRNA variant, thereby producing a mature engineered tRNA variant. In some embodiments, the subject can be a human. In some embodiments, the human can be aged from about birth to about 40 years old. In some embodiments, the human can be aged about 6 months to about 15 years old. In some embodiments, the human can be an embryo or a fetus. In some embodiments, the disease or condition can comprise Rett Syndrome. In some embodiments, the disease or condition can comprise cystic fibrosis. In some embodiments, the disease or condition can comprise retinitis pigmentosa. In some embodiments, the disease or condition can comprise deafness. In some embodiments, the deafness can comprise autosomal dominant 17 deafness, autosomal dominant 13 deafness, or autosomal dominant 11 deafness. In some embodiments, the premature stop codon can be an opal stop codon. In some embodiments, the administering can be oral administering, rectal administering, or parenteral administering. In some embodiments, the administering can be the parenteral administering, and where the parenteral administering can be an intravenous administering, an intra-arterial administering, an intrathecal administering, an intraocular administering, an otic administering, an intracerebroventricular administering, or an intraperitoneal administering. In some embodiments, the polypeptide can comprise an MeCP2 polypeptide. In some embodiments, the polypeptide can comprise a FoxG1 polypeptide. In some embodiments, the polypeptide can comprise a CDKL5 polypeptide. In some embodiments, the polypeptide can comprise a MYH9 polypeptide. In some embodiments, the polypeptide can

comprise a COL11A2 polypeptide. In some embodiments, the polypeptide can comprise a MYO7A polypeptide. In some embodiments, the administering can be performed at least twice during a time period. In some embodiments, the time period can be about 24 hours. In some embodiments, the composition can be a composition of preventing the disease or condition, and where the preventing can comprise a prophylactic administering of the engineered tRNA variant or the polynucleotide encoding the engineered tRNA variant to the subject. In some embodiments, the polynucleotide encoding the engineered tRNA variant can be administered to the subject. In some embodiments, the polynucleotide can be a viral vector. In some embodiments, the viral vector can be an adenoviral vector, an adeno-associated viral (AAV) vector, or a lentiviral vector. In some embodiments, the AAV vector can comprise a genome comprising a replication gene and inverted terminal repeats from a first AAV serotype and a capsid protein from a second AAV serotype. In some embodiments, the AAV vector can comprise an AAV 2/5 vector, an AAV 2/6 vector, an AAV 2/7 vector, an AAV2/8 vector, or an AAV 2/9 vector. In some embodiments, the AAV vector can be an AAV 2/5 vector. In some embodiments, the engineered tRNA variant or the polynucleotide encoding the engineered tRNA can be present in a delivery system. In some embodiments, the delivery system can comprise a liposome, a charged polymer, an uncharged polymer, a nanoparticle, a surfactant, a penetrating enhancer, a gene transfer agent, a phospholipid, a micelle, a synthetic vector, a macromolecule, a dendrimer, a biopolymer, a viral particle, or any combination thereof. In some embodiments, the composition can further comprise administering a composition comprising a polynucleotide sequence, or a vector encoding the polynucleotide sequence, where the polynucleotide sequence can comprise: (i) a recruiting region and (ii) a targeting region, where the polynucleotide sequence recruits an editing entity via at least a portion of the recruiting region, and where the editing entity when contacted with the polynucleotide sequence and the mRNA performs a chemical modification on a base of a nucleotide of the premature stop codon of the mRNA, thereby converting the premature stop codon into a sense codon. In some embodiments, the editing entity can comprise: (a) an ADAR polypeptide; (b) an APOBEC polypeptide; (c) a biologically active fragment of (a) or (b); or (d) a fusion protein comprising the biological active fragment of (c). In some embodiments, the disease or condition can comprise Rett Syndrome, where the mRNA encodes an MeCP2 polypeptide, where the premature stop codon can be an opal stop codon, where the engineered tRNA can be an arginyl-tRNA, where the subject can be a

human aged 0-6 years old, and where when the engineered tRNA variant can be administered to the subject as an AAV vector, the engineered tRNA variant during translation of the mRNA encoding the MeCP2 polypeptide at least partially transforms interpretation of the premature stop codon into an arginine sense codon and produces a substantially full-length MeCP2 polypeptide, thereby at least partially treating the Rett Syndrome. In some embodiments, the engineered tRNA variant can comprise a chemical modification can comprise a methyl group, a fluoro group, a methoxyethyl group, an ethyl group, a phosphate group, an amide group, an ester group, or any combination thereof. In some embodiments, the composition can be isolated. Another aspect of the present disclosure provides a pharmaceutical composition; the pharmaceutical composition can comprise any of the compositions mentioned herein and a pharmaceutically acceptable excipient, a diluent, or a carrier. Another aspect of the present disclosure provides an isolated cell comprising any of the compositions mentioned herein. Another aspect of the present disclosure provides an isolated plurality of cells comprising the any of the compositions mentioned herein. Another aspect of the present disclosure provides a vector comprising the engineered tRNA variant, or the polynucleotide encoding the engineered tRNA variant, of any of the compositions mentioned herein. In some embodiments, the vector can further optionally comprise, (e.g. in a 5' to 3' order): (a) a first promoter; (b) a first quantification cassette; (c) a second promoter; (d) a second quantification cassette; (e) a third promoter; (f) a transgene comprising the polynucleotide encoding the engineered tRNA variant; or (g) a reporter gene encoding a detectable polypeptide. In some embodiments, the polynucleotide further can comprise a 5' ITR upstream of the promoter and a 3' ITR downstream of the reporter gene. In some embodiments, herein the detectable polypeptide can comprise mCherry, green fluorescent protein (GFP), or β-galactosidase. In some embodiments, herein the first promoter and the second promoter can be U6 promoters, where the U6 promoters can be optionally methylated, or where the U6 promoters can be optionally mouse U6 promoters. In some embodiments, herein the third promoter can be a human cytomegalovirus (CMV) promoter. In some embodiments, the vector can be purified or isolated.

[9] Some embodiments relate to a kit comprising: (a) any of the compositions mentioned herein, the pharmaceutical composition mentioned herein, the isolated cell(s) as mentioned herein, or the vector as mentioned herein; and (b) a container configured to store the composition.

[10] Another aspect of the present disclosure provides a method of generating the vector as mentioned herein, the method comprising: introducing the engineered tRNA variant, or the polynucleotide encoding the engineered tRNA variant, into a transgene of a backbone vector sequence.

[11] Another aspect of the present disclosure provides a method of generating the pharmaceutical composition described herein, the method comprising: introducing the engineered tRNA variant, or the polynucleotide encoding the engineered tRNA variant, into the pharmaceutically acceptable excipient, a diluent, or a carrier.

[12] Disclosed herein are methods of treating a disease or condition in a subject in need thereof. In some embodiments, a method can comprise administering to a subject an engineered tRNA or a vector encoding an engineered tRNA. In some embodiments, an engineered tRNA can recognize a premature stop codon in an mRNA encoding a polypeptide. In some embodiments, an engineered tRNA during translation of an mRNA can at least partially transforms interpretation of a premature stop codon into a sense codon and can produce a substantially full-length polypeptide at an efficiency of from about 1% to about 100% (e.g. at least about 80%), relative to a comparable polypeptide produced using a comparable mRNA that lacks the premature stop codon. In some embodiments, an efficiency can be determined by: (a) transfecting a first vector encoding an engineered tRNA and a second vector encoding a screening mRNA encoding a first green fluorescent protein into a first human cell, where a screening mRNA encoding a first green fluorescent protein can comprise a premature stop codon; (b) transfecting a third vector encoding a comparable screening mRNA encoding a second green fluorescent protein into a second human cell, where the comparable screening mRNA may not comprise the premature stop codon; and (c) comparing an amount of fluorescence emitted from the first human cell and the second human cell. In some embodiments, an engineered tRNA can be acylated with an amino acid selected from the group consisting of: lysine, arginine, histidine, glycine, alanine, valine, leucine, isoleucine, methionine, phenylalanine, tryptophan, proline, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartate, glutamate, pyrolysine, and selenocysteine. In some embodiments, an engineered tRNA can be acylated with a non-canonical amino acid. In some embodiments, an engineered tRNA during translation of an mRNA can insert an amino acid into a nascent MeCP2 polypeptide chain encoded by an mRNA in vivo in response to a premature stop codon, where the amino acid when inserted can be

sufficient to produce an at least partially functional MeCP2 polypeptide, as compared to a comparable MeCP2 polypeptide produced using a comparable mRNA that lacks a premature stop codon. In some embodiments, an mRNA can comprise at least two premature stop codons. In some embodiments, at least two premature stop codons can be the same stop codon. In some embodiments, at least two premature stop codons can be different stop codons. In some embodiments, an engineered tRNA can be a lysyl-tRNA, an arginyl-tRNA, a histidyl-tRNA, a glycyl-tRNA, an alanyl-tRNA, a valyl-tRNA, a leucyl-tRNA, an isoleucyl-tRNA, methionyl-tRNA, a phenylalanyl-tRNA, a tryptophanyl-tRNA, a prolyl-tRNA, a seryl-tRNA, a threonyl-tRNA, a cysteinyl-tRNA, a tyrosyl-tRNA, an asparaginyl tRNA, a glutaminyl-tRNA, a pyrrolysyl tRNA, a selenocytstyl tRNA, an aspartyl-tRNA, or a glutamyl-tRNA. In some embodiments, an engineered tRNA can be an engineered pre-tRNA. In some embodiments, an engineered tRNA can comprise an intronic sequence. In some embodiments, an intronic sequence can be spliced within a cell containing the engineered tRNA, thereby producing a mature engineered tRNA. In some embodiments, a subject can be a human. In some embodiments, a human can be aged from about birth to about 40 years old. In some embodiments, a human can be aged about 6 months to about 15 years old. In some embodiments, a human can be an embryo or a fetus. In some embodiments, a disease or condition can comprise Rett Syndrome. In some embodiments, a disease or condition can comprise cystic fibrosis. In some embodiments, a disease or condition can comprise retinitis pigmentosa. In some embodiments, a disease or condition can comprise deafness. In some embodiments, deafness can comprise autosomal dominant 17 deafness, autosomal dominant 13 deafness, or autosomal dominant 11 deafness. In some embodiments, a premature stop codon can be an opal stop codon. In some embodiments, an administering can be oral administering, rectal administering, or parenteral administering. In some embodiments, an administering can be a parenteral administering, where the parenteral administering can be an intravenous administering, an intra-arterial administering, an intrathecal administering, an intraocular administering, an otic administering, an intracerebroventricular administering, or an intraperitoneal administering. In some embodiments, a polypeptide can comprise an MeCP2 polypeptide. In some embodiments, a polypeptide can comprise a FoxG1 polypeptide. In some embodiments, a polypeptide can comprise a CDKL5 polypeptide. In some embodiments, a polypeptide can comprise a MYH9 polypeptide. In some embodiments, a polypeptide can comprise a COL11A2 polypeptide. In some embodiments, a polypeptide can comprise a MYO7A

polypeptide. In some embodiments, an administering can be performed at least twice during a time period. In some embodiments, a time period can be about 24 hours. In some embodiments, a method can be a method of preventing the disease or condition, where preventing can comprise a prophylactic administering of an engineered tRNA or a vector encoding an engineered tRNA to a subject. In some embodiments, a vector encoding an engineered tRNA can be administered to a subject. In some embodiments, a vector can be a viral vector. In some embodiments, a viral vector can be an adenoviral vector, an adeno-associated viral vector, or a lentiviral vector. In some embodiments, an engineered tRNA or a vector encoding an tRNA can be present in a delivery system. In some embodiments, a delivery system can comprise a liposome, a charged polymer, an uncharged polymer, a nanoparticle, a surfactant, a penetrating enhancer, a gene transfer agent, a phospholipid, a micelle, a synthetic vector, a macromolecule, a dendrimer, a biopolymer, a viral particle, or any combination thereof. In some embodiments, a method can further comprise administering a composition comprising a polynucleotide sequence, or a vector encoding the polynucleotide sequence, where a polynucleotide sequence can comprise: (i) a recruiting region and (ii) a targeting region, where a polynucleotide sequence can recruit an editing entity via at least a portion of a recruiting region, and where an editing entity when contacted with a polynucleotide sequence and an mRNA can perform a chemical modification on a base of a nucleotide of a premature stop codon of an mRNA, thereby converting a premature stop codon into a sense codon. In some embodiments, an editing entity can be an ADAR polypeptide or an APOBEC polypeptide. In some embodiments, a disease or condition can comprise Rett Syndrome, where an mRNA can encode an MeCP2 polypeptide, where a stop codon can be an opal stop codon, where an engineered tRNA can be an arginyl-tRNA, where a subject can be a human aged 0-6 years old, and where when an engineered tRNA can be administered to a subject as an AAV vector, an engineered tRNA during translation of an mRNA encoding an MeCP2 polypeptide can at least partially transforms interpretation of a premature stop codon into an arginine sense codon and can produce a substantially full-length MeCP2 polypeptide, thereby at least partially treating the Rett Syndrome.

[13] Also disclosed herein are methods of treating a disease or condition in a subject in need thereof. In some embodiments, a method can comprise administering to a subject an engineered tRNA or a vector encoding an engineered tRNA. In some embodiments, an engineered tRNA can recognize a premature stop codon in an mRNA encoding a polypeptide. In

some embodiments, an engineered tRNA during translation of an mRNA can at least partially transforms interpretation of a premature stop codon into a sense codon and produces a substantially full-length polypeptide in vivo at an efficiency of at least about 10%, relative to a comparable polypeptide produced using a comparable mRNA that lacks a premature stop codon, thereby at least partially treating a disease or condition in the subject. In some embodiments, an engineered tRNA can be acylated with an amino acid selected from the group consisting of: lysine, arginine, histidine, glycine, alanine, valine, leucine, isoleucine, methionine, phenylalanine, tryptophan, proline, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartate, glutamate, pyrolysine, and selenocysteine. In some embodiments, an engineered tRNA can be acylated with a non-canonical amino acid. In some embodiments, an engineered tRNA during translation of an mRNA can insert an amino acid into a nascent MeCP2 polypeptide chain encoded by an mRNA in vivo in response to a premature stop codon, where the amino acid when inserted can be sufficient to produce an at least partially functional MeCP2 polypeptide, as compared to a comparable MeCP2 polypeptide produced using a comparable mRNA that lacks a premature stop codon. In some embodiments, an mRNA can comprise at least two premature stop codons. In some embodiments, at least two premature stop codons can be the same stop codon. In some embodiments, at least two premature stop codons can be different stop codons. In some embodiments, an engineered tRNA can be a lysyl-tRNA, an arginyl-tRNA, a histidyl-tRNA, a glycyl-tRNA, an alanyl-tRNA, a valyl-tRNA, a leucyl-tRNA, an isoleucyl-tRNA, methionyl-tRNA, a phenylalanyl-tRNA, a tryptophanyl-tRNA, a prolyl-tRNA, a seryl-tRNA, a threonyl-tRNA, a cysteinyl-tRNA, a tyrosyl-tRNA, an asparaginyl tRNA, a glutaminyl-tRNA, an aspartyl-tRNA, a pyrrolysyl tRNA, a selenocytstyl tRNA, or a glutamyl-tRNA. In some embodiments, an engineered tRNA can be an engineered pre-tRNA. In some embodiments, an engineered tRNA can comprise an intronic sequence. In some embodiments, an intronic sequence can be spliced within a cell containing the engineered tRNA, thereby producing a mature engineered tRNA. In some embodiments, a subject can be a human. In some embodiments, a human can be aged from about birth to about 40 years old. In some embodiments, a human can be aged about 6 months to about 15 years old. In some embodiments, a human can be an embryo or a fetus. In some embodiments, a disease or condition can comprise Rett Syndrome. In some embodiments, a disease or condition can comprise cystic fibrosis. In some embodiments, a disease or condition can comprise retinitis pigmentosa. In some embodiments, a disease or

condition can comprise deafness. In some embodiments, deafness can comprise autosomal dominant 17 deafness, autosomal dominant 13 deafness, or autosomal dominant 11 deafness. In some embodiments, a stop codon can be an opal stop codon. In some embodiments, an administering can be oral administering, rectal administering, or parenteral administering. In some embodiments, an administering can be a parenteral administering, where the parenteral administering can be an intravenous administering, an intra-arterial administering, an intrathecal administering, an intraocular administering, an otic administering, an intracerebroventricular administering, or an intraperitoneal administering. In some embodiments, a polypeptide can comprise an MeCP2 polypeptide. In some embodiments, a polypeptide can comprise a FoxG1 polypeptide. In some embodiments, a polypeptide can comprise a CDKL5 polypeptide. In some embodiments, a polypeptide can comprise a MYH9 polypeptide. In some embodiments, a polypeptide can comprise a COL11A2 polypeptide. In some embodiments, a polypeptide can comprise a MYO7A polypeptide. In some embodiments, an administering can be performed at least twice during a time period. In some embodiments, a time period can be about 24 hours. In some embodiments, a method can be a method of preventing the disease or condition, where preventing can comprise a prophylactic administering of an engineered tRNA or a vector encoding an engineered tRNA to a subject. In some embodiments, a vector encoding an engineered tRNA can be administered to a subject. In some embodiments, a vector can be a viral vector. In some embodiments, a viral vector can be an adenoviral vector, an adeno-associated viral vector, or a lentiviral vector. In some embodiments, an engineered tRNA or a vector encoding an tRNA can be present in a delivery system. In some embodiments, a delivery system can comprise a liposome, a charged polymer, an uncharged polymer, a nanoparticle, a surfactant, a penetrating enhancer, a gene transfer agent, a phospholipid, a micelle, a synthetic vector, a macromolecule, a dendrimer, a biopolymer, a viral particle, or any combination thereof. In some embodiments, a method can further comprise administering a composition comprising a polynucleotide sequence, or a vector encoding the polynucleotide sequence, where a polynucleotide sequence can comprise: (i) a recruiting region and (ii) a targeting region, where a polynucleotide sequence can recruit an editing entity via at least a portion of a recruiting region, and where an editing entity when contacted with a polynucleotide sequence and an mRNA can perform a chemical modification on a base of a nucleotide of a premature stop codon of an mRNA, thereby converting a premature stop codon into a sense codon. In some embodiments, an

editing entity can be an ADAR polypeptide or an APOBEC polypeptide. In some embodiments, a disease or condition can comprise Rett Syndrome, where an mRNA can encode an MeCP2 polypeptide, where a stop codon can be an opal stop codon, where an engineered tRNA can be an arginyl-tRNA, where a subject can be a human aged 0-6 years old, and where when an engineered tRNA can be administered to a subject as an AAV vector, an engineered tRNA during translation of an mRNA encoding an MeCP2 polypeptide can at least partially transforms interpretation of a premature stop codon into an arginine sense codon and can produce a substantially full-length MeCP2 polypeptide, thereby at least partially treating the Rett Syndrome.

[14] Also disclosed herein are compositions that can comprise: an engineered tRNA or a vector encoding the engineered tRNA; and an excipient, a diluent, or a carrier. In some embodiments, an engineered tRNA can recognize a premature stop codon in an mRNA encoding a polypeptide. In some embodiments, an engineered tRNA during translation of an mRNA at least partially transforms interpretation of a premature stop codon into a sense codon and can produce a substantially full-length polypeptide at an efficiency of at least about 80%, relative to a comparable polypeptide produced using a comparable mRNA that lacks a premature stop codon. In some embodiments, efficient can be determined by: (a) transfecting a first vector encoding am engineered tRNA and a second vector encoding a screening mRNA encoding a first green fluorescent protein into a first human cell, where a screening mRNA encoding a first green fluorescent protein can comprise a premature stop codon; (b) transfecting a third vector encoding a comparable screening mRNA encoding a second green fluorescent protein into a second human cell, where a comparable screening mRNA may not comprise a premature stop codon; and (c) comparing an amount of fluorescence emitted from a first human cell and a second human cell. In some embodiments, an engineered tRNA can be acylated with an amino acid selected from the group consisting of: lysine, arginine, histidine, glycine, alanine, valine, leucine, isoleucine, methionine, phenylalanine, tryptophan, proline, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartate, glutamate, pyrolysine, and selenocysteine. In some embodiments, an engineered tRNA can be acylated with a non-canonical amino acid. In some embodiments, an engineered tRNA during translation of an mRNA can insert an amino acid into a nascent polypeptide chain encoded by an mRNA in response to a premature stop codon, where the amino acid when inserted can be sufficient to produce an at least partially functional polypeptide, as

compared to the comparable polypeptide produced using a comparable mRNA that can lack a premature stop codon. In some embodiments, a screening mRNA encoding a first green fluorescent protein can can comprise at least two premature stop codons. In some embodiments, at least two premature stop codons can be the same stop codon. In some embodiments, at least two premature stop codons can be different stop codons. In some embodiments, an engineered tRNA can be a lysyl-tRNA, an arginyl-tRNA, a histidyl-tRNA, a glycyl-tRNA, an alanyl-tRNA, a valyl-tRNA, a leucyl-tRNA, an isoleucyl-tRNA, methionyl-tRNA, a phenylalanyl-tRNA, a tryptophanyl-tRNA, a prolyl-tRNA, a seryl-tRNA, a threonyl-tRNA, a cysteinyl-tRNA, a tyrosyl-tRNA, an asparaginyl tRNA, a glutaminyl-tRNA, an aspartyl-tRNA, a pyrrolysyl tRNA, a selenocytstyl tRNA, or a glutamyl-tRNA. In some embodiments, an engineered tRNA can be an engineered pre-tRNA. In some embodiments, an engineered tRNA can comprise an intronic sequence. In some embodiments, an intronic sequence can be spliced within a cell containing the engineered tRNA, thereby producing a mature engineered tRNA. In some embodiments, a first human cell or a second human cell can be a HEK293 cell. In some embodiments, a composition can comprise a vector encoding an engineered tRNA. In some embodiments, a vector can be a viral vector. In some embodiments, a viral vector can be an adenoviral vector, an adeno-associated viral vector, or a lentiviral vector. In some embodiments, an engineered tRNA or a vector encoding an engineered tRNA can be present in a liposome, a nanoparticle, or a viral particle. In some embodiments, an engineered tRNA can be an arginyl-tRNA, where a premature stop codon can be an opal stop codon, where an engineered tRNA can be present in a vector, where a vector can be an adeno-associated viral vector, and where a first human cell and a second human cell can be HEK293 cells.

[15] Also disclosed herein are kits that can comprise a composition as described herein in a container. Some embodiments include a method of making the kit comprising contacting the composition with the container.

[16] Additional aspects and advantages of the present disclosure will become readily apparent to those skilled in this art from the following detailed description, wherein only illustrative embodiments of the present disclosure are shown and described. As will be realized, the present disclosure is capable of other and different embodiments, and its several details are capable of modifications in various obvious respects, all without departing from the disclosure.

Accordingly, the drawings and description are to be regarded as illustrative in nature, and not as restrictive.

INCORPORATION BY REFERENCE

[17] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference. To the extent publications and patents or patent applications incorporated by reference contradict the disclosure contained in the specification, the specification is intended to supersede and/or take precedence over any such contradictory material.

BRIEF DESCRIPTION OF THE DRAWINGS

[18] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[19] **FIG. 1** schematically illustrates plasmid constructs encoding one or two copies of an engineered tRNA suppressor according to some embodiments.

[20] **FIG. 2A** schematically illustrates a consensus secondary structure and sequence of a human Arg tRNA.

[21] **FIG. 2B** schematically illustrates sites in a consensus secondary structure and sequence of human Arg tRNA that were excluded from mutagenesis to engineer suppressor tRNAs, according to some embodiments.

[22] **FIG. 3A** schematically illustrates an Arginine (Arg) tRNA suppressor with an anticodon sequence of ‘CCT’ that has been modified to ‘TCA’ for suppression of a premature opal stop codon in an mRNA according to some embodiments.

[23] **FIG. 3B** schematically illustrates an Arg tRNA suppressor with the anticodon sequence of ‘TCT’ that has been modified to ‘TCA’ for suppression of a premature opal stop codon in an mRNA according to some embodiments.

[24] **FIG. 4** schematically illustrates a workflow of transfecting cells stably expressing an engineered GFP (containing a premature opal stop codon), with a plasmid vector encoding an

engineered tRNA suppressor and an mCherry fluorescent reporter, according to some embodiments.

[25] **FIG. 5A** schematically illustrates an experimental workflow for screening engineered tRNA suppressor activity in human embryonic kidney (HEK) cells according to some embodiments, including **FIG. 5B** and **FIG. 6**. This schematic shows temporally the experimental setup depicted in **FIG. 4**.

[26] **FIG. 5B** shows premature stop codon suppression by engineered Arg tRNA suppressor molecules in two HEK cell lines carrying two different Arg-to-stop codon mutations in the stably integrated GFP reporter, according to some embodiments.

[27] **FIG. 5C** shows correlation analysis of premature stop codon suppression by engineered Arg tRNA suppressor molecules between the two HEK cell lines carrying different Arg-to-stop mutations) according to some embodiments.

[28] **FIG. 6** shows premature stop codon suppression by engineered Arg tRNA suppressor molecules in different HEK cell lines carrying the R74X or R97X Arg-to-stop codon mutations, according to some embodiments.

[29] **FIG. 7** schematically illustrates a workflow of a dual plasmid screening system for evaluating engineered tRNA suppressor molecules described herein according to some embodiments, including **FIGs. 8-9B**.

[30] **FIG. 8A** shows exemplary results of premature stop codon suppression by engineered Arg tRNA suppressor molecules, measured in terms of percentage readthrough (fraction of tRNA/mCherry producing cells that show green fluorescence), in HEK cells expressing engineered GFP containing two different Arg-to-stop codon mutations using the dual plasmid screening system shown in **FIG. 7** according to some embodiments.

[31] **FIG. 8B** shows correlation analysis of premature stop codon suppression by engineered Arg tRNA suppressor molecules described herein between the two different engineered GFP reporters containing the Arg-to-stop mutations: R974X and R97X used in **FIG. 8A** according to some embodiments.

[32] **FIG. 9A** shows efficiency of premature stop codon suppression in HEK cell lines carrying two different Arg-to-stop mutations (R74X and R97X, where X is the opal stop codon) by the dual plasmid screening system according to some embodiments.

[33] **FIG. 9B** shows correlation analysis of premature stop codon suppression by engineered Arg tRNA suppressor molecules described herein between the two different engineered GFP reporters containing the Arg-to-stop mutations: R974X and R97X used in **FIG. 9A** according to some embodiments.

[34] **FIG. 10A** shows a cartoon mRNA for luciferase in K562 cells that encode wild-type (WT) mRNA (top), an opal premature stop codon at position 41, and an opal stop codon at position 73, according to some embodiments.

[35] **FIG. 10B** schematically illustrates a broken-luciferase transfection process, according to some embodiments.

[36] **FIG. 10C** shows efficiency of suppression of engineered Arg tRNA suppressor molecules described herein in K562 cell lines containing the two different premature opal stop codons shown in **FIG. 10A** using a broken luciferase screening system according to some embodiments.

[37] **FIG. 11A** shows a cartoon mRNA for green fluorescent protein (GFP) in HEK293 cells that encode WT mRNA (top), an opal premature stop codon at amino acid position 74 and an opal stop codon at amino acid position 97, according to some embodiments.

[38] **FIG. 11B** shows percentages of cells with readthrough of premature termination codons (PTCs) by engineered Arg tRNA suppressor molecules according to some embodiments.

[39] **FIG. 11C** shows readthrough of premature termination codons (PTCs) by engineered Arg tRNA suppressor molecules described herein in the cells carrying two different Arg-to-stop mutations using a broken GFP screening system according to some embodiments.

[40] **FIG. 11D** shows potency of suppression of engineered Arg tRNA suppressor molecules described herein in the cells carrying two different Arg-to-stop mutations using a broken GFP screening system according to some embodiments.

[41] **FIG. 11E** schematically illustrates a vector map, according to some embodiments.

[42] **FIG. 11F** schematically illustrates a dual transfection system used to generate the results shown in **FIG. 11B**.

[43] **FIG. 11G** schematically illustrates a process of suppressing an opal stop codon in a broken GFP using an Arg tRNA suppressor carrying an mCherry tag compared to an unbroken GFP, according to some embodiments.

[44] **FIG. 12** shows examples of efficiency levels of different engineered Arg tRNA molecules described herein by flow cytometry according to some embodiments.

[45] **FIG. 13** shows examples of alternative stop codons for which the engineered tRNA molecules can be useful.

[46] **FIG. 14A** schematically illustrates a vector design for engineered tRNA constructs, according to some embodiments.

[47] **FIG. 14B** schematically illustrates a vector design for engineered tRNA constructs used for transfecting primary neurons, according to some embodiments.

[48] **FIG. 15A** illustrates a representative western blot example of stop codon readthrough, according to some embodiments.

[49] **FIG. 15B** is a histogram of different engineered tRNAs stop codon readthrough, according to some embodiments.

[50] **FIGs. 16A-16F** shows exemplary flow cytometry results of neuronal cell cultures from a R255X Rett mouse model treated with engineered tRNAs and variants thereof, according to some embodiments.

[51] **FIGs. 17A-17D** shows exemplary flow cytometry MeCP2 signal using D4F3 antibody, according to some embodiments.

[52] **FIGs. 17E-17H** shows exemplary flow cytometry MeCP2 signal using NeuN antibody MAB377, according to some embodiments.

[53] **FIG. 18A** is a histogram depicting percentages of neurons with MeCP2 signal, according to some embodiments.

[54] **FIG. 18B** is a histogram depicting total MeCP2 signal in neurons transduced by lentivirus carrying engineered tRNA or variant thereof, according to some embodiments.

[55] **FIGs. 19A-19G** schematically illustrate different vector designs with one or more copies of an engineered tRNA with or without an exogenous promoter, according to some embodiments.

[56] **FIG. 20A** is a graph depicting ratios of cells with engineered tRNA readthrough using different vectors, according to some embodiments.

[57] **FIG. 20B** is a graph depicting total engineered tRNA readthrough in cells using different vectors, according to some embodiments.

[58] **FIG. 21A-21C** schematically illustrate different AAV-9 vector designs one or more copies of an engineered tRNA, according to some embodiments.

[59] **FIG. 22A** illustrates schematic examples of a single strand AAV9 vector with one or more copies of an engineered tRNA, according to some embodiments.

[60] **FIG. 22B** illustrates schematic examples of a self-complementary AAV9 vector with one or more copies of an engineered tRNA, according to some embodiments.

[61] **FIG. 23A** schematically illustrates vector constructs of AAV backbones with engineered tRNA or variants thereof placed at different distances from inverted terminal repeats, according to some embodiments.

[62] **FIG. 23B-23C** schematically illustrate vector constructs of AAV backbones with engineered tRNA or variants thereof placed in different orientations, according to some embodiments.

[63] **FIG. 24** shows the gating strategy used in **FIG. 25 - FIG. 28**.

[64] **FIG. 25** shows example flow cytometry dot plots of self-complementary AAV2/9 constructs carrying 0 copies, 1 copy, 3 copies, or 6 copies of SEQ ID NO: 3.

[65] **FIG. 26** shows a plot of cell viability for single-stranded and self-complementary AAV2/9 constructs carrying 0 copies, 1 copy, 3 copies, or 6 copies of SEQ ID NO: 3.

[66] **FIG. 27A** and **27B** show plots of infectivity for single-stranded and self-complementary AAV2/9 constructs carrying 0 copies, 1 copy, 3 copies, or 6 copies of SEQ ID NO: 3.

[67] **FIG. 28A** and **28B** show plots of PTC readthrough for single-stranded and self-complementary AAV2/9 constructs carrying 0 copies, 1 copy, 3 copies, or 6 copies of SEQ ID NO: 3.

[68] **FIG. 29A** and **28B** show plots of the percentage cells that exhibited PTC suppression (A) and the total percent of PTCs that were suppressed (B) for plasmids encoding for 2 copies or 3 copies of CCT and encoding for a 6bp spacer, a 500 bp spacer, a 100 bp spacer, a 200 bp spacer, or a 500 bp spacer separating tRNA payloads.

[69] **FIG. 30A** and **30B** show plots of % GFP and GFP MFI % of ST21, respectively, in accordance with some embodiments.

[70] **FIG. 31** shows exemplary strategies to improve editing efficiency of a gRNA, according to some embodiments.

[71] **FIG. 32** illustrates exemplary gRNA designs, according to some embodiments.

[72] **FIG. 33** shows an example of a luciferase gain of function assay, according to some embodiments.

[73] **FIG. 34** shows exemplary results of experiments to screen exemplary gRNA designs, according to some embodiments.

[74] **FIG. 35** shows exemplary screening assays, cell lines, and analytics assays and tools, according to some embodiments.

[75] **FIG. 36** illustrates an exemplary construct to generate ADAR1 KO cell line, according to some embodiments.

[76] **FIG. 37** illustrates an exemplary construct to generate ADAR1 KO cell line, according to some embodiments.

[77] **FIG. 38** illustrates a schematic process to integrate ADAR2 into an ADAR1 KO cell, according to some embodiments.

[78] **FIG. 39** illustrates a schematic of a digital droplet PCR (ddPCR) process, according to some embodiments.

[79] **FIG. 40** shows exemplary analysis using ddPCR to design PCR primers and probe sets, according to some embodiments.

[80] **FIG. 41** shows exemplary system to quantify gRNA expression, according to some embodiments.

[81] **FIG. 42** shows examples of high cellular levels of gRNAs after transfection into target cells, according to some embodiments.

[82] **FIG. 43** shows exemplary gRNA constructs and their efficiency in editing Rab7a in HEK293 cells, according to some embodiments.

[83] **FIG. 44 shows** examples of gRNA constructs and their efficiency in editing Rab7a in HEK293 cells, according to some embodiments.

[84] **FIG. 45** illustrates exemplary approaches in utilizing gRNA design with high-throughput screening methods, according to some embodiments.

[85] **FIG. 46** shows exemplary luciferase with opal premature mutations, according to some embodiments.

[86] **FIG. 47A-47E** show exemplary vector maps that can comprise polynucleotides described herein.

[87] **FIG. 48A-48B** show exemplary vector maps that can comprise polynucleotides described herein.

[88] **FIG. 49** shows an example of a mechanism of mammalian nonsense mediated decay.

DETAILED DESCRIPTION

[89] Premature stop codons leading to mutations in proteins have been implicated in severe neurodevelopmental disorders, such as Rett Syndrome. Translation of a mRNA molecule that contains a premature stop codon can cause premature termination of the translation process to produce a truncated polypeptide or protein. As used herein, "premature stop codon" can be used interchangeably with "premature termination codon" (PTC). The unintended truncation of proteins vital to neurodevelopment, particularly in children, can cause severe and life-altering diseases or disorders.

[90] The protein methyl-CpG binding protein 2 (MeCP2), encoded by *MECP2* (NCBI Gene ID: 4204) can be important for the function of neurons and can play a role in maintaining connections between neurons. In addition, MeCP2 can be a regulator of many genes that encode proteins that drive normal brain function. Rett Syndrome can be caused by loss-of-function mutations in MeCP2, which include premature stop codons in the MECP2 gene. In a healthy individual, the MeCP2 protein can contain an Arginine (Arg) at amino acid positions 168, 186, 198, 255, 270, 294 and 453 with reference to SEQ ID NO: 49, or with reference to SEQ ID NO: 50. In Rett Syndrome patients, a mutation in the MeCP2 protein that causes premature termination of translation of the MeCP2 protein at amino acid position 168, 255, 270, 294, 198, 186 and 453 causes loss-of-function of MeCP2, and collectively account for more than 25% of Rett-causing mutations. Arginine (Arg) in MECP2 can also be found at positions R8 (for isoform 2 only), R9 (for isoform 1 only), R84, R85, R89, R91, R106, R111, R115, R133, R162, R167, R188, R190,R211, R250, R253, R268, R306, R309, R344, R354, R420, R458, R468, R471, R478, and R484. The present disclosure provides engineered tRNAs and engineered tRNA variants that are capable of premature stop codon read-through of a premature stop codon that can be present at any position in the MeCP2 protein, including any one of: R168X, R255X, R270X, R294X, R198X, R186X, R453X, R8X (in isoform 2), R9X (in isoform 1), R84X, R85X, R89X, R91X, R106X, R111X, R115X, R133X, R162X, R167X, R188 X, R190X, R211X, R250X, R253X, R268X, R306X, R309X, R344X, R354X, R420X, R458X, R468X, R471X,

R478X, R484X, or any combination thereof. It has been shown that MeCP2 re-activation in adult MeCP2-deficient mice with advanced neurological deficit can robustly reverse disease.

[91] An example of a protein sequence of a first isomer of an MeCP2 protein can be the amino acid sequence of SEQ ID NO: 49. In some embodiments, the MeCP2 protein can comprise the first isomer of MeCP2. An example of a protein sequence of a second isomer of an MECP2 protein can be the amino acid sequence of SEQ ID NO: 50. In some embodiments, the MeCP2 protein can comprise the second isomer of MeCP2.

[92] Provided herein are engineered transfer RNA (tRNA) molecules that suppress premature stop codons to enable readthrough of the premature stop codon in a template messenger RNA (mRNA). These are also referred to as "engineered tRNA suppressor molecules" or "engineered tRNA suppressors." As used herein, "suppression" and "readthrough" can be used interchangeably to reference the activity of the engineered tRNAs and variants thereof disclosed herein. Vectors encoding the engineered tRNA molecules of the present disclosure are also provided, which in some cases, are viral vectors. The engineered tRNA molecules or vectors encoding the engineered tRNA molecules can be packaged into a virus for virus particle-mediated delivery of the engineered tRNA molecules to a target cell or tissue *in vivo*. In some cases, the virus can be an adeno-associated virus (AAV). Compositions described herein comprising the engineered tRNA molecule(s) or the vector(s) encoding the engineered tRNA molecule(s) can employ an AAV (IV/CNS) vector for delivery to a subject. AAV vector delivery can achieve long-term benefit with single dose and can provide opportunity for multiplexed targeting. Methods can include identifying AAV serotypes that can promote central neuronal tropism and biodistribution with CNS/IV dosing. The engineered tRNAs of the present disclosure can comprise an engineered anticodon for recognition of a premature stop codon. Also provided herein are engineered tRNA variants, which comprise mutations relative to their parent engineered tRNA sequence that can stabilize or improve one or more regions of the engineered tRNA structure. For example, engineered tRNA variants of the present disclosure can have mutations in the acceptor stem, the D-loop, the D-stem, the T-loop, the T-stem, the variable loop, the anticodon stem, the anticodon loop, or any combination thereof. Also disclosed herein are various constructs for packaging engineered tRNA suppressor molecules in viral vectors, including packaging of: multiple engineered tRNA suppressor payloads, markers such as GFP or mCherry, stuffer sequences, or any combination thereof.

[93] Methods of delivering the engineered tRNA or engineered tRNA variant or vector encoding the engineered tRNA, or engineered tRNA variant optionally packaged into a virus, are provided herein. In some cases, delivery of the engineered tRNA or engineered tRNA variant, or vector encoding the engineered tRNA or engineered tRNA variant can be effective to treat a disease, condition, or disorder described herein (e.g., Rett Syndrome).

I. Compositions

[94] Disclosed herein are compositions comprising engineered tRNA molecules, engineered tRNA variants, engineered pre-tRNA molecules, engineered pre-tRNA variants, or vectors encoding the engineered tRNA, engineered pre-tRNA molecules, engineered tRNA variants, or engineered pre-tRNA variants. An engineered tRNA or engineered tRNA variant as described herein can recognize a premature stop codon in an mRNA encoding a polypeptide and at least partially transform interpretation of a premature stop codon into a sense codon, such as, for example by adding the correct (e.g., non-disease-causing) amino acid to the growing peptide. A "premature" stop codon can refer to a stop codon that, when present, results in an unintended truncation of a polypeptide relative to a wild-type counterpart. Such truncation, also known as stop codon readthrough or premature stop codon readthrough (PTC), can produce a substantially full-length polypeptide at an efficiency of from about 1% to about 100% relative to a comparable polypeptide produced using a comparable mRNA that lacks the premature stop codon. In some cases, an efficiency can be at least about: 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% 90%, 95%, or 99%. In some cases, an efficiency can be at least about: 1% to about 10%, 5% to about 20%, 10% to about 35%, 25% to about 50%, 40% to about 70%, 60% to about 80%, 75% to about 90% or about 85% to about 100%.

[95] In some cases, an efficiency of premature terminal codon (PTC) readthrough can comprise an in vivo efficiency of PTC readthrough. In some cases, in vivo efficiency of PTC readthrough can be determined by at least partially treating a disease or condition. For example, in vivo efficiency of PTC readthrough can be measured by at least partially improving the ability to hear, improving the ability to see, improving motor ability, cognitive ability or any combination thereof. In some cases, an efficiency of PTC readthrough can comprise an in vitro efficiency of PTC readthrough, such as an in vitro efficiency of PTC suppression readthrough as determined by: (a) transfecting a first vector encoding an engineered tRNA or variant thereof and a second vector encoding a screening mRNA encoding a first green fluorescent protein into a

first human cell, where the screening mRNA encoding the first green fluorescent protein can comprise a premature stop codon (this can be referred to herein as a (e.g., "broken" GFP); (b) transfecting a third vector encoding a comparable screening mRNA encoding a second green fluorescent protein into a second human cell, wherein the comparable screening mRNA does not comprise a premature stop codon; and (c) comparing an amount of fluorescence emitted from the first human cell and the second human cell. In some cases, a green fluorescent protein can comprise at least two premature stop codons. In some instances, the premature stop codons can be the same stop codons, or different stop codons.

[96] An in vivo efficiency of PTC readthrough can be from about 1% to 100%. An in vivo efficiency of PTC readthrough can be from about 10% to 100%. An in vivo efficiency of PTC readthrough can be from about 20% to 100%. An in vivo efficiency of PTC readthrough can be from about 30% to 100%. An in vivo efficiency of PTC readthrough can be from about 40% to 100%. An in vivo efficiency of PTC readthrough can be from about 50% to 100%. An in vivo efficiency of PTC readthrough can be from about 60% to 100%. An in vivo efficiency of PTC readthrough can be from about 70% to 100%. An in vivo efficiency of PTC readthrough can be from about 75% to 100%. An in vivo efficiency of PTC readthrough can be from about 80% to 100%. An in vivo efficiency of PTC readthrough can be from about 85% to 100%. An in vivo efficiency of PTC readthrough can be from about 90% to 100%. An in vivo efficiency of PTC readthrough can be from about 95% to 100%. An in vivo efficiency of PTC readthrough can be from about 20% to about 40%. An in vivo efficiency of PTC readthrough can be from about 20% to 60%. An in vivo efficiency of PTC readthrough can be from about 10% to 70%.

[97] An in vitro efficiency of PTC readthrough can be from about 1% to 100%. An in vitro efficiency of PTC readthrough can be from about 10% to 100%. An in vitro efficiency of PTC readthrough can be from about 20% to 100%. An in vitro efficiency of PTC readthrough can be from about 30% to 100%. An in vitro efficiency of PTC readthrough can be from about 40% to 100%. An in vitro efficiency of PTC readthrough can be from about 50% to 100%. An in vitro efficiency of PTC readthrough can be from about 60% to 100%. An in vitro efficiency of PTC readthrough can be from about 70% to 100%. An in vitro efficiency of PTC readthrough can be from about 75% to 100%. An in vitro efficiency of PTC readthrough can be from about 80% to 100%. An in vitro efficiency of PTC readthrough can be from about 85% to 100%. An in vitro efficiency of PTC readthrough can be from about 90% to 100%. An in vitro efficiency of PTC

readthrough can be from about 95% to 100%. An in vitro efficiency of PTC readthrough can be from about 20% to about 40%. An in vitro efficiency of PTC readthrough can be from about 20% to 60%. An in vitro efficiency of PTC readthrough can be from about 10% to 70%.

[98] Some embodiments relate to a polypeptide. In some embodiments, the polypeptide can include a fragment of the polypeptide. In some embodiments, the polypeptide can include at least a fragment of the polypeptide. In some embodiments, the polypeptide can include a full-length polypeptide. In some embodiments, the polypeptide can be encoded by an mRNA. In some embodiments, the fragment of the polypeptide can be encoded by an mRNA with a premature stop codon. In some embodiments the full-length polypeptide can be encoded by an mRNA without a premature stop codon.

[99] The engineered tRNAs and variants thereof disclosed herein are capable of premature stop codon readthrough, as disclosed herein. For example, the engineered tRNAs and variants thereof are capable of premature stop codon readthrough of an Arg-to-opal stop codon mutation in the MECP2 gene. In some embodiments, the engineered tRNAs and variants thereof can enable premature stop codon readthrough of the R168X mutation in the MECP2 gene. In some embodiments, the engineered tRNAs and variants thereof can enable premature stop codon readthrough of the R255X mutation in the MECP2 gene. In some embodiments, the engineered tRNAs and variants thereof can enable premature stop codon readthrough of the R270X mutation in the MECP2 gene. In some embodiments, the engineered tRNAs and variants thereof can enable premature stop codon read-through of the R294X mutation in the MECP2 gene. In some embodiments, the engineered tRNAs and variants thereof can enable premature stop codon read-through of the R198X mutation in the MECP2 gene. In some embodiments, the engineered tRNAs and variants thereof can enable premature stop codon readthrough of the R453X mutation in the MECP2 gene.

[100] In some embodiments, the mRNA encoding the polypeptide corresponds to MeCP2.

[101] In some cases, an engineered tRNA or engineered tRNA variant can be acylated with a canonical amino acid. In some cases, an engineered tRNA or engineered tRNA variant can be acylated with a non-canonical amino acid. A non-canonical amino acid can comprise p-Acetylphenylalanine, p-Propargyloxyphenylalanine, p-Azidophenylalanine, O-methyltyrosine, p-Iodophenylalanine, 3-Iodotyrosine, Biphenylalanine, 2-Aminocaprylic acid, p-Benzoylphenylalanine, o-Nitrobenzylcysteine, o-Nitrobenzylserine, 4,5-Dimethoxy-2-

nitrobenzylserine, o-Nitrobenzyllysine, Dansylalanine, Acetyllysine, Methylhistidine, 2-Aminononanoic acid, 2-Aminodecanoic acid, 2-Aminodecanoic acid, Cbz-lysine, Boc-lysine, or Allyloxycarbonyllysine.

[102] In some embodiments, an engineered tRNA or engineered tRNA variant can be acylated with an amino acid selected provided in **Table 1**.

Table 1. Amino Acids, one and three letter codes

Amino acid	Three letter code	One letter code
alanine	ala	A
arginine	arg	R
asparagine	asn	N
aspartic acid	asp	D
asparagine or aspartic acid	asx	B
cysteine	cys	C
glutamic acid	glu	E
glutamine	gln	Q
glutamine or glutamic acid	glx	Z
glycine	gly	G
histidine	his	H
isoleucine	ile	I
leucine	leu	L
lysine	lys	K
methionine	met	M
phenylalanine	phe	F
proline	pro	P
serine	ser	S

threonine	**thr**	**T**
tryptophan	**trp**	**W**
tyrosine	**tyr**	**Y**
valine	**val**	**V**

[103] In some cases, the engineered tRNA or engineered tRNA variant can be acylated with a lysine. In some cases, the engineered tRNA or engineered tRNA variant can be acylated with an arginine. In some cases, the engineered tRNA or engineered tRNA variant can be acylated with a histidine. In some cases, the engineered tRNA or engineered tRNA variant can be acylated with a glycine. In some cases, the engineered tRNA or engineered tRNA variant can be acylated with an alanine. In some cases, the engineered tRNA or engineered tRNA variant can be acylated with a valine. In some cases, the engineered tRNA or engineered tRNA variant can be acylated with a leucine. In some cases, the engineered tRNA or engineered tRNA variant can be acylated with an isoleucine. In some cases, the engineered tRNA or engineered tRNA variant can be acylated with a methionine. In some cases, the engineered tRNA or engineered tRNA variant can be acylated with a phenylalanine. In some cases, the engineered tRNA or engineered tRNA variant can be acylated with a tryptophan. In some cases, the engineered tRNA or engineered tRNA variant can be acylated with a proline. In some cases, the engineered tRNA or engineered tRNA variant can be acylated with a serine. In some cases, the engineered tRNA or engineered tRNA variant can be acylated with a threonine. In some cases, the engineered tRNA or engineered tRNA variant can be acylated with a cysteine. In some cases, the engineered tRNA or engineered tRNA variant can be acylated with a tyrosine. In some cases, the engineered tRNA or engineered tRNA variant can be acylated with an asparagine. In some cases, the engineered tRNA or engineered tRNA variant can be acylated with a glutamine. In some cases, the engineered tRNA or engineered tRNA variant can be acylated with an aspartate. In some cases, the engineered tRNA or engineered tRNA variant can be acylated with a glutamate. In some cases, the engineered tRNA or engineered tRNA variant can be acylated with a pyrolysine. In some cases, the engineered tRNA or engineered tRNA variant can be acylated with a selenocysteine.

[104] In some cases, an engineered tRNA or engineered tRNA variant can be a lysyl-tRNA, an arginyl-tRNA, a histidyl-tRNA, a glycyl-tRNA, an alanyl-tRNA, a valyl-tRNA, a leucyl-

tRNA, an isoleucyl-tRNA, methionyl-tRNA, a phenylalanyl-tRNA, a tryptophanyl-tRNA, a prolyl-tRNA, a seryl-tRNA, a threonyl-tRNA, a cysteinyl-tRNA, a tyrosyl-tRNA, an asparaginyl tRNA, a glutaminyl-tRNA, an aspartyl-tRNA, a pyrrolysyl tRNA, a selenocytstyl tRNA, or a glutamyl-tRNA.

[105] This disclosure contemplates incorporating specific hybrid tRNAs or orthogonal tRNA/tRNA synthetase pairs. In some cases, an engineered tRNA or engineered tRNA variant can include a designer tRNAs (such as hybrid tRNAs made from two different naturally occurring tRNAs) or orthogonal tRNAs from other species. In some cases, a synthetic or chimeric orthogonal tRNA-tRNA synthetase pair can be used or included. In some cases, synthetic tRNAs that can interact with naturally occurring tRNA synthetases are included or used. In some cases, a pyrrolysyl tRNA or a selenocytstyl tRNA can be used in genetic code expansion to incorporate non-canonical amino acids into an engineered tRNA or engineered tRNA variant.

[106] In some cases, an engineered tRNA or engineered tRNA variant can be a lysine-tRNA, an arginine-tRNA, a histidine-tRNA, a glycine-tRNA, an alanine-tRNA, a valine-tRNA, a leucine-tRNA, an isoleucine-tRNA, methionine-tRNA, a phenylalanine-tRNA, a tryptophanine-tRNA, a proline-tRNA, a serine-tRNA, a threonine-tRNA, a cysteinine-tRNA, a tyrosine-tRNA, an asparaginine tRNA, a glutaminine-tRNA, an aspartine-tRNA, or a glutamine-tRNA.

[107] In some cases, an engineered tRNA or engineered tRNA variant can be derived from a human tRNA. In some cases, an engineered tRNA or engineered tRNA variant can be derived from a non-human tRNA. In some cases, an engineered tRNA or engineered tRNA variant can be derived from a tRNA that can be orthogonal to a human tRNA. An engineered tRNA or engineered tRNA variant can be acylated by an amino-acyl synthetase that can recognize the engineered tRNA or engineered tRNA variant and acylate the engineered tRNA or engineered tRNA variant with an amino acid. In some cases, the engineered tRNA or engineered tRNA variant can be an engineered pre-tRNA or an engineered pre-tRNA variant. Such an engineered pre-tRNA or variant thereof can comprise an intronic sequence. In some cases, an intronic sequence can be spliced to produce a mature engineered tRNA or variant thereof. In some cases, an intronic sequence can be spliced within a cell containing an engineered tRNA or variant thereof. In some cases, once the intronic sequence is spliced the mature engineered tRNA or engineered tRNA variant can at least partially transform an interpretation of a premature stop

codon into a sense codon. In some cases, an engineered pre-tRNA or variant thereof with an intron can be more efficient at transforming an interpretation of a premature stop codon into a sense codon as compared to an engineered pre-tRNA or variant thereof without an intron. The efficiency can be measured by transforming a vector encoding an engineered suppressor pre-tRNA or variant thereof with an intron into a primary cell line comprising a premature stop codon to which the engineered suppressor pre-tRNA or variant thereof recognizes and comparing the level of premature stop codon readthrough against another comparable cell that has been transformed with a vector encoding an engineered suppressor pre-tRNA or variant thereof without an intron. In some cases, determining the amount of full-length protein can be used to measure premature stop codon readthrough.

[108] An engineered tRNA or variant thereof can be engineered with an anticodon sequence that base pairs with a stop codon, instead of a codon encoding for the amino acid of interest. For example, an engineered tRNA or variant thereof targeting a premature stop codon at a position in the growing polypeptide in which an Arginine (amino acid of interest) can be normal (e.g., not causing disease). In some cases, an engineered Arg tRNA or variant thereof with an anticodon sequence that base pairs with the premature stop codon can base pair with the stop codon enabling the engineered tRNA or variant thereof charged with the Arg to add the Arg to the growing polypeptide molecule, thus, effecting readthrough of the premature stop codon.

[109] Engineered tRNAs or engineered tRNA variants of the present disclosure can be engineered to recognize and suppress an amber stop codon (UAG), an ochre stop codon (UAA), or an opal stop codon (UGA), or a combination thereof. In some cases, an engineered tRNA or variant thereof can be engineered to suppress the readthrough of an amber stop codon (UAG). In some cases, an engineered tRNA or variant thereof can be engineered to suppress the readthrough of an ochre stop codon (UAA). In some cases, an engineered tRNA or variant thereof can be engineered to suppress the readthrough of an opal stop codon (UGA). In some cases, the present disclosure provides for compositions of multiple engineered tRNAs or variants thereof capable of recognizing and suppressing more than one of the amber stop codon, the ochre stop codon, and the opal stop codon (**FIG. 13**). An engineered tRNA or variant thereof that can recognize and suppress an amber stop codon can comprise an Arginine (Arg) tRNA isodecoder. An engineered tRNA or variant thereof that can recognize and suppress an amber stop codon can comprise a Glutamine (Gln) tRNA isodecoder (**FIG. 13**). An engineered tRNA or variant thereof

that can recognize and suppress an ochre stop codon can comprise an Arginine (Arg) tRNA isodecoder. An engineered tRNA or variant thereof that can recognize and suppress an ochre stop codon can comprise a Glutamine (Gln) tRNA isodecoder (**FIG. 13**). In some embodiments, an engineered tRNA or variant thereof that can recognize and suppress an amber stop codon can have a sequence similarity of about 70% to about 100% to a naturally occurring Gln tRNA isodecoder. In some embodiments, an engineered tRNA or variant thereof that can recognize and suppress an ochre stop codon can have a sequence similarity of about 70% to about 100% to a naturally occurring Gln tRNA isodecoder. In some embodiments, an engineered tRNA or variant thereof that can recognize and suppress an amber stop codon can have a sequence similarity of about 70% to about 100% to a naturally occurring Arg tRNA isodecoder. In some embodiments, an engineered tRNA or variant thereof that can recognize and suppress an ochre stop codon can have a sequence similarity of about 70% to about 100% to a naturally occurring Arg tRNA isodecoder.

[110] An engineered tRNA or variant thereof with an anticodon configured to base pair with any one of the stop codons described herein can be charged with any one of the amino acids (canonical or noncanonical) described herein. FIG. 2A illustrates a consensus secondary structure for human Arg tRNAs. FIG. 2B illustrates sites in a consensus secondary structure and sequence of human Arg tRNA that were excluded from mutagenesis to generate engineer tRNAs disclosed herein.

[111] In some embodiments, an mRNA targeted by the engineered tRNA or variant thereof can comprise one, two, three, four, or five premature stop codons. Accordingly, an engineered tRNA or variant thereof as described herein can produce readthrough of the one or more premature stop codons, at least partially restoring a substantially full-length polypeptide. In some cases, at least partially restoring a substantially full-length polypeptide can comprise at least partially treating a disease or condition. In some cases, an engineered tRNA or variant thereof can restore about 30% to about 99% of PTC readthroughs. In some cases, an engineered tRNA or variant thereof can restore about 40% to about 45%, about 40% to about 50%, about 40% to about 55%, about 40% to about 60%, about 40% to about 65%, about 40% to about 70%, about 40% to about 75%, about 40% to about 80%, about 40% to about 85%, about 40% to about 90%, about 40% to about 95%, about 45% to about 50%, about 45% to about 55%, about 45% to about 60%, about 45% to about 65%, about 45% to about 70%, about 45% to about 75%, about 45% to

about 80%, about 45% to about 85%, about 45% to about 90%, about 45% to about 95%, about 50% to about 55%, about 50% to about 60%, about 50% to about 65%, about 50% to about 70%, about 50% to about 75%, about 50% to about 80%, about 50% to about 85%, about 50% to about 90%, about 50% to about 95%, about 55% to about 60%, about 55% to about 65%, about 55% to about 70%, about 55% to about 75%, about 55% to about 80%, about 55% to about 85%, about 55% to about 90%, about 55% to about 95%, about 60% to about 65%, about 60% to about 70%, about 60% to about 75%, about 60% to about 80%, about 60% to about 85%, about 60% to about 90%, about 60% to about 95%, about 65% to about 70%, about 65% to about 75%, about 65% to about 80%, about 65% to about 85%, about 65% to about 90%, about 65% to about 95%, about 70% to about 75%, about 70% to about 80%, about 70% to about 85%, about 70% to about 90%, about 70% to about 95%, about 75% to about 80%, about 75% to about 85%, about 75% to about 90%, about 75% to about 95%, about 80% to about 85%, about 80% to about 90%, about 80% to about 95%, about 85% to about 90%, about 85% to about 95%, or about 90% to about 95% of PTC readthroughs.. In some cases, an engineered tRNA or variant thereof can restore at least about 40% of PTC readthroughs. In some cases, two or more stop codons can be the same stop codon. In some cases, two or more stop codons can be different stop codons. In some instances, one type of engineered tRNA or variant thereof can be used to at least partially restore a full-length polypeptide when a target mRNA contains two or more stop codons that are the same stop codon. In some instances, more than one type of engineered tRNA or variant thereof can be used to at least partially restore a full-length polypeptide when a target mRNA contains two or more stop codons that are different stop codons. In some embodiments, the engineered tRNA or engineered tRNA variant can reduce or prevent nonsense-mediated decay of the target mRNA.

[112] The engineered tRNAs disclosed herein can be modified to produce an engineered tRNA variant. For example, the engineered tRNAs disclosed herein can be modified relative to a reference tRNA. In some cases, the modification can be a mutation in a sequence of the engineered tRNA, such as an insertion or a substitution of a nucleotide. In some cases, the sequence that can be mutated can be a DNA sequence, a tRNA sequence or a pre-tRNA sequence. In some cases, the modification can be a chemical modification of a nucleotide in the sequence of the engineered tRNA. The chemical modification can comprise a methyl group, a fluoro group, a methoxyethyl group, an ethyl group, a phosphate group, an amide group, an ester group, or any combination thereof. In some cases, the engineered tRNA or the variant thereof can

comprise a chemical modification comprising a methyl group, a fluoro group, a methoxyethyl group, an ethyl group, a phosphate group, an amide group, an ester group, or any combination thereof.

[113] The reference tRNA can be a wild-type tRNA or an engineered tRNA, such that the engineered tRNA has more than one mutation. In this context, the wild-type tRNA or the engineered tRNA can be referred to as a "backbone" or "parental" tRNA.

[114] Mutations can be made in any region of the engineered tRNA including, but not limited to, the acceptor stem, anticodon stem, D-loop, D stem, and T-loop, T-stem, or the variable region or loop to produce the engineered tRNA variants of the present disclosure. For example, substitutions of nucleotides in the acceptor stem and anticodon stem to increase Watson-Crick base pairing can stabilize the acceptor and anticodon stems of the engineered tRNA. Non-limiting examples of mutations to the engineered tRNAs disclosed herein are provided in **Table 2**.

Table 2. Exemplary Mutations to Engineered tRNA to produce Engineered tRNA variants

Modifications to SEQ ID NO: 3		
72T>C	**13T>C**	**31C>A**
2C>G	**22A>G**	**39G>T**
71G>C	**15A>G**	**37A>G**
6A>G	**28T>C**	**44G>A**
67T>C	**42A>G**	**50T>C**
64A>G		

Modifications to SEQ ID NO: 6			
6C>T	**67G>A**	**49C>G**	**31A>C**
65G>C	**50C>T**	**64G>A**	**39T>G**
71T>G	**4T>C**	**69A>G**	**44A>G**
6C>A	**67G>T**	**12G>C**	**46G>A**
23C>G	**27T>C**	**43A>G**	**2G>C**
28T>C	**42A>G**	**40T>C**	**71T>C**

*N>N' means that N can be substituted for N'. The number preceding N>N' indicates the nucleotide position with reference to the DNA sequence encoding the engineered tRNA. Thymine ("T") can be only present in the DNA context, and when in reference to the tRNA sequence, should be understood to refer to uracil ("U").

[115] Disclosed herein, in some embodiments, are engineered tRNA variants. The engineered tRNA variant can comprise a mutation in a T-loop, a T-stem, a D-loop, a D-stem, a variable loop, an anticodon stem, or an anticodon loop. The mutation can be relative to the nucleic acid sequence of any one of SEQ ID NOS: 3-22 or 103-122. In some embodiments, the mutation can comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 mutations, or a range of mutations defined by any two of the aforementioned numbers of mutations. In some embodiments, the mutation can comprise at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, or at least 15 mutations. In some embodiments, the mutation can comprise no more than 1, no more than 2, no more than 3, no more than 4, no more than 5, no more than 6, no more than 7, no more than 8, no more than 9, no more than 10, no more than 11, no more than 12, no more than 13, no more than 14, or no more than 15 mutations. In some embodiments, the mutation can comprise a mutation in a T-loop, a T-stem, a D-loop, a D-stem, a variable loop, an anticodon stem, an anticodon loop, or a combination thereof. In some embodiments, the mutation can comprise a mutation in a T-loop. In some embodiments, the mutation can comprise a mutation in a T-stem. In some embodiments, the mutation can comprise a mutation in a D-loop. In some embodiments, the mutation can comprise a mutation in a D-stem. In some embodiments, the mutation can comprise a mutation in a variable loop. In some embodiments, the mutation can comprise a mutation in an anticodon stem. In some embodiments, the mutation can comprise a mutation in an anticodon loop. In some embodiments, the mutation can comprise a mutation in a T-loop and a T-stem. In some embodiments, the mutation can comprise a mutation in a T-loop and a D-loop. In some embodiments, the mutation can comprise a mutation in a T-loop and a D-stem. In some embodiments, the mutation can comprise a mutation in a T-loop and a variable loop. In some embodiments, the mutation can comprise a mutation in a T-loop and an anticodon stem. In some embodiments, the mutation can comprise a mutation in a T-loop and an anticodon loop. In some embodiments, the mutation can comprise a mutation in a T-stem and a D-loop. In some embodiments, the mutation can comprise a mutation in a T-stem and a D-stem. In some embodiments, the mutation can comprise a mutation in a T-stem and a variable loop. In some embodiments, the mutation can comprise a mutation in a T-stem and an anticodon stem. In some embodiments, the mutation can comprise a mutation in a T-stem and an anticodon stem. In some embodiments, the mutation can comprise a mutation in a D-loop and a D-stem. In some

embodiments, the mutation can comprise a mutation in a D-loop and a variable loop. In some embodiments, the mutation can comprise a mutation in a D-loop and an anticodon stem. In some embodiments, the mutation can comprise a mutation in a D-loop and an anticodon stem. In some embodiments, the mutation can comprise a mutation in a D-stem and a variable loop. In some embodiments, the mutation can comprise a mutation in a D-stem and an anticodon stem. In some embodiments, the mutation can comprise a mutation in a D-stem and an anticodon stem. In some embodiments, the mutation can comprise a mutation in an anticodon stem and an anticodon stem. In some embodiments, the mutation can comprise a mutation in an anticodon stem and an anticodon stem.

[116] In some cases, the engineered tRNA variant can be a variant of the engineered tRNA variant can comprise 72C, 2G, 71C, 6G, 67C, 64G, 13C, 22G, 15G, 28C, 42G, 31A, 39T, 37G, 44A, or 50C, or any combination thereof, in relation to SEQ ID NO: 3. In some cases, the engineered tRNA variant can comprise 72C. In some cases, the engineered tRNA variant can comprise 2G. In some cases, the engineered tRNA variant can comprise 71G. In some cases, the engineered tRNA variant can comprise 6G. In some cases, the engineered tRNA variant can comprise 67C. In some cases, the engineered tRNA variant can comprise 64G. In some cases, the engineered tRNA variant can comprise 13C. In some cases, the engineered tRNA variant can comprise 22G. In some cases, the engineered tRNA can comprise 15G. In some cases, the engineered tRNA variant can comprise 28C. In some cases, the engineered tRNA variant can comprise 42G. In some cases, the engineered tRNA variant can comprise 31A. In some cases, the engineered tRNA variant can comprise 39T. In some cases, the engineered tRNA variant can comprise 37G. In some cases, the engineered tRNA variant can comprise 44A. In some cases, the engineered tRNA variant can comprise 50C. In some cases, the engineered tRNA variant can have a sequence identity of about 70% to about 100% to SEQ ID NO: 3. In some cases, the engineered tRNA variant can comprise one of the engineered tRNA variants comprising the sequence of SEQ ID NO: 23, 24, 25, 25, 27, 28, 29, 30, 31 or 32. In some cases, the engineered tRNA variant can have a sequence identity of about 70% to about 100% to an engineered tRNA variant comprising the sequence of SEQ ID NO: 23, 24, 25, 25, 27, 28, 29, 30, 31 or 32.

[117] In some cases, the engineered tRNA variant can be a variant of the engineered tRNA TCT1 comprising 2C, 6T, 65C, 71G, 71C, 6A, 23G, 28C, 67A, 50T, 4C, 67T, 27C, 42G, 49G, 64A, 69G, 12C, 43G, 40C, 31C, 39G, 44G or 46A, or any combination thereof, relative to the

sequence of SEQ ID NO: 6. In some cases, the engineered tRNA variant can comprise 6T. In some cases, the engineered tRNA variant can comprise 65C. In some cases, the engineered tRNA variant can comprise 2C. In some cases, the engineered tRNA variant can comprise 71C. In some cases, the engineered tRNA variant can comprise 46A. In some cases, the engineered tRNA variant can comprise 71G. In some cases, the engineered tRNA variant can comprise 6A. In some cases, the engineered tRNA variant can comprise 23G. In some cases, the engineered tRNA variant can comprise 28C. In some cases, the engineered tRNA variant can comprise 67A. In some cases, the engineered tRNA variant can comprise 50T. In some cases, the engineered tRNA variant can comprise 4C. In some cases, the engineered tRNA variant can comprise 67T. In some cases, the engineered tRNA variant can comprise 27C. In some cases, the engineered tRNA variant can comprise 42G. In some cases, the engineered tRNA variant can comprise 49G. In some cases, the engineered tRNA variant can comprise 64A. In some cases, the engineered tRNA variant can comprise 69G. In some cases, the engineered tRNA variant can comprise 12C. In some cases, the engineered tRNA variant can comprise 43G. In some cases, the engineered tRNA variant can comprise 40C. In some cases, the engineered tRNA variant can comprise 31C. In some cases, the engineered tRNA variant can comprise 39G. In some cases, the engineered tRNA variant can comprise 44G. In some cases, the engineered tRNA variant can have a sequence identity of about 70% to about 100% to the sequence of SEQ ID NO: 6. In some cases, the engineered tRNA variant comprise one of the sequences of SEQ ID NO: 35-48. In some cases, the engineered tRNA variant can comprise a sequence selected from SEQ ID NO: 3, SEQ ID NO: 32, SEQ ID NO: 6, or SEQ ID NO: 45. In some cases, the engineered tRNA variant includes SEQ ID NO: 3, SEQ ID NO: 32, SEQ ID NO: 6, or SEQ ID NO: 45, and SEQ ID NOS: 1 and 2. In some cases, the engineered tRNA variant can comprise SEQ ID NO: 3. In some cases, the engineered tRNA variant can comprise SEQ ID NO: 32. In some cases, the engineered tRNA variant can comprise SEQ ID NO: 6. In some cases, the engineered tRNA variant can comprise SEQ ID NO: 45. In some cases, the engineered tRNA variant can have a sequence identity of about 70% to about 100% to any of the engineered tRNA variants comprising sequences of SEQ ID NO: 35-48. In some cases, the engineered tRNA variant can have a sequence identity of 70%, 75%, 80%, 85%, 90%, 95%, or 100% compared to any of the engineered tRNA variants comprising sequences of SEQ ID NO: 35-48.

[118] In some cases, the engineered tRNA variant can comprise more than or equal to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acid substitutions. In some cases, the engineered tRNA variant can comprise no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acid substitutions. In some cases, the engineered tRNA or variant thereof does not comprise a substitute in the anticodon. In some cases, the engineered tRNAs or engineered tRNA variants described herein can be provided in **Table 3**. In some embodiments, an engineered tRNA or a variant thereof can be flanked by a 5' end sequence comprising the sequence of SEQ ID NO: 1 and/or a 3' end sequence comprising the sequence of SEQ ID NO: 2. In some embodiments, the 5' end sequence can be the leader sequence of the engineered tRNA or a variant thereof. In some embodiments, the 3' end sequence can be the poly-T termination signal and a trailer sequence of the engineered tRNA or a variant thereof.

Table 3. tRNA Sequences

Name	SEQ ID NO:	5' to 3' tRNA sequence (DNA)	SEQ ID NO:	5' to 3' tRNA sequence (RNA)
5' end of tRNA	1	GGTGGTATTGCCCGGAGTAGAAGCACCTGTGACTAACTGTGCCAA	101	GGUGGUAUUGCCCGGAGUAGAAGCACCUGUGACUAACUGUGCCAA
3' end of tRNA	2	TTTTTTGATGGCCAGCTCCGAGGATCTAGCGTACTGAGTCGCCCAGTCT	102	UUUUUUGAUGGCCAGCUCCGAGGAUCUAGCGUACUGAGUCGCCCAGUCU
CCT2	3	GCCCCAGTGGCCTAATGGATAAGGCACTGGCCTTCAAAGCCAGGGATTGTGGGTTCGAGTCCCACCTGGGGTG	103	GCCCCAGUGGCCUAAUGGAUAAGGCACUGGCCUUCAAAGCCAGGGAUUGUGGGUUCGAGUCCCACCUGGGGUG
CCT1	4	GCCCCAGTGGCCTAATGGATAAGGCACTGGCCTTCAAAGCCAGGGATTGTGGGTTCGAGTCCCACCTGGGGTA	104	GCCCCAGUGGCCUAAUGGAUAAGGCACUGGCCUUCAAAGCCAGGGAUUGUGGGUUCGAGUCCCACCUGGGGUA
CCT3	5	GCCCCGGTGGCCTAATGGATAAGGCATTGGCCTTCAAAGCCAGGGATTGTGGGTTCGAGTCCCACCCGGGGTA	105	GCCCCGGUGGCCUAAUGGAUAAGGCAUUGGCCUUCAAAGCCAGGGAUUGUGGGUUCGAGUCCCACCCGGGGUA
TCT1	6	GGCTCCGTGGCGCAATGGATAGCGCATTGGACTTCAAATTCAAAGGTTCCGGGTTCGAGTCCCGGCGGAGTCG	106	GGCUCCGUGGCGCAAUGGAUAGCGCAUUGGACUUCAAAUUCAAAGGUUCCGGGUUCGAGUCCCGGCGGAGUCG
CCT4	7	GCCCCAGTGGCCTAATGGATAAGGCATTGGCCTTCAAAGCCAGG	107	GCCCCAGUGGCCUAAUGGAUAAGGCAUUGGCCUUCAAAGCCAGGGA

		GATTGTGGGTTCGAGTCCCATCTGGGGTG		UUGUGGGUUCGAGUCCCAUCUGGGGUG
CCT5	8	GCCCCAGTGGCCTGATGGATAAGGTACTGGCCTTCAAAGCCAGGGATTGTGGGTTCGAGTTCCACCTGGGGTA	108	GCCCCAGUGGCCUGAUGGAUAAGGUACUGGCCUUCAAAGCCAGGGAUUGUGGGUUCGAGUUCCACCUGGGGUA
TCT2	9	GGCTCTGTGGCGCAATGGATAGCGCATTGGACTTCAAATTCAAAGGTTGTGGGTTCGAATCCCACCAGAGTCG	109	GGCUCUGUGGCGCAAUGGAUAGCGCAUUGGACUUCAAAUUCAAAGGUUGUGGGUUCGAAUCCCACCAGAGUCG
TCT3	10	GGCTCTGTGGCGCAATGGATAGCGCATTGGACTTCAAATTCAAAGGTTGTGGGTTCGAGTCCCACCAGAGTCG	110	GGCUCUGUGGCGCAAUGGAUAGCGCAUUGGACUUCAAAUUCAAAGGUUGUGGGUUCGAGUCCCACCAGAGUCG
TCT4	11	GTCTCTGTGGCGCAATGGACGAGCGCGCTGGACTTCAAATCCAGAGGTTCCGGGTTCGAGTCCCGGCAGAGATG	111	GUCUCUGUGGCGCAAUGGACGAGCGCGCUGGACUUCAAAUCCAGAGGUUCCGGGUUCGAGUCCCGGCAGAGAUG
TCT5	12	GGCTCTGTGGCGCAATGGATAGCGCATTGGACTTCAAATTCAAAGGTTGCGGGTTCGAGTCCCTCCAGAGTCG	112	GGCUCUGUGGCGCAAUGGAUAGCGCAUUGGACUUCAAAUUCAAAGGUUGCGGGUUCGAGUCCCUCCAGAGUCG
TCG1	13	GGCCGCGTGGCCTAATGGATAAGGCGTCTGACTTCAGATCAGAAGATTGCAGGTTCGAGTCCTGCCGCGGTCG	113	GGCCGCGUGGCCUAAUGGAUAAGGCGUCUGACUUCAGAUCAGAAGAUUGCAGGUUCGAGUCCUGCCGCGGUCG
TCG2	14	GACCACGTGGCCTAATGGATAAGGCGTCTGACTTCAGATCAGAAGATTGAGGGTTCGAATCCCTCCGTGGTTA	114	GACCACGUGGCCUAAUGGAUAAGGCGUCUGACUUCAGAUCAGAAGAUUGAGGGUUCGAAUCCCUCCGUGGUUA
TCG3	15	GACCGCGTGGCCTAATGGATAAGGCGTCTGACTTCAGATCAGAAGATTGAGGGTTCGAGTCCCTTCGTGGTCG	115	GACCGCGUGGCCUAAUGGAUAAGGCGUCUGACUUCAGAUCAGAAGAUUGAGGGUUCGAGUCCCUUCGUGGUCG
TCG4	16	GACCACGTGGCCTAATGGATAAGGCGTCTGACTTCAGATCAGAAGATTGAGGGTTCGAATCCCTTCGTGGTTA	116	GACCACGUGGCCUAAUGGAUAAGGCGUCUGACUUCAGAUCAGAAGAUUGAGGGUUCGAAUCCCUUCGUGGUUA
TCG5	17	GACCACGTGGCCTAATGGATAAGGCGTCTGACTTCAGATCAGAAGATTGAGGGTTCGAATCCCTTCGTGGTTG	117	GACCACGUGGCCUAAUGGAUAAGGCGUCUGACUUCAGAUCAGAAGAUUGAGGGUUCGAAUCCCUUCGUGGUUG
TCG6	18	GGCCGTGTGGCCTAATGGATAAGGCGTCTGACTTCAGATCAAAA	118	GGCCGUGUGGCCUAAUGGAUAAGGCGUCUGACUUCAGAUCAAAAGA

		GATTGCAGGTTTGAGTTCTGCCACGGTCG		UUGCAGGUUUGAGUUCUGCCACGGUCG
CCG1	19	GGCCGCGTGGCCTAATGGATAAGGCGTCTGATTTCAGATCAGAAGATTGAGGGTTCGAGTCCCTTCGTGGTCG	119	GGCCGCGUGGCCUAAUGGAUAAGGCGUCUGAUUUCAGAUCAGAAGAUUGAGGGUUCGAGUCCCUUCGUGGUCG
CCG2	20	GACCCAGTGGCCTAATGGATAAGGCATCAGCCTTCAGAGCTGGGGATTGTGGGTTCGAGTCCCATCTGGGTCG	120	GACCCAGUGGCCUAAUGGAUAAGGCAUCAGCCUUCAGAGCUGGGGAUUGUGGGUUCGAGUCCCAUCUGGGUCG
ACG1	21	GGGCCAGTGGCGCAATGGATAACGCGTCTGACTTCAGATCAGAAGATTCCAGGTTCGACTCCTGGCTGGCTCG	121	GGGCCAGUGGCGCAAUGGAUAACGCGUCUGACUUCAGAUCAGAAGAUUCCAGGUUCGACUCCUGGCUGGCUCG
ACG2	22	GGGCCAGTGGCGCAATGGATAACGCGTCTGACTTCAGATCAGAAGATTCTAGGTTCGACTCCTGGCTGGCTCG	122	GGGCCAGUGGCGCAAUGGAUAACGCGUCUGACUUCAGAUCAGAAGAUUCUAGGUUCGACUCCUGGCUGGCUCG
CCT2.1	23	GCCCCAGTGGCCTAATGGATAAGGCACTGGCCTTCAAAGCCAGGGATTGTGGGTTCGAGTCCCACCTGGGGCG	123	GCCCCAGUGGCCUAAUGGAUAAGGCACUGGCCUUCAAAGCCAGGGAUUGUGGGUUCGAGUCCCACCUGGGGCG
CCT2.2	24	GGCCCAGTGGCCTAATGGATAAGGCACTGGCCTTCAAAGCCAGGGATTGTGGGTTCGAGTCCCACCTGGGCTG	124	GGCCCAGUGGCCUAAUGGAUAAGGCACUGGCCUUCAAAGCCAGGGAUUGUGGGUUCGAGUCCCACCUGGGCUG
CCT2.3	25	GCCCCGGTGGCCTAATGGATAAGGCACTGGCCTTCAAAGCCAGGGATTGTGGGTTCGAGTCCCACCCGGGGTG	125	GCCCCGGUGGCCUAAUGGAUAAGGCACUGGCCUUCAAAGCCAGGGAUUGUGGGUUCGAGUCCCACCCGGGGUG
CCT2.4	26	GCCCCAGTGGCCCAATGGATAGGGCACTGGCCTTCAAAGCCAGGGATTGTGGGTTCGAGTCCCACCTGGGGTG	126	GCCCCAGUGGCCCAAUGGAUAGGGCACUGGCCUUCAAAGCCAGGGAUUGUGGGUUCGAGUCCCACCUGGGGUG
CCT2.5	27	GCCCCAGTGGCCTAGTGGATAAGGCACTGGCCTTCAAAGCCAGGGATTGTGGGTTCGAGTCCCACCTGGGGTG	127	GCCCCAGUGGCCUAGUGGAUAAGGCACUGGCCUUCAAAGCCAGGGAUUGUGGGUUCGAGUCCCACCUGGGGUG
CCT2.6	28	GCCCCAGTGGCCTAATGGATAAGGCACCGGCCTTCAAAGCCGGGGATTGTGGGTTCGAGTCCCACCTGGGGTG	128	GCCCCAGUGGCCUAAUGGAUAAGGCACCGGCCUUCAAAGCCGGGGAUUGUGGGUUCGAGUCCCACCUGGGGUG
CCT2.7	29	GCCCCAGTGGCCTAATGGATAAGGCACTGGACTTCAAATCCAGG	129	GCCCCAGUGGCCUAAUGGAUAAGGCACUGGACUUCAAAUCCAGGGA

		GATTGTGGGTTCGAGTCCCACC TGGGGTG		UUGUGGGUUCGAGUCCCACCUGG GGUG
CCT2.8	30	GCCCCAGTGGCCTAATGGATAA GGCACTGGCCTTCAGAGCCAGG GATTGTGGGTTCGAGTCCCACC TGGGGTG	130	GCCCCAGUGGCCUAAUGGAUAAG GCACUGGCCUUCAGAGCCAGGGA UUGUGGGUUCGAGUCCCACCUGG GGUG
CCT2.9	31	GCCCCAGTGGCCTAATGGATAA GGCACTGGCCTTCAAAGCCAGA GATTGTGGGTTCGAGTCCCACC TGGGGTG	131	GCCCCAGUGGCCUAAUGGAUAAG GCACUGGCCUUCAAAGCCAGAGA UUGUGGGUUCGAGUCCCACCUGG GGUG
CCT2.10	32	GCCCCAGTGGCCTAATGGATAA GGCACTGGCCTTCAAAGCCAGG GATTGCGGGTTCGAGTCCCGCC TGGGGTG	132	GCCCCAGUGGCCUAAUGGAUAAG GCACUGGCCUUCAAAGCCAGGGA UUGCGGGUUCGAGUCCCGCCUGG GGUG
CCT1.1	33	GCCCCAGTGGCCTAATGGATAA GGCACTGGCCTTCAAAGCCAGG GATTGTGGGTTCGAGTCCCACC TGGGGTG	133	GCCCCAGUGGCCUAAUGGAUAAG GCACUGGCCUUCAAAGCCAGGGA UUGUGGGUUCGAGUCCCACCUGG GGUG
CCT3.1	34	GCCCCGGTGGCCTAATGGATAA GGCATTGGCCTTCAAAGCCAGG GATTGTGGGTTCGAGTCCCACC CGGGGTG	134	GCCCCGGUGGCCUAAUGGAUAAG GCAUUGGCCUUCAAAGCCAGGGA UUGUGGGUUCGAGUCCCACCCGG GGUG
TCT1.1	35	GGCTCTGTGGCGCAATGGATAG CGCATTGGACTTCAAATTCAAA GGTTCCGGGTTCGAGTCCCGGC AGAGTCG	135	GGCUCUGUGGCGCAAUGGAUAGC GCAUUGGACUUCAAAUUCAAAGG UUCCGGGUUCGAGUCCCGGCAGA GUCG
TCT1.2	36	GGCTCCGTGGCGCAATGGATAG CGCATTGGACTTCAAATTCAAA GGTTGCGGGTTCGAGTCCCGCC GGAGTCG	136	GGCUCCGUGGCGCAAUGGAUAGC GCAUUGGACUUCAAAUUCAAAGG UUGCGGGUUCGAGUCCCGCCGGA GUCG
TCT1.3	37	GGCTCCGTGGCGCAATGGATAG CGCATTGGACTTCAAATTCAAA GGTTCTGGGTTCGAGTCCCAGC GGAGTCG	137	GGCUCCGUGGCGCAAUGGAUAGC GCAUUGGACUUCAAAUUCAAAGG UUCUGGGUUCGAGUCCCAGCGGA GUCG
TCT1.4	38	GGCTCCGTGGCGCAATGGATAG CGCATTGGACTTCAAATTCAAA GGTTCCGGGTTCGAGTCCCGGC GGAGCCG	138	GGCUCCGUGGCGCAAUGGAUAGC GCAUUGGACUUCAAAUUCAAAGG UUCCGGGUUCGAGUCCCGGCGGA GCCG
TCT1.5	39	GCCTCCGTGGCGCAATGGATAG CGCATTGGACTTCAAATTCAAA GGTTCCGGGTTCGAGTCCCGGC GGAGGCG	139	GCCUCCGUGGCGCAAUGGAUAGC GCAUUGGACUUCAAAUUCAAAGG UUCCGGGUUCGAGUCCCGGCGGA GGCG
TCT1.6	40	GGCCCCGTGGCGCAATGGATAG CGCATTGGACTTCAAATTCAAA	140	GGCCCCGUGGCGCAAUGGAUAGC GCAUUGGACUUCAAAUUCAAAGG

		GGTTCCGGGTTCGAGTCCCGGCGGGGTCG		UUCCGGGUUCGAGUCCCGGCGGGGUCG
TCT1.7	41	GGCTCAGTGGCGCAATGGATAGCGCATTGGACTTCAAATTCAAAGGTTCCGGGTTCGAGTCCCGGCTGAGTCG	141	GGCUCAGUGGCGCAAUGGAUAGCGCAUUGGACUUCAAAUUCAAAGGUUCCGGGUUCGAGUCCCGGCUGAGUCG
TCT1.8	42	GGCTCCGTGGCCCAATGGATAGGGCATTGGACTTCAAATTCAAAGGTTCCGGGTTCGAGTCCCGGCGGAGTCG	142	GGCUCCGUGGCCCAAUGGAUAGGGCAUUGGACUUCAAAUUCAAAGGUUCCGGGUUCGAGUCCCGGCGGAGUCG
TCT1.9	43	GGCTCCGTGGCGCAATGGATAGCGCACTGGACTTCAAATTCAGAGGTTCCGGGTTCGAGTCCCGGCGGAGTCG	143	GGCUCCGUGGCGCAAUGGAUAGCGCACUGGACUUCAAAUUCAGAGGUUCCGGGUUCGAGUCCCGGCGGAGUCG
TCT1.10	44	GGCTCCGTGGCGCAATGGATAGCGCATCGGACTTCAAATTCGAAGGTTCCGGGTTCGAGTCCCGGCGGAGTCG	144	GGCUCCGUGGCGCAAUGGAUAGCGCAUCGGACUUCAAAUUCGAAGGUUCCGGGUUCGAGUCCCGGCGGAGUCG
TCT1.11	45	GGCTCCGTGGCGCAATGGATAGCGCATTGGACTTCAAATCCAAAGGTTCCGGGTTCGAGTCCCGGCGGAGTCG	145	GGCUCCGUGGCGCAAUGGAUAGCGCAUUGGACUUCAAAUCCAAAGGUUCCGGGUUCGAGUCCCGGCGGAGUCG
TCT1.12	46	GGCTCCGTGGCGCAATGGATAGCGCATTGGCCTTCAAAGTCAAAGGTTCCGGGTTCGAGTCCCGGCGGAGTCG	146	GGCUCCGUGGCGCAAUGGAUAGCGCAUUGGCCUUCAAAGUCAAAGGUUCCGGGUUCGAGUCCCGGCGGAGUCG
TCT1.13	47	GGCTCCGTGGCGCAATGGATAGCGCATTGGACTTCAAATTCAAGGGTTCCGGGTTCGAGTCCCGGCGGAGTCG	147	GGCUCCGUGGCGCAAUGGAUAGCGCAUUGGACUUCAAAUUCAAGGGUUCCGGGUUCGAGUCCCGGCGGAGUCG
TCT1.14	48	GGCTCCGTGGCGCAATGGATAGCGCATTGGACTTCAAATTCAAAGATTCCGGGTTCGAGTCCCGGCGGAGTCG	148	GGCUCCGUGGCGCAAUGGAUAGCGCAUUGGACUUCAAAUUCAAAGAUUCCGGGUUCGAGUCCCGGCGGAGUCG

[119] Some embodiments refer to a DNA sequence (e.g. any of SEQ ID NOS: 1-48). In some embodiments, the DNA sequence can be interchangeable with a similar RNA sequence (e.g. any of SEQ ID NOs: 101-148). Some embodiments refer to an RNA sequence. In some embodiments, the RNA sequence can be interchangeable with a similar DNA sequence. In some embodiments, Us and Ts can be interchanged in a sequence provided herein. Some embodiments refer to a DNA sequence such as a DNA sequence provided in **Table 3**. In some embodiments, the DNA sequence can be interchangeable with a similar RNA sequence provided herein, such as

a corresponding RNA sequence in **Table 3**. Some embodiments refer to an RNA sequence such as an RNA sequence provided in **Table 3**. In some embodiments, the RNA sequence can be interchangeable with a similar DNA sequence provided herein, such as a corresponding DNA sequence in **Table 3**. In some embodiments, an engineered tRNA described herein can comprise the DNA sequence provided in **Table 3**. In some embodiments, an engineered tRNA described herein has the RNA sequence provided in **Table 3**. In general, Us and Ts can be interchanged between the sequences, and still be useful for the methods and compositions described herein. Some embodiments describing an engineered tRNA or tRNA sequence can include a DNA or RNA sequence in **Table 3**. Some embodiments describing an engineered tRNA or tRNA sequence can include a DNA sequence in **Table 3**. Some embodiments describing an engineered tRNA or tRNA sequence can include an RNA sequence in **Table 3**. In some cases, a reference to any of SEQ ID NOS: 1-48 can include a reference to a corresponding sequence within SEQ ID NOS: 101-148, or vice versa. In some cases, a reference made herein to an engineered tRNA or engineered tRNA variant comprising a DNA sequence of **Table 3** can include a tRNA comprising an RNA sequence that is encoded by a polynucleotide comprising the DNA sequence.

[120] In some embodiments, A can be a nucleobase comprising adenosine. In some embodiments, A can be a nucleoside comprising a ribose or a deoxyribose, and adenosine. In some embodiments, A can be a nucleotide comprising a phosphate, a ribose or a deoxyribose, and adenosine. In some embodiments, T can be a nucleobase comprising thymine. In some embodiments, T can be a nucleoside comprising a ribose or a deoxyribose, and thymine. In some embodiments, T can be a nucleotide comprising a phosphate, a ribose or a deoxyribose, and thymine. In some embodiments, U can be a nucleobase comprising uracil. In some embodiments, U can be a nucleoside comprising a ribose or a deoxyribose, and uracil. In some embodiments, U can be a nucleotide comprising a phosphate, a ribose or a deoxyribose, and uracil. In some embodiments, C can be a nucleobase comprising cytosine. In some embodiments, C can be a nucleoside comprising a ribose or a deoxyribose, and cytosine. In some embodiments, C can be a nucleotide comprising a phosphate, a ribose or a deoxyribose, and cytosine. In some embodiments, G can be a nucleobase comprising guanine. In some embodiments, G can be a nucleoside comprising a ribose or a deoxyribose, and guanine. In some embodiments, G can be a nucleotide comprising a phosphate, a ribose or a deoxyribose, and guanine.

[121] In some embodiments, the engineered tRNA or a variant thereof can comprise a sequence that has about 70% to about 99% identity to any of the sequences of SEQ ID NO: 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, or 22, as measured using BLAST. In some cases, the sequence identity between an engineered tRNA and any of the sequences of SEQ ID NO: 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, or 22, can be from about 70% to about 80%, from 80% to about 90%, from about 85% to about 95%, from about 90% to about 95%, from about 95% to about 99%. In some cases, the sequence identity between an engineered tRNA and any of the sequences of SEQ ID NO: 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, or 22, can be at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more. In some cases, the sequence identity between an engineered tRNA and any of the sequences of SEQ ID NO: 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, or 22, can be at most about 99%, 98%, 97%, 69%, 59%, 94%, 93%, 92%, 91%, 89%, 88%, 87%, 86%, 85%, 84%, 83%, 82%, 81%, 80%, 79%, 78%, 77%, 76%, 75%, 74%, 73%, 72%, 71%, 70%, or less. For example, an engineered tRNA comprising the sequence of SEQ ID NO: 4 can have a sequence identity of about 98.6% to SEQ ID NO: 3.

[122] In some embodiments, the engineered tRNA or a variant thereof can comprise a sequence that has about 70% to about 99% identity to any of the sequences of SEQ ID NO: 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, or 122, as measured using BLAST. In some cases, the sequence identity between an engineered tRNA and any of the sequences of SEQ ID NO: 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, or 122, can be from about 70% to about 80%, from 80% to about 90%, from about 85% to about 95%, from about 90% to about 95%, from about 95% to about 99%. In some cases, the sequence identity between an engineered tRNA and any of the sequences of SEQ ID NO: 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, or 122, can be at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more. In some cases, the sequence identity between an engineered tRNA and any of the sequences of SEQ ID NO: 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, or 122, can be at most about 99%, 98%, 97%, 69%, 59%, 94%, 93%, 92%, 91%, 89%, 88%, 87%, 86%, 85%, 84%, 83%, 82%,

81%, 80%, 79%, 78%, 77%, 76%, 75%, 74%, 73%, 72%, 71%, 70%, or less. For example, an engineered tRNA comprising the sequence of SEQ ID NO: 104 can have a sequence identity of about 98.6% to SEQ ID NO: 103.

[123] In some cases, the engineered tRNA or a variant thereof can comprise a sequence that has about 70% to about 99% identity to any of the sequences of SEQ ID NOS: 3, 4, 5, or 6, as measured using BLAST. In some cases, the sequence identity between an engineered tRNA and any of the sequences of SEQ ID NO: 3, 4, 5, or 6 can be from about 70% to about 80%, from 80% to about 90%, from about 85% to about 95%, from about 90% to about 95%, from about 95% to about 99%. In some cases, the sequence identity between an engineered tRNA and any of the sequences of SEQ ID NO: 3, 4, 5, or 6 can be at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more. In some cases, the sequence identity between an engineered tRNA and any of the sequences of SEQ ID NO: 3, 4, 5, or 6 can be at most about 99%, 98%, 97%, 69%, 59%, 94%, 93%, 92%, 91%, 89%, 88%, 87%, 86%, 85%, 84%, 83%, 82%, 81%, 80%, 79%, 78%, 77%, 76%, 75%, 74%, 73%, 72%, 71%, 70%, or less. For example, an engineered tRNA variant comprising the sequence of SEQ ID NO: 35 can comprise a sequence identity of about 97% to the sequence of SEQ ID NO: 6.

[124] In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has about 70% to about 99% identity to any of the sequences of SEQ ID NO: 3, 6, 7, 32, or 45. In some cases, the sequence identity between an engineered tRNA and any of the sequences of SEQ ID NO: 3, 6, 7, 32, or 45 can be from about 70% to about 80%, from 80% to about 90%, from about 85% to about 95%, from about 90% to about 95%, from about 95% to about 99%.

[125] In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has about 70% to about 100% identity to an engineered tRNA or engineered tRNA variant sequence disclosed herein (e.g. comprising a sequence disclosed in **Table 3**). In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has about 70% to about 99% identity to an engineered tRNA or engineered tRNA variant sequence. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 70% identity compared to an engineered tRNA or engineered tRNA variant sequence. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 75% identity compared to an engineered tRNA or engineered tRNA variant sequence. In some cases, an

engineered tRNA or a variant thereof can comprise a sequence that has at least 80% identity compared to an engineered tRNA or engineered tRNA variant sequence. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 85% identity compared to an engineered tRNA or engineered tRNA variant sequence. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 90% identity compared to an engineered tRNA or engineered tRNA variant sequence. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 91% identity compared to an engineered tRNA or engineered tRNA variant sequence. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 92% identity compared to an engineered tRNA or engineered tRNA variant sequence. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 93% identity compared to an engineered tRNA or engineered tRNA variant sequence. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 94% identity compared to an engineered tRNA or engineered tRNA variant sequence. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 95% identity compared to an engineered tRNA or engineered tRNA variant sequence. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 96% identity compared to an engineered tRNA or engineered tRNA variant sequence. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 97% identity compared to an engineered tRNA or engineered tRNA variant sequence. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 98% identity compared to an engineered tRNA or engineered tRNA variant sequence. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 99% identity compared to an engineered tRNA or engineered tRNA variant sequence. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has 100% identity compared to an engineered tRNA or engineered tRNA variant sequence. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has no more than 75% identity compared to an engineered tRNA or engineered tRNA variant sequence. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has no more than 80% identity compared to an engineered tRNA or engineered tRNA variant sequence. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has no more than 85% identity compared

to an engineered tRNA or engineered tRNA variant sequence. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has no more than 90% identity compared to an engineered tRNA or engineered tRNA variant sequence. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has no more than 91% identity compared to an engineered tRNA or engineered tRNA variant sequence. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has no more than 92% identity compared to an engineered tRNA or engineered tRNA variant sequence. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has no more than 93% identity compared to an engineered tRNA or engineered tRNA variant sequence. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has no more than 94% identity compared to an engineered tRNA or engineered tRNA variant sequence. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has no more than 95% identity compared to an engineered tRNA or engineered tRNA variant sequence. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has no more than 96% identity compared to an engineered tRNA or engineered tRNA variant sequence. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has no more than 97% identity compared to an engineered tRNA or engineered tRNA variant sequence. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has no more than 98% identity compared to an engineered tRNA or engineered tRNA variant sequence. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has no more than 99% identity compared to an engineered tRNA or engineered tRNA variant sequence. In some embodiments, the % identity can be over a range (e.g. 70-100% of the length) of nucleotides of an engineered tRNA or engineered tRNA variant sequence. In some embodiments, the % identity can be over a range of 70% of the length of nucleotides of an engineered tRNA or engineered tRNA variant sequence. In some embodiments, the % identity can be over a range of 75% of the length of nucleotides of an engineered tRNA or engineered tRNA variant sequence. In some embodiments, the % identity can be over a range of 80% of the length of nucleotides of an engineered tRNA or engineered tRNA variant sequence. In some embodiments, the % identity can be over a range of 85% of the length of nucleotides of an engineered tRNA or engineered tRNA variant sequence. In some embodiments, the % identity can over a range of 90% of the length of nucleotides of an engineered tRNA or engineered tRNA variant sequence. In some embodiments, the % identity

can be over a range of 95% of the length of nucleotides of an engineered tRNA or engineered tRNA variant sequence. In some embodiments, the % identity can be over a range of 100% of the length of nucleotides of an engineered tRNA or engineered tRNA variant sequence. The tRNA or engineered tRNA variant sequence can be a sequence of **Table 3**. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 1. The engineered tRNA or engineered tRNA variant can include SEQ ID NO: 1 or 101, and any of SEQ ID NOS: 3-48. The engineered tRNA or engineered tRNA variant can include SEQ ID NO: 1 or 101, and any of SEQ ID NOs: 103-148. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 2. The engineered tRNA or engineered tRNA variant can include any of SEQ ID NOS: 3-48, in addition to SEQ ID NO: 2. The engineered tRNA or engineered tRNA variant can include any of SEQ ID NOs: 103-148, in addition to SEQ ID NO: 102. The engineered tRNA or engineered tRNA variant can comprise or consist of SEQ ID NO: 1, any of SEQ ID NOS: 3-48, and SEQ ID NO: 2. The engineered tRNA or engineered tRNA variant can comprise or consist of SEQ ID NO: 101, any of SEQ ID NOs: 103-148, and SEQ ID NO: 102. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 3. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 4. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 5. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 6. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 7. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 8. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 9. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 10. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 11. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 12. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 13. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 14. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 15. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can

comprise SEQ ID NO: 16. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 17. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 18. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 19. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 20. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 21. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 22. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 23. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 24. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 25. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 26. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 27. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 28. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 29. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 30. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 31. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 32. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 33. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 34. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 35. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 36. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 37. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 38. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 39. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 40. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 41. In some embodiments, the engineered tRNA or engineered tRNA

variant sequence can comprise SEQ ID NO: 42. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 43. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 44. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 45. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 46. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 47. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 48. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 101. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 102. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 103. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 104. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 105. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 106. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 107. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 108. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 109. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 110. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 111. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 112. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 113. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 114. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 115. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 116. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 117. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 118. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 119. In some embodiments, the engineered tRNA or

engineered tRNA variant sequence can comprise SEQ ID NO: 120. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 121. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 122. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 123. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 124. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 125. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 126. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 127. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 128. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 129. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 130. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 131. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 132. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 133. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 134. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 135. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 136. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 137. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 138. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 139. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 140. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 141. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 142. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 143. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 144. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 145. In some embodiments, the

engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 146. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 147. In some embodiments, the engineered tRNA or engineered tRNA variant sequence can comprise SEQ ID NO: 148.

[126] In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has about 70% to about 99% identity to the sequence of SEQ ID NO: 3. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has about 70% to about 99% identity to the sequence of SEQ ID NO: 103. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 75% identity compared to the sequence of SEQ ID NO: 3 or 103. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 80% identity compared to the sequence of SEQ ID NO: 3 or 103. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 85% identity compared to the sequence of SEQ ID NO: 3 or 103. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 90% identity compared to the sequence of SEQ ID NO: 3 or 103. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 91% identity compared to the sequence of SEQ ID NO: 3 or 103. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 92% identity compared to the sequence of SEQ ID NO: 3 or 103. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 93% identity compared to the sequence of SEQ ID NO: 3 or 103. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 94% identity compared to the sequence of SEQ ID NO: 3 or 103. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 95% identity compared to the sequence of SEQ ID NO: 3 or 103. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 96% identity compared to the sequence of SEQ ID NO: 3 or 103. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 97% identity compared to the sequence of SEQ ID NO: 3 or 103. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 98% identity compared to the sequence of SEQ ID NO: 3 or 103. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 99% identity compared to the sequence of SEQ ID NO: 3 or 103. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has 100% identity compared

to the sequence of SEQ ID NO: 3 or 103. In some embodiments, the % identity is over a range (e.g. 70-100% of the length) of nucleotides of SEQ ID NO: 3 or 103. In some embodiments, the % identity can be over a range of 70% of the length of nucleotides of SEQ ID NO: 3 or 103. In some embodiments, the % identity can be over a range of 75% of the length of nucleotides of SEQ ID NO: 3 or 103. In some embodiments, the % identity can be over a range of 80% of the length of nucleotides of SEQ ID NO: 3 or 103. In some embodiments, the % identity can be over a range of 85% of the length of nucleotides of SEQ ID NO: 3 or 103. In some embodiments, the % identity is over a range of 90% of the length of nucleotides of SEQ ID NO: 3 or 103. In some embodiments, the % identity can be over a range of 95% of the length of nucleotides of SEQ ID NO: 3 or 103. In some embodiments, the % identity can be over a range of 100% of the length of nucleotides of SEQ ID NO: 3 or 103.

[127] In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has about 70% to about 99% identity to the sequence of SEQ ID NO: 32. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has about 70% to about 99% identity to the sequence of SEQ ID NO: 132. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 75% identity compared to the sequence of SEQ ID NO: 32 or 132. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 80% identity compared to the sequence of SEQ ID NO: 32 or 132. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 85% identity compared to the sequence of SEQ ID NO: 32 or 132. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 90% identity compared to the sequence of SEQ ID NO: 32 or 132. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 91% identity compared to the sequence of SEQ ID NO: 32 or 132. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 92% identity compared to the sequence of SEQ ID NO: 32 or 132. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 93% identity compared to the sequence of SEQ ID NO: 32 or 132. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 94% identity compared to the sequence of SEQ ID NO: 32 or 132. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 95% identity compared to the sequence of SEQ ID NO: 32 or 132. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least

96% identity compared to the sequence of SEQ ID NO: 32 or 132. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 97% identity compared to the sequence of SEQ ID NO: 32 or 132. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 98% identity compared to the sequence of SEQ ID NO: 32 or 132. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 99% identity compared to the sequence of SEQ ID NO: 32 or 132. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has 100% identity compared to the sequence of SEQ ID NO: 32 or 132. In some embodiments, the % identity can be over a range (e.g. 70-100% of the length) of nucleotides of SEQ ID NO: 32 or 132. In some embodiments, the % identity can be over a range of 70% of the length of nucleotides of SEQ ID NO: 32 or 132. In some embodiments, the % identity can be over a range of 75% of the length of nucleotides of SEQ ID NO: 32 or 132. In some embodiments, the % identity can be over a range of 80% of the length of nucleotides of SEQ ID NO: 32 or 132. In some embodiments, the % identity can be over a range of 85% of the length of nucleotides of SEQ ID NO: 32 or 132. In some embodiments, the % identity can be over a range of 90% of the length of nucleotides of SEQ ID NO: 32 or 132. In some embodiments, the % identity can be over a range of 95% of the length of nucleotides of SEQ ID NO: 32 or 132. In some embodiments, the % identity can be over a range of 100% of the length of nucleotides of SEQ ID NO: 32 or 132.

[128] In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has about 70% to about 99% identity to the sequence of SEQ ID NO: 6. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has about 70% to about 99% identity to the sequence of SEQ ID NO: 106. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 75% identity compared to the sequence of SEQ ID NO: 6 or 106. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 80% identity compared to the sequence of SEQ ID NO: 6 or 106. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 85% identity compared to the sequence of SEQ ID NO: 6 or 106. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 90% identity compared to the sequence of SEQ ID NO: 6 or 106. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 91% identity compared to the sequence of SEQ ID NO: 6 or 106. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at

least 92% identity compared to the sequence of SEQ ID NO: 6 or 106. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 93% identity compared to the sequence of SEQ ID NO: 6 or 106. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 94% identity compared to the sequence of SEQ ID NO: 6 or 106. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 95% identity compared to the sequence of SEQ ID NO: 6 or 106. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 96% identity compared to the sequence of SEQ ID NO: 6 or 106. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 97% identity compared to the sequence of SEQ ID NO: 6 or 106. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 98% identity compared to the sequence of SEQ ID NO: 6 or 106. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 99% identity compared to the sequence of SEQ ID NO: 6 or 106. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has 100% identity compared to the sequence of SEQ ID NO: 6 or 106. In some embodiments, the % identity can be over a range (e.g. 70-100% of the length) of nucleotides of SEQ ID NO: 6 or 106. In some embodiments, the % identity can be over a range of 70% of the length of nucleotides of SEQ ID NO: 6 or 106. In some embodiments, the % identity can be over a range of 75% of the length of nucleotides of SEQ ID NO: 6 or 106. In some embodiments, the % identity can be over a range of 80% of the length of nucleotides of SEQ ID NO: 6 or 106. In some embodiments, the % identity can be over a range of 85% of the length of nucleotides of SEQ ID NO: 6 or 106. In some embodiments, the % identity can be over a range of 90% of the length of nucleotides of SEQ ID NO: 6 or 106. In some embodiments, the % identity can be over a range of 95% of the length of nucleotides of SEQ ID NO: 6 or 106. In some embodiments, the % identity can be over a range of 100% of the length of nucleotides of SEQ ID NO: 6 or 106.

[129] In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has about 70% to about 99% identity to the sequence of SEQ ID NO: 45. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has about 70% to about 99% identity to the sequence of SEQ ID NO: 145. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 75% identity compared to the sequence of SEQ ID NO: 45 or 145. In some cases, an engineered tRNA or a variant thereof can comprise a

sequence that has at least 80% identity compared to the sequence of SEQ ID NO: 45 or 145. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 85% identity compared to the sequence of SEQ ID NO: 45 or 145. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 90% identity compared to the sequence of SEQ ID NO: 45 or 145. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 91% identity compared to the sequence of SEQ ID NO: 45 or 145. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 92% identity compared to the sequence of SEQ ID NO: 45 or 145. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 93% identity compared to the sequence of SEQ ID NO: 45 or 145. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 94% identity compared to the sequence of SEQ ID NO: 45 or 145. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 95% identity compared to the sequence of SEQ ID NO: 45 or 145. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 96% identity compared to the sequence of SEQ ID NO: 45 or 145. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 97% identity compared to the sequence of SEQ ID NO: 45 or 145. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 98% identity compared to the sequence of SEQ ID NO: 45 or 145. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 99% identity compared to the sequence of SEQ ID NO: 45 or 145. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has 100% identity compared to the sequence of SEQ ID NO: 45 or 145. In some embodiments, the % identity can be over a range (e.g. 70-100% of the length) of nucleotides of SEQ ID NO: 45 or 145. In some embodiments, the % identity can be over a range of 70% of the length of nucleotides of SEQ ID NO: 45 or 145. In some embodiments, the % identity can be over a range of 75% of the length of nucleotides of SEQ ID NO: 45 or 145. In some embodiments, the % identity can be over a range of 80% of the length of nucleotides of SEQ ID NO: 45 or 145. In some embodiments, the % identity can be over a range of 85% of the length of nucleotides of SEQ ID NO: 45 or 145. In some embodiments, the % identity can be over a range of 90% of the length of nucleotides of SEQ ID NO: 45 or 145. In some embodiments, the % identity can be over a range of 95% of the

length of nucleotides of SEQ ID NO: 45 or 145. In some embodiments, the % identity can be over a range of 100% of the length of nucleotides of SEQ ID NO: 45 or 145.

[130] In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has about 70% to about 99% identity to the sequence of SEQ ID NO: 7. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has about 70% to about 99% identity to the sequence of SEQ ID NO: 107. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 75% identity compared to the sequence of SEQ ID NO: 7 or 107. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 80% identity compared to the sequence of SEQ ID NO: 7 or 107. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 85% identity compared to the sequence of SEQ ID NO: 7 or 107. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 90% identity compared to the sequence of SEQ ID NO: 7 or 107. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 91% identity compared to the sequence of SEQ ID NO: 7 or 107. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 92% identity compared to the sequence of SEQ ID NO: 7 or 107. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 93% identity compared to the sequence of SEQ ID NO: 7 or 107. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 94% identity compared to the sequence of SEQ ID NO: 7 or 107. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 95% identity compared to the sequence of SEQ ID NO: 7 or 107. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 96% identity compared to the sequence of SEQ ID NO: 7 or 107. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 97% identity compared to the sequence of SEQ ID NO: 7 or 107. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 98% identity compared to the sequence of SEQ ID NO: 7 or 107. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has at least 99% identity compared to the sequence of SEQ ID NO: 7 or 107. In some cases, an engineered tRNA or a variant thereof can comprise a sequence that has 100% identity compared to the sequence of SEQ ID NO: 7 or 107. In some embodiments, the % identity can be over a range (e.g. 70-100% of the length) of nucleotides of SEQ ID NO: 7 or 107. In some

embodiments, the % identity can be over a range of 70% of the length of nucleotides of SEQ ID NO: 7 or 107. In some embodiments, the % identity can be over a range of 75% of the length of nucleotides of SEQ ID NO: 7 or 107. In some embodiments, the % identity can be over a range of 80% of the length of nucleotides of SEQ ID NO: 7 or 107. In some embodiments, the % identity can be over a range of 85% of the length of nucleotides of SEQ ID NO: 7 or 107. In some embodiments, the % identity can be over a range of 90% of the length of nucleotides of SEQ ID NO: 7 or 107. In some embodiments, the % identity can be over a range of 95% of the length of nucleotides of SEQ ID NO: 7 or 107. In some embodiments, the % identity can be over a range of 100% of the length of nucleotides of SEQ ID NO: 7 or 107.

[131] The engineered tRNA variants comprising the modifications described herein, in some cases, can exhibit improved suppression efficiency as compared with a corresponding parental tRNA. In some cases, suppression efficiency can be more than or equal to about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 21%, 22%, 23%, 24%, 25%, 26%, 27%, 28%, 29%, or 30% more than the suppression efficiency for a corresponding parental tRNA. In some cases, suppression efficiency can be from about 1% to about 30%, from about 2% to about 29%, from about 3% to about 28%, from about 4% to about 27%, from about 5% to about 26%, from about 6% to about 25%, from about 7% to about 24%, from about 8% to about 23%, from about 9% to about 22%, from about 10% to about 21%, from about 11% to about 20%, from about 12% to about 19%, from about 13% to about 18%, from about 14% to about 17%, or from about 15% to about 16%, more than the suppression efficiency for a corresponding parental tRNA.

[132] In some cases, the engineered tRNA variants comprising the modifications described herein can exhibit better stability as compared with the stability of a corresponding parental tRNA. Changing a base pair to C-G instead of A-T or non-Watson Crick can at least have a stabilizing effect on the engineered tRNA as a C-G bond can be stronger than an A-T bond or a non-Watson Crick bond. Stability can be measured in different ways, for example, in terms of thermodynamic stability or shielding an engineered tRNA from degradation. Changing a base pair to G-C bond can at least provide a thermodynamic as well as a degradation shield, therefore stabilizing an engineered tRNA from degradation No current plans to measure.

[133] Some embodiments include a composition comprising: an engineered tRNA variant comprising one or more mutations with reference to a sequence provided in any of SEQ ID NOS:

3-22. In some embodiments, the engineered tRNA variant can comprise at least 70%, at least 80%, at least 85%, at least 90%, at least 92%, at least 95%, at least 97%, at least 99%, or 100% sequence identity to any one of SEQ ID NO: 23 - SEQ ID NO: 48. Some embodiments can include a composition that can comprise: an engineered tRNA variant or a polynucleotide encoding the engineered tRNA variant. In some embodiments, the engineered tRNA variant can comprise one or more mutations in a sequence of the engineered tRNA as compared with a reference tRNA comprising a sequence provided in any of SEQ ID NOS: 3-22. In some embodiments, the engineered tRNA variant can recognize a premature stop codon in an mRNA encoding a polypeptide and at least partially transforms interpretation of the premature stop codon into a sense codon during translation of the mRNA to produce a substantially full-length polypeptide.

[134] In some embodiments, the engineered tRNA variant can comprise a sequence that can be at least 70% identical to SEQ ID NO: 6. In some embodiments, the engineered tRNA variant can comprise a sequence that can be at least 75% identical to SEQ ID NO: 6. In some embodiments, the engineered tRNA variant can comprise a sequence that can be at least 80% identical to SEQ ID NO: 6. In some embodiments, the engineered tRNA variant can comprise a sequence that can be at least 85% identical to SEQ ID NO: 6. In some embodiments, the engineered tRNA variant can comprise a sequence that can be at least 90% identical to SEQ ID NO: 6. In some embodiments, the engineered tRNA variant can comprise a sequence that can be at least 95% identical to SEQ ID NO: 6.

[135] In some embodiments, the engineered tRNA variant can comprise a substitution at position 2, 4, 6, 12, 23, 27, 28, 31, 39, 40, 42, 43, 44, 46, 49, 50, 64, 65, 67, 69, or 71, of the sequence of SEQ ID NO: 6. In some embodiments, the engineered tRNA variant can comprise a substitution at position 2, 4, 6, 12, 23, 27, 28, 31, 39, 40, 42, 43, 44, 46, 49, 50, 64, 65, 67, 69, or 71, of the sequence of SEQ ID NO: 106. In some embodiments, the engineered tRNA variant can comprise a substitution at position 2 of SEQ ID NO: 6 or 106. In some embodiments, the engineered tRNA variant can comprise a substitution at position 4 of SEQ ID NO: 6 or 106. In some embodiments, the engineered tRNA variant can comprise a substitution at position 6 of SEQ ID NO: 6 or 06. In some embodiments, the engineered tRNA variant can comprise a substitution at position 12 of SEQ ID NO: 6 or 106. In some embodiments, the engineered tRNA variant can comprise a substitution at position 23 of SEQ ID NO: 6 or 106. In some

embodiments, the engineered tRNA variant can comprise a substitution at position 27 of SEQ ID NO: 6 or 106. In some embodiments, the engineered tRNA variant can comprise a substitution at position 28 of SEQ ID NO: 6 or 106. In some embodiments, the engineered tRNA variant can comprise a substitution at position 31 of SEQ ID NO: 6 or 106. In some embodiments, the engineered tRNA variant can comprise a substitution at position 39 of SEQ ID NO: 6 or 106. In some embodiments, the engineered tRNA variant can comprise a substitution at position 40 of SEQ ID NO: 6 or 106. In some embodiments, the engineered tRNA variant can comprise a substitution at position 42 of SEQ ID NO: 6 or 106. In some embodiments, the engineered tRNA variant can comprise a substitution at position 43 of SEQ ID NO: 6 or 106. In some embodiments, the engineered tRNA variant can comprise a substitution at position 44 of SEQ ID NO: 6 or 106. In some embodiments, the engineered tRNA variant can comprise a substitution at position 46 of SEQ ID NO: 6 or 106. In some embodiments, the engineered tRNA variant can comprise a substitution at position 49 of SEQ ID NO: 6 or 106. In some embodiments, the engineered tRNA variant can comprise a substitution at position 50 of SEQ ID NO: 6 or 106. In some embodiments, the engineered tRNA variant can comprise a substitution at position 64 of SEQ ID NO: 6 or 106. In some embodiments, the engineered tRNA variant can comprise a substitution at position 65 of SEQ ID NO: 6 or 106. In some embodiments, the engineered tRNA variant can comprise a substitution at position 67 of SEQ ID NO: 6 or 106. In some embodiments, the engineered tRNA variant can comprise a substitution at position 69 of SEQ ID NO: 6 or 106. In some embodiments, the engineered tRNA variant can comprise a substitution at position 71 of SEQ ID NO: 6 or 106. In some embodiments. In some embodiments, the substitution at position 2 can be to a C. In some embodiments. In some embodiments, the substitution at position 4 can be to a C. In some embodiments, the substitution at position 6 can be to a T. In some embodiments, the substitution at position 6 can be to an A. In some embodiments, the substitution at position 12 can be to a C. In some embodiments, the substitution at position 23 can be to a G. In some embodiments, the substitution at position 27 can be to a C. In some embodiments, the substitution at position 28 can be to a C. In some embodiments, the substitution at position 31 can be to a C. In some embodiments, the substitution at position 39 can be to a G. In some embodiments, the substitution at position 40 can be to a C. In some embodiments, the substitution at position 42 can be to a G. In some embodiments, the substitution at position 43 can be to a G. In some embodiments, the

substitution at position 44 can be to a G. In some embodiments, the substitution at position 46 can be to an A. In some embodiments, the substitution at position 49 can be to a G. In some embodiments, the substitution at position 50 can be to a T. In some embodiments, the substitution at position 64 can be to an A. In some embodiments, the substitution at position 65 can be to a C. In some embodiments, the substitution at position 67 can be to an A. In some embodiments, the substitution at position 67 can be to a T. In some embodiments, the substitution at position 69 can be to a G. In some embodiments, the substitution at position 71 can be to a C. In some embodiments, the substitution at position 71 can be to a G. In some embodiments, the sequence of the engineered tRNA variant can include multiple substitutions. In some embodiments, the sequence of the engineered tRNA variant can be identical to SEQ ID NO: 6 or 106, except for the substitution(s). In some embodiments, the engineered tRNA variant can exhibit an increased stability in vivo, as compared with a comparable tRNA comprising the sequence provided in SEQ ID NO: 6, as determined by a proxy measurement, a half-life measurement, an amino acid charging efficiency measurement, or a measurement of binding to a synthetase or ribosomal machinery.

[136] In some embodiments, the engineered tRNA variant can comprise a sequence that can be at least 70% identical to SEQ ID NO: 3. In some embodiments, the engineered tRNA variant can comprise a sequence that can be at least 75% identical to SEQ ID NO: 3. In some embodiments, the engineered tRNA variant can comprise a sequence that can be at least 80% identical to SEQ ID NO: 3. In some embodiments, the engineered tRNA variant can comprise a sequence that can be at least 85% identical to SEQ ID NO: 3. In some embodiments, the engineered tRNA variant can comprise a sequence that can be at least 90% identical to SEQ ID NO: 3. In some embodiments, the engineered tRNA variant can comprise a sequence that can be at least 95% identical to SEQ ID NO: 3.

[137] In some embodiments, the engineered tRNA variant can comprise a substitution at position 2, 6, 13, 15, 22, 28, 31, 37, 39, 42, 44, 50, 64, 67, 71, or 72, of SEQ ID NO: 3 or 103. In some embodiments, the engineered tRNA variant can comprise a substitution at position 2 of SEQ ID NO: 3 OR 103. In some embodiments, the engineered tRNA variant can comprise a substitution at position 6 of SEQ ID NO: 3 OR 103. In some embodiments, the engineered tRNA variant can comprise a substitution at position 13 of SEQ ID NO: 3 OR 103. In some embodiments, the engineered tRNA variant can comprise a substitution at position 15 of SEQ ID

NO: 3 OR 103. In some embodiments, the engineered tRNA variant can comprise a substitution at position 22 of SEQ ID NO: 3 OR 103. In some embodiments, the engineered tRNA variant can comprise a substitution at position 28 of SEQ ID NO: 3 OR 103. In some embodiments, the engineered tRNA variant can comprise a substitution at position 31 of SEQ ID NO: 3 OR 103. In some embodiments, the engineered tRNA variant can comprise a substitution at position 37 of SEQ ID NO: 3 OR 103. In some embodiments, the engineered tRNA variant can comprise a substitution at position 39 of SEQ ID NO: 3 OR 103. In some embodiments, the engineered tRNA variant can comprise a substitution at position 42 of SEQ ID NO: 3 OR 103. In some embodiments, the engineered tRNA variant can comprise a substitution at position 44 of SEQ ID NO: 3 OR 103. In some embodiments, the engineered tRNA variant can comprise a substitution at position 50 of SEQ ID NO: 3 OR 103. In some embodiments, the engineered tRNA variant can comprise a substitution at position 64 of SEQ ID NO: 3 OR 103. In some embodiments, the engineered tRNA variant can comprise a substitution at position 67 of SEQ ID NO: 3 OR 103. In some embodiments, the engineered tRNA variant can comprise a substitution at position 71 of SEQ ID NO: 3 OR 103. In some embodiments, the engineered tRNA variant can comprise a substitution at position 72 of SEQ ID NO: 3 OR 103. In some embodiments, the substitution at position 2 can be to a G. In some embodiments, the substitution at position 6 can be to a G. In some embodiments, the substitution at position 13 can be to a C. In some embodiments, the substitution at position 15 can be to a G. In some embodiments, the substitution at position 22 can be to a G. In some embodiments, the substitution at position 28 can be to a C. In some embodiments, the substitution at position 31 can be to an A. In some embodiments, the substitution at position 37 can be to a G. In some embodiments, the substitution at position 39 can be to a T. In some embodiments, the substitution at position 42 can be to a G. In some embodiments, the substitution at position 44 can be to an A. In some embodiments, the substitution at position 50 can be to a C. In some embodiments, the substitution at position 64 can be to a G. In some embodiments, the substitution at position 67 can be to a C. In some embodiments, the substitution at position 71 can be to a C. In some embodiments, the substitution at position 72 can be to a C. In some embodiments, the sequence of the engineered tRNA variant includes multiple substitutions. In some embodiments, the sequence of the engineered tRNA variant can be identical to SEQ ID NO: 3 or SEQ ID NO: 103, except for the substitution(s). In some embodiments, the engineered tRNA variant exhibits an increased

stability in vivo, as compared with a comparable tRNA comprising the sequence provided in SEQ ID NO: 3 or SEQ ID NO: 103, as determined by a proxy measurement, a half-life measurement, an amino acid charging efficiency measurement, or a measurement of binding to a synthetase or ribosomal machinery.

[138] In some embodiments, the engineered tRNA variant can comprise a sequence that can be at least 70% identical to SEQ ID NO: 5, and can comprise a substitution at position 73 of SEQ ID NO: 5. In some embodiments, the substitution at position 73 can be to a G. In some embodiments, the sequence of the engineered tRNA variant can include multiple substitutions. In some embodiments, the sequence of the engineered tRNA variant can be identical to SEQ ID NO: 5, except for the substitutions. In some embodiments, the engineered tRNA variant can exhibit an increased stability in vivo, as compared with a comparable tRNA comprising the sequence provided in SEQ ID NO: 5, as determined by a proxy measurement, a half-life measurement, an amino acid charging efficiency measurement, or a measurement of binding to a synthetase or ribosomal machinery.

[139] Disclosed herein, in some embodiments, are compositions comprising an engineered tRNA variant exhibits an increased stability. In some embodiments, the increased stability can comprise increased thermodynamic stability.

Additional RNA Editing Systems

[140] In some embodiments, a stop codon readthrough using an engineered tRNA or variant thereof can be complemented by a nucleic acid editing technique. For example, a clustered regularly interspaced palindromic repeats (CRISPR)-Cas based system can be used to edit a gene encoding an mRNA to remove a premature stop codon. In some cases, an RNA editing system can be used to directly edit a premature stop codon in an mRNA.

[141] An RNA editing system can comprise an engineered polynucleotide sequence that can comprise: (i) a recruiting region and/or (ii) a targeting region. Such a polynucleotide sequence can recruit an RNA editing entity, for example, via at least a portion of the recruiting region. In some cases, an RNA editing system may not comprise a recruiting region. An editing entity, when contacted with the engineered polynucleotide sequence and a target mRNA can perform a chemical modification on a base of a nucleotide of a premature stop codon of the mRNA, thereby converting the premature stop codon into a sense codon.

[142] An RNA editing entity as described herein can include a double-stranded RNA-specific adenosine deaminase, such as ADAR (encoded by *ADAR*, NCBI Gene ID: 103), including ADAR1 or ADAR2. The editing entity can comprise an apolipoprotein B mRNA editing enzyme (APOBEC), such as APOBEC subunit A (NCBI Gene ID: 200315), B (NCBI Gene ID: 9582), C (NCBI Gene ID: 27350), D (NCBI Gene ID: 140564), H (NCBI Gene ID: 164668). The RNA editing entities can be endogenous. The RNA editing entities can be exogenously delivered as a part of the RNA editing system.

[143] In some cases, conversion of a premature stop codon into a sense codon can be performed with an efficiency of the editing of the premature stop codon into a sense codon. Efficiency of RNA editing can be determined by comparing the amount of the full-length polypeptide of two comparable cells or cell lines that comprise a premature stop codon encoding for the polypeptide. One cell or cell line can comprise the RNA editing system, while the other cell or cell line may not have the RNA editing system. A comparison of the amount of the full-length polypeptide between the two cells can be determined. In some embodiments, an edit of a base of a nucleotide of a target RNA by an RNA editing entity can be determined in an *in vitro* assay comprising: (i) directly or indirectly introducing (e.g., transfecting) a target RNA into a primary cell line, (ii) directly or indirectly introducing (e.g., transfecting) an engineered polynucleotide (e.g., a polynucleotide with a recruiting region and/or a targeting region) into a primary cell line, and (iii) sequencing the target RNA. In some cases, transfecting the target RNA into a primary cell line can comprise transfecting a plasmid encoding for the target RNA into a primary cell line. In some instances, transfecting an engineered polynucleotide into a primary cell line can comprise transfecting a polynucleotide (e.g., plasmid) that encodes for an engineered polynucleotide into a primary cell line. In some cases, sequencing can comprise Sanger sequencing of a target RNA after the target RNA can comprise been converted to cDNA by reverse transcriptase.

[144] In some cases, an efficiency of RNA editing can comprise an in vivo efficiency of RNA editing. In some cases, in vivo efficiency of RNA editing can be determined by at least partially treating a disease or condition. For example, in vivo efficiency of RNA editing can be measured by at least partially improving the ability to hear, improving the ability to see, improving motor ability, cognitive ability or any combination thereof. In some cases, an efficiency of RNA editing can comprise an in vitro efficiency of RNA editing

[145] An in vivo efficiency of RNA editing can be from about 1% to 100%. An in vivo efficiency of RNA editing can be from about 10% to 100%. An in vivo efficiency of RNA editing can be from about 20% to 100%. An in vivo efficiency of RNA editing can be from about 30% to 100%. An in vivo efficiency of RNA editing can be from about 40% to 100%. An in vivo efficiency of RNA editing can be from about 50% to 100%. An in vivo efficiency of RNA editing can be from about 60% to 100%. An in vivo efficiency of RNA editing can be from about 70% to 100%. An in vivo efficiency of RNA editing can be from about 75% to 100%. An in vivo efficiency of RNA editing can be from about 80% to 100%. An in vivo efficiency of RNA editing can be from about 85% to 100%. An in vivo efficiency of RNA editing can be from about 90% to 100%. An in vivo efficiency of RNA editing can be from about 95% to 100%. An in vivo efficiency of RNA editing can be from about 20% to about 40%. An in vivo efficiency of RNA editing can be from about 20% to 60%. An in vivo efficiency of RNA editing can be from about 10% to 70%.

[146] An in vitro efficiency of RNA editing can be from about 1% to 100%. An in vitro efficiency of RNA editing can be from about 10% to 100%. An in vitro efficiency of RNA editing can be from about 20% to 100%. An in vitro efficiency of RNA editing can be from about 30% to 100%. An in vitro efficiency of RNA editing can be from about 40% to 100%. An in vitro efficiency of RNA editing can be from about 50% to 100%. An in vitro efficiency of RNA editing can be from about 60% to 100%. An in vitro efficiency of RNA editing can be from about 70% to 100%. An in vitro efficiency of RNA editing can be from about 75% to 100%. An in vitro efficiency of RNA editing can be from about 80% to 100%. An in vitro efficiency of RNA editing can be from about 85% to 100%. An in vitro efficiency of RNA editing can be from about 90% to 100%. An in vitro efficiency of RNA editing can be from about 95% to 100%. An in vitro efficiency of RNA editing can be from about 20% to about 40%. An in vitro efficiency of RNA editing can be from about 20% to 60%. An in vitro efficiency of RNA editing can be from about 10% to 70%.

Vectors Encoding the Engineered tRNA and Variants thereof and Pre-tRNA

[147] In some embodiments, a vector can encode for an engineered tRNA or variant thereof or an engineered pre-tRNA or variant thereof. In some embodiments, a composition can comprise a vector. In some cases, a vector can comprise a plasmid or a viral vector. In some cases, a vector encoding the engineered tRNA or variant thereof or engineered pre-tRNA or

variant thereof can be administered to a subject. A viral vector can comprise an adenoviral vector, an adeno-associated viral (AAV) vector, a retroviral vector, a lentiviral vector, a portion of any of these, or any combination thereof. In some cases, a vector can comprise DNA, such as double-stranded DNA or single-stranded DNA. A vector can comprise RNA. A vector can comprise a recombinant vector. In some instances, a vector can be modified from a naturally occurring vector. A vector can comprise DNA, such as double stranded DNA or single stranded DNA. A vector can comprise RNA. In some cases, the RNA can comprise a base modification. The vector can comprise a recombinant vector. The vector can be a vector that can be modified from a naturally occurring vector. The vector can comprise at least a portion of a non-naturally occurring vector. Any vector can be utilized. A viral vector can comprise an adenoviral vector, an adeno-associated viral vector (AAV), a lentiviral vector, a retroviral vector, a portion of any of these, or any combination thereof. In some cases, a vector can comprise an AAV vector. A vector can be modified to include a modified VP1 protein (such as an AAV vector modified to include a VP1 protein). In an aspect an AAV vector can be a recombinant AAV (rAAV) vector. rAAVs can be composed of substantially similar capsid sequence and structure as found in wild-type AAVs (wtAAVs). However, rAAVs encapsidate genomes that are substantially devoid of AAV protein-coding sequences and have therapeutic gene expression cassettes, such as subject polynucleotides, designed in their place. In some cases, sequences of viral origin can be the ITRs, which can be needed to guide genome replication and packaging during vector production. Suitable AAV vectors can be selected from any AAV serotype or combination of serotypes. For example, an AAV vector can be any one of: AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV 10, AAV11, AAV12, or any combination thereof. In some cases, a vector can be selected based on its natural tropism. In some cases, a vector serotype can be selected based on its ability to cross the blood brain barrier. AAV9 and AAV10 have been shown to cross the blood brain barrier to transduce neurons and glia. In an aspect, an AAV vector can be AAV2, AAV5, AAV6, AAV8, or AAV9. In some cases, an AAV vector can be a chimera of at least two serotypes. In an aspect, an AAV vector can be of serotypes AAV2 and AAV5. In some cases, a chimeric AAV vector can comprise rep and ITR sequences from AAV2 and a cap sequence from AAV5. In some embodiments, an AAV vector can be self-complementary. In some cases, an AAV vector can comprise an inverted terminal repeat. In other cases, an AAV vector can comprise a self-complementary inverted terminal repeat (scITR) sequence. In some

cases, rep, cap, and ITR sequences can be mixed and matched from all the of the different AAV serotypes provided herein. In some cases, a suitable AAV vector can be further modified to encompass modifications such as in a capsid or rep protein. Modifications can also include deletions, insertions, mutations, and combinations thereof. In some cases, a modification to a vector can be made to reduce immunogenicity to allow for repeated dosing. In some cases, a serotype of a vector that can be utilized can be changed when repeated dosing can be performed to reduce and/or eliminate immunogenicity.

[148] A vector can be employed to deliver a nucleic acid. A vector can comprise DNA, such as double stranded DNA or single stranded DNA. A vector can comprise RNA. In some cases, the RNA can comprise a base modification. The vector can comprise a recombinant vector. The vector can be a vector that can be modified from a naturally occurring vector. The vector can comprise at least a portion of a non-naturally occurring vector. Any vector can be utilized. A viral vector can comprise an adenoviral vector, an adeno-associated viral vector (AAV), a lentiviral vector, a retroviral vector, a portion of any of these, or any combination thereof. In some cases, a vector can comprise an AAV vector. A vector can comprise a single strand backbone (e.g., virus backbones shown in **FIG. 22A)**. In some cases, a vector can comprise a backbone that can comprise a self-complementary region (e.g., virus backbones shown in **FIG. 22B)**. A vector can be modified to include a modified VP1 protein (such as an AAV vector modified to include a VP1 protein). In an aspect an AAV vector can be a recombinant AAV (rAAV) vector. rAAVs can be composed of substantially similar capsid sequence and structure as found in wild-type AAVs (wtAAVs). However, rAAVs encapsidate genomes that are substantially devoid of AAV protein-coding sequences and have therapeutic gene expression cassettes, such as subject polynucleotides, designed in their place. In some cases, sequences of viral origin can be the ITRs, which can be needed to guide genome replication and packaging during vector production. Suitable AAV vectors can be selected from any AAV serotype or combination of serotypes. For example, an AAV vector can be any one of: AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV 10, AAV11, AAV12, or any combination thereof. In some cases, a vector can be selected based on its natural tropism. In some cases, a vector serotype can be selected based on its ability to cross the blood brain barrier. AAV9 and AAV10 have been shown to cross the blood brain barrier to transduce neurons and glia. In an aspect, an AAV vector can be AAV2, AAV5, AAV6, AAV8, or AAV9. In some

cases, an AAV vector can be a chimera of at least two serotypes. In an aspect, an AAV vector can be of serotypes AAV2 and AAV5. In some cases, a chimeric AAV vector can comprise rep and ITR sequences from AAV2 and a cap sequence from AAV5. In some embodiments, an AAV vector can be self-complementary. In some cases, an AAV vector can comprise an inverted terminal repeat. In other cases, an AAV vector can comprise a self-complementary inverted terminal repeat (scITR) sequence. In some cases, rep, cap, and ITR sequences can be mixed and matched from all the of the different AAV serotypes provided herein. In some cases, a suitable AAV vector can be further modified to encompass modifications such as in a capsid or rep protein. Modifications can also include deletions, insertions, mutations, and combinations thereof. In some cases, a modification to a vector can be made to reduce immunogenicity to allow for repeated dosing. In some cases, a serotype of a vector that can be utilized can be changed when repeated dosing can be performed to reduce and/or eliminate immunogenicity.

[149] In some cases, the AAV vector can be from an AAV having: a serotype comprising AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11 or AAV12, or a pseudotype comprising AAV-DJ, AAV-DJ/8, AAV-Rh10, AAV-Rh74, AAV-retro, AAV-PHP.B, AAV8-PHP.eB, AAV-PHP.S or AAV-2i8. In some cases, the AAV vector can comprise a genome comprising a replication gene and inverted terminal repeats from a first AAV serotype and a capsid protein from a second AAV serotype. In some cases, the AAV vector can comprise an AAV 2/5 vector, an AAV 2/6 vector, an AAV 2/7 vector, an AAV2/8 vector, or an AAV 2/9 vector. In some cases, the AAV vector can be an AAV 2/5 vector.

[150] In some embodiments, an AAV vector can comprise from 2 to 6 copies of an engineered tRNA or a variant thereof per viral genome. In some cases, an AAV vector can comprise an engineered tRNA, an engineered tRNA variant, or a combination thereof. In some cases, an AAV vector can comprise from 1 to 2, from 1 to 3, from 1 to 4, from 1 to 5, from 1 to 6, from 1 to 7, from 1 to 8, from 1 to 9, from 1 to 10, from 2 to 3, from 2 to 4, from 2 to 5, from 2 to 6, from 2 to 7, from 2 to 8, from 2 to 9, or from 2 to 10 copies per viral genome. In some cases, an AAV vector can comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 copies per viral genome. In some embodiments, an AAV vector can comprise from 1 to 5, from 1 to 10, from 1 to 15, from 1 to 20, from 1 to 25, from 1 to 30, from 1 to 35, from 1 to 40, from 1 to 45, or from 1 to 50 copies per viral genome.

[151] In some cases, an engineered tRNA or variant thereof, a vector encoding the engineered tRNA or variant thereof, or both can be present in a delivery system. In some cases, a delivery system can comprise a viral particle, a liposome, a nanoparticle, an exosome, an extracellular vesicle, a nanomesh, a charged polymer, an uncharged polymer, a surfactant, a penetrating enhancer, a gene transfer agent, a phospholipid, a micelle, a synthetic vector, a macromolecule, a dendrimer, a biopolymer, or any combination thereof.

[152] In some cases, an engineered tRNA or variant thereof or a vector encoding the engineered tRNA or variant thereof can be present in a viral particle, a liposome, a nanoparticle, an exosome, an extracellular vesicle, a nanomesh, or any combination thereof. In some cases, a vector can be present inside a polypeptide coat.

[153] In some embodiments, an engineered tRNA or variant thereof, an engineered pre-tRNA or variant thereof, or both can be comprised in a composition such as a pharmaceutical composition. In some embodiments, a vector encoding an engineered tRNA or variant thereof, a vector encoding an engineered pre-tRNA or variant thereof, or any combination thereof, can be comprised in a composition such as a pharmaceutical composition. In some cases, a composition can comprise an excipient, a diluent or a carrier.

[154] In some embodiments, compositions disclosed herein can be in unit dose forms or multiple-dose forms. For example, a pharmaceutical composition described herein can be in unit dose form. Unit dose forms, as used herein, can refer to physically discrete units suitable for administration to human or non-human subjects (e.g., animals). In some cases, unit dose forms can be packaged individually. Each unit dose can contain a predetermined quantity of an active ingredient(s) that can be sufficient to produce the desired therapeutic effect in association with pharmaceutical carriers, diluents, excipients, or any combination thereof. Examples of unit dose forms can include, ampules, syringes, and individually packaged tablets and capsules. In some instances, a unit dose form can be comprised in a disposable syringe. In some instances, unit-dosage forms can be administered in fractions or multiples thereof. A multiple-dose form can be a plurality of identical unit dose forms packaged in a single container, which can be administered in segregated a unit dose form. Examples of a multiple-dose form can include vials, bottles of tablets or capsules, or bottles of pints or gallons. In some instances, a multiple-dose form can comprise the same pharmaceutically active agents. In some instances, a multiple-dose form can comprise different pharmaceutically active agents.

[155] A composition described herein can comprise an excipient. An excipient can be added to a stem cell or can be co-isolated with the stem cell from its source. An excipient can comprise a cryo-preservative, such as DMSO, glycerol, polyvinylpyrrolidone (PVP), or any combination thereof. An excipient can comprise a cryo-preservative, such as a sucrose, a trehalose, a starch, a salt of any of these, a derivative of any of these, or any combination thereof. An excipient can comprise a pH agent (for example, to minimize oxidation or degradation of a component of the composition), a stabilizing agent (for example, to prevent modification or degradation of a component of the composition), a buffering agent (for example, to enhance temperature stability), a solubilizing agent (for example, to increase protein solubility), or any combination thereof. An excipient can comprise a surfactant, a sugar, an amino acid, an antioxidant, a salt, a non-ionic surfactant, a solubilizer, a trigylceride, an alcohol, or any combination thereof. An excipient can comprise sodium carbonate, acetate, citrate, phosphate, poly-ethylene glycol (PEG), human serum albumin (HSA), sorbitol, sucrose, trehalose, polysorbate 80, sodium phosphate, sucrose, disodium phosphate, mannitol, polysorbate 20, histidine, citrate, albumin, sodium hydroxide, glycine, sodium citrate, trehalose, arginine, sodium acetate, acetate, HCl, disodium edetate, lecithin, glycerine, xanthan rubber, soy isoflavones, polysorbate 80, ethyl alcohol, water, teprenone, or any combination thereof. An excipient can be an excipient described in the Handbook of Pharmaceutical Excipients, American Pharmaceutical Association (1986).

[156] A composition described herein can comprise a naturally-occurring or non-naturally-occurring carrier, inert (for example, a detectable agent or label) or active, such as an adjuvant, diluent, binder, stabilizer, buffers, salts, lipophilic solvents, preservative, adjuvant or the like (e.g. and include. In some cases, a carrier can comprise a pharmaceutically acceptable carrier. Carriers also include pharmaceutical excipients and additives proteins, peptides, amino acids, lipids, and carbohydrates (e.g., sugars, including monosaccharides, di-, tri-, tetra-oligosaccharides, and oligosaccharides; derivatized sugars such as alditols, aldolic acids, esterified sugars and the like; and polysaccharides or sugar polymers), which can be present singly or in combination, comprising alone or in combination 1-99.99% by weight or volume. Exemplary protein excipients include serum albumins such as human serum albumin (HSA), recombinant human albumin (rHA), gelatin, casein, and the like. Representative amino acid components, antibody components, or both, which can also function in a buffering capacity,

include alanine, arginine, glycine, arginine, betaine, histidine, glutamic acid, aspartic acid, cysteine, lysine, leucine, isoleucine, valine, methionine, phenylalanine, aspartame, and the like. Carbohydrate excipients can be also intended within the scope of this technology, examples of which include but can be not limited to monosaccharides such as fructose, maltose, galactose, glucose, D-mannose, sorbose, and the like; disaccharides, such as lactose, sucrose, trehalose, cellobiose, and the like; polysaccharides, such as raffinose, melezitose, maltodextrins, dextrans, starches, and the like; and alditols, such as mannitol, xylitol, maltitol, lactitol, xylitol sorbitol (glucitol), and myoinositol.

[157] A composition can comprise a diluent. Non-limiting examples of diluents can include water, glycerol, methanol, ethanol, and other similar biocompatible diluents. In some cases, a diluent can be an aqueous acid such as acetic acid, citric acid, maleic acid, hydrochloric acid, phosphoric acid, nitric acid, sulfuric acid, or similar. In other cases, a diluent can be selected from a group comprising alkaline metal carbonates such as calcium carbonate; alkaline metal phosphates such as calcium phosphate; alkaline metal sulphates such as calcium sulphate; cellulose derivatives such as cellulose, microcrystalline cellulose, cellulose acetate; magnesium oxide, dextrin, fructose, dextrose, glyceryl palmitostearate, lactitol, choline, lactose, maltose, mannitol, simethicone, sorbitol, starch, pregelatinized starch, talc, xylitol and/or anhydrates, hydrates and/or pharmaceutically acceptable derivatives thereof or combinations thereof.

[158] In some cases, a composition described herein can be administered to prevent a disease or condition as described herein. For example, a composition as described herein can be administered prophylactically to prevent an incidence of a disease or condition. Prevention can at least partially reduce an appearance, onset, or incidence of one or more symptoms of a disease or condition.

[159] Disclosed herein are nucleic acids. The nucleic acid can comprise a plasmid or vector described in any of **FIGs. 46A-46F** or SEQ ID NOS: 52-56. The nucleic acid can be delivered or enclosed within a viral vector such as an AAV described herein. Some versions of any of such nucleic acid can be used in a method described herein. For example, an AAV comprising the nucleic acid can be administered.

Guide RNAs

Guide RNA design

[160] Disclosed herein, in some embodiments, are engineered polynucleotides such as guide RNAs (gRNAs). Some embodiments of the methods disclosed herein include manufacturing or use of a gRNA, for example administration of a gRNA to a subject in need thereof.

[161] Various parameters that can affect editing efficiency of a gRNA and exemplary strategies to improve editing efficiency of a gRNA are shown in **FIG. 31**. A plurality of engineered gRNAs can be designed and screened by for example: changing a gRNA guide length, introducing a bulge in a gRNA (e.g., placing an A/C mismatch in a gRNA), or placing a GLURD domain in a gRNA. Exemplary gRNA designs are shown in **FIG. 32**.

[162] Medium throughput screening assays can be used to screen a guide RNA (gRNA) or a tRNAs (e.g., an engineered tRNA or a variant thereof). A medium throughput screening can comprise a gain of function (e.g., luciferase gain of signal) assays. **FIG. 33** shows an example of a luciferase gain of function assay where a start codon is attenuated by mis-translating the start codon using a t-RNA which can increase the production of a downstream luciferase. **FIG. 34** shows some results of experiments that were performed to screen exemplary gRNA designs.

[163] In some cases, such as where a recruiting sequence is absent, an engineered polynucleotide can be capable of associating with a subject RNA editing entity (e.g., ADAR) to facilitate editing of a target RNA and/or modulate expression of a polypeptide encoded by a subject target RNA. This can be achieved through structural features. Structural features can comprise any one of a: mismatch, symmetrical bulge, asymmetrical bulge, symmetrical internal loop, asymmetrical internal loop, hairpins, wobble base pairs, a structured motif, circularized RNA, chemical modification, or any combination thereof. In an aspect, a double stranded RNA (dsRNA) substrate, for example hybridized polynucleotide strands, can be formed upon hybridization of an engineered polynucleotide of the present disclosure to a target RNA. Described herein is a feature, which can correspond to one of several structural features that can be present in a dsRNA substrate of the present disclosure. Examples of features can include a mismatch, a bulge (symmetrical bulge or asymmetrical bulge), an internal loop (symmetrical internal loop or asymmetrical internal loop), or a hairpin (a recruiting hairpin or a hairpin comprising a non-targeting domain). Engineered polynucleotides of the present disclosure can have from 1 to 50 features. Engineered polynucleotides of the present disclosure can have from 1 to 5, from 5 to 10, from 10 to 15, from 15 to 20, from 20 to 25, from 25 to 30, from 30 to 35,

from 35 to 40, from 40 to 45, from 45 to 50, from 5 to 20, from 1 to 3, from 4 to 5, from 2 to 10, from 20 to 40, from 10 to 40, from 20 to 50, from 30 to 50, from 4 to 7, or from 8 to 10 features.

[164] As disclosed herein, a structured motif can comprise two or more features in a dsRNA substrate.

[165] A double stranded RNA (dsRNA) substrate can be formed upon hybridization of an engineered guide RNA of the present disclosure to a target RNA. As disclosed herein, a mismatch can refer to a nucleotide in a guide RNA that is unpaired to an opposing nucleotide in a target RNA within the dsRNA. A mismatch can comprise any two nucleotides that do not base pair, are not complementary, or both. In some embodiments, a mismatch can be an A/C mismatch. An A/C mismatch can comprise a C in an engineered guide RNA of the present disclosure opposite an A in a target RNA. An A/C mismatch can comprise an A in an engineered guide RNA of the present disclosure opposite an C in a target RNA. In an embodiment, a G/G mismatch can comprise a G in an engineered guide RNA of the present disclosure opposite a G in a target RNA. In some embodiments, a mismatch positioned 5' of the edit site can facilitate base-flipping of the target A to be edited. A mismatch can also help confer sequence specificity. In an embodiment, a mismatch can comprise a G/G mismatch. In an embodiment, a mismatch can comprise an A/C mismatch, wherein the A is in the target RNA and the C is in the targeting sequence of the engineered polynucleotide. In another embodiment, the A in the A/C mismatch can be the base of the nucleotide in the target RNA edited by a subject RNA editing entity.

[166] In an aspect, a structural feature can form in an engineered polynucleotide independently. In other cases, a structural feature can form when an engineered polynucleotide binds to a target RNA. A structural feature can also form when an engineered polynucleotide associates with other molecules such as a peptide, a nucleotide, or a small molecule. In certain embodiments, a structural feature of an engineered polynucleotide can be formed independent of a target RNA, and its structure can change as a result of the engineered polypeptide hybridization with a target RNA region. In certain embodiments, a structural feature is present when an engineered polynucleotide is in association with a target RNA.

[167] In some cases, a structural feature can be or can include a hairpin. In some cases, an engineered polynucleotide can lack a hairpin domain. In other cases, an engineered polynucleotide can contain a hairpin domain or more than one hairpin domain. A hairpin can be located anywhere in a polynucleotide. As disclosed herein, a hairpin can be an RNA duplex

wherein a single RNA strand can comprise folded in upon itself to form the RNA duplex. The single RNA strand can fold upon itself due to having nucleotide sequences upstream and downstream of the folding region base pairs to each other. A hairpin can have from 10 to 500 nucleotides in length of the entire duplex structure. The stem-loop structure of a hairpin can be from 3 to 15 nucleotides long. A hairpin can be present in any of the engineered polynucleotides disclosed herein. The engineered polynucleotides disclosed herein can have from 1 to 10 hairpins. In some embodiments, the engineered polynucleotides disclosed herein have 1 hairpin. In some embodiments, the engineered polynucleotides disclosed herein have 2 hairpins. As disclosed herein, a hairpin can refer to a recruitment hairpin or a hairpin or a non-recruitment hairpin. A hairpin can be located anywhere within the engineered polynucleotides of the present disclosure. In some embodiments, one or more hairpins is present at the 3' end of an engineered polynucleotide of the present disclosure, at the 5' end of an engineered polynucleotide of the present disclosure or within the targeting sequence of an engineered polynucleotide of the present disclosure, or any combination thereof.

[168] In some aspects, a structural feature can comprise a recruitment hairpin, as disclosed herein. A recruitment hairpin can recruit an RNA editing entity, such as ADAR. In some embodiments, a recruitment hairpin can comprise a GluR2 domain. In some embodiments, a recruitment hairpin can comprise an Alu domain.

[169] In yet another aspect, a structural feature can comprise a non-recruitment hairpin. A non-recruitment hairpin, as disclosed herein, can exhibit functionality that improves localization of the engineered polynucleotide to the target RNA. In some embodiments, the non-recruitment hairpin improves nuclear retention. In some embodiments, the non-recruitment hairpin can comprise a hairpin from U7 snRNA.

[170] In another aspect, a structural feature can comprise a wobble base. A wobble base pair can refer to two bases that weakly pair. For example, a wobble base pair of the present disclosure can refer to a G paired with a U.

[171] A hairpin of the present disclosure can be of any length. In an aspect, a hairpin can be from about 5-200 or more nucleotides. In some cases, a hairpin can comprise about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87,

88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397, 398, 399, or 400 or more nucleotides. In other cases, a hairpin can also comprise 5 to 10, 5 to 20, 5 to 30, 5 to 40, 5 to 50, 5 to 60, 5 to 70, 5 to 80, 5 to 90, 5 to 100, 5 to 110, 5 to 120, 5 to 130, 5 to 140, 5 to 150, 5 to 160, 5 to 170, 5 to 180, 5 to 190, 5 to 200, 5 to 210, 5 to 220, 5 to 230, 5 to 240, 5 to 250, 5 to 260, 5 to 270, 5 to 280, 5 to 290, 5 to 300, 5 to 310, 5 to 320, 5 to 330, 5 to 340, 5 to 350, 5 to 360, 5 to 370, 5 to 380, 5 to 390, or 5 to 400 nucleotides. A hairpin can be a structural feature formed from a single strand of RNA with sufficient complementarity to itself to hybridize into a double stranded RNA motif/structure consisting of double-stranded hybridized RNA separated by a nucleotide loop.

[172] In some cases, a structural feature can be a bulge. A bulge can comprise a single (intentional) nucleic acid mismatch between the target strand and an engineered polynucleotide strand. In other cases, more than one consecutive mismatch between strands constitutes a bulge if the bulge region, mismatched stretch of bases, is flanked on both sides with hybridized, complementary dsRNA regions. A bulge can be located at any location of a polynucleotide. In some cases, a bulge can be located from about 30 to about 70 nucleotides from a 5' hydroxyl or 3' hydroxyl.

[173] In some embodiments, a double stranded RNA (dsRNA) substrate can be formed upon hybridization of an engineered polynucleotide of the present disclosure to a target RNA. As disclosed herein, a bulge can refer to the structure formed upon formation of the dsRNA substrate, where nucleotides in either the engineered polynucleotide or the target RNA are not complementary to their positional counterparts on the opposite strand. A bulge can change the secondary or tertiary structure of the dsRNA substrate. A bulge can have from 1 to 4 nucleotides on the engineered polynucleotide side of the dsRNA substrate or the target RNA side of the dsRNA substrate. In some embodiments, the engineered polynucleotides of the present disclosure can have 2 bulges. In some embodiments, the engineered polynucleotides of the present disclosure can have 3 bulges. In some embodiments, the engineered polynucleotides of the present disclosure can have 4 bulges. In some embodiments, the presence of a bulge in a dsRNA substrate can position ADAR to selectively edit the target A in the target RNA and reduce off-target editing of non-targets. In some embodiments, the presence of a bulge in a dsRNA substrate can recruit additional ADAR. Bulges in dsRNA substrates disclosed herein can recruit other proteins, such as other RNA editing entities. In some embodiments, a bulge positioned 5' of the edit site can facilitate base-flipping of the target A to be edited. A bulge can also help confer sequence specificity. A bulge can help direct ADAR editing by constraining it in an orientation that yield selective editing of the target A.

[174] In some aspects, a double stranded RNA (dsRNA) substrate can be formed upon hybridization of an engineered polynucleotide of the present disclosure to a target RNA. A bulge can be a symmetrical bulge or an asymmetrical bulge. A bulge can be formed by 1 to 4 participating nucleotides on either the guide RNA side or the target RNA side of the dsRNA substrate. In some cases, a symmetrical bulge can be formed when the same number of nucleotides is present on each side of the bulge. A symmetrical bulge can have from 2 to 4 nucleotides on the engineered polynucleotide side of the dsRNA substrate or the target RNA side of the dsRNA substrate. For example, a symmetrical bulge in a dsRNA substrate of the present disclosure can have the same number of nucleotides on the engineered polynucleotide side and the target RNA side of the dsRNA substrate. A symmetrical bulge of the present disclosure can be formed by 2 nucleotides on the engineered polynucleotide side of the dsRNA target and 2 nucleotides on the target RNA side of the dsRNA substrate. A symmetrical bulge of the present disclosure can be formed by 3 nucleotides on the engineered polynucleotide side of the dsRNA

target and 3 nucleotides on the target RNA side of the dsRNA substrate. A symmetrical bulge of the present disclosure can be formed by 4 nucleotides on the engineered polynucleotide side of the dsRNA target and 4 nucleotides on the target RNA side of the dsRNA substrate.

[175] In some cases, a double stranded RNA (dsRNA) substrate can be formed upon hybridization of an engineered guide RNA of the present disclosure to a target RNA. A bulge can be a symmetrical bulge or an asymmetrical bulge. In some cases, an asymmetrical bulge can be formed when a different number of nucleotides is present on each side of the bulge. An asymmetrical bulge can have from 1 to 4 participating nucleotides on either the guide RNA side or the target RNA side of the dsRNA substrate. For example, an asymmetrical bulge in a dsRNA substrate of the present disclosure can have different numbers of nucleotides on the engineered guide RNA side and the target RNA side of the dsRNA substrate. An asymmetrical bulge of the present disclosure can be formed by 0 nucleotides on the engineered guide RNA side of the dsRNA substrate and 1 nucleotide on the target RNA side of the dsRNA substrate. An asymmetrical bulge of the present disclosure can be formed by 0 nucleotides on the target RNA side of the dsRNA substrate and 1 nucleotide on the engineered guide RNA side of the dsRNA substrate. An asymmetrical bulge of the present disclosure can be formed by 0 nucleotides on the engineered guide RNA side of the dsRNA substrate and 2 nucleotides on the target RNA side of the dsRNA substrate. An asymmetrical bulge of the present disclosure can be formed by 0 nucleotides on the target RNA side of the dsRNA substrate and 2 nucleotides on the engineered guide RNA side of the dsRNA substrate. An asymmetrical bulge of the present disclosure can be formed by 0 nucleotides on the engineered guide RNA side of the dsRNA substrate and 3 nucleotides on the target RNA side of the dsRNA substrate. An asymmetrical bulge of the present disclosure can be formed by 0 nucleotides on the target RNA side of the dsRNA substrate and 3 nucleotides on the engineered guide RNA side of the dsRNA substrate. An asymmetrical bulge of the present disclosure can be formed by 0 nucleotides on the engineered guide RNA side of the dsRNA substrate and 4 nucleotides on the target RNA side of the dsRNA substrate. An asymmetrical bulge of the present disclosure can be formed by 0 nucleotides on the target RNA side of the dsRNA substrate and 4 nucleotides on the engineered guide RNA side of the dsRNA substrate. An asymmetrical bulge of the present disclosure can be formed by 1 nucleotide on the engineered guide RNA side of the dsRNA substrate and 2 nucleotides on the target RNA side of the dsRNA substrate. An asymmetrical bulge of the present disclosure can be formed by 1

nucleotide on the target RNA side of the dsRNA substrate and 2 nucleotides on the engineered guide RNA side of the dsRNA substrate. An asymmetrical bulge of the present disclosure can be formed by 1 nucleotide on the engineered guide RNA side of the dsRNA substrate and 3 nucleotides on the target RNA side of the dsRNA substrate. An asymmetrical bulge of the present disclosure can be formed by 1 nucleotide on the target RNA side of the dsRNA substrate and 3 nucleotides on the engineered guide RNA side of the dsRNA substrate. An asymmetrical bulge of the present disclosure can be formed by 1 nucleotide on the engineered guide RNA side of the dsRNA substrate and 4 nucleotides on the target RNA side of the dsRNA substrate. An asymmetrical bulge of the present disclosure can be formed by 1 nucleotide on the target RNA side of the dsRNA substrate and 4 nucleotides on the engineered guide RNA side of the dsRNA substrate. An asymmetrical bulge of the present disclosure can be formed by 2 nucleotides on the engineered guide RNA side of the dsRNA substrate and 3 nucleotides on the target RNA side of the dsRNA substrate. An asymmetrical bulge of the present disclosure can be formed by 2 nucleotides on the target RNA side of the dsRNA substrate and 3 nucleotides on the engineered guide RNA side of the dsRNA substrate. An asymmetrical bulge of the present disclosure can be formed by 2 nucleotides on the engineered guide RNA side of the dsRNA substrate and 4 nucleotides on the target RNA side of the dsRNA substrate. An asymmetrical bulge of the present disclosure can be formed by 2 nucleotides on the target RNA side of the dsRNA substrate and 4 nucleotides on the engineered guide RNA side of the dsRNA substrate. An asymmetrical bulge of the present disclosure can be formed by 3 nucleotides on the engineered guide RNA side of the dsRNA substrate and 4 nucleotides on the target RNA side of the dsRNA substrate. An asymmetrical bulge of the present disclosure can be formed by 3 nucleotides on the target RNA side of the dsRNA substrate and 4 nucleotides on the engineered guide RNA side of the dsRNA substrate. In some cases, a structural feature is a loop.

[176] In an aspect, a double stranded RNA (dsRNA) substrate can be formed upon hybridization of an engineered polynucleotide of the present disclosure to a target RNA. As disclosed herein, an internal loop can refer to the structure formed upon formation of the dsRNA substrate, where nucleotides in either the engineered polynucleotide or the target RNA are not complementary to their positional counterparts on the opposite strand and where one side of the internal loop, either on the target RNA side or the engineered polynucleotide side of the dsRNA substrate, can comprise greater than 5 nucleotides. An internal loop can be a symmetrical internal

loop or an asymmetrical internal loop. Internal loops present in the vicinity of the edit site can help with base flipping of the target A in the target RNA to be edited. In some cases, a double stranded RNA (dsRNA) substrate can be formed upon hybridization of an engineered polynucleotide of the present disclosure to a target RNA. An internal loop can be a symmetrical internal loop or an asymmetrical internal loop. In some cases, a symmetrical internal loop can be formed when the same number of nucleotides is present on each side of the internal loop. For example, a symmetrical internal loop in a dsRNA substrate of the present disclosure can have the same number of nucleotides on the engineered polynucleotide side and the target RNA side of the dsRNA substrate. A symmetrical internal loop of the present disclosure can be formed by 5 nucleotides on the engineered polynucleotide side of the dsRNA target and 5 nucleotides on the target RNA side of the dsRNA substrate. A symmetrical internal loop of the present disclosure can be formed by 6 nucleotides on the engineered polynucleotide side of the dsRNA target and 6 nucleotides on the target RNA side of the dsRNA substrate. A symmetrical internal loop of the present disclosure can be formed by 7 nucleotides on the engineered polynucleotide side of the dsRNA target and 7 nucleotides on the target RNA side of the dsRNA substrate. A symmetrical internal loop of the present disclosure can be formed by 8 nucleotides on the engineered polynucleotide side of the dsRNA target and 8 nucleotides on the target RNA side of the dsRNA substrate. A symmetrical internal loop of the present disclosure can be formed by 9 nucleotides on the engineered polynucleotide side of the dsRNA target and 9 nucleotides on the target RNA side of the dsRNA substrate. A symmetrical internal loop of the present disclosure can be formed by 10 nucleotides on the engineered polynucleotide side of the dsRNA target and 10 nucleotides on the target RNA side of the dsRNA substrate.

[177] In an aspect, a double stranded RNA (dsRNA) substrate can be formed upon hybridization of an engineered polynucleotide of the present disclosure to a target RNA. An internal loop can be a symmetrical internal loop or an asymmetrical internal loop. In some cases, an asymmetrical internal loop is formed when a different number of nucleotides is present on each side of the internal loop. For example, an asymmetrical internal loop in a dsRNA substrate of the present disclosure can have different numbers of nucleotides on the engineered polynucleotide side and the target RNA side of the dsRNA substrate. An asymmetrical internal loop of the present disclosure can be formed by 5 nucleotides on the engineered polynucleotide side of the dsRNA substrate and 6 nucleotides on the target RNA side of the dsRNA substrate.

An asymmetrical internal loop of the present disclosure can be formed by 5 nucleotides on the target RNA side of the dsRNA substrate and 6 nucleotides on the engineered polynucleotide side of the dsRNA substrate. An asymmetrical internal loop of the present disclosure can be formed by 5 nucleotides on the engineered polynucleotide side of the dsRNA substrate and 7 nucleotides on the target RNA side of the dsRNA substrate. An asymmetrical internal loop of the present disclosure can be formed by 5 nucleotides on the target RNA side of the dsRNA substrate and 7 nucleotides on the engineered polynucleotide side of the dsRNA substrate. An asymmetrical internal loop of the present disclosure can be formed by 5 nucleotides on the engineered polynucleotide side of the dsRNA substrate and 8 nucleotides internal loop the target RNA side of the dsRNA substrate. An asymmetrical internal loop of the present disclosure can be formed by 5 nucleotides on the target RNA side of the dsRNA substrate and 8 nucleotides on the engineered polynucleotide side of the dsRNA substrate. An asymmetrical internal loop of the present disclosure can be formed by 5 nucleotides on the engineered polynucleotide side of the dsRNA substrate and 9 nucleotides internal loop the target RNA side of the dsRNA substrate. An asymmetrical internal loop of the present disclosure can be formed by 5 nucleotides on the target RNA side of the dsRNA substrate and 9 nucleotides on the engineered polynucleotide side of the dsRNA substrate. An asymmetrical internal loop of the present disclosure can be formed by 5 nucleotides on the engineered polynucleotide side of the dsRNA substrate and 10 nucleotides internal loop the target RNA side of the dsRNA substrate. An asymmetrical internal loop of the present disclosure can be formed by 5 nucleotides on the target RNA side of the dsRNA substrate and 10 nucleotides on the engineered polynucleotide side of the dsRNA substrate. An asymmetrical internal loop of the present disclosure can be formed by 6 nucleotides on the engineered polynucleotide side of the dsRNA substrate and 7 nucleotides internal loop the target RNA side of the dsRNA substrate. An asymmetrical internal loop of the present disclosure can be formed by 6 nucleotides on the target RNA side of the dsRNA substrate and 7 nucleotides on the engineered polynucleotide side of the dsRNA substrate. An asymmetrical internal loop of the present disclosure can be formed by 6 nucleotides on the engineered polynucleotide side of the dsRNA substrate and 8 nucleotides internal loop the target RNA side of the dsRNA substrate. An asymmetrical internal loop of the present disclosure can be formed by 6 nucleotides on the target RNA side of the dsRNA substrate and 8 nucleotides on the engineered polynucleotide side of the dsRNA substrate. An asymmetrical internal loop of the present disclosure can be formed by 6

nucleotides on the engineered polynucleotide side of the dsRNA substrate and 9 nucleotides internal loop the target RNA side of the dsRNA substrate. An asymmetrical internal loop of the present disclosure can be formed by 6 nucleotides on the target RNA side of the dsRNA substrate and 9 nucleotides on the engineered polynucleotide side of the dsRNA substrate. An asymmetrical internal loop of the present disclosure can be formed by 6 nucleotides on the engineered polynucleotide side of the dsRNA substrate and 10 nucleotides internal loop the target RNA side of the dsRNA substrate. An asymmetrical internal loop of the present disclosure can be formed by 6 nucleotides on the target RNA side of the dsRNA substrate and 10 nucleotides on the engineered polynucleotide side of the dsRNA substrate. An asymmetrical internal loop of the present disclosure can be formed by 7 nucleotides on the engineered polynucleotide side of the dsRNA substrate and 8 nucleotides internal loop the target RNA side of the dsRNA substrate. An asymmetrical internal loop of the present disclosure can be formed by 7 nucleotides on the target RNA side of the dsRNA substrate and 8 nucleotides on the engineered polynucleotide side of the dsRNA substrate. An asymmetrical internal loop of the present disclosure can be formed by 7 nucleotides on the engineered polynucleotide side of the dsRNA substrate and 9 nucleotides internal loop the target RNA side of the dsRNA substrate. An asymmetrical internal loop of the present disclosure can be formed by 7 nucleotides on the target RNA side of the dsRNA substrate and 9 nucleotides on the engineered polynucleotide side of the dsRNA substrate. An asymmetrical internal loop of the present disclosure can be formed by 7 nucleotides on the engineered polynucleotide side of the dsRNA substrate and 10 nucleotides internal loop the target RNA side of the dsRNA substrate. An asymmetrical internal loop of the present disclosure can be formed by 7 nucleotides on the target RNA side of the dsRNA substrate and 10 nucleotides on the engineered polynucleotide side of the dsRNA substrate. An asymmetrical internal loop of the present disclosure can be formed by 8 nucleotides on the engineered polynucleotide side of the dsRNA substrate and 9 nucleotides internal loop the target RNA side of the dsRNA substrate. An asymmetrical internal loop of the present disclosure can be formed by 8 nucleotides on the target RNA side of the dsRNA substrate and 9 nucleotides on the engineered polynucleotide side of the dsRNA substrate. An asymmetrical internal loop of the present disclosure can be formed by 8 nucleotides on the engineered polynucleotide side of the dsRNA substrate and 10 nucleotides internal loop the target RNA side of the dsRNA substrate. An asymmetrical internal loop of the present disclosure can be formed by 8 nucleotides on the

target RNA side of the dsRNA substrate and 10 nucleotides on the engineered polynucleotide side of the dsRNA substrate. An asymmetrical internal loop of the present disclosure can be formed by 9 nucleotides on the engineered polynucleotide side of the dsRNA substrate and 10 nucleotides internal loop the target RNA side of the dsRNA substrate. An asymmetrical internal loop of the present disclosure can be formed by 9 nucleotides on the target RNA side of the dsRNA substrate and 10 nucleotides on the engineered polynucleotide side of the dsRNA substrate.

[178] Structural features that comprise a bulge or loop can be of any size. In some cases, a bulge or loop can comprise at least: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 bases. In some cases, a bulge or loop can comprise at least about 1-10, 5-15, 10-20, 15-25, or 20-30 bases in total.

[179] In some cases, a structural feature is a structured motif. As disclosed herein, a structured motif can comprise two or more structural features in a dsRNA substrate. A structured motif can comprise of any combination of structural features, such as in the above claims, to generate an ideal substrate for ADAR editing at a precise location(s). These structural motifs could be artificially engineered to maximized ADAR editing, and/or these structural motifs can be modeled to recapitulate known ADAR substrates.

[180] In some cases, a structural feature can comprise an at least partial circularization of a polynucleotide. In some cases, a polynucleotide provided herein can be circularized or in a circular configuration. In some aspects, an at least partially circular polynucleotide can lack a 5' hydroxyl or a 3' hydroxyl.

<u>Guide RNA screening and quantitation</u>

[181] A plurality of screening assays, cell lines, and analytics tools were developed to screen or analyze an efficiency of various constructs comprising gRNAs or tRNA. **FIG. 35** shows exemplary screening assays, cell lines, and analytics assays and tools. Knock out (KO) cell lines were generated. In some cases, the KO cell line can lack ADAR1. In some cases, the KO cell line lacks ADAR2. Examples of deleted regions to generate ADAR1 KO cell lines and protein expression analysis in exemplary ADAR1 KO cell lines are shown in **FIG. 36**. Examples of deleted regions to generate ADAR2 KO cell lines and mRNA levels in exemplary ADAR2 KO cell lines are shown in **FIG. 37**. In some cases, ADAR1 can be integrated stably into an

ADAR2 KO cell line. In some cases, ADAR2 can be integrated stably into an ADAR1 KO cell line (e.g. **FIG. 38**).

[182] In some cases, digital droplet PCR is used to quantify gene expression, splice variants, gRNAs, extent of editing, copy number of variants, etc. A schematic of a digital droplet PCR (ddPCR) process is depicted in **FIG. 39**. Exemplary analysis using ddPCR that was used to design PCR primers and probe sets are shown in **FIG. 40**. In some cases, cellular expression of gRNA is quantified ($gRNA^{Q)}$ using an assay for example by using ddPCR. A ddPCR assay can be designed to quantify cellular expression of gRNAs using a universal tag that can be placed at the ends (e.g., 3' end or 5'end) of a gRNA (e.g. **FIG. 41**). **FIG. 42** shows examples of high cellular levels of gRNAs after transfection into target cells. Quantification of cellular expression levels of gRNA can be used to analyze (e.g., establish relationship between gRNA and editing efficiency) RNA editing in the cell.

[183] In some cases, a hairpin structure is introduced in the gRNA to increase stability. Exemplary gRNA constructs and their efficiency in editing Rab7a in HEK293 cells are shown in **FIG. 43**. Data suggests that adding a U6+27 loop to a gRNA can increase editing efficiency in the presence of ADAR2. Other examples of gRNA constructs and their efficiency in editing Rab7a in HEK293 cells are shown in **FIG. 44**.

[184] In some cases, rational and semi-rational gRNA designs are combined with high-throughput screening. **FIG. 45** illustrates exemplary approaches in utilizing gRNA design with high-throughput screening methods.

II. Methods

Methods of Delivery

[185] In some cases, a composition described herein (e.g. a pharmaceutical composition) can be administered to enable the delivery of an engineered tRNA or variant thereof or a vector encoding an engineered tRNA or variant thereof to the desired site of biological action. For example, a nucleic acid encoding for an engineered tRNA or variant thereof described herein can be comprised in a viral vector and can be administered by intravenous administration. Administration disclosed herein to an area in need of treatment or therapy can be achieved by, for example, and not by way of limitation, oral administration, topical administration, intravenous administration, inhalation administration, or any combination thereof. In some embodiments, delivery can include injection, catheterization, gastrostomy tube administration, intraosseous

administration, ocular administration, intracerebroventricular administration, otic administration, transdermal administration, oral administration, rectal administration, nasal administration, intravaginal administration, intracavernous administration, transurethral administration, sublingual administration, intracranial injection, intracranial injection into the parenchyma, intra-cisternal magna (ICM), intra-cerebroventricular (ICV) or a combination thereof. Delivery can include direct application to the affected tissue or region of the body. In some cases, topical administration can comprise administering a lotion, a solution, an emulsion, a cream, a balm, an oil, a paste, a stick, an aerosol, a foam, a jelly, a foam, a mask, a pad, a powder, a solid, a tincture, a butter, a patch, a gel, a spray, a drip, a liquid formulation, an ointment to an external surface of a surface, such as a skin. In some embodiments, administration can comprise an injection. In some embodiments, delivery can comprise an injection. Delivery can include a parenchymal injection, an intra-thecal injection, an intra-ventricular injection, or an intra-cisternal injection. A composition provided herein can be administered by any method. A method of administration can be by intraarterial injection, intracisternal injection, intramuscular injection, intraparenchymal injection, intraperitoneal injection, intraspinal injection, intrathecal injection, intravenous injection, intraventricular injection, stereotactic injection, subcutaneous injection, epidural, or any combination thereof. Delivery can include parenteral administration. Examples of parenteral administration can include an intravenous administering, an intra-arterial administering, an intrathecal administering, an intraocular administering, an otic administering, an intracerebroventricular administering, or an intraperitoneal administering). In some cases, delivery can be from a device. In some instances, delivery can be administered by a pump, an infusion pump, or a combination thereof. In some embodiments, delivery can be by an enema, an eye drop, a nasal spray, or any combination thereof. In some instances, a subject can administer the composition in the absence of supervision. In some instances, a subject can administer the composition under the supervision of a medical professional (e.g., a physician, nurse, physician's assistant, orderly, hospice worker, etc.). In some embodiments, a medical professional can administer the composition.

[186] In some cases, administering can be oral ingestion. In some cases, delivery can be a capsule or a tablet. Oral ingestion delivery can comprise a tea, an elixir, a food, a drink, a beverage, a syrup, a liquid, a gel, a capsule, a tablet, an oil, a tincture, or any combination thereof. In some embodiments, a food can be a medical food. In some instances, a capsule can

comprise hydroxymethylcellulose. In some embodiments, a capsule can comprise a gelatin, hydroxypropylmethyl cellulose, pullulan, or any combination thereof. In some cases, capsules can comprise a coating, for example, an enteric coating. In some embodiments, a capsule can comprise a vegetarian product or a vegan product such as a hypromellose capsule. In some embodiments, delivery can comprise inhalation by an inhaler, a diffuser, a nebulizer, a vaporizer, or a combination thereof.

[187] In some cases, a composition can be administered/applied as a single unit dose or as divided unit doses. In some cases, the compositions described herein can be administered at a first time point and a second time point. In some cases, a composition can be administered such that a first administration can be administered before the other with a difference in administration time of 1 hour, 2 hours, 4 hours, 8 hours, 12 hours, 16 hours, 20 hours, 1 day, 2 days, 4 days, 7 days, 2 weeks, 4 weeks, 2 months, 3 months, 4 months, 5 months, 6 months, 7 months, 8 months, 9 months, 10 months, 11 months, 1 year or more.

[188] Administration or application of a composition disclosed herein can be performed for a treatment duration of at least about at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 days consecutive or nonconsecutive days. In some cases, a treatment duration can be from about 1 to about 30 days, from about 2 to about 30 days, from about 3 to about 30 days, from about 4 to about 30 days, from about 5 to about 30 days, from about 6 to about 30 days, from about 7 to about 30 days, from about 8 to about 30 days, from about 9 to about 30 days, from about 10 to about 30 days, from about 11 to about 30 days, from about 12 to about 30 days, from about 13 to about 30 days, from about 14 to about 30 days, from about 15 to about 30 days, from about 16 to about 30 days, from about 17 to about 30 days, from about 18 to about 30 days, from about 19 to about 30 days, from about 20 to about 30 days, from about 21 to about 30 days, from about 22 to about 30 days, from about 23 to about 30 days, from about 24 to about 30 days, from about 25 to about 30 days, from about 26 to about 30 days, from about 27 to about 30 days, from about 28 to about 30 days, or from about 29 to about 30 days.

[189] Administration or application of a composition disclosed herein can be performed for a treatment duration of at least about 1 week, at least about 1 month, at least about 1 year, at least

about 2 years, at least about 3 years, at least about 4 years, at least about 5 years, at least about 6 years, at least about 7 years, at least about 8 years, at least about 9 years, at least about 10 years, at least about 15 years, at least about 20 years, or more. Administration can be performed repeatedly over a lifetime of a subject, such as once a month or once a year for the lifetime of a subject. Administration can be performed repeatedly over a substantial portion of a subject's life, such as once a month or once a year for at least about 1 year, 5 years, 10 years, 15 years, 20 years, 25 years, 30 years, or more.

[190] Administration or application of composition disclosed herein can be performed at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24 times a day. In some cases, administration or application of composition disclosed herein can be performed at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or 21 times a week. In some cases, administration or application of composition disclosed herein can be performed at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, or 90 times a month.

[191] In some cases, a subject can receive a diagnosis of a disease. In some cases, a subject may not have received a diagnosis of a disease. A diagnosis can include a blood test, a clinical diagnosis based on one or more symptoms, or any combination thereof. A diagnostic (such as a blood test) can confirm a presence or an absence of a mutation (e.g. a premature stop codon) in an mRNA encoding a polypeptide. In some cases, a diagnostic test can comprise sequencing (e.g. Sanger sequencing or sequencing by synthesis) a biological sample from a subject. A presence or an absence of a mutation in a portion of an mRNA can include a plurality of mutations (such as from about 1 to about 200 mutations). A clinical diagnosis can be based on one or more symptoms. A symptom can include loss of speech, loss of purposeful use of hands, involuntary hand movements, loss of mobility, gait disturbances, loss of muscle tone, seizures, scoliosis, sleep disturbances, slowed growth rate, breathing issues, or any combination thereof. In some cases, a symptom can comprise loss of hearing, muffing of speech and sounds, difficulty understanding words, trouble hearing constants, a loss of vision, a restricted vision field, a cloudiness of vision, a blurred vision, eye discomfort or any combination thereof. In some instances, a symptom can comprise a cough, a cough with phlegm, shortness of breath, fatty

stools, greasy stools, infertility, weight loss, salty skin, difficulty breathing or any combination thereof.

[192] Disclosed herein, in some embodiments is use of a viral vector such as an AAV vector. The viral vector can comprise a viral genome comprising an engineered tRNA. The viral vector can comprise a viral genome comprising an engineered tRNA variant. The viral vector can comprise a viral genome comprising a construct described herein. In some cases, the method can comprise administering the viral vector.

[193] In some embodiments, at least 1 x 10^3 viral genomes are administered. In some embodiments, at least 1 x 10^4 viral genomes are administered. In some embodiments, at least 1 x 10^5 viral genomes are administered. In some embodiments, at least 1 x 10^6 viral genomes are administered. In some embodiments, at least 1 x 10^7 viral genomes are administered. In some embodiments, at least 1 x 10^8 viral genomes are administered. In some embodiments, at least 1 x 10^9 viral genomes are administered. In some embodiments, at least 1 x 10^{10} viral genomes are administered. In some embodiments, at least 1 x 10^{11} viral genomes are administered. In some embodiments, at least 1 x 10^{12} viral genomes are administered. In some embodiments, at least 1 x 101^3 viral genomes are administered. In some embodiments, at least 1 x 10^{14} viral genomes are administered. In some embodiments, at least 1 x 10^{15} viral genomes are administered. In some embodiments, at least 1 x 10^{16} viral genomes are administered. In some embodiments, at least 1 x 10^{17} viral genomes are administered. In some embodiments, at least 1 x 10^{18} viral genomes are administered. In some embodiments, at least 1 x 10^{19} viral genomes are administered. In some embodiments, at least 1 x 10^{20} viral genomes are administered. In some embodiments, no more than 1 x 10^3 viral genomes are administered. In some embodiments, no more than 1 x 10^4 viral genomes are administered. In some embodiments, no more than 1 x 10^5 viral genomes are administered. In some embodiments, no more than 1 x 10^6 viral genomes are administered. In some embodiments, no more than 1 x 10^7 viral genomes are administered. In some embodiments, no more than 1 x 10^8 viral genomes are administered. In some embodiments, no more than 1 x 10^9 viral genomes are administered. In some embodiments, no more than 1 x 10^{10} viral genomes are administered. In some embodiments, no more than 1 x 10^{11} viral genomes are administered. In some embodiments, no more than 1 x 10^{12} viral genomes are administered. In some embodiments, no more than 1 x 101^3 viral genomes are administered. In some embodiments, no more than 1 x 10^{14} viral genomes are administered. In some embodiments, no more than 1 x 10^{15}

viral genomes are administered. In some embodiments, no more than 1 x 10^{16} viral genomes are administered. In some embodiments, no more than 1 x 10^{17} viral genomes are administered. In some embodiments, no more than 1 x 10^{18} viral genomes are administered. In some embodiments, no more than 1 x 10^{19} viral genomes are administered. In some embodiments, no more than 1 x 10^{20} viral genomes are administered. In some embodiments, from 1 x 10^{12} to 1 x 10^{15} viral genomes are administered. In some embodiments, from 1 x 10^{11} to 1 x 10^{16} viral genomes are administered. In some embodiments, from 1 x 10^{10} to 1 x 10^{17} viral genomes are administered.

[194] Disclosed herein, in some embodiments, are compositions comprising an engineered tRNA or variant thereof. Disclosed herein, in some embodiments, are compositions comprising an engineered tRNA variant. In some embodiments, the composition can comprise a pharmaceutical composition. The pharmaceutical composition can include the engineered tRNA or variant thereof and a pharmaceutically acceptable excipient, carrier or diluent. The pharmaceutical composition can include the engineered tRNA variant and a pharmaceutically acceptable excipient, carrier or diluent. The pharmaceutical composition can be in a dose unit form.

Methods of Treatment

[195] Provided here are methods of treating a disease, disorder or condition. Provided here are methods of preventing a disease, disorder or condition. Provided here are methods of treatment of a disease or disorder, or prevention of the disease or disorder.

[196] Provided here are methods of treating a disease, disorder or condition that can be associated with a mutation in an mRNA sequence that encodes for a premature stop codon instead of a desired amino acid. In some cases, the stop codon can be opal (TGA; UGA), ochre (TAA; UAA), or an amber (TAG; UAG) stop codon. In some cases, the disease-causing mutation in the mRNA sequence can comprise an opal stop codon (TGA; UGA) in the place of a codon encoding Arg (e.g., CGU, CGC, CGA, CGG, AGA, or AAG). In some cases, the disease-causing mutation in the mRNA sequence can comprise an ochre (TAA; UAA) or an ochre (TAG; UAG) stop codon in the place of a codon encoding Glutamine (e.g., CCA, CAG). In some cases, the premature stop codon results in a truncated version of the polypeptide or protein. In some cases, the disease, disorder, or condition can be caused by an increased level of a truncated version of the polypeptide, or a decreased level of substantially full-length polypeptide.

[197] For example, Rett Syndrome can be caused by an Arg-to-stop mutation in a polypeptide sequence of MeCP2. In a healthy individual, the MeCP2 protein contains an Arginine (Arg) at amino acid positions 168, 255, 270, 294, 198, 186 and 453 with reference to SEQ ID NO: 49, or with reference to SEQ ID NO: 50. In Rett Syndrome patients, a mutation in the MeCP2 protein that causes premature termination of translation of the MeCP2 protein at amino acid position 168, 255, 270, 294, 198, 186 and 453 causes loss-of-function of MeCP2, and collectively account for more than 25% of Rett-causing mutations. Arginine (Arg) in MECP2 can also be found at positions R8 (for isoform 2 only), R9 (for isoform 1 only), R84, R85, R89, R91, R106, R111, R115, R133, R162, R167, R188, R190,R211, R250, R253, R268, R306, R309, R344, R354, R420, R458, R468, R471, R478, and R484. The present disclosure provides engineered tRNAs and engineered tRNA variants that are capable of premature stop codon read-through of a premature stop codon that can be present at any position in the MeCP2 protein, including any one of: R168X, R255X, R270X, R294X, R198X, R186X, R453X, R8X (in isoform 2), R9X (in isoform 1), R84X, R85X, R89X, R91X, R106X, R111X, R115X, R133X, R162X, R167X, R188 X, R190X, R211X, R250X, R253X, R268X, R306X, R309X, R344X, R354X, R420X, R458X, R468X, R471X, R478X, R484X, or any combination thereof.. In some cases, Rett Syndrome in a subject can be treated by administering the compositions described herein to the subject, wherein the composition can comprise an engineered tRNA or variant thereof with an anticodon sequence that base pairs with the disease-causing premature stop codon (e.g., UGA). During translation, the engineered tRNA or variant thereof can be charged with an Arg, which can be transferred to the growing MeCP2 polypeptide, thereby at least substantially restoring MeCP2 expression in the subject. In some cases, the substantially restored MeCP2 polypeptide can be functional as compared with a WT MeCP2 protein. In some cases, restoring the MeCP2 polypeptide can be performed by the composition described herein at an efficacy greater than at least 10%, relative to a comparable polypeptide produced using a comparable mRNA that lacks the premature stop codon.

[198] In some cases, an mRNA can comprise one, two, three, four or five premature stop codons. Accordingly, an engineered tRNA or variant thereof as described herein can produce a readthrough of the premature stop codon, at least partially restoring a substantially full-length polypeptide and at least partially treating the disease or condition. In some cases, a pregnant female subject can be administered a composition as described herein. A pregnant female subject

can be administered the composition at one or more stages of the pregnancy. A female subject can be administered the composition before onset of a pregnancy, during a prenatal period. In some cases, an embryo or a fetus within a pregnant female subject can be administered a composition as described herein. In some cases, an embryo can be administered a composition as described herein in an *in vitro* setting.

[199] A disease or condition can comprise Rett syndrome, autism, West syndrome, Lennox-Gastaut syndrome, epileptic encephalopathy (EEP), Pitt-Hopkins syndrome, or any combination thereof. In some cases, a disease or condition can comprise cystic fibrosis, deafness (e.g. autosomal dominant 17 deafness, autosomal dominant 13 deafness, autosomal dominant 11 deafness) retinitis pigmentosa or any combination thereof. In some cases, a disease or condition can comprise Tay-Sachs, Parkinson's, Cystic Fibrosis, Usher syndrome, Wolman disease, a liver disease (Alpha-1 antitrypsin (AAT) deficiency), or any combination thereof. A disease or condition can comprise a neurodegenerative disease, a muscular disorder, a metabolic disorder, an ocular disorder (e.g. an ocular disease), a cancer, or any combination thereof. The disease or condition can comprise cystic fibrosis, albinism, Alzheimer disease, Amyotrophic lateral sclerosis, Asthma, β-thalassemia, Cadasil syndrome, Charcot-Marie-Tooth disease, Chronic Obstructive Pulmonary Disease (COPD), dementia, Distal Spinal Muscular Atrophy (DSMA), Dystrophic Epidermolysis bullosa, Epidermylosis bullosa, Fabry disease, Factor V Leiden associated disorders, Familial Adenomatous, Polyposis, Galactosemia, Gaucher's Disease, Glucose-6-phosphate dehydrogenase, Haemophilia, Hereditary Hematochromatosis, Hunter Syndrome, Huntington's disease, Hurler Syndrome, Inflammatory Bowel Disease (IBD), Inherited polyagglutination syndrome, Leber congenital amaurosis, Lesch-Nyhan syndrome, Lynch syndrome, Marfan syndrome, Mucopolysaccharidosis, Myotonic dystrophy types I and II, neurofibromatosis, Niemann-Pick disease type A, B and C, NY-eso1 related cancer, Parkinson's disease, Peutz-Jeghers Syndrome, Phenylketonuria, Pompe's disease, Primary Ciliary Disease, Prothrombin mutation related disorders, such as the Prothrombin G20210A mutation, Pulmonary Hypertension, Retinitis Pigmentosa, Sandhoff Disease, Severe Combined Immune Deficiency Syndrome (SCID), Sickle Cell Anemia, Spinal Muscular Atrophy, Stargardt's Disease, X-linked immunodeficiency, various forms of cancer (e.g., BRCA1 and 2 linked breast cancer and ovarian cancer). The disease or condition can comprise a muscular dystrophy, an ornithine transcarbamylase deficiency, a breast cancer, an ovarian cancer, a prostate cancer, a lung cancer,

a skin cancer, Stargardt macular dystrophy, Charcot-Marie-Tooth disease, or any combination thereof. A disease or condition can comprise a muscular dystrophy. A muscular dystrophy can include myotonic, Duchenne, Becker, Limb-girdle, facioscapulohumeral, congenital, oculopharyngeal, distal, Emery-Dreifuss, or any combination thereof. A disease or condition can comprise pain, such as chronic pain. Pain can include neuropathic pain, nociceptive pain, or a combination thereof. Nociceptive pain can include visceral pain, somatic pain, or a combination thereof.

[200] In some cases, a disease or condition can be Rett Syndrome. Rett syndrome can comprise a spectrum of related disorders. Rett syndrome can comprise an atypical Rett syndrome. Rett syndrome can comprise a congenital variant, a zappella variant, a hanefeld variant or any combination thereof. A disease or condition can comprise any number of diseases caused by one or more mutations in MECP2, CDKL5, FOXG1, STXBP1, TCF4, SCN2A, WDR45, MEF2C, or any combination thereof. In some embodiments, Rett Syndrome can be caused by a premature stop codon present in an mRNA encoding a polypeptide such as MeCP2, FoxG1, or CDKL5. For example, a subject suffering from Rett Syndrome can have an R168X, R225X, R270X, R294X, R198X, or R453X mutation in an mRNA encoding an MeCP2 polypeptide, where X can be a premature stop codon. Accordingly, an engineered tRNA or variant thereof as described herein can recognize the premature stop codon in an mRNA and insert an amino acid into a nascent MeCP2 polypeptide in response to the stop codon, thereby at least partially restoring a substantially full-length MeCP2 polypeptide. In some cases, at least partially restoring can be measured by determining the amount of the full-length MeCP2 polypeptide in a subject treated with the engineered tRNA or variant thereof compared to a subject not treated with the engineered tRNA or variant thereof. In some cases, a subject suffering from Rett Syndrome can have an R59X, R134X, R550X, R559X, R952X, R970X, Q118X, Q347X, Q459X, Q464X, Q652X, Q805X, Q832X, Q834X, Q865X, or Q902X mutation in an mRNA encoding an CDKL5 polypeptide, where X can be a premature stop codon. In some cases, a subject suffering from Rett Syndrome can have a Q46X, Q86X, or Q196X mutation in an mRNA encoding an FOXG1 polypeptide, where X can be a premature stop codon. Accordingly, an engineered tRNA or variant thereof as described herein can recognize the premature stop codon in an mRNA and insert an amino acid into a nascent CDKL5 or FOXG1 polypeptide in response to the stop codon, thereby at least partially restoring a substantially full-

length the polypeptide. In some cases, at least partially restoring can be measured by determining the amount of the full-length polypeptide in a subject treated with the engineered tRNA or variant thereof compared to a subject not treated with the engineered tRNA or variant thereof.

[201] In some cases, the polypeptide includes a FoxG1 polypeptide. In some cases, an engineered tRNA or variant thereof as described herein can recognize a premature stop codon in a FoxG1 mRNA and inserts an amino acid into a nascent FoxG1 polypeptide in response to the stop codon, thereby at least partially restoring a substantially full-length the polypeptide. In some cases, the premature stop codon in FoxG1 can be at Q46X, Q86X, or Q196X, where X represents a stop codon mutation.

[202] In some embodiments, at least one symptom of Rett Syndrome is alleviated. For example, a measurement relating to a symptom of Rett Syndrome can be improved in comparison to a baseline measurement. Examples of symptoms of Rett Syndrome can include slowed growth, slowed brain growth, microcephaly, a decrease or loss of movement or coordination, reduced hand control, decreased walking ability, rigid or spastic movement, a decreased ability to speak, decreased eye, disinterestedness, repetitive hand movement, unusual eye movements, difficulty breathing, irritability, fear, anxiety, a cognitive defect, seizures, an abnormal electroencephalogram, scoliosis, irregular heartbeat, or an abnormal sleep pattern.

[203] In some embodiments, the disease or condition is Rett Syndrome and at least one symptom of Rett Syndrome is alleviated. In some cases, the symptom of Rett Syndrome can be alleviated by reading through a stop codon using an engineered tRNA or engineered tRNA variant provided herein. In some cases, the symptom of Rett Syndrome can be alleviated by reading through a stop codon as little as 5% of the time, as little as 10% of the time, as little as 15% of the time, as little as 20% of the time, or as little as 25% of the time. In some cases, the symptom of Rett Syndrome can be alleviated by reading through a stop codon at least 5% of the time, at least 10% of the time, at least 15% of the time, at least 20% of the time, or at least 25% of the time.

[204] In some cases, the polypeptide includes a CDKL5 polypeptide. In some cases, an engineered tRNA or variant thereof as described herein can recognize a premature stop codon in a CDKL5 mRNA and inserts an amino acid into a nascent CDKL5 polypeptide in response to the stop codon, thereby at least partially restoring a substantially full-length the polypeptide. In some cases, the premature stop codon in CDKL5 can be at R59X, R134X, R550X, R559X, R952X,

R970X, Q118X, Q347X, Q459X, Q464X, Q652X, Q805X, Q832X, Q834X, Q865X, or Q902X, where X represents a stop codon mutation.

[205] In some cases, a disease or condition can be deafness. Some forms of deafness can be caused by the presence of a premature stop codon present in an mRNA encoding a polypeptide such as MYH9, COL11A2, or MYO7A. In some embodiments, an autosomal dominant 17 form of deafness can result from an R1933X mutation in an mRNA encoding a MYH9 polypeptide. In some embodiments, an autosomal dominant 13 form of deafness can result from an R845X mutation in an mRNA encoding a COL11A2 polypeptide. In some embodiments, an autosomal dominant 11 form of deafness can result from an R666X mutation in an mRNA encoding a MYO7A polypeptide.

[206] In some cases, a disease or condition can be Usher syndrome. Some forms of Usher syndrome such as Usher syndrome type IB can be associated with MYO7A mutations. Usher syndrome can be associated with combined deafness and blindness. MYO7A encodes for Myosin VIIA which can encode for a myosin which can be an actin binding molecular motor. In some cases, a mutation can comprise a C628X mutation, a R1861X mutation, or both. In some cases, a mutation can comprise a A26E mutation, a P132L mutation, a R241G mutation, a L366P mutation, a I1045T mutation, a L1863P mutation, a G1218R mutation, a R1240Q mutation, a A1492V mutation, a A2204P mutation or any combination thereof.

[207] In some cases, a disease or condition can be cystic fibrosis caused by the presence of a premature stop codon present in an mRNA encoding a polypeptide. In some cases, a disease or condition can be retinitis pigmentosa caused by the presence of a premature stop codon present in an mRNA encoding a polypeptide.

[208] Treatment can comprise administration to a subject one or more compositions as described herein. Treatment can comprise administration of a co-therapy to a subject. In some cases, a co-therapy can comprise a cancer treatment (e.g. radiotherapy, chemotherapy, CAR-T therapy, immunotherapy, hormone therapy, cryoablation). In some cases, a co-therapy can comprise surgery, antibiotics, antivirals, or any combination thereof. In some instances, a co-therapy can comprise a mucus thinner, cystic fibrosis transmembrane conductance regulator (CFTR) modulator therapies, a lung transplant, bronchodilator, airway clearance, an anti-inflammatory medication, nebulizer treatment, an oral pancreatic enzyme, a stool softener. In some cases, a co-therapy can comprise elexacaftor, ivacaftor and tezacaftor, lumacaftor, or any

combination thereof. In some embodiments, a co-therapy can comprise physical therapy, hydrotherapy, occupational therapy, speech-language therapy, feeding assistance, an antiepileptic drug, an antireflux drug, levocarnitine or any combination thereof. A co-therapy can comprise surgery, a steroid therapy, a nonsteroidal anti-inflammatory drug (NSAID) or any combination thereof. In some instances, a co therapy can help repair vision loss (e.g. glasses, or corrective eye surgery) or help treat loss of hearing (e.g. a hearing aid). In some cases, a co-therapy can comprise an RNA or DNA editing technology. Treatment can include curing a disease or condition. Treatment can include substantially reducing one or more symptoms of a disease or condition.

[209] Disclosed herein, in some embodiments, are methods that include administration of an engineered tRNA or variant thereof to a subject. Some embodiments include administration of an engineered tRNA variant to a subject. In some embodiments, the engineered tRNA or variant thereof recognizes a premature stop codon in an mRNA encoding a polypeptide, wherein the engineered tRNA or variant thereof during translation of the mRNA at least partially transforms interpretation of the premature stop codon into a sense codon and produces a substantially full-length polypeptide in vivo at an efficiency of at least about 10%, relative to a comparable polypeptide produced using a comparable mRNA that lacks the premature stop codon, as optionally determined by: transfecting a first vector encoding the engineered tRNA or variant thereof and a second vector encoding a screening mRNA encoding a first marker into a first human cell, wherein the screening mRNA encoding the first marker can comprise the premature stop codon; transfecting a third vector encoding a comparable screening mRNA encoding a second marker into a second human cell, wherein the comparable screening mRNA may not comprise the premature stop codon; and comparing an amount of a detectable signal emitted from the first human cell and the second human cell.

Methods of Diagnosis

[210] Methods and compositions as described herein can include identifying a presence or an absence of a disease or condition, treating a disease or condition, or any combination thereof. The method can include administering to a subject in need a pharmaceutically acceptable amount of a composition or compound such as an engineered tRNA or variant thereof described herein.

[211] In some embodiments, a subject can be or can comprise been given a diagnosis with a disease or the condition prior to administration. In some embodiments, the diagnosis can be or

can comprise been determined by an *in vitro* diagnostic test. In some embodiments, a treatment can be provided upon diagnosis.

Inhibition of Nonsense-Mediated Decay

[212] In some embodiments, an engineered tRNA suppresses nonsense-mediated decay (NMD) of an mRNA including a target mRNA. In some embodiments, an engineered tRNA variant suppresses NMD. Such suppression of NMD by an engineered tRNA or engineered tRNA variant can be included in any of the methods described herein. Some embodiments can include a method of inhibiting NMD, comprising contacting a cell with an engineered tRNA, or a variant thereof. In some embodiments, the contact can result in a prevention or decrease of NMD of a target mRNA in the cell. In some embodiments, the contact can result in an increase in a target mRNA measurement in the cell, relative to a baseline measurement. Some embodiments can include a method of inhibiting NMD, comprising administering to a subject in need thereof, an engineered tRNA, or a variant thereof. In some embodiments, the administration can result in a prevention or decrease of NMD of a target mRNA in the subject. In some embodiments, the administration can result in an increase in a target mRNA measurement in the subject, relative to a baseline measurement. The increase in the target mRNA measurement can be in a first sample taken from the subject, relative to a baseline target mRNA measurement in a first sample taken from the subject. The first and/or second samples can comprise a tissue or fluid sample described herein.

[213] In some cases, the engineered tRNA or variant thereof can decrease NMD of the target mRNA by: 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% 90%, 95%, 99% or 100%, or a range of any two of the aforementioned percentages. In some cases, the engineered tRNA or variant thereof can decrease NMD of the target mRNA by at least about: 1% to about 10%, 5% to about 20%, 10% to about 35%, 25% to about 50%, 40% to about 70%, 60% to about 80%, 75% to about 90% or about 85% to about 100%.

[214] NMD is in some cases can be a quality control pathway that degrades premature termination codon (PTC)-containing mRNAs. Exon-exon junctions have been characterized for determining whether or not an mRNA is targeted for NMD. During mRNA splicing, an exon junction complex (EJC) can form 20-24 nucleotides upstream from an exon-exon junction. The EJC can remain bound to an mRNA after splicing until displaced during a second round of

translation. If no PTC is present, EJCs can be removed during translation, rendering the mRNA resistant to degradation by NMD. If a PTC is present at least 50 nucleotides upstream of the EJC, a ribosome can stall and the EJC can remain stably associated with the mRNA. This complex can be a dynamic structure with a heterogenous protein composition that can recruit Upf3, a nucleocytoplasmic shuttling factor, and Upf2, a perinuclear protein, both of which can be involved in NMD. An RNA-dependent helicase such as Upf1 can be recruited to the mRNA by translation release factors and bridge the terminated ribosome and the downstream EJC to form an active NMD complex that can trigger decay of the mRNA. This degradation can involve decapping followed by 5' to 3' decay and deadenylation followed by 3' to 5' decay. In the case of PTC readthrough, the bridge between the termination complex and the EJC can in some cases not form and the ribosome can detach the EJC protein complex from a mutant transcript, which can no longer be a target for NMD. This can help explain how stop codon readthrough can antagonize NMD and can stabilize mRNA. An engineered tRNA or variant thereof can interfere with any of these aspects of NMD described herein.

[215] PTC suppression can inhibit NMD by enabling (1) continuation of translation and/or (2) disassociation of the last EJC from the mRNA (see, e.g., **FIG. 49** - adapted from Keeling and Bedwell, Wiley Interdiscip Rev RNA (2011)).

[216] PTC suppression and read-through of a target mRNA containing a PTC/nonsense mutation by suppressor tRNAs could be one of the ways to escape the NMD pathway and stabilize/increase the levels of the target mRNA. For example, transfection of a suppressor tRNA into Ulrich Disease fibroblasts can comprise been shown to result in up-regulation of a target mRNA. Sako et al, NASS (2006).

[217] PTC readthrough with suppressor tRNAs described herein can inhibit nonsense-mediated decay (NMD) of a target mRNA. This can increase intracellular levels of the target mRNA. Subsequent suppression of the PTC in the target mRNA can produce full-length functional protein.

Methods of Manufacture

[218] Provided herein are methods of generating the engineered tRNAs or variants thereof, the polynucleotide encoding the engineered tRNAs or variants thereof, or the pharmaceutical compositions described herein. The method can include aspects disclosed herein. In some cases, a vector can be produced that can comprise a plurality of promoters, a genetic sequence encoding

one or more engineered tRNAs or variants thereof. The vector can comprise a first promoter (e.g., an hU6, or a mU6 promoter), a second promoter (e.g., an hU6, or a mU6 promoter), a third promoter (e.g., a human cytomegalovirus (CMV) promoter), a transgene comprising a polynucleotide encoding an engineered tRNA or a variant thereof, and a reporter gene encoding a detectable polypeptide. The polynucleotide can comprise a 5' ITR upstream of the promoter. In some cases, the polynucleotide can comprise a 3' ITR downstream of the reporter gene. In some cases, the reporter gene can comprise one or more genes encoding for mCherry, green fluorescent protein (GFP), or β-galactosidase. The first promoter or the second promoter can comprise a U6 promoter (e.g., a human U6 (hU6). The U6 promoter can be a human U6 promoter or a mouse U6 promoter. The U6 promoter can be methylated. The third promoter can be a human cytomegalovirus (CMV) promoter. In some cases, the vector can be produced by purification or isolation. In some cases, the promoter can be a U7 promoter. In some cases, the promoter can be a PolIII promoter.

Packaging

[219] The present disclosure provides viral vectors packaging engineered tRNAs or variants thereof, promoters, stuffer sequences, and any combination thereof. In some embodiments, the viral vectors of the present disclosure can package one or more copies of the engineered tRNA or variant thereof. In some embodiments, the viral vectors of the present disclosure can package 2 copies of the engineered tRNAs or variants thereof. In some embodiments, the viral vectors of the present disclosure can package 3 copies of the engineered tRNAs or variants thereof. In some embodiments, the viral vectors of the present disclosure can package 4 copies of the engineered tRNAs or variants thereof. In some embodiments, the viral vectors of the present disclosure can package 6 copies of the engineered tRNAs or variants thereof. In some embodiments, the viral vectors of the present disclosure can package more than 6 copies of the engineered tRNAs or variants thereof. Viral vectors of the present disclosure can package from 1 to 200, from 1 to 100, from 50 to 100, from 20 to 40, from 10 to 50, or from 1 to 70 copies of the engineered tRNAs or variants thereof. In some embodiments, the viral vectors of the present disclosure can package at least 1, 10, 20, 30, 40, 50, 70, 100, 200, 300, 400 or more copies of the engineered tRNAs or variants thereof. In some embodiments, the viral vectors of the present disclosure can package at most about 400, 300, 200, 100, 70, 50, 40, 30, 20, 10, 2, or less copies of the engineered tRNAs or variants thereof.

[220] One or more engineered tRNAs or variants thereof can be packaged in a vector, including but not limited to a plasmid, an AAV vector, a lentivirus vector, or any other vector system. The vectors disclosed herein can also encode for a marker or a reporter gene, such as GFP or mCherry. The vectors disclosed herein can also encode for an upstream exogenous promoter, such as human U6 (hU6). In some embodiments, engineered tRNA or variants thereof, markers, and/or stuffer sequences are packaged in a viral vector are under the control of an exogenous promotor (e.g., a mouse U6 (mU6) or a human U6 (hU6) promoter). In some embodiments, engineered tRNAs suppressors or variants thereof, markers, and/or stuffer sequences are packaged in a viral vector without an exogenous promoter. In some cases, a vector can comprise a plurality of promoters, a genetic sequence encoding one or more engineered tRNAs or variants thereof. The vector can comprise a first promoter (e.g., an hU6, or a mU6 promoter), a second promoter (e.g., an hU6, or a mU6 promoter), a third promoter (e.g., a human cytomegalovirus (CMV) promoter), a transgene comprising a polynucleotide encoding an engineered tRNA or a variant thereof, and a reporter gene encoding a detectable polypeptide. The polynucleotide can comprise a 5' ITR upstream of the promoter. In some cases, the polynucleotide can comprise a 3' ITR downstream of the reporter gene. In some cases, the reporter gene can comprise one or more genes encoding for mCherry, green fluorescent protein (GFP), or β-galactosidase. The first promoter or the second promoter can comprise a U6 promoter (e.g., a human U6 (hU6). The U6 promoter can be a human U6 promoter or a mouse U6 promoter. The U6 promoter can be methylated. The third promoter can be a human cytomegalovirus (CMV) promoter. In some cases, the vector can be purified or isolated.

[221] The vectors of the present disclosure can also package a stuffer sequence, to ensure successful production of virus particles that package the entire genome to be delivered. For example, a naturally occurring stuffer sequence (e.g., DNA from strawberry or lambda phage) can be included in the vector. In some embodiments, the stuffer sequence can be a fully synthetic sequence. In some embodiments, vectors provided herein package from 1 to 6 engineered tRNAs or variants thereof and a stuffer sequence. In some embodiments, vectors provided herein package an exogenous hU6 promoter, from 1 to 6 engineered tRNAs and a stuffer sequence. A spacer sequence can include a stuffer sequence or a filler sequence. A spacer sequence, a stuffer sequence, or a filler sequence can be referred to interchangeably in some embodiments. In some cases, the vector can comprise one or more spacer sequences to place a sequence of an

engineered tRNA or a variant thereof in different distances from an ITR. The spacer sequence can comprise a 6 nucleotides (nts) long sequence. The spacer sequence can comprise 10, 20, 30, 40, 50, 100, 200, 300, 400, 500, 600, 1000 nucleotides or any number of nucleotides in between any of the two numbers of nucleotides mentioned herein. For example, the space sequence can comprise between about 10 nts to about 100 nts, about 100 nts to about 500 nts, about 50 to about 600 nts, or about 200 nts to about 1000 nts. In some cases, the spacer sequence can comprise less than 6 nts or more than 1000 nts. In some cases, the spacer sequence can comprise a stuffer sequence. The stuffer sequence can be about 25, about 50, about 100, about 150, about 200, about 250, or about 300 nucleotides in length. The stuffer sequence can be 25, 50, 100, 150, 200, 250, or 300 nucleotides in length, or a range of lengths encompassing any two of the aforementioned numbers of nucleotides. In some embodiments, the stuffer sequence can comprise at least 25 nucleotides, at least 50 nucleotides, at least 100 nucleotides, at least 150 nucleotides, at least 200 nucleotides, at least 250 nucleotides, or at least 300 nucleotides. In some embodiments, the stuffer sequence is at least about 50 nucleotides, at least about 100 nucleotides, at least about 150 nucleotides, or at least about 200 nucleotides. In some embodiments, the stuffer sequence can comprise at least 250 nucleotides, at least 300 nucleotides, at least 350 nucleotides, at least 400 nucleotides, at least 450 nucleotides, or at least 500 nucleotides. In some embodiments, the stuffer sequence can comprise no more than 25 nucleotides, no more than 50 nucleotides, no more than 100 nucleotides, no more than 150 nucleotides, no more than 200 nucleotides, no more than 250 nucleotides, or no more than 300 nucleotides. In some embodiments, the stuffer sequence is no more than about 50 nucleotides, no more than about 100 nucleotides, no more than about 150 nucleotides, or no more than about 200 nucleotides. In some embodiments, the stuffer sequence can comprise no more than 250 nucleotides, no more than 300 nucleotides, no more than 350 nucleotides, no more than 400 nucleotides, no more than 450 nucleotides, or no more than 500 nucleotides.

[222] In some embodiments, the stuffer sequence is 5’ of one or more copies of the engineered tRNA variant within the polynucleotide. In some embodiments, the stuffer sequence is 3’ of one or more copies of the engineered tRNA variant within the polynucleotide. In some embodiments, the stuffer sequence separates two or more copies of the engineered tRNA variant within a polynucleotide. In some embodiments, multiple stuffer sequences separate multiple copies of the engineered tRNA variant within a polynucleotide. For example, a polynucleotide

encoding an engineered tRNA or engineered tRNA variant can comprise a first copy of the engineered tRNA or engineered tRNA variant, followed by a first stuffer sequence, followed by a second copy of the engineered tRNA or engineered tRNA variant, followed by a second stuffer sequence, followed by a third copy of the engineered tRNA or engineered tRNA variant (in a 5' to 3' direction). There also can be one or more stuffer sequences 5' of the first copy of the engineered tRNA or engineered tRNA variant, or 3' of the third copy of the engineered tRNA or engineered tRNA variant.

[223] SEQ ID NO: 57 includes an example of a polynucleotide comprising a filler sequence. Some embodiments include a polynucleotide having at least 75% identity, at least 80% identity, at least 85% identity, at least 90% identity, at least 95% identity, or 100% identity to SEQ ID NO: 57, or a fragment thereof (e.g. a fragment including any of the spacer sequences provided therein). In Some embodiments, the fragment of SEQ ID NO: 57 is 10, 20, 30, 40, 50, 75, 100, 200, 300, 400, 500, 750, 1000, 1500, 2000, 2500 or more nucleotides long, or a range of nucleotides defined by any two of the aforementioned numbers.

[224] In some cases, changing the distance of an engineered tRNA or a variant thereof from an ITR can increase an efficiency of a stop codon readthrough of the engineered tRNA or a variant thereof. In some cases, an orientation of a sequence of an engineered tRNA or a variant thereof in a vector construct can affect an efficiency of a stop codon readthrough of the engineered tRNA or a variant thereof. In some cases, a vector can comprise one or more engineered tRNA sequences or an engineered tRNA variant sequences in a vector placed in different orientations.

III. Kits

[225] In some embodiments, a kit can comprise a composition described herein and a container. For example, a kit can comprise a pharmaceutical composition, which can comprise an engineered tRNA or variant thereof, a polynucleotide (e.g., vector) encoding the engineered tRNA or variant thereof, or both. In some instances, a container can be plastic, glass, metal, or any combination thereof. In some cases, a kit can comprise instructions for use, such as instructions for administration to a subject in need thereof.

[226] In some instances, a packaged product comprising a composition described herein can be properly labeled. In some instances, the pharmaceutical composition described herein can be

manufactured according to good manufacturing practice (cGMP) and labeling regulations. In some cases, a pharmaceutical composition disclosed herein can be aseptic.

[227] Disclosed herein, in some embodiments, are kits comprising an engineered tRNA or variant thereof. Disclosed herein, in some embodiments, are kits comprising an engineered tRNA variant. In some embodiments, the kits can comprise a pharmaceutical composition described herein (e.g. an engineered tRNA or variant thereof and a pharmaceutically acceptable excipient, carrier or diluent, optionally in a dose unit form). In some embodiments, the kit can comprise a packaging or a container. In some embodiments, the kit can comprise a packaging. In some embodiments, the kit can comprise a container.

[228] Some embodiments relate to methods of making the kit. The method can include contacting the composition with a packaging or container. The method can include contacting the composition with a packaging. The method can include contacting the composition with a container.

IV. Definitions

[229] Unless defined otherwise, all terms of art, notations and other technical and scientific terms or terminology used herein are intended to have the same meaning as can be commonly understood by one of ordinary skill in the art to which the claimed subject matter pertains. In some cases, terms with commonly understood meanings are defined herein for clarity and/or for ready reference, and the inclusion of such definitions herein should not necessarily be construed to represent a substantial difference over what can be generally understood in the art.

[230] Throughout this application, various embodiments can be presented in a range format. It should be understood that the description in range format can be merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the disclosure. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

[231] As used in the specification and claims, the singular forms "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a sample" includes a plurality of samples, including mixtures thereof.

[232] The terms "determining," "measuring," "evaluating," "assessing," "assaying," and "analyzing" are often used interchangeably herein to refer to forms of measurement. The terms include determining if an element can be present or not (for example, detection). These terms can include quantitative, qualitative or quantitative and qualitative determinations. Assessing can be relative or absolute. "Detecting the presence of" can include determining the amount of something present in addition to determining whether it can be present or absent depending on the context.

[233] The term "*in vivo*" can be used to describe an event that takes place in a subject's body.

[234] The term "*ex vivo*" can be used to describe an event that takes place outside of a subject's body. An *ex vivo* assay can be not performed on a subject. Rather, it can be performed upon a sample separate from a subject. An example of an *ex vivo* assay performed on a sample can be an "*in vitro*" assay.

[235] The term "*in vitro*" can be used to describe an event that takes places contained in a container for holding laboratory reagent such that it can be separated from the biological source from which the material can be obtained. *In vitro* assays can encompass cell-based assays in which living or dead cells are employed. *In vitro* assays can also encompass a cell-free assay in which no intact cells are employed.

[236] As used herein, the term "about" a number can refer to that number plus or minus 10% of that number. The term "about" a range can refer to that range minus 10% of its lowest value and plus 10% of its greatest value.

[237] As used herein, the terms "treatment" or "treating" are used in reference to a pharmaceutical or other intervention regimen for obtaining beneficial or desired results in the recipient. Beneficial or desired results include but are not limited to a therapeutic benefit and/or a prophylactic benefit. A therapeutic benefit can refer to eradication or amelioration of symptoms or of an underlying disorder being treated. Also, a therapeutic benefit can be achieved with the eradication or amelioration of one or more of the physiological symptoms associated with the underlying disorder such that an improvement can be observed in the subject, notwithstanding

that the subject can still be afflicted with the underlying disorder. A prophylactic effect includes delaying, preventing, or eliminating the appearance of a disease or condition, delaying or eliminating the onset of symptoms of a disease or condition, slowing, halting, or reversing the progression of a disease or condition, or any combination thereof. For prophylactic benefit, a subject at risk of developing a particular disease, or to a subject reporting one or more of the physiological symptoms of a disease can undergo treatment, even though a diagnosis of this disease may not have been made.

[238] The term "subject," "host," "individual," and "patient" are as used interchangeably herein to refer to animals, typically mammalian animals. Any suitable subject can be administered a composition as described herein or treated by a method as described herein. Non-limiting examples of mammals include humans, non-human primates (e.g., apes, gibbons, chimpanzees, orangutans, monkeys, macaques, and the like), domestic animals (e.g., dogs and cats), farm animals (e.g., horses, cows, goats, sheep, pigs) and experimental animals (e.g., mouse, rat, rabbit, guinea pig). In some embodiments a mammal can be a human. A mammal can be any age or at any stage of development (e.g., an adult, teen, child, infant, or a mammal in utero). In some cases, a human can be an embryo, a fetus, a child, or an adult. In some cases, a human can be from about: 1 day to about 7 days old, 1 week to about 5 weeks old, 1 month to about 12 months old, 1 year to about 10 years old, 6 months to about 15 years old, 5 years to about 25 years old, 20 years to about 50 years old, 40 years to about 80 years old, 75 years to about 100 years old, or about 90 years to about 130 years old. A mammal can be male or female. In some embodiments, the subject can male. In some embodiments, the subject can female. In some embodiments a subject can be a human. In some embodiments, a subject can comprise or can be suspected of having a disease or condition, such as Rett syndrome. In some cases, a subject can be a pregnant subject, such as a pregnant human at an age appropriate for reproduction. In some embodiments, the patient can about 20 years of age. In some embodiments, the patient can 10-30 years of age.

[239] In some embodiments, the patient is at least 5 years of age, at least 10 years of age, at least 15 years of age, at least 20 years of age, at least 25 years of age, at least 30 years of age, at least 35 years of age, at least 50 years of age, at least 75 years of age, or at least 95 years of age. In some embodiments, the patient is no more than 5 years of age, no more than 10 years of age, no more than 15 years of age, no more than 20 years of age, no more than 25 years of age, no

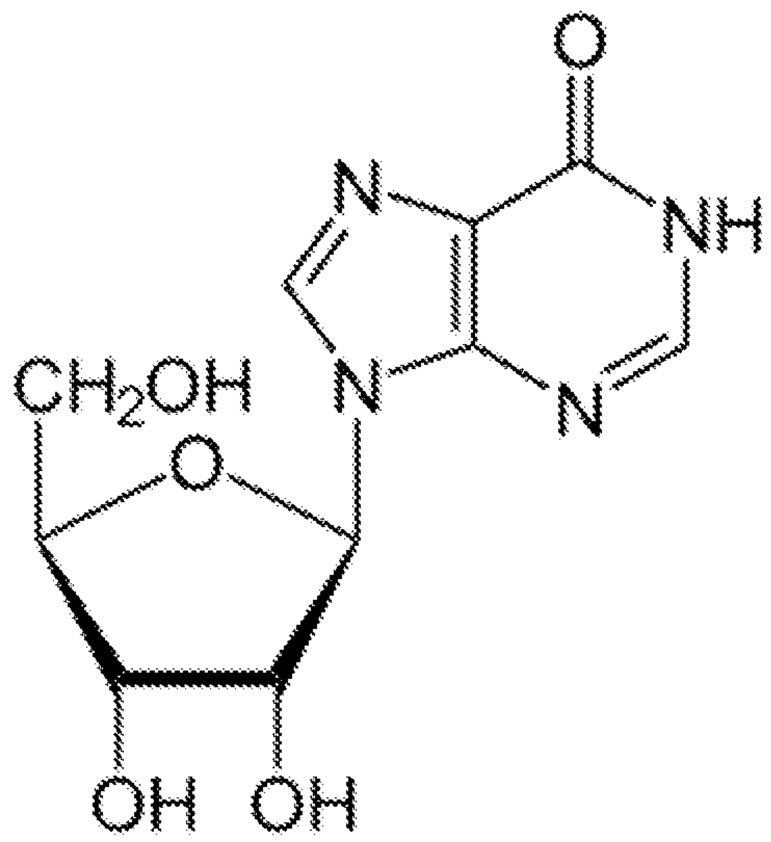
more than 30 years of age, no more than 35 years of age, no more than 50 years of age, no more than 75 years of age, or no more than 95 years of age.

[240] "Eukaryotic cells" comprise all of the life kingdoms except monera. They can be easily distinguished through a membrane-bound nucleus. Animals, plants, fungi, and protists can be eukaryotes or organisms whose cells can be organized into complex structures by internal membranes and a cytoskeleton. A characteristic membrane-bound structure can be the nucleus. The term "host" can include a eukaryotic host, including, for example, yeast, higher plant, insect and mammalian cells. Non-limiting examples of eukaryotic cells or hosts include simian, bovine, porcine, murine, rat, avian, reptilian and human. Other examples of eukaryotic cells can include cell lines. In some cases, a primary cell or a cell can be a neuron, a photoreceptor cell (e.g. a S cone cell, a L cone cell, a M cone cell, a rod cell), a retinal pigment epithelium cell, a glia cell (e.g. an astrocyte, an oligodendrocyte, a microglia), a muscle cell (e.g. a myoblast, a myotube), a hepatocyte, a lung epithelial cell, or a fibroblast (e.g. dermal fibroblast). In some cases, a cell can be a horizontal cell, a ganglion cell, or a bipolar cell. In some cases, a cell line can be a mammalian cell line, such as HEK293T, HEK293, NCI-60, MCF-7, HL-60, RD, LHCN differentiated, LHCN undifferentiated, Saos-2, CHO, or HeLa cells. In some cases, a cell line can be an insect cell line, such as Sf9.

[241] The term "protein", "peptide" and "polypeptide" can be used interchangeably and in their broadest sense can refer to a compound of two or more subunit amino acids, amino acid analogs or peptidomimetics. The subunits can be linked by peptide bonds. In another embodiment, the subunit can be linked by other bonds, e.g., ester, ether, etc. A protein or peptide can contain at least two amino acids and no limitation can be placed on the maximum number of amino acids which can comprise a protein's or peptide's sequence. As used herein the term "amino acid" can refer to either natural amino acids, unnatural amino acids, or synthetic amino acids, including glycine and both the D and L optical isomers, amino acid analogs and peptidomimetics. As used herein, the term "fusion protein" can refer to a protein comprised of domains from more than one naturally occurring or recombinantly produced protein, where generally each domain serves a different function. In this regard, the term "linker" can refer to a protein fragment that can be used to link these domains together – optionally to preserve the conformation of the fused protein domains, prevent unfavorable interactions between the fused protein domains which can compromise their respective functions, or both.

[242] The terms "polynucleotide" and "oligonucleotide" can be used interchangeably and can refer to a polymeric form of nucleotides of any length, either deoxyribonucleotides or ribonucleotides or analogs thereof. Polynucleotides can have any three-dimensional structure and can perform any function, known or unknown. The following are non-limiting examples of polynucleotides: a gene or gene fragment (for example, a probe, primer, EST or SAGE tag), exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, RNAi, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes and primers. A polynucleotide can comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs. If present, modifications to the nucleotide structure can be imparted before or after assembly of the polynucleotide. The sequence of nucleotides can be interrupted by non-nucleotide components. A polynucleotide can be further modified after polymerization, such as by conjugation with a labeling component. The term can also refer to both double- and single-stranded molecules. Unless otherwise specified or required, any embodiment of this invention that can be a polynucleotide encompasses both the double-stranded form and each of two complementary single-stranded forms known or predicted to make up the double-stranded form.

[243] A polynucleotide can be composed of a specific sequence of nucleotides. A nucleotide can comprise a nucleoside and a phosphate group. A nucleotide can comprise a sugar (e.g., ribose or 2'deoxyribose) and a nucleobase, such as a nitrogenous base. Non-limiting examples of nucleobases include adenine (A), cytosine (C), guanine (G), thymine (T), uracil (U), and inosine (I). In some embodiments, I can be formed when hypoxanthine can be attached to ribofuranose via a P-N9-glycosidic bond, resulting in the chemical structure:



Inosine can be read by the translation machinery as guanine (G).

[244] The term "polynucleotide sequence" can be the alphabetical representation of a polynucleotide molecule. This alphabetical representation can be input into databases in a computer having a central processing unit and used for bioinformatics applications such as functional genomics and homology searching.

[245] "Homology" or "identity" or "similarity" can refer to sequence similarity between two peptides or between two nucleic acid molecules. Homology can be determined by comparing a position in each sequence which can be aligned for purposes of comparison. When a position in the compared sequence can be occupied by the same base or amino acid, then the molecules can be homologous at that position. A degree of homology between sequences can be a function of the number of matching or homologous positions shared by the sequences. An "unrelated" or "non-homologous" sequence shares less than 40% identity, or alternatively less than 25% identity, with one of the sequences of the disclosure. Sequence homology can refer to a% identity of a sequence to a reference sequence. As a practical matter, whether any particular sequence can be at least 50%, 60%, 70%, 80%, 85%, 90%, 92%, 95%, 96%, 97%, 98% or 99% identical to any sequence described herein (which can correspond with a particular nucleic acid sequence described herein), such particular polypeptide sequence can be determined conventionally using known computer programs such the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, Wis. 53711). When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence,

the parameters can be set such that the percentage of identity can be calculated over the full-length of the reference sequence and that gaps in sequence homology of up to 5% of the total reference sequence can be allowed.

[246] In some cases, the identity between a reference sequence (query sequence, i.e., a sequence of the disclosure) and a subject sequence, also referred to as a global sequence alignment, can be determined using the FASTDB computer program-based on the algorithm of Brutlag et al. (Comp. App. Biosci. 6:237-245 (1990)). In some embodiments, parameters for a particular embodiment in which identity can be narrowly construed, used in a FASTDB amino acid alignment, can include: Scoring Scheme=PAM (Percent Accepted Mutations) 0, k-tuple=2, Mismatch Penalty=1, Joining Penalty=20, Randomization Group Length=0, Cutoff Score=1, Window Size=sequence length, Gap Penalty=5, Gap Size Penalty=0.05, Window Size=500 or the length of the subject sequence, whichever can be shorter. According to this embodiment, if the subject sequence can be shorter than the query sequence due to N- or C-terminal deletions, not because of internal deletions, a manual correction can be made to the results to take into consideration the fact that the FASTDB program does not account for N- and C-terminal truncations of the subject sequence when calculating global percent identity. For subject sequences truncated at the N- and C-termini, relative to the query sequence, the percent identity can be corrected by calculating the number of residues of the query sequence that can be lateral to the N- and C-terminal of the subject sequence, which can be not matched/aligned with a corresponding subject residue, as a percent of the total bases of the query sequence. A determination of whether a residue can be matched/aligned can be determined by results of the FASTDB sequence alignment. This percentage can be then subtracted from the percent identity, calculated by the FASTDB program using the specified parameters, to arrive at a final percent identity score. This final percent identity score can be used for the purposes of this embodiment. In some cases, only residues to the N- and C-termini of the subject sequence, which can be not matched/aligned with the query sequence, can be considered for the purposes of manually adjusting the percent identity score. That is, only query residue positions outside the farthest N- and C-terminal residues of the subject sequence can be considered for this manual correction. For example, a 90-residue subject sequence can be aligned with a 100-residue query sequence to determine percent identity. The deletion occurs at the N-terminus of the subject sequence, and therefore, the FASTDB alignment does not show a matching/alignment of the first 10 residues at

the N-terminus. The 10 unpaired residues represent 10% of the sequence (number of residues at the N- and C-termini not matched/total number of residues in the query sequence) so 10% can be subtracted from the percent identity score calculated by the FASTDB program. If the remaining 90 residues were perfectly matched, the final percent identity can be 90%. In another example, a 90-residue subject sequence can be compared with a 100-residue query sequence. This time the deletions can be internal deletions, so there can be no residues at the N- or C-termini of the subject sequence which can be not matched/aligned with the query. In this case, the percent identity calculated by FASTDB can be not manually corrected. Once again, only residue positions outside the N- and C-terminal ends of the subject sequence, as displayed in the FASTDB alignment, which can be not matched/aligned with the query sequence can be manually corrected for.

[247] In some cases, the identity between a reference sequence (query sequence, i.e., a sequence of the disclosure) and a subject sequence, also referred to as a global sequence alignment, can be determined using the FASTDB computer program based on the algorithm of Brutlag et al. (Comp. App. Biosci. 6:237-245 (1990)). In some embodiments, parameters for a particular embodiment in which identity can be narrowly construed, used in a FASTDB amino acid alignment, can include: Scoring Scheme=PAM (Percent Accepted Mutations) 0, k-tuple=2, Mismatch Penalty=1, Joining Penalty=20, Randomization Group Length=0, Cutoff Score=1, Window Size=sequence length, Gap Penalty=5, Gap Size Penalty=0.05, Window Size=500 or the length of the subject sequence, whichever can be shorter. According to this embodiment, if the subject sequence can be shorter than the query sequence due to N- or C-terminal deletions, not because of internal deletions, a manual correction can be made to the results to take into consideration the fact that the FASTDB program does not account for N- and C-terminal truncations of the subject sequence when calculating global percent identity. For subject sequences truncated at the N- and C-termini, relative to the query sequence, the percent identity can be corrected by calculating the number of residues of the query sequence that can be lateral to the N- and C-terminal of the subject sequence, which can be not matched/aligned with a corresponding subject residue, as a percent of the total bases of the query sequence. A determination of whether a residue can be matched/aligned can be determined by results of the FASTDB sequence alignment. This percentage can be then subtracted from the percent identity, calculated by the FASTDB program using the specified parameters, to arrive at a final percent

identity score. This final percent identity score can be used for the purposes of this embodiment. In some cases, only residues to the N- and C-termini of the subject sequence, which can be not matched/aligned with the query sequence, can be considered for the purposes of manually adjusting the percent identity score. That is, only query residue positions outside the farthest N- and C-terminal residues of the subject sequence can be considered for this manual correction. For example, a 90-residue subject sequence can be aligned with a 100-residue query sequence to determine percent identity. The deletion occurs at the N-terminus of the subject sequence, and therefore, the FASTDB alignment does not show a matching/alignment of the first 10 residues at the N-terminus. The 10 unpaired residues represent 10% of the sequence (number of residues at the N- and C-termini not matched/total number of residues in the query sequence) so 10% can be subtracted from the percent identity score calculated by the FASTDB program. If the remaining 90 residues were perfectly matched, the final percent identity can be 90%. In another example, a 90-residue subject sequence can be compared with a 100-residue query sequence. This time the deletions can be internal deletions, so there can be no residues at the N- or C-termini of the subject sequence which can be not matched/aligned with the query. In this case, the percent identity calculated by FASTDB can be not manually corrected. Once again, only residue positions outside the N- and C-terminal ends of the subject sequence, as displayed in the FASTDB alignment, which can be not matched/aligned with the query sequence can be manually corrected for.

[248] "Transfer ribonucleic acid" or "tRNA" can be a nucleic acid molecule that helps translate mRNA to protein. tRNA can have a distinctive folded structure, comprising three hairpin loops; one of these loops can comprise a "stem" portion that encodes an anticodon. The anticodon can recognize the corresponding codon on the mRNA. Each tRNA can be "charged with" an amino acid corresponding to the mRNA codon. "Charging can be accomplished by the enzyme tRNA synthetase. Upon tRNA recognition of the codon corresponding to its anticodon, the tRNA can transfer the amino acid with which it can be charged to the growing amino acid chain to form a polypeptide or protein. Endogenous tRNA can be charged by endogenous tRNA synthetase. Accordingly, endogenous tRNA are typically charged with canonical amino acids. Orthogonal tRNA, derived from an external source, can require a corresponding orthogonal tRNA synthetase. Such orthogonal tRNAs can be charged with both canonical and non-canonical amino acids. Non-limiting examples of non-canonical amino acids can be found in "Adding New

Chemistries to the Genetic Code" By C. Liu and P. Schultz, Annu. Re. Biochem. 79:413-44 (2010). In some embodiments, the amino acid with which the tRNA can be charged can be detectably labeled to enable detection *in vivo*. Suitable techniques for labeling include, but are not limited to, click chemistry wherein an azide/alkyne containing unnatural amino acid can be added by the orthogonal tRNA/synthetase pair and, thus, can be detected using alkyne/azide comprising fluorophore or other such molecule.

[249] The term "engineered tRNA," as used herein can refer to transfer RNAs having at least one difference in the sequence of the engineered tRNA relative to a comparable wild type tRNA, for example, in the anticodon region. The term "engineered tRNA variant" or "variant thereof" as used herein can refer to transfer RNAs having a mutation in their sequence as compared to a reference engineered tRNA. In some embodiments, the engineered tRNA or variants thereof are referred to as "suppressor tRNAs or variants thereof," "tRNA suppressor" or "engineered tRNA suppressor," which generally can refer to an engineered tRNA or variant thereof capable of suppressing premature stop codon readthrough in an mRNA.

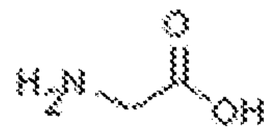
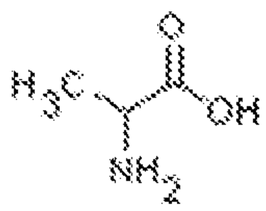
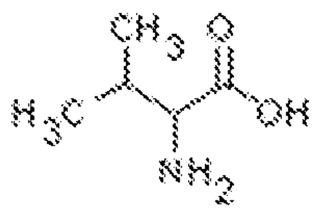
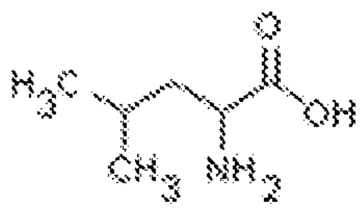
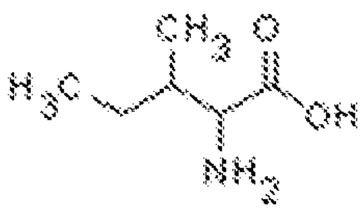
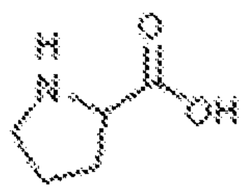
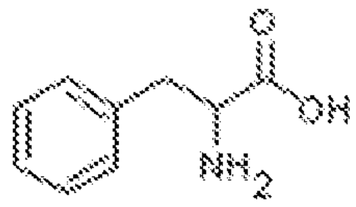
[250] The term "tRNA isodecoder" can be used interchangeably herein to refer to tRNA molecules that, when aminoacylated, are aminoacylated with the same amino acid. In some cases, the tRNA isodecoder can refer to tRNA molecules that share the same anticodon sequence.

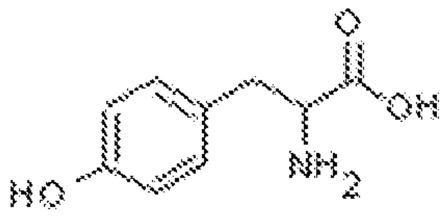
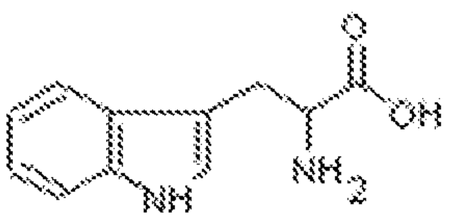
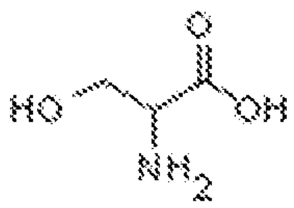
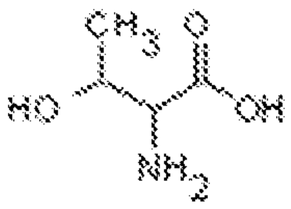
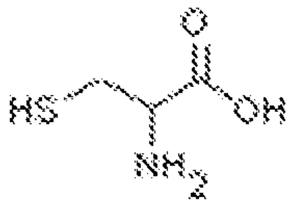
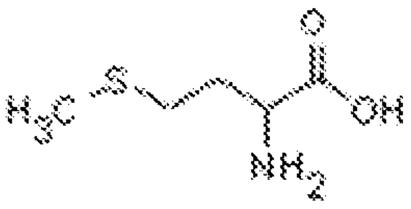
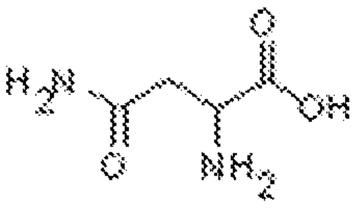
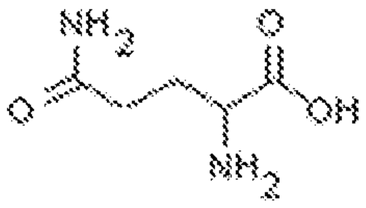
[251] "Messenger RNA" or "mRNA" are referred to herein are RNA molecules comprising a sequence that encodes a polypeptide or protein. In general, RNA can be transcribed from DNA. In some cases, precursor mRNA containing non-protein coding regions in the sequence can be transcribed from DNA and then processed to remove all or a portion of the non-coding regions (introns) to produce mature mRNA. As used herein, the term "pre-mRNA" can refer to the RNA molecule transcribed from DNA before undergoing processing to remove the non-protein coding regions.

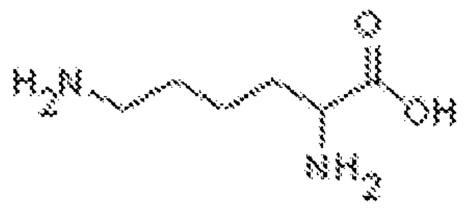
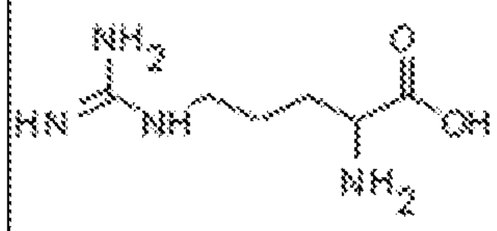
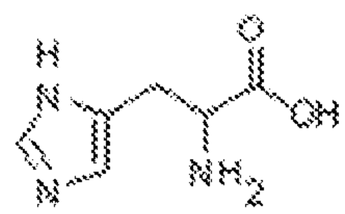
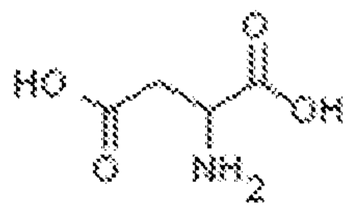
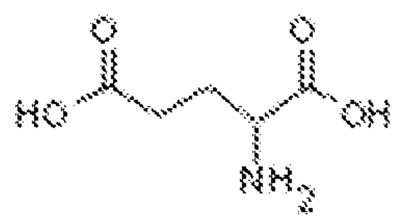
[252] The term "stop codon" can refer to a three-nucleotide contiguous sequence within messenger RNA that signals a termination of translation. Non-limiting examples include in RNA, UAG (amber), UAA (ochre), UGA (umber, also known as opal) and in DNA TAG, TAA or TGA. Unless otherwise noted, the term can also include nonsense mutations within DNA or RNA that introduce a premature stop codon, causing any resulting protein to be abnormally shortened. tRNA that correspond to the various stop codons are known by their stop codon corresponding names: amber (UAG), ochre (UAA), and opal (UGA).

[253] "Canonical amino acids" refer to those 20 amino acids that occur in nature, including for example, the amino acids shown in **Table 4.**

Table 4. Naturally occurring amino acids indicated with the three letter abbreviations, one letter abbreviations, structures, and corresponding codons

Non-polar, aliphatic residues				
Glycine	**Gly**	**G**		**GGU GGC GGA GGG**
Alanine	**Ala**	**A**		**GCU GCC GCA GCG**
Valine	**Val**	**V**		**GUU GUC GUA GUG**
Leucine	**Leu**	**L**		**UUA UUG CUU CUC CUA CUG**
Isoleucine	**Ile**	**I**		**AUU AUC AUA**
Proline	**Pro**	**P**		**CCU CCC CCA CCG**
Aromatic residues				
Phenylalanine	**Phe**	**F**		**UUU UUC**

Tyrosine	**Tyr**	**Y**		**UAU UAC**
Tryptophan	**Trp**	**W**		**UGG**
Polar, non-charged residues				
Serine	**Ser**	**S**		**UCU UCC UCA UCG AGU AGC**
Threonine	**Thr**	**T**		**ACU ACC ACA ACG**
Cysteine	**Cys**	**C**		**UGU UGC**
Methionine	**Met**	**M**		**AUG**
Asparagine	**Asn**	**N**		**AAU AAC**
Glutamine	**Gln**	**Q**		**CAA CAG**

Positively charged residues				
Lysine	**Lys**	**K**		**AAA AAG**
Arginine	**Arg**	**R**		**CGU CGC CGA CGG AGA AGG**
Histidine	**His**	**H**		**CAU CAC**
Negatively charged residues				
Aspartate	**Asp**	**D**		**GAU GAC**
Glutamate	**Glu**	**E**		**GAA GAG**

[254] The term "effective amount" can refer to a quantity sufficient to achieve a desired effect. In the context of therapeutic or prophylactic applications, the effective amount can depend on the type and severity of the condition at issue and the characteristics of the individual subject, such as general health, age, sex, body weight, and tolerance to pharmaceutical compositions. In some embodiments, an effective amount can be an amount that can be required to at least partially treat a patient with a disease such as Rett Syndrome, or Deafness. In some cases, the skilled artisan will be able to determine appropriate amounts depending on these and other factors.

[255] In the case of an *in vitro* application, in some embodiments the effective amount will depend on the size and nature of the application in question. It will also depend on the nature and sensitivity of the *in vitro* target and the methods in use. In some cases, the skilled artisan will be

able to determine the effective amount based on these and other considerations. The effective amount can comprise one or more administrations of a composition depending on the embodiment.

[256] As used herein the term "restoring" in relation to expression of a protein can refer to the ability to establish expression of full-length protein where previously protein expression was truncated due to mutation such as a premature stop codon.

[257] The term "mutation" as used herein, can refer to an alteration to a nucleic acid sequence or a polypeptide sequence that can be relative to a reference sequence. A mutation can occur in a DNA molecule, a RNA molecule (e.g., tRNA, mRNA), or in a polypeptide or protein, or any combination thereof. The reference sequence can be obtained from a database such as the NCBI Reference Sequence Database (RefSeq) database. Specific changes that can constitute a mutation can include a substitution, a deletion, an insertion, an inversion, or a conversion in one or more nucleotides or one or more amino acids. Non-limiting examples of mutations in a nucleic acid sequence that, without the mutation, encodes for a polypeptide sequence, include: "missense" mutations that can result in the substitution of one codon for another, a "nonsense" mutations that can change a codon from one encoding a particular amino acid to a stop codon (which can result in truncated translation of proteins), or a "silent" mutations that can be those which have no effect on the resulting protein. The mutation can be a "point mutation," which can refer to a mutation affecting only one nucleotide in a DNA or RNA sequence. The mutation can be a "splice site mutations," which can be present pre-mRNA (prior to processing to remove introns) resulting in mistranslation and often truncation of proteins from incorrect delineation of the splice site. The mutation can be a fusion gene. A fusion pair or a fusion gene can result from a mutation, such as a translocation, an interstitial deletion, a chromosomal inversion, or any combination thereof. A mutation can constitute variability in the number of repeated sequences, such as triplications, quadruplications, or others. For example, a mutation can be an increase or a decrease in a copy number associated with a given sequence (i.e., copy number variation, or CNV). A mutation can include two or more sequence changes in different alleles or two or more sequence changes in one allele. A mutation can include two different nucleotides at one position in one allele, such as a mosaic. A mutation can include two different nucleotides at one position in one allele, such as a chimeric. A mutation can be present in a malignant tissue. A mutation can

comprise a single nucleotide variation (SNV). A mutation can comprise a sequence variant, a sequence variation, a sequence alteration, or an allelic variant.

[258] A presence or an absence of a mutation can indicate an increased risk to develop a disease or condition. A presence or an absence of a mutation can indicate a presence of a disease or condition. A mutation can be present in a benign tissue. Absence of a mutation can indicate that a tissue or sample can be benign. As an alternative, absence of a mutation may not indicate that a tissue or sample can be benign. Methods as described herein can comprise identifying a presence of a mutation in a sample.

[259] The term "encode," as used herein, can refer to an ability of a polynucleotide to provide information or instructions sequence sufficient to produce a corresponding gene expression product. In a non-limiting example, mRNA can encode for a polypeptide during translation, whereas DNA can encode for an mRNA molecule during transcription.

[260] The term "complementary" or "complementarity" can refer to the ability of a nucleic acid to form one or more bonds with a corresponding nucleic acid sequence by, for example, hydrogen bonding (e.g., traditional Watson-Crick), covalent bonding, or other similar methods. In Watson-Crick base pairing, a double hydrogen bond forms between nucleobases T and A, whereas a triple hydrogen bond forms between nucleobases C and G. For example, the sequence A-G-T can be complementary to the sequence T-C-A. 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, respectively). "Perfectly complementary" can mean 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 can refer to a degree of complementarity that can be at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%. 97%, 98%, 99%, or 100% over a region of 10, 15, 20, 25, 30, 35, 40, 45, 50, or more nucleotides, or can refer to two nucleic acids that hybridize under stringent conditions (i.e., stringent hybridization conditions). Nucleic acids can include nonspecific sequences. As used herein, the term "nonspecific sequence" or "not specific" can refer to a nucleic acid sequence that contains a series of residues that can be not designed to be complementary to or can be only partially complementary to any other nucleic acid sequence.

[261] The terms "hairpin," "hairpin loop," "stem-loop," and/or "loop" used alone or in combination with "motif" can refer to a structure in a single stranded polynucleotide molecule that forms and resembles a hairpin or a loop. This structure can form when a first nucleotide sequence of the polynucleotide molecule and a second nucleotide sequence in the polynucleotide molecule base-pair or otherwise bond to form the hairpin or loop structure. Such structures are illustrated in FIG. 2, which shows the various stem-loop structures in naturally occurring or engineered tRNAs according to some embodiments.

[262] As used herein, the term "domain" can refer to a particular region of a protein, polynucleotide (e.g. an engineered tRNA) or polypeptide and can be associated with a particular function. For example, "a domain which associates with an RNA hairpin motif" can refer to the domain of a protein that binds one or more RNA hairpin.

[263] It can be to be inferred without explicit recitation and unless otherwise intended, that when the present disclosure relates to a polypeptide, protein, polynucleotide or antibody, an equivalent or a biologically equivalent of such can be intended within the scope of this disclosure. As used herein, the term "biological equivalent thereof" can be intended to be synonymous with "equivalent thereof" when referring to a reference protein, antibody, polypeptide or nucleic acid, intends those having minimal homology while still maintaining desired structure or functionality. Unless specifically recited herein, it can be contemplated that any polynucleotide, polypeptide or protein mentioned herein also includes equivalents thereof. For example, an equivalent intends at least about 70% homology or identity, or at least 80% homology or identity and alternatively, or at least about 85%, or alternatively at least about 90%, or alternatively at least about 95%, or alternatively 98% percent homology or identity and exhibits substantially equivalent biological activity to the reference protein, polypeptide or nucleic acid. Alternatively, when referring to polynucleotides, an equivalent thereof can be a polynucleotide that hybridizes under stringent conditions to the reference polynucleotide or its complement.

[264] An "RNA editing entity" can comprise an endogenous enzyme that can edit a target RNA sequence. In some instances, an RNA editing entity can comprise a recombinant enzyme. In some cases, an RNA editing entity can comprise a fusion polypeptide. In some embodiments, an RNA editing entity can comprise APOBEC1, APOBEC2, APOBEC3A, APOBEC3B, APOBEC3C, APOBEC3D ("APOBEC3E" now can refer to this), APOBEC3F, APOBEC3G,

APOBEC3H, APOBEC4, Activation-induced (cytidine) deaminase (AID), ADAR1, ADAR1p110, ADAR1p150, ADAR2, ADAR3, or any combination thereof. In some cases, an RNA editing entity can comprise at least about 80% sequence homology to APOBEC1, APOBEC2, APOBEC3A, APOBEC3B, APOBEC3C, APOBEC3D ("APOBEC3E" now can refer to this), APOBEC3F, APOBEC3G, APOBEC3H, APOBEC4, Activation-induced (cytidine) deaminase (AID), ADAR1, ADAR1p110, ADAR1p150, ADAR2, ADAR3, or any combination thereof. In some cases, an RNA editing entity can be a virus-encoded RNA-dependent RNA polymerase. In some cases, an RNA editing entity can be a virus-encoded RNA-dependent RNA polymerase from measles, mumps, or parainfluenza. In some instances, an RNA editing entity can be an enzyme from *Trypanosoma brucei* capable of adding or deleting a nucleotide or nucleotides in a target RNA. In some instances, an RNA editing entity can be an enzyme from *Trypanosoma brucei* capable of adding or deleting a Uracil or more than one Uracil in a target RNA.

[265] The term "ADAR" as used herein can refer to an adenosine deaminase that can convert adenosines (A) to inosines (I) in an RNA sequence. ADAR1 and ADAR2 can be two exemplary species of ADAR that can be involved in mRNA editing *in vivo*. Non-limiting exemplary sequences for ADAR1 can be found under the following reference numbers: HGNC: 225; Entrez Gene: 103; Ensembl: ENSG 00000160710; OMIM: 146920; UniProtKB: P55265; and GeneCards: GC01M154554, as well as biological equivalents thereof. Non-limiting exemplary sequences for ADAR2 can be found under the following reference numbers: HGNC: 226; Entrez Gene: 104; Ensembl: ENSG00000197381; OMIM: 601218; UniProtKB: P78563; and GeneCards: GC21P045073, as well as biological equivalents thereof.

[266] The term "APOBEC" as used herein can refer to any protein that falls within the family of evolutionarily conserved cytidine deaminases involved in mRNA editing – catalyzing a C to U conversion – and equivalents thereof. In some respects, the term APOBEC can refer to any one of APOBEC1, APOBEC2, APOBEC3A, APOBEC3B, APOBEC3C, APOBEC3E, APOBEC3F, APOBEC3G, APOBEC3H, APOBEC4, or equivalents each thereof. Non-limiting exemplary sequences of fusion proteins comprising one or more APOBEC domains can be provided herein both fused to an ADAR domain or fused to alternative domains to render them suitable for use in an RNA editing system. To this end, APOBECs can be considered an equivalent of ADAR – catalyzing editing albeit by a different conversion. Thus, not to be bound

by theory, Applicants believe that all embodiments contemplated herein for use with an ADAR based editing system can be adapted for use in an APOBEC based RNA editing system.

[267] The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described.

V. Embodiments

[268] Provided are the following non-limiting embodiments:

1. A method of treating a disease or condition in a subject in need thereof, comprising:

administering to the subject an engineered tRNA or variant thereof or a polynucleotide encoding the engineered tRNA or variant thereof; and

producing a substantially full-length polypeptide *in vivo* at an efficiency of at least about 10%, relative to a comparable polypeptide produced using an mRNA that lacks a premature stop codon,

wherein the engineered tRNA or the variant thereof is capable of reading through the premature stop codon in an mRNA encoding for the substantially full-length polypeptide.

2. A method of treating a disease or condition in a subject in need thereof, comprising:

administering to the subject an engineered tRNA or variant thereof or a polynucleotide encoding the engineered tRNA or variant thereof, thereby at least partially treating the disease or condition in the subject;

wherein the engineered tRNA or variant thereof recognizes a premature stop codon in an mRNA encoding a polypeptide, wherein the engineered tRNA or variant thereof during translation of the mRNA at least partially transforms interpretation of the premature stop codon into a sense codon and produces a substantially full-length polypeptide *in vivo* at an efficiency of at least about 10%, relative to a comparable polypeptide produced using a comparable mRNA that lacks the premature stop codon, as determined by:

a) transfecting a first vector encoding the engineered tRNA or variant thereof and a second vector encoding a screening mRNA encoding a first marker into a first human cell, wherein the screening mRNA encoding the first marker comprises the premature stop codon;

b) transfecting a third vector encoding a comparable screening mRNA encoding a second marker into a second human cell, wherein the comparable screening mRNA does not comprise the premature stop codon; and

c) comparing an amount of a detectable signal emitted from the first human cell and the second human cell.

3. The composition of embodiment 2, wherein the first human cell or the second human cell is a HEK293 cell.
4. The method any one of embodiments 1-3, further comprising restoring at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 85%, at least about 90%, at least about 92%, at least about 95%, at least about 97%, at least about 99%, or 100% expression of the substantially full-length polypeptide.
5. The method of embodiment 3, wherein the engineered tRNA or variant thereof produces the substantially full-length polypeptide at an efficiency of at least about 35%, relative to the comparable polypeptide produced using the comparable mRNA that lacks the premature stop codon.
6. The method of any one of embodiments 1-5, wherein the engineered tRNA or variant thereof is acylated with an amino acid selected from the group consisting of: lysine, arginine, histidine, glycine, alanine, valine, leucine, isoleucine, methionine, phenylalanine, tryptophan, proline, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartate, glutamate, pyrolysine, and selenocysteine.
7. The method of any one of embodiments 1-6, wherein the engineered tRNA or variant thereof is acylated with arginine.
8. The method of any one of embodiments 1-7, wherein the engineered tRNA or variant thereof is acylated with a non-canonical amino acid.
9. The method of any one of embodiments 1-8, wherein the mRNA encoding the polypeptide corresponds to at least a fragment of MeCP2.
10. The method of any one of embodiments 1-9, wherein the engineered tRNA or variant thereof, during translation of the mRNA, inserts an amino acid into a nascent MeCP2 polypeptide chain encoded by the mRNA *in vivo* in response to the premature stop codon, wherein the amino acid when inserted is sufficient to produce an at least partially

functional MeCP2 polypeptide, as compared to a comparable MeCP2 polypeptide produced using the comparable mRNA that lacks the premature stop codon.

11. The method of any one of embodiments 1-10, wherein the mRNA comprises at least two premature stop codons.
12. The method of embodiment 11, wherein the at least two premature stop codons are the same stop codon.
13. The method of embodiment 11, wherein the at least two premature stop codons are different stop codons.
14. The method of any one of embodiments 1-13, wherein the premature stop codon results from an R to an X at amino acid position 168, 255, 270, 294, 198, 186, 453, 8 (in isoform 2), 9 (in isoform 1), 84, 85, 89, 91, 106, 111, 115, 133, 162, 167, 188, 190, 211, 250, 253, 268, 306, 309, 344, 354, 420, 458, 468, 471, 478, or 484, or any combination thereof, in a sequence corresponding to a MeCP2 polypeptide, wherein X is a premature stop codon.
15. The method of any one of embodiments 1-14, wherein the premature stop codon is an opal stop codon.
16. The method of any one of embodiments 1-14, wherein the premature stop codon is an amber stop codon.
17. The method of any one of embodiments 1-14, wherein the premature stop codon is an ochre stop codon.
18. The method of any one of embodiments 1-17, wherein the engineered tRNA or variant thereof is a lysyl-tRNA, an arginyl-tRNA, a histidyl-tRNA, a glycyl-tRNA, an alanyl-tRNA, a valyl-tRNA, a leucyl-tRNA, an isoleucyl-tRNA, methionyl-tRNA, a phenylalanyl-tRNA, a tryptophanyl-tRNA, a prolyl-tRNA, a seryl-tRNA, a threonyl-tRNA, a cysteinyl-tRNA, a tyrosyl-tRNA, an asparaginyl tRNA, a glutaminyl-tRNA, an aspartyl-tRNA, a pyrrolysyl tRNA, a selenocytstyl tRNA or a glutamyl-tRNA.
19. The method of any one of embodiments 1-18, wherein the engineered tRNA or variant thereof is an engineered pre-tRNA.
20. The method of embodiment 19, wherein the engineered tRNA or variant thereof comprises an intronic sequence.

21. The method of embodiment 20, wherein the intronic sequence is spliced within a cell containing the engineered tRNA or variant thereof, thereby producing a mature engineered tRNA or variant thereof.
22. The method of any one of embodiments 1-21, wherein the subject is a human.
23. The method of any one of embodiments 1-21, where the subject is a non-human animal.
24. The method of embodiment 22, wherein the human is aged from about birth to about 40 years old.
25. The method of embodiment 22 or 25, wherein the human is aged about 6 months to about 15 years old.
26. The method of embodiment 22, wherein the human is an embryo or a fetus.
27. The method of any one of embodiments 1-26, wherein the disease or condition comprises Rett Syndrome.
28. The method of any one of embodiments 1-26, wherein the disease or condition comprises cystic fibrosis.
29. The method of any one of embodiments 1-26, wherein the disease or condition comprises retinitis pigmentosa.
30. The method of any one of embodiments 1-26, wherein the disease or condition comprises deafness.
31. The method of embodiment 30, wherein the deafness comprises autosomal dominant 17 deafness, autosomal dominant 13 deafness, or autosomal dominant 11 deafness.
32. The method of any one of embodiments 1-31, wherein the administering is oral administering, rectal administering, or parenteral administering.
33. The method of embodiment 32, wherein the administering is the parenteral administering, and wherein the parenteral administering is an intravenous administering, an intra-arterial administering, an intrathecal administering, an intraocular administering, an otic administering, an intracerebroventricular administering, or an intraperitoneal administering.
34. The method of any one of embodiments 1-31, wherein the administering is intra-cisternal magna (ICM).
35. The method of any one of embodiments 1-31, wherein the administering is intra-cerebroventricular (ICV).

36. The method of any one of embodiments 1-35, wherein the polypeptide comprises an MeCP2 polypeptide, or a fragment thereof.
37. The method of any one of embodiments 1-35, wherein the polypeptide comprises a FoxG1 polypeptide, or a fragment thereof.
38. The method of any one of embodiments 1-35, wherein the polypeptide comprises a CDKL5 polypeptide, or a fragment thereof.
39. The method of any one of embodiments 1-35, wherein the polypeptide comprises a MYH9 polypeptide, or a fragment thereof.
40. The method of any one of embodiments 1-35, wherein the polypeptide comprises a COL11A2 polypeptide, or a fragment thereof.
41. The method of any one of embodiments 1-35, wherein the polypeptide comprises a MYO7A polypeptide, or a fragment thereof.
42. The method of any one of embodiments 1-41, wherein the administering is performed at least twice during a time period.
43. The method of embodiment 42, wherein the time period is about 24 hours.
44. The method of any one of embodiments 1-43, wherein the method is a method of preventing the disease or condition, and wherein the preventing comprises a prophylactic administration of the engineered tRNA or variant thereof or the polynucleotide encoding the engineered tRNA or variant thereof to the subject.
45. The method of any one of embodiments 1-44, wherein the polynucleotide encoding the engineered tRNA or variant thereof is administered to the subject.
46. The method of embodiment 44 or 45, wherein the polynucleotide encoding the engineered tRNA or variant thereof is comprised in a vector.
47. The method of embodiment 46, wherein the vector is a viral vector.
48. The method of embodiment 46 or 47, wherein the vector comprises a plasmid, adenoviral vector, an adeno-associated viral (AAV) vector, a lentiviral (LV) vector.
49. The method of embodiment 48, wherein the AAV vector is from an AAV having:
    a) a serotype comprising AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11 or AAV12, or
    b) a pseudotype comprising AAV-DJ, AAV-DJ/8, AAV-Rh10, AAV-Rh74, AAV-retro, AAV-PHP.B, AAV8-PHP.eB, AAV-PHP.S or AAV-2i8.

50. The method of embodiment 48 or 49, wherein the AAV vector comprises a genome comprising a replication gene and inverted terminal repeats from a first AAV serotype and a capsid protein from a second AAV serotype.
51. The method of any one of embodiments 48-50, wherein the AAV vector comprises an AAV 2/5 vector, an AAV 2/6 vector, an AAV 2/7 vector, an AAV2/8 vector, or an AAV 2/9 vector.
52. The method of any one of embodiments 48-51, wherein the AAV vector is an AAV 2/5 vector.
53. The method of any one of embodiments 1-52, wherein the engineered tRNA or variant thereof, or the polynucleotide encoding the engineered tRNA or variant thereof is present in a delivery system.
54. The method of embodiment 53, wherein the delivery system comprises a liposome, a charged polymer, an uncharged polymer, a nanoparticle, a surfactant, a penetrating enhancer, a gene transfer agent, a phospholipid, a micelle, a synthetic vector, a macromolecule, a dendrimer, a biopolymer, a viral particle, or any combination thereof.
55. The method of any one of embodiments 1-54, further comprising administering a composition comprising a polynucleotide sequence comprising or encoding for: (i) a recruiting region and (ii) a targeting region, wherein the polynucleotide sequence recruits an RNA editing entity, and wherein the editing entity when contacted with the polynucleotide sequence and the mRNA performs a chemical modification on a base of a nucleotide of the premature stop codon of the mRNA, thereby converting the premature stop codon into a sense codon.
56. The method of embodiment 55, wherein the RNA editing entity comprises:
    a) an ADAR polypeptide;
    b) an APOBEC polypeptide;
    c) a biologically active fragment of (a) or (b); or
    d) a fusion protein comprising: the biological active fragment of (c), the ADAR polypeptide of (a), or the APOBEC polypeptide of (b).
57. The method of embodiment 1, wherein the disease or condition comprises Rett Syndrome, wherein the mRNA encodes an MeCP2 polypeptide, wherein the premature stop codon is an opal stop codon, wherein the engineered tRNA or variant thereof is an

arginyl-tRNA, wherein the subject is a human aged 0-6 years old, and wherein when the engineered tRNA or variant thereof is administered to the subject as an AAV vector, the engineered tRNA or variant thereof during translation of the mRNA encoding the MeCP2 polypeptide at least partially transforms interpretation of the premature stop codon into an arginine sense codon and produces a substantially full-length MeCP2 polypeptide, thereby at least partially treating the Rett Syndrome.

58. The method of any one of embodiments 1-57, wherein the engineered tRNA or variant thereof comprises a chemical modification comprising a methyl group, a fluoro group, a methoxyethyl group, an ethyl group, a phosphate group, an amide group, an ester group, or any combination thereof.
59. The method of any one of embodiments 1-58, wherein the subject was given a diagnosis with the disease or the condition prior to administration.
60. The method of embodiment 59, wherein the diagnosis was determined by an *in vitro* diagnostic test.
61. A composition comprising:
    a) an engineered tRNA or variant thereof of any one of embodiments 1-60; and a pharmaceutically acceptable excipient, diluent, or carrier.
62. A kit comprising the composition of embodiment 61 in a container.
63. A method of treating a disease or condition in a subject in need thereof, comprising: administering to the subject an engineered tRNA variant or a polynucleotide encoding the engineered tRNA variant, wherein the engineered tRNA variant comprises one or more mutations in a sequence of a reference tRNA provided in any one of SEQ ID NOS: 3-22, and wherein the engineered tRNA variant recognizes a premature stop codon in an mRNA encoding a polypeptide and at least partially transforms interpretation of the premature stop codon into a sense codon during translation of the mRNA to produce a substantially full-length polypeptide *in vivo*.
64. The method of embodiment 63, wherein the one or more mutations comprises a substitution of at least two nucleotides in an acceptor stem or an anticodon stem of the engineered tRNA variant that do not Watson-Crick base pair to each other with at least two nucleotides that do Watson-Crick base pair.

65. The method of embodiment 63 or 64, wherein the one or more mutations comprises a substitution of a thymine with a cytosine at nucleotide position 72 with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA.
66. The method of embodiment 63 or 64, wherein the engineered tRNA variant comprises a cytosine at nucleotide position 2 and a guanine at nucleotide position 71 with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA.
67. The method of embodiment 63 or 64, wherein the one or more mutations comprises a an adenine substituted with a guanine at nucleotide position 6 and a thymine substituted with a cytosine at nucleotide position 67, wherein the nucleotide position 6 and the nucleotide position 67 are with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA.
68. The method of embodiment 63 or 64, wherein the one or more mutations comprises a thymine substituted with a cytosine at nucleotide position 13 and an adenine substituted with a guanine at nucleotide position 22, where the nucleotide position 13 and the nucleotide position 22 are with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA.
69. The method of embodiment 63 or 64, wherein the one or more mutations comprises an adenine substituted with to a guanine at nucleotide position 15 with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA.
70. The method of embodiment 63 or 64, wherein the one or more mutations comprises a thymine substituted with a cytosine at nucleotide position 28 and an adenine substituted with a guanine at nucleotide position 42, where the nucleotide position 28 and the nucleotide position 42 are with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA.
71. The method of embodiment 63 or 64, wherein the one or more mutations comprises a cytosine substituted with an adenine at nucleotide position 31 and a guanine substituted with a thymine at nucleotide position 39, where the nucleotide position 31 and the nucleotide position 39 are with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA.
72. The method of embodiment 63 or 64, wherein the one or more mutations comprises an adenine substituted with a guanine at nucleotide position 37 with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA.

73. The method of embodiment 63 or 64, wherein the one or more mutations comprises a guanine substituted with an adenine at nucleotide position 44 with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA.
74. The method of embodiment 63 or 64, wherein the one or more mutations comprises a thymine substituted with a cytosine at nucleotide position 50 and an adenine substituted with a guanine at nucleotide position 64, where the nucleotide position 50 and the nucleotide position 64 are with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA.
75. The method of embodiment 63 or 64, wherein the one or more mutations comprises a cytosine substituted with a thymine at nucleotide position 6 and a guanine substituted with an adenine at nucleotide position 67, where the nucleotide position 6 and the nucleotide position 67 are with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA.
76. The method of embodiment 63 or 64, wherein the one or more mutations comprises a cytosine substituted with a guanine at nucleotide position 49 and a guanine substituted with a cytosine at nucleotide position 65, where the nucleotide position 49 and the nucleotide position 65 are with reference to SEQ ID NO: 6.
77. The method of embodiment 63 or 64, wherein the one or more mutations comprises a cytosine substituted with a thymine at nucleotide position 50 and a guanine substituted with an adenine at nucleotide position 64, where the nucleotide position 50 and the nucleotide position 64 are with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA.
78. The method of embodiment 63 or 64, wherein the one or more mutations comprises a thymine substituted with a cytosine at nucleotide position 71 with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA.
79. The method of embodiment 63 or 64, wherein the one or more mutations comprises a guanine substituted with a cytosine at nucleotide position 2 and a thymine substituted with a guanine at nucleotide position 71, where the nucleotide position 2 and the nucleotide position 72 are with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA.

80. The method of embodiment 63 or 64, wherein the one or more mutations comprises a thymine substituted with a cytosine at nucleotide position 4 and an adenine substituted with a guanine at nucleotide position 69, where the nucleotide position 4 and the nucleotide position 69 are with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA.

81. The method of embodiment 63 or 64, wherein the one or more mutations comprises a cytosine substituted with an adenine at nucleotide position 6 and a guanine substituted with a thymine at nucleotide position 67, where the nucleotide position 6 and the nucleotide position 67 are with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA.

82. The method of embodiment 63 or 64, wherein the one or more mutations comprises a guanine substituted with a cytosine at nucleotide position 12 and a cytosine substituted with a guanine at nucleotide position 23, where the nucleotide position 12 and the nucleotide position 23 are with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA.

83. The method of embodiment 63 or 64, wherein the one or more mutations comprises a thymine substituted with a cytosine at nucleotide position 27 and an adenine substituted with a guanine at nucleotide position 43, where the nucleotide position 27 and the nucleotide position 43 are with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA.

84. The method of embodiment 63 or 64, wherein the one or more mutations comprises a thymine substituted with a cytosine at nucleotide position 28 and an adenine substituted with a guanine at nucleotide position 42, where the nucleotide position 28 and the nucleotide position 42 are with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA.

85. The method of embodiment 63 or 64, wherein the one or more mutations comprises a thymine substituted with a cytosine at nucleotide position 40 with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA.

86. The method of embodiment 63 or 64, wherein the one or more mutations comprises an adenine substituted with a cytosine at nucleotide position 31 and a thymine substituted with a guanine at nucleotide position 39, where the nucleotide position 31 and the

nucleotide position 39 are with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA.

87. The method of embodiment 63 or 64, wherein the one or more mutations comprises an adenine substituted with a guanine at nucleotide position 44 with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA.

88. The method of embodiment 63 or 64, wherein the one or more mutations comprises a guanine substituted with an adenine at nucleotide position 46 with reference to SEQ ID NO: 6, or a 5' end of the reference tRNA.

89. The method of any one of embodiments 63-88, wherein the polypeptide is produced at an efficiency of at least about 10%, relative to a comparable polypeptide produced using a comparable mRNA that lacks the premature stop codon, as determined by:

a) transfecting a first vector encoding the engineered tRNA variant and a second vector encoding a screening mRNA encoding a first green fluorescent protein into a first human cell, wherein the screening mRNA encoding the first green fluorescent protein comprises the premature stop codon;

b) transfecting a third vector encoding a comparable screening mRNA encoding a second green fluorescent protein into a second human cell, wherein the comparable screening mRNA does not comprise the premature stop codon; and

c) comparing an amount of fluorescence emitted from the first human cell and the second human cell.

90. The method of any one of embodiments 63-89, wherein the polypeptide is produced at an efficiency of at least about 80%, relative to a comparable polypeptide produced using a comparable mRNA that lacks the premature stop codon, thereby at least partially treating the disease or condition in the subject.

91. The method of any one of embodiments 63-90, wherein the engineered tRNA variant is acylated with an amino acid selected from the group consisting of: lysine, arginine, histidine, glycine, alanine, valine, leucine, isoleucine, methionine, phenylalanine, tryptophan, proline, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartate, glutamate, pyrolysine, and selenocysteine.

92. The method of any one of embodiments 63-91, wherein the engineered tRNA variant is acylated with a non-canonical amino acid.

93. The method of any one of embodiments 63-92, wherein the engineered tRNA variant during translation of the mRNA inserts an amino acid into a nascent MeCP2 polypeptide chain encoded by the mRNA *in vivo* in response to the premature stop codon, wherein the amino acid when inserted is sufficient to produce an at least partially functional MeCP2 polypeptide, as compared to a comparable MeCP2 polypeptide produced using the comparable mRNA that lacks the premature stop codon.
94. The method of any one of embodiments 63-93, wherein the mRNA comprises at least two premature stop codons.
95. The method of embodiment 94, wherein the at least two premature stop codons are the same stop codon.
96. The method of embodiment 94, wherein the at least two premature stop codons are different stop codons.
97. The method of any one of embodiments 63-96, wherein the engineered tRNA variant is a lysyl-tRNA, an arginyl-tRNA, a histidyl-tRNA, a glycyl-tRNA, an alanyl-tRNA, a valyl-tRNA, a leucyl-tRNA, an isoleucyl-tRNA, methionyl-tRNA, a phenylalanyl-tRNA, a tryptophanyl-tRNA, a prolyl-tRNA, a seryl-tRNA, a threonyl-tRNA, a cysteinyl-tRNA, a tyrosyl-tRNA, an asparaginyl tRNA, a glutaminyl-tRNA, an aspartyl-tRNA, a pyrrolysyl tRNA, a selenocytstyl tRNA, or a glutamyl-tRNA.
98. The method of any one of embodiments 63-97, wherein the engineered tRNA variant is an engineered pre-tRNA.
99. The method of embodiment 98, wherein the engineered tRNA variant comprises an intronic sequence.
100. The method of embodiment 98, wherein the intronic sequence is spliced within a cell containing the engineered tRNA variant, thereby producing a mature engineered tRNA variant.
101. The method of any one of embodiments 63-100, wherein the subject is a human.
102. The method of embodiment 101, wherein the human is aged from about birth to about 40 years old.
103. The method of embodiment 101, wherein the human is aged about 6 months to about 15 years old.
104. The method of embodiment 101, wherein the human is an embryo or a fetus.

105. The method of any one of embodiments 63-104, wherein the disease or condition comprises Rett Syndrome.

106. The method of any one of embodiments 63-104, wherein the disease or condition comprises cystic fibrosis.

107. The method of any one of embodiments 63-104, wherein the disease or condition comprises retinitis pigmentosa.

108. The method of any one of embodiments 63-104, wherein the disease or condition comprises deafness.

109. The method of embodiment 108, wherein the deafness comprises autosomal dominant 17 deafness, autosomal dominant 13 deafness, or autosomal dominant 11 deafness.

110. The method of any one of embodiments 63-109, wherein the premature stop codon is an opal stop codon.

111. The method of any one of embodiments 63-110, wherein the administering is oral administering, rectal administering, or parenteral administering.

112. The method of embodiment 63-111, wherein the administering is the parenteral administering, and wherein the parenteral administering is an intravenous administering, an intra-arterial administering, an intrathecal administering, an intraocular administering, an otic administering, an intracerebroventricular administering, intracranial, intracranial into the parenchyma, or an intraperitoneal administering.

113. The method of any one of embodiments 63-112, wherein the polypeptide comprises an MeCP2 polypeptide.

114. The method of any one of embodiments 63-112, wherein the polypeptide comprises a FoxG1 polypeptide.

115. The method of any one of embodiments 63-112, wherein the polypeptide comprises a CDKL5 polypeptide.

116. The method of any one of embodiments 63-112, wherein the polypeptide comprises a MYH9 polypeptide.

117. The method of any one of embodiments 63-112, wherein the polypeptide comprises a COL11A2 polypeptide.

118. The method of any one of embodiments 63-112, wherein the polypeptide comprises a MYO7A polypeptide.

119. The method of any one of embodiments 63-112, wherein the administering is performed at least twice during a time period.

120. The method of embodiment 119, wherein the time period is about 24 hours.

121. The method of any one of embodiments 63-120, wherein the method is a method of preventing the disease or condition, and wherein the preventing comprises a prophylactic administering of the engineered tRNA variant or the polynucleotide encoding the engineered tRNA variant to the subject.

122. The method of any one of embodiments 63-121, wherein the polynucleotide encoding the engineered tRNA variant is administered to the subject.

123. The method of embodiment 63-122, wherein the vector is a viral vector.

124. The method of embodiment 123, wherein the viral vector is an adenoviral vector, an adeno-associated viral (AAV) vector, or a lentiviral vector.

125. The method of embodiment 124, wherein the AAV vector comprises a genome comprising a replication gene and inverted terminal repeats from a first AAV serotype and a capsid protein from a second AAV serotype.

126. The method of 124 or 125, wherein the AAV vector comprises an AAV 2/5 vector, an AAV 2/6 vector, an AAV 2/7 vector, an AAV2/8 vector, or an AAV 2/9 vector.

127. The method of any one of embodiments 124-126, wherein the AAV vector is an AAV 2/5 vector.

128. The method of embodiment any one of embodiments 63-127, wherein the engineered tRNA variant or the polynucleotide encoding the engineered tRNA is present in a delivery system.

129. The method of embodiment 128, wherein the delivery system comprises a liposome, a charged polymer, an uncharged polymer, a nanoparticle, a surfactant, a penetrating enhancer, a gene transfer agent, a phospholipid, a micelle, a synthetic vector, a macromolecule, a dendrimer, a biopolymer, a viral particle, or any combination thereof.

130. The method of any one of embodiments 63-129, further comprising administering a composition comprising a polynucleotide sequence comprising or encoding an RNA editing polynucleotide comprising: (i) a recruiting region and (ii) a targeting region,

wherein the polynucleotide sequence recruits an editing entity via at least a portion of the recruiting region, and wherein the editing entity when contacted with the RNA editing polynucleotide sequence and the mRNA performs a chemical modification on a base of a nucleotide of the premature stop codon of the mRNA, thereby converting the premature stop codon into a sense codon.

131. The method of embodiment 130, wherein the editing entity comprises:
    a) an ADAR polypeptide;
    b) an APOBEC polypeptide;
    c) a biologically active fragment of (a) or (b); or
    d) a fusion protein comprising the biological active fragment of (c), the ADAR polypeptide of (a), or the APOBEC polypeptide of (b).

132. The method of embodiment 63, wherein the disease or condition comprises Rett Syndrome, wherein the mRNA encodes an MeCP2 polypeptide, wherein the premature stop codon is an opal stop codon, wherein the engineered tRNA is an arginyl-tRNA, wherein the subject is a human aged 0-6 years old, and wherein when the engineered tRNA variant is administered to the subject as an AAV vector, the engineered tRNA variant during translation of the mRNA encoding the MeCP2 polypeptide at least partially transforms interpretation of the premature stop codon into an arginine sense codon and produces a substantially full-length MeCP2 polypeptide, thereby at least partially treating the Rett Syndrome.

133. The method of any one of embodiments 63-132, wherein the engineered tRNA variant comprises a chemical modification comprises a methyl group, a fluoro group, a methoxyethyl group, an ethyl group, a phosphate group, an amide group, an ester group, or any combination thereof.

134. The method of any one of embodiments 63-133, wherein the engineered tRNA variant or the polynucleotide encoding the engineered tRNA variant is comprised in a pharmaceutical unit dose form further comprising a pharmaceutically acceptable carrier excipient, a diluent, or a carrier.

135. The method of any one of embodiments 63-134, wherein the subject was given a diagnosis with the disease or the condition.

136. The method of embodiment 135, wherein the diagnosis was determined by an *in vitro* diagnostic test.

137. A composition comprising: an engineered tRNA variant comprising one or more mutations with reference to a sequence provided in any of SEQ ID NOS: 3-22.

138. The composition of embodiment 137, wherein the engineered tRNA variant has at least 70%, at least 80%, at least 85%, at least 90%, at least 92%, at least 95%, at least 97%, at least 99%, or 100% sequence identity to any one of SEQ ID NO: 23 - SEQ ID NO: 48.

139. A composition that comprises:

an engineered tRNA variant or a polynucleotide encoding the engineered tRNA variant, wherein the engineered tRNA variant comprises one or more mutations in a sequence of the engineered tRNA as compared with a reference tRNA comprising a sequence provided in any of SEQ ID NOS: 3-22, and wherein the engineered tRNA variant recognizes a premature stop codon in an mRNA encoding a polypeptide and at least partially transforms interpretation of the premature stop codon into a sense codon during translation of the mRNA to produce a substantially full-length polypeptide.

140. The composition of any one of embodiments 137-139, wherein the engineered tRNA variant comprises a sequence that is at least 70% identical to SEQ ID NO: 6, and has a substitution at position 2, 4, 6, 12, 23, 27, 28, 31, 39, 40, 42, 43, 44, 46, 49, 50, 64, 65, 67, 69, or 71, of SEQ ID NO: 6.

141. The composition of embodiment 140, wherein the substitution at position 2 is to a C, the substitution at position 4 is to a C, the substitution at position 6 is to a T, the substitution at position 6 is to an A, the substitution at position 12 is to a C, the substitution at position 23 is to a G, the substitution at position 27 is to a C, the substitution at position 28 is to a C, the substitution at position 31 is to a C, the substitution at position 39 is to a G, the substitution at position 40 is to a C, the substitution at position 42 is to a G, the substitution at position 43 is to a G, the substitution at position 44 is to a G, the substitution at position 46 is to an A, the substitution at position 49 is to a G, the substitution at position 50 is to a T, the substitution at position 64 is to an A, the substitution at position 65 is to a C, the substitution at position 67 is to an A, the substitution at position 67 is to a T, the

substitution at position 69 is to a G, the substitution at position 71 is to a C, or the substitution at position 71 is to a G.

142. The composition of embodiment 140 or 141, wherein the sequence of the engineered tRNA variant includes multiple substitutions.

143. The composition of any one of embodiments 140-142, wherein the sequence of the engineered tRNA variant is identical to SEQ ID NO: 6, except for the substitution(s).

144. The composition of any one of embodiments 140-143, wherein the engineered tRNA variant exhibits an increased stability *in vivo*, as compared with a comparable tRNA comprising the sequence provided in SEQ ID NO: 6, as determined by a proxy measurement, a half life measurement, an amino acid charging efficiency measurement, or a measurement of binding to a synthetase or ribosomal machinery.

145. The composition of any one of embodiments 137-139, wherein the engineered tRNA variant comprises a sequence that is at least 70% identical to SEQ ID NO: 3, and has a substitution at position 2, 6, 13, 15, 22, 28, 31, 37, 39, 42, 44, 50, 64, 67, 71, or 72, of SEQ ID NO: 3.

146. The composition of embodiment 145, wherein the substitution at position 2 is to a G, the substitution at position 6 is to a G, the substitution at position 13 is to a C, the substitution at position 15 is to a G, the substitution at position 22 is to a G, the substitution at position 28 is to a C, the substitution at position 31 is to a A, the substitution at position 37 is to a G, the substitution at position 39 is to a T, the substitution at position 42 is to a G, the substitution at position 44 is to an A, the substitution at position 50 is to a C, the substitution at position 64 is to a G, the substitution at position 67 is to a C, the substitution at position 71 is to a C, or the substitution at position 72 is to a C.

147. The composition of embodiment 145 or 146, wherein the sequence of the engineered tRNA variant includes multiple substitutions.

148. The composition of any one of embodiments 145-147, wherein the sequence of the engineered tRNA variant is identical to SEQ ID NO: 3, except for the substitution(s).

149. The composition of any one of embodiments 145-148, wherein the engineered tRNA variant exhibits an increased stability *in vivo*, as compared with a comparable tRNA comprising the sequence provided in SEQ ID NO: 3, as determined by a proxy

measurement, a half life measurement, an amino acid charging efficiency measurement, or a measurement of binding to a synthetase or ribosomal machinery.

150. The composition of any one of embodiments 137-139, wherein the engineered tRNA variant comprises a sequence that is at least 70% identical to SEQ ID NO: 5, and has a substitution at position 73 of SEQ ID NO: 5.

151. The composition of embodiment 150, wherein the substitution at position 73 is to a G.

152. The composition of embodiment 150 or 151, wherein the sequence of the engineered tRNA variant includes multiple substitutions.

153. The composition of any one of embodiments 150-152, wherein the sequence of the engineered tRNA variant is identical to SEQ ID NO: 5, except for the substitutions.

154. The composition of any one of embodiments 150-153, wherein the engineered tRNA variant exhibits an increased stability *in vivo*, as compared with a comparable tRNA comprising the sequence provided in SEQ ID NO: 5, as determined by a proxy measurement, a half-life measurement, an amino acid charging efficiency measurement, or a measurement of binding to a synthetase or ribosomal machinery.

155. The composition of embodiment 150, wherein the one or more mutations comprises a substitution at least two nucleotides in an acceptor stem or an anticodon stem of the engineered tRNA variant that do not Watson-Crick base pair to each other with at least two nucleotides that do Watson-Crick base pair.

156. The composition of any one of embodiments 150-153, wherein the engineered tRNA variant comprises a cytosine at nucleotide position 2 and a guanine at nucleotide position 71 with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA.

157. The composition of any one of embodiments 137-139, wherein the one or more mutations comprises a substitution of a thymine with a cytosine at nucleotide position 72 with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA.

158. The composition of any one of embodiments 137-139, wherein the one or more mutations comprises a cytosine substituted with a guanine at nucleotide position 22 and a guanine substituted with a cytosine at nucleotide position 71, wherein the nucleotide position 22 and nucleotide position 71 are with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA.

159. The composition of any one of embodiments 137-139, wherein the one or more mutations comprises a an adenine substituted with a guanine at nucleotide position 6 and a thymine substituted with a cytosine at nucleotide position 67, wherein the nucleotide position 6 and the nucleotide position 67 are with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA.

160. The composition of any one of embodiments 137-139, wherein the one or more mutations comprises a thymine substituted with a cytosine at nucleotide position 13 and an adenine substituted with a guanine at nucleotide position 22, where the nucleotide position 13 and the nucleotide position 22 are with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA.

161. The composition of any one of embodiments 137-139, wherein the one or more mutations comprises an adenine substituted with to a guanine at nucleotide position 15 with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA.

162. The composition of any one of embodiments 137-139, wherein the one or more mutations comprises a thymine substituted with a cytosine at nucleotide position 28 and an adenine substituted with a guanine at nucleotide position 42, where the nucleotide position 28 and the nucleotide position 42 are with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA.

163. The composition of any one of embodiments 137-139, wherein the one or more mutations comprises a cytosine substituted with an adenine at nucleotide position 31 and a guanine substituted with a thymine at nucleotide position 39, where the nucleotide position 31 and the nucleotide position 39 are with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA.

164. The composition of any one of embodiments 137-139, wherein the one or more mutations comprises an adenine substituted with a guanine at nucleotide position 37 with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA.

165. The composition of any one of embodiments 137-139, wherein the one or more mutations comprises a guanine substituted with an adenine at nucleotide position 44 with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA.

166. The composition of any one of embodiments 137-139, wherein the one or more mutations comprises an adenine substituted with a guanine at nucleotide position 73 with reference to SEQ ID NO: 3 or a 5' end of the reference tRNA.

167. The composition of any one of embodiments 137-139, wherein the one or more mutations comprises a cytosine substituted with a thymine at nucleotide position 50 and a guanine substituted with an adenine at nucleotide position 64, where the nucleotide position 50 and the nucleotide position 64 are with reference to SEQ ID NO: 4 or a 5' end of the reference tRNA.

168. The composition of any one of embodiments 137-139, wherein the one or more mutations comprises a cytosine substituted with a thymine at nucleotide position 6 and a guanine substituted with an adenine at nucleotide position 67, where the nucleotide position 6 and the nucleotide position 67 are with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA.

169. The composition of any one of embodiments 137-139, wherein the one or more mutations comprises a cytosine substituted with a guanine at nucleotide position 49 and a guanine substituted with a cytosine at nucleotide position 65, where the nucleotide position 49 and the nucleotide position 65 are with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA.

170. The composition of any one of embodiments 137-139, wherein the one or more mutations comprises a cytosine substituted with a thymine at nucleotide position 50 and a guanine substituted with an adenine at nucleotide position 64, where the nucleotide position 50 and the nucleotide position 64 are with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA.

171. The composition of any one of embodiments 137-139, wherein the one or more mutations comprises a thymine substituted with a guanine at nucleotide position 71 with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA.

172. The composition of any one of embodiments 137-139, wherein the one or more mutations comprises a thymine substituted with a cytosine at nucleotide position 4 and an adenine substituted with a guanine at nucleotide position 69, where the nucleotide position 4 and the nucleotide position 69 are with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA.

173. The composition of any one of embodiments 137-139, wherein the one or more mutations comprises a cytosine substituted with an adenine at nucleotide position 6 and a guanine substituted with a thymine at nucleotide position 67, where the nucleotide position 6 and the nucleotide position 67 are with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA.

174. The composition of any one of embodiments 137-139, wherein the one or more mutations comprises a guanine substituted with a cytosine at nucleotide position 12 and a cytosine substituted with a guanine at nucleotide position 23, where the nucleotide position 12 and the nucleotide position 23 are with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA.

175. The composition of any one of embodiments 137-139, wherein the one or more mutations comprises a thymine substituted with a cytosine at nucleotide position 27 and an adenine substituted with a guanine at nucleotide position 43, where the nucleotide position 27 and the nucleotide position 43 are with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA.

176. The composition of any one of embodiments 137-139, wherein the one or more mutations comprises a thymine substituted with a cytosine at nucleotide position 28 and an adenine substituted with a guanine at nucleotide position 42, where the nucleotide position 28 and the nucleotide position 42 are with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA.

177. The composition of any one of embodiments 137-139, wherein the one or more mutations comprises a thymine substituted with a cytosine at nucleotide position 40 with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA.

178. The composition of any one of embodiments 137-139, wherein the one or more mutations comprises an adenine substituted with a cytosine at nucleotide position 31 and a thymine substituted with a guanine at nucleotide position 39, where the nucleotide position 31 and the nucleotide position 39 are with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA.

179. The composition of any one of embodiments 137-139, wherein the one or more mutations comprises an adenine substituted with a guanine at nucleotide position 44 with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA.

180. The composition of any one of embodiments 137-139, wherein the one or more mutations comprises a guanine substituted with an adenine at nucleotide position 46 with reference to SEQ ID NO: 6 or a 5' end of the reference tRNA.

181. The composition of any one of embodiments 137-180, wherein the polypeptide is produced at an efficiency of at least about 10%, relative to a comparable polypeptide produced using a comparable mRNA that lacks the premature stop codon, as determined by:

a) transfecting a first vector encoding the engineered tRNA or variant thereof and a second vector encoding a screening mRNA encoding a first green fluorescent protein into a first human cell, wherein the screening mRNA encoding the first green fluorescent protein comprises the premature stop codon;
b) transfecting a third vector encoding a comparable screening mRNA encoding a second green fluorescent protein into a second human cell, wherein the comparable screening mRNA does not comprise the premature stop codon; and
c) comparing an amount of fluorescence emitted from the first human cell and the second human cell.

182. The composition of embodiment 181, wherein the polypeptide is produced at an efficiency of at least about 80%, relative to a comparable polypeptide produced using a comparable mRNA that lacks the premature stop codon, thereby at least partially treating the disease or condition in the subject.

183. The composition of any one of embodiments 137-182, wherein the engineered tRNA variant is acylated with an amino acid selected from the group consisting of: lysine, arginine, histidine, glycine, alanine, valine, leucine, isoleucine, methionine, phenylalanine, tryptophan, proline, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartate, glutamate, pyrolysine, and selenocysteine.

184. The composition of any one of embodiments 137-182, wherein the engineered tRNA variant is acylated with a non-canonical amino acid.

185. The composition of any one of embodiments 137-182, wherein the engineered tRNA variant during translation of the mRNA inserts an amino acid into a nascent MeCP2 polypeptide chain encoded by the mRNA *in vivo* in response to the premature stop codon, wherein the amino acid when inserted is sufficient to produce an at least

partially functional MeCP2 polypeptide, as compared to a comparable MeCP2 polypeptide produced using the comparable mRNA that lacks the premature stop codon.

186. The composition of any one of embodiments 137-185, wherein the mRNA comprises at least two premature stop codons.

187. The composition of embodiment 186, wherein the at least two premature stop codons are the same stop codon.

188. The composition of embodiment 186, wherein the at least two premature stop codons are different stop codons.

189. The composition of any one of embodiments 137-188, wherein the engineered tRNA variant is a lysyl-tRNA, an arginyl-tRNA, a histidyl-tRNA, a glycyl-tRNA, an alanyl-tRNA, a valyl-tRNA, a leucyl-tRNA, an isoleucyl-tRNA, methionyl-tRNA, a phenylalanyl-tRNA, a tryptophanyl-tRNA, a prolyl-tRNA, a seryl-tRNA, a threonyl-tRNA, a cysteinyl-tRNA, a tyrosyl-tRNA, an asparaginyl tRNA, a glutaminyl-tRNA, an aspartyl-tRNA, a pyrrolysyl tRNA, a selenocytstyl tRNA, or a glutamyl-tRNA.

190. The composition of any one of embodiments 137-189, wherein the engineered tRNA variant is an engineered pre-tRNA.

191. The composition of embodiment 190, wherein the engineered tRNA variant comprises an intronic sequence.

192. The composition of embodiment 191, wherein the intronic sequence is spliced within a cell containing the engineered tRNA variant, thereby producing a mature engineered tRNA variant.

193. The composition of any one of embodiments 137-192, wherein the subject is a human.

194. The composition of embodiment 193, wherein the human is aged from about birth to about 40 years old.

195. The composition of embodiment 193, wherein the human is aged about 6 months to about 15 years old.

196. The composition of embodiment 193, wherein the human is an embryo or a fetus.

197. The composition of any one of embodiments 137-196, wherein the disease or condition comprises Rett Syndrome.

198. The composition of any one of embodiments 137-196, wherein the disease or condition comprises cystic fibrosis.

199. The composition of any one of embodiments 137-196, wherein the disease or condition comprises retinitis pigmentosa.

200. The composition of any one of embodiments 137-196, wherein the disease or condition comprises deafness.

201. The composition of embodiment 200, wherein the deafness comprises autosomal dominant 17 deafness, autosomal dominant 13 deafness, or autosomal dominant 11 deafness.

202. The composition of any one of embodiments 137-201, wherein the premature stop codon is an opal stop codon.

203. The composition of any one of embodiments 137-202, wherein the administering is oral administering, rectal administering, or parenteral administering.

204. The composition of any one of embodiments 137-203, wherein the administering is the parenteral administering, and wherein the parenteral administering is an intravenous administering, an intra-arterial administering, an intrathecal administering, an intraocular administering, an otic administering, an intracerebroventricular administering, or an intraperitoneal administering.

205. The composition of any one of embodiments 137-204, wherein the polypeptide comprises an MeCP2 polypeptide.

206. The composition of any one of embodiments 137-204, wherein the polypeptide comprises a FoxG1 polypeptide.

207. The composition of any one of embodiments 137-204, wherein the polypeptide comprises a CDKL5 polypeptide.

208. The composition of any one of embodiments 137-204, wherein the polypeptide comprises a MYH9 polypeptide.

209. The composition of any one of embodiments 137-204, wherein the polypeptide comprises a COL11A2 polypeptide.

210. The composition of any one of embodiments 137-204, wherein the polypeptide comprises a MYO7A polypeptide.

211. The composition of any one of embodiments 137-204, wherein the administering is performed at least twice during a time period.

212. The composition of embodiment 211, wherein the time period is about 24 hours.

213. The composition of any one of embodiments 137-212, wherein the composition is a composition of preventing the disease or condition, and wherein the preventing comprises a prophylactic administering of the engineered tRNA variant or the polynucleotide encoding the engineered tRNA variant to the subject.

214. The composition of any one of embodiments 137-213, wherein the polynucleotide encoding the engineered tRNA variant is administered to the subject.

215. The composition of embodiment 214, wherein the polynucleotide is a viral vector.

216. The composition of embodiment 215, wherein the viral vector is an adenoviral vector, an adeno-associated viral (AAV) vector, or a lentiviral vector.

217. The method of embodiment 216, wherein the AAV vector comprises a genome comprising a replication gene and inverted terminal repeats from a first AAV serotype and a capsid protein from a second AAV serotype.

218. The method of 216 or 217, wherein the AAV vector comprises an AAV 2/5 vector, an AAV 2/6 vector, an AAV 2/7 vector, an AAV2/8 vector, or an AAV 2/9 vector.

219. The method of any one of embodiments 216-218, wherein the AAV vector is an AAV 2/5 vector.

220. The composition of embodiment any one of embodiments 137-219, wherein the engineered tRNA variant or the polynucleotide encoding the engineered tRNA is present in a delivery system.

221. The composition of embodiment 220, wherein the delivery system comprises a liposome, a charged polymer, an uncharged polymer, a nanoparticle, a surfactant, a penetrating enhancer, a gene transfer agent, a phospholipid, a micelle, a synthetic vector, a macromolecule, a dendrimer, a biopolymer, a viral particle, or any combination thereof.

222. The composition of any one of embodiments 137-221, further comprising administering a composition comprising a polynucleotide sequence, or a vector encoding the polynucleotide sequence, wherein the polynucleotide sequence comprises: (i) a recruiting region and (ii) a targeting region, wherein the polynucleotide sequence recruits an editing entity via at least a portion of the recruiting region, and wherein the editing entity when

contacted with the polynucleotide sequence and the mRNA performs a chemical modification on a base of a nucleotide of the premature stop codon of the mRNA, thereby converting the premature stop codon into a sense codon.

223. The composition of embodiment 222, wherein the editing entity comprises:

a) an ADAR polypeptide;

b) an APOBEC polypeptide;

c) a biologically active fragment of (a) or (b); or

d) a fusion protein comprising the biological active fragment of (c), the ADAR polypeptide of (a), or the APOBEC polypeptide of (b).

224. The composition of embodiment 223, wherein the disease or condition comprises Rett Syndrome, wherein the mRNA encodes an MeCP2 polypeptide, wherein the premature stop codon is an opal stop codon, wherein the engineered tRNA is an arginyl-tRNA, wherein the subject is a human aged 0-6 years old, and wherein when the engineered tRNA variant is administered to the subject as an AAV vector, the engineered tRNA variant during translation of the mRNA encoding the MeCP2 polypeptide at least partially transforms interpretation of the premature stop codon into an arginine sense codon and produces a substantially full-length MeCP2 polypeptide, thereby at least partially treating the Rett Syndrome.

225. The composition of any one of embodiments 137-224, wherein the engineered tRNA variant comprises a chemical modification comprises a methyl group, a fluoro group, a methoxyethyl group, an ethyl group, a phosphate group, an amide group, an ester group, or any combination thereof.

226. The composition of any one of embodiments 137-225, wherein the composition is isolated.

227. A pharmaceutical composition comprising the composition of embodiments 137-226 and a pharmaceutically acceptable excipient, a diluent, or a carrier.

228. An isolated cell comprising the composition of any one of embodiments 137-227.

229. An isolated plurality of cells comprising the composition of any one of embodiments 137-228.

230. A vector comprising the engineered tRNA variant, or the polynucleotide encoding the engineered tRNA variant, of the composition of any one of embodiments 137-229.

231. The vector of embodiment 230, further comprising, (e.g. in a 5' to 3' order):

a) a first promoter;

b) a first quantification cassette;

c) a second promoter;

d) a second quantification cassette;

e) a third promoter;

f) a transgene comprising the polynucleotide encoding the engineered tRNA variant;

g) a reporter gene encoding a detectable polypeptide; or

h) any combination thereof.

232. The vector of embodiment 231, wherein the polynucleotide further comprises a 5' ITR upstream of the promoter and a 3' ITR downstream of the reporter gene.

233. The vector of embodiment 231 or 232, wherein herein the detectable polypeptide comprises mCherry, green fluorescent protein (GFP), or β-galactosidase.

234. The vector of embodiment 231, wherein herein the first promoter and the second promoter are U6 promoters, wherein the U6 promoters are optionally methylated, or wherein the U6 promoters are optionally mouse U6 promoters.

235. The vector of embodiment 231, wherein herein the third promoter is a human cytomegalovirus (CMV) promoter.

236. The vector of any one of embodiments 231-235 that is purified or isolated.

237. A kit comprising:

a) the composition of any one of embodiments 137-226, the pharmaceutical composition of embodiment 227, the isolated cell(s) of embodiment 228 or 229, or the vector of any one of embodiments 230-236; and

b) a container configured to store the composition.

238. A method of generating the vector of any of embodiments 230-236, the method comprising: introducing the engineered tRNA variant, or the polynucleotide encoding the engineered tRNA variant, into a transgene of a backbone vector sequence.

239. A method of generating the pharmaceutical composition of embodiment 227, the method comprising: introducing the engineered tRNA variant, or the polynucleotide encoding the engineered tRNA variant, into the pharmaceutically acceptable excipient, a diluent, or a carrier.

VI. Examples

[269] The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

Example 1: Engineered Arginine tRNAs

[270] There are five naturally occurring human Arginine (Arg) tRNA isodecoders. In this example, each of the five Arg isodecoders were mutated in the anticodon sequence to recognize an opal stop codon and were cloned into a backbone vector (hereafter referred to as Arg-tRNA(UGA) isodecoders). These vectors, shown in **FIG. 1**, contained one copy (at left) or two copies (at right) of each Arg-tRNA(UGA) isodecoder. One copy was under the human U6 promoter and the other was under the mouse U6 promoter (mU6). However, both copies of the Arg-tRNA(UGA) isodecoders retained their endogenous promoters. Each construct also contained a CMV-promoter driving expression of an mCherry tag to enable assessments of transfection efficiency. Each vector further contained inverted terminal repeats (ITRs) regions which were used for quantification by qPCR using primers specific to the ITR region. **FIG. 1** (at right) shows the construct described herein with two copies of the genes encoding the engineered tRNA (UGA) isodecoder; and **FIG. 1** (at left) shows an example of a vector comprising only one copy of the gene encoding the engineered tRNA (UGA) isodecoder under human U6 promoter but not under the mouse U6 promoter. The vectors described herein were used to generate a library of engineered Arg tRNA molecules to screen for suppression efficiency.

[271] **FIG. 2A** illustrates a consensus secondary structure for human Arg tRNAs. **FIG. 2B** illustrates sites in a consensus secondary structure and sequence of human Arg tRNA that were excluded from mutagenesis to generate engineer tRNAs disclosed herein. The consensus secondary structure and sequence of human Arg tRNA. Crossed-out residues (with black lines) indicate highly conserved positions/nucleotides which were excluded while choosing putative sites for targeted mutagenesis to make engineered suppressor tRNAs, according to some embodiments.

Example 2. mRNA Editing Efficiencies of Engineered tRNA Molecules

Broken Luciferase screening in K562 cells

[272] K562 cells were engineered to stably express a luciferase gene broken by either R41X (middle lane in **FIG. 10A**) or R73X (bottom lane in **FIG. 10A**) premature opal stop

codons, where X represents the opal stop codon (**FIG. 10A**). **FIG. 39** shows an example of loss of luciferase activity due to some such mutations compared to wild type (WT luciferase as well as shown in percent of wild type activity. A schematic of the broken Luciferase transfection process can be provided in **FIG. 10B**. The amino acid position can be relative to full-length template mRNA encoding luciferase. These engineered cells were nucleofected (SF buffer, FF120 program, 1 μg DNA per well, 200K cells per well) with an Arg-tRNA(UGA) isodecoder, described above in duplicate for each of two biological replicates. Forty-eight hours post transfection, luciferase was measured using 50 μl of culture media by the Promega Nano-Glo® Luciferase Assay System (N1110) and mRNA was isolated from cellular lysates by the Qiagen RNeasy Plus kit (74136) according to manufacturer instructions. A schematic representation of a vector design used for the engineered tRNA and variants thereof carrying an mCherry is shown in **FIG. 14A**. The vector carried two copies of an engineered tRNA or variant thereof placed upstream of two U6 promoters. One was a human U6 (hU6) promoter and the other was a mouse U6 (mU6) promoter. The screenings were performed using transient transfections. Luciferase readings were obtained by Varioskan LUX with a measurement time of 1000 ms. mRNA expression of mCherry was obtained by quantitative PCR (Biorad CFX Connect) using TaqMan Fast Advanced Master mix (ThermoFisher 4444557) and a FAM conjugated TaqMan probe (Mr07319439_mr). mCherry mRNA expression was measured in duplicate technical replicates. One technical replicate contained the housekeeping gene Hprt and the other technical replicate contained the housekeeping gene Gapdh (VIC-conjugated TaqMan probes). These technical replicates were averaged and then used to normalize the luciferase reading from its respective well as a control for transfection efficiency. Each data point represents the mean with standard error of the mean (+/- SEM) of two biological replicates as shown in **FIG. 10C**.

Broken GFP screen in HEK293 cells.

[273] HEK293s were transfected with an Arg-tRNA(UGA) isodecoders (300 ng) (e.g., from a set of twenty Arg-tRNA(UGA) isodecoders) using the vector shown in FIG. 14A as described above as well as constructs containing a GFP gene (ST21 backbone) broken by either R74X or R97X premature stop codons (300 ng), where X represents the opal stop codon (**FIG. 11A**). The amino acid position can be relative to full-length template mRNA encoding GFP. The ST21 (e.g. SEQ ID NO: 51) vector map is provided in FIG. 11E. Transfection was achieved using the TransIT293 reagent (Mirus Bio MIR2705) according to manufacturer instructions in a 2:1 ratio

with DNA. 30K to 60K cells were plated per well and the number of cells per well was kept constant within each biological replicate. Forty-eight and seventy-two hours later, cells were stained with the viability dye, Zombie Violet, at a 1:2000 dilution for 15 minutes before analysis by flow cytometry. Cells were selected for (e.g., gated using Flow cytometry FACS machine) to remove debris, doublets, and dead cells (Zombie Violet positive). The rank order of each Arg-tRNA(UGA) isodecoders was identical between the forty-eight and seventy-two hour time points, however the intensity of GFP expression was higher at the forty-eight hour time point. As such, the depicted graphs in **FIGs. 11B-11D** all reflect the forty-eight hour data. Each data point represents the mean with standard error of the mean (+/- SEM) of three biological replicates, as shown in **FIGs. 11B-11D**. In **FIGs. 11B-11D**, live cells were further selected for (e.g., by Flow cytometry FACS gating) based on mCherry to select the population of live cells that had received the Arg tRNA suppressor molecules, and then by GFP to select the population of Arg tRNA suppressor molecule containing cells (tRNA+) that exhibited readthrough of the engineered broken GFP gene. **FIG. 11F** schematically shows a dual transfection system, where HEK293s were transfected with an Arg-tRNA(UGA) isodecoders (300 ng) (e.g., from a set of twenty Arg-tRNA(UGA) isodecoders) as well as constructs containing a GFP gene (ST21 backbone) broken by either R74X or R97X premature stop codons (300 ng), where X represent an opal stop codon; R74X or R97X are shown in **FIG. 11A**. The analysis of the results are shown in **FIG. 11B**. In **Fig. 11B**, the y-axis depicts the percentage of GFP positive (GFP+) cells, which can be indicating that readthrough can be occurring, of the cells that have received the Arg tRNA suppressor molecules (tRNA+ cells). In 14 of the 20 V1.0 isodecoders, 50% or more of cells that express the engineered tRNA have PTC suppression present. In **Fig. 11C,** the y-axis shows the total percent of PTCs that were suppressed by normalizing GFP mean fluorescence intensity (MFI) in cells transfected with the broken GFP and the engineered Arg tRNA suppressor molecules to GFP MFI in cells transfected with unbroken GFP (control cells). In 8 of the 20 V1.0 isodecoders, 70% or more the PTCs are suppressed in the cells exhibiting readthrough. **FIG. 11G** schematically shows the process of suppressing an opal stop codon in a broken GFP using an Arg tRNA suppressor carrying an mCherry tag compared to an unbroken GFP, as used to generate the results shown in **FIG. 11C**. In **Fig. 11D**, the y-axis shows the GFP MFI in cells transfected with the broken GFP and the engineered Arg tRNA suppressor molecules normalized to cells transfected with the engineered Arg tRNA suppressor molecules as measured by mCherry

expression. Using this proxy for tRNA potency, 5 of the 20 V1.0 isodecoders can take relatively less tRNA to suppress PTCs than they otherwise can. The fluorescent imaging data of four of the 20 engineered tRNAs (each comprising one of SEQ ID NO: 4, SEQ ID NO: 22, SEQ ID NO: 6, or SEQ ID NO: 7) shown in **FIG. 11D** are provided in **FIG. 12**. The sequence of the ST21 backbone plasmid that was used is included as SEQ ID NO: 51.

[274] **FIG. 12** shows flow cytometry results to identify low efficiency, medium efficiency, or high efficiency suppressor activity of the Arg-tRNA(UGA) isodecoders (e.g., engineered tRNAs comprising one of SEQ ID NO: 4, SEQ ID NO: 22, SEQ ID NO: 6, or SEQ ID NO: 7) based on GFP production in the cells. Live cells were selected for (e.g., by Flow cytometry FACS gating) based on by mCherry to select the population of live cells that had received the Arg tRNA suppressor molecules, and then by GFP to select the population of Arg tRNA suppressor molecule containing cells (tRNA+) that exhibited readthrough of the engineered broken GFP gene. The y-axis in **FIG. 12** shows mCherry expression, indicating cells transfected with the engineered Arg tRNA suppressor molecules and the x-axis shows GFP expression, indicating cells transfected with the broken GFP. High GFP expression indicates successful readthrough of a pre-mature stop codon in the broken GFP gene by an Arg tRNA suppressor molecule. In other words, higher activity of the suppressor tRNA (Arg-tRNA(UGA) isodecoders) can result in higher levels of GFP production from the broken GFP gene produced by relatively less tRNA molecules present. The proxy for potency can be observed by examining the curve of the graphs. An analysis of the fluorescent imaging results of the 20 engineered tRNA are provided in **FIG. 11D**, as described herein.

Example 3. Mutations in Arg Isodecoders Yield Better Suppression Efficiency

[275] To elucidate the role of various mutations in the or variants thereof (or Arg tRNA isodecoders) described herein, ten mutations were introduced in the engineered tRNA sequence of the engineered tRNA SEQ ID NO: 3 with an anticodon sequence of 'CCT' to generate engineered tRNA variants SEQ ID NOS: 23-32, which are pictorially illustrated in **FIG. 3A**. Mutations believed to stabilize the acceptor stem include, but are not limited to a G1T72 to G1C72, a C2G71 to G2C71, and/or a A6T67 to G6C67. Mutations believed to stabilize the D-stem region of the engineered tRNA include, but are not limited to, T13A22 to C13G22. Mutations believed to stabilize the anticodon stem include, but are not limited to, T28A42 to C28G42. Mutations believed to stabilize the T-stem include, but are not limited to, T50A64 to

C50G64. Mutations that match the consensus A-box promoter sequence of human tRNAs include A15 to G15. Mutations that match the consensus anticodon stem sequence of human Arg tRNAs include a C31G39 to A31T39. The consensus sequence can be generated upon alignment of all the human Arg tRNA isodecoders (e.g., shown in **FIG. 2**). 14 out of the 20 unique Arg tRNA sequences have an A31T39, thus rendering this base-pair part of the consensus sequence. However, CCT isodecoders have a C31G39, which was modified to match the consensus A31T39 in order to evaluate the suppression efficiency. Mutations that match the consensus human Arg sequence include G44A. Mutations including A37G was made that changed the +1 position immediately downstream of the anticodon. The anticodon region can comprise bases 34 to 36. The +1 position can be considered the position 37, accordingly the mutation A37G was made to evaluate the effect of changing the base right next to the anticodon region on suppression efficiency.

[276] Similarly, fourteen mutations were introduced in the engineered tRNA sequence of SEQ ID NO: 6 with an anticodon sequence of "TCT" to generate fourteen engineered tRNA variants that each included a sequence of SEQ ID NOS: 35-48, which are pictorially illustrated in **FIG. 3B**. The sequence of the parent tRNA SEQ ID NO: 6 and the fourteen variants thereof are provided in **Table 3** as SEQ ID NOS: 35-48. It can be believed that mutations that stabilize the acceptor stem include, but are not limited to, G2T71 to G2C71, G2T71 to C2G71, T4A69 to C4G69. Some exemplary mutations believed to stabilize the anticodon stem include T28A42 to C28G42, G30T40 to G30C40, T27A43 to C27G43, or A31T39 to C31G39 (e.g., in the engineered tRNA variants SEQ ID NOS: 37, 42, 48, 29, 31) as compared to the consensus Arg tRNA sequence. In SEQ ID NO: 36, C49 can be changed to a G49 to partially match the consensus Arg tRNA sequence and mutation in SEQ ID NO: 27 A15 changes to G15 to match consensus A-box sequence for all human tRNAs and not just the Arg tRNA sequence. The variant tRNA sequences are provided in **Table 3**. Mutations that match the consensus sequence in the engineered tRNA sequence included, but are not limited to, C49 to G49, C50G64 to T50A64, G12C23 to C12G23, or a G46A. Mutations made to SEQ ID NO: 6 to match SEQ ID NO: 3 include C6G67 to A6T67, A44G and T27A43 to C27G43. Mutations to match other TCT isodcoder sequences (e.g. SEQ ID NOS: 9-012) include C6G67 to T6A67. Variants containing the above-described mutations were made to evaluate the effect of these changes on suppression efficiency of the engineered tRNAs.

Example 4. Screening Engineered tRNA molecules for Suppression Efficiency

[277] Engineered suppressor tRNAs, as described herein were cloned into a plasmid vector comprising an mCherry tag, as described in **Example 1**. An exemplary workflow of the process can be shown in **FIG. 4**. Plasmids were transfected into HEK293 (or HEK cells), a cell line derived from human embryonic kidney cells. A broken green fluorescent protein (GFP) gene was stably integrated into the HEK cells chromosomal DNA comprising a premature stop codon by a nonsense mutation (e.g., at R74X or at R97X), using a PiggyBac (PB) transposon system. In the following examples, HEK cells were transfected using PB transposase to transposon ratio of 1:5 or 1:2.5. Each data point represents the mean with standard deviation (+/- SD) of one biological replicate.

[278] **FIG. 5A** shows a schematic workflow of screening suppressor tRNAs in HEK cells carrying a broken GFP gene as shown in **FIG. 4**. HEK cells (seeded at a concentration of 20,000 cells/well) were transfected at 48 hours using 300 nanogram (ng) of a plasmid encoding the engineered (suppressor) tRNA or a control plasmid without a suppressor tRNA. ST21 backbones with an engineered tRNA comprising the sequence of SEQ ID NO: 3 or 6 with an unbroken GFP plasmid were used as positive controls (or benchmarks). Inactive tRNA plasmids (e.g., comprising the sequence of SEQ ID NO: 8, or the sequence of SEQ ID NO: 18) as well as GFP-encoding plasmids (ST21) were used as negative controls. Flow gating was set based on single stain and untransfected cells. Read-through was assessed 48 hours post-transfection using flow cytometry to measure the fraction of tRNA-mCherry positive cells that were also positive for GFP. The measurements were performed in a population of live cells.

[279] **FIG. 5B** shows the percentage ratio of tRNA and GFP positive cells in R74X (black squares) and R97X (gray squares) for the engineered suppressor tRNA variants comprising engineered tRNA variants from the engineered tRNA SEQ ID NO: 3, or 6, as well as the positive and negative control plasmids. All variants showed retained suppressor activity. The engineered tRNA variant comprising the sequence of SEQ ID NO: 32 (502) showed a suppressor activity (stop codon readthrough) of about 70%. Variants with at least about 60% suppressor activity are shown using a gray square 501. 40,000 cells/ well and a transposase: transposon ratio of 1:5 were used for this study. Data shows one replicate for each GFP construct.

[280] A correlation in suppressor activity between R74X and R79X mutations, where X represents the opal stop codon, was investigated to find differences due to the position of the

mutation. **FIG. 5C** shows a correlation with R^2 value of about 0.88. The results show that the suppressor activity was substantially independent of the position of the arginine mutation. Additionally, some high performing engineered tRNA variants each included one of the sequences of SEQ ID NOS: 32, 39, 40, 45, 47, and 34. The engineered tRNA variants with at least about 60% suppressor activity (gray square 501 in **FIG. 5B**) are marked in **FIG. 5C** using an oval shape 511.

[281] Similar experiments were repeated in three stable cells lines including a 1:5 PB transposase to transposon ratio in cells with stably integrate broken GFP with a premature stop codon comprising a R74X and /or R97X, and a 1:2.5 PB transposase to transposon ratio in cells to integrate GFP with a premature stop codon comprising a R74X. A ratio of the percentage readthrough of GFP and mCherry positive cells, denoting cells that carry the engineered suppressor tRNA variant, was compared to (or normalized by) a positive control (e.g., comprising the sequence of SEQ ID NO: 3). Data points represent mean+/- standard deviation from the mean (SD) across 3 different cell lines - R74X(1:2.5), R74X(1:5) and the R97X(1:5). Results are shown in **FIG. 6**. The mean values are shown in dark squares with error bars representing SD values. All variants showed retained suppressor activity in all three cell lines.

Example 5. Dual-Plasmid Screening of Engineered tRNA Molecules

[282] DNA sequences encoding the engineered tRNA were incorporated into a vector comprising mCherry tag, as described herein. A second plasmid comprising a broken GFP tag was also provided. HEK cells were co-transfected with the engineered tRNA plasmid and the GFP plasmid. Cells with stable integration of plasmids were selected for integration of the broken GFP constructs using puromycin treatment (5 μg/mL) for 10-12 days. Cells with stable integration of the plasmid carrying an engineered tRNA expressed the engineered tRNA and mCherry. Cells with stable integration of both plasmids expressed GFP and mCherry. **FIG. 7** shows a schematic of the process of co-transfecting cells with two plasmids and selecting the cells with stable integration of both plasmids. Flow cytometry was performed at 48 hours post transfection.

Example 6. Measuring Suppression Efficiency using Dual-Plasmid System

[283] 40,000 HEK cells were inoculated in a well. 24 hours post-inoculation cells were transfected with 300 nanograms (ng) of each of the plasmids, described in example 5. Cells were

subjected to flow cytometry at 48 hours post transfection. Geometric mean fluorescent intensity of GFP and mCherry in cells with stable integration of both plasmids were measured. **FIG. 8A** shows the exemplary results of premature stop codon suppression by engineered Arg tRNA suppressor molecules, measured in terms of percentage readthrough (fraction of tRNA/mCherry producing cells that show green fluorescence), in in two different HEK cells lines expressing engineered GFP containing carrying two different Arg-to-stop codon mutations (R74X and R97X, where X represents the opal stop codon) using the dual plasmid screening system shown in **FIG.** 7. Very high correlation with R^2 of about 0.99 was established (**FIG. 8B**) between cells with different arginine mutations in positions 74 and 97 (R74X and R97X). The high correlation shows that the engineered tRNA suppressor activity was substantially retained at different arginine locations. These results demonstrated that several of the engineered tRNAs or engineered tRNA variants, shown using a gray square 801 (e.g., including the sequence of SEQ ID NO: 34, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 30, SEQ ID NO: 32, or SEQ ID NO: 5) upon transfection into cells, yielded high percentages of cells (more than 90% of tRNA+ cells) with PTC readthrough.

[284] The results from the same experiment, described herein, were normalized based on mCherry expression levels to evaluate an efficiency of the premature stop codon suppression. Accordingly, the suppression efficiency can be evaluated by normalizing the mean fluorescence intensity of the GFP signal (produced by readthrough of the stop mutation in the engineered GFP) to the mean fluorescence intensity of the mCherry signal (that correlates with tRNA expression levels). **FIG. 9A** shows the efficiency of premature stop codon suppression in HEK cell lines carrying two different Arg-to-stop mutations (R74X and R97X, where X represents the opal stop codon) by the dual plasmid screening system. High correlation with R^2 of about 0.89 was established (**FIG. 9B**) between the two different engineered GFP reporters containing the Arg-to-stop mutations: R74X and R97X. The high correlation shows that the engineered tRNA suppressor efficiency was substantially retained at different arginine locations. Overall, as described in the examples disclosed herein, results of suppressor tRNA activities in HEK cells disclosed herein show a set of mutations other than anticodon mutations that can preserve and/or enhance suppressor tRNA activity in arginine isodecoders. A plurality of engineered tRNAs or variants thereof each comprising a sequence of SEQ ID NO: 3, 32, 34, 39, 45, and 47, showed high arginine suppressor activity (or stop codon readthrough) across both stable and transient

expression of broken GFP reporter systems. Results of a tRNA with the sequence of SEQ ID NO: 32 shown in **FIG. 5B** (502) **and FIG. 6** show the mutation (T50A64 —> C50G64) present in SEQ ID NO: 32 can further stabilize the SEQ ID NO: 3 tRNA and can lead to improved suppression activity (e.g., efficiency). In another example, in the naturally occurring isodecoder SEQ ID NO: 6 at the same location a G-C base pair change to an A-T base pair (e.g., in an engineered tRNA variant comprising the sequence of SEQ ID NO: 37) can decrease tRNA's arginine suppressor activity (e.g., arginine suppression efficiency). In some examples, the A73G mutation included in SEQ ID NO: 33 or SEQ ID NO: 34 improves suppressor efficiency. It can also be shown in the results (**FIGs. 5B,6, 8A** and **9A**) that changing a G-T non Watson-Crick pair to a G-C Watson crick pair in the acceptor stem or to a C- G Watson crick pair in the anticodon stem as made in SEQ ID NO: 39 or SEQ ID NO: 45 can stabilize the acceptor/anticodon stems; the C-G bond can be shown to be strongly preferred at 2-71 position compared to G-C.

Example 7. Rett Syndrome model

[285] Selected engineered tRNAs were tested in primary neural cultures harvested from R255X Rett model mice. **FIG. 14B** shows a cartoon depiction of the vector design used in the primary neural cultures. The vector comprised a copy of the selected engineered tRNA or variant thereof placed upstream of a human U6 (hU6) promoter. The vector was packaged into a lentivirus (pSMPUW).

[286] The selected engineered tRNAs included eight of the engineered tRNAs each comprising one of the sequences selected from SEQ ID NOS: 6, 9, 10, 11, 20, 3, 7, and 13, that produced high luciferase signal in the broken luciferase screening experiment as well as high GFP signals in the broken GFP screening experiment. Two of the engineered tRNAs, each individually comprising the sequence of SEQ ID NOS: 19 or 8, that produced the lowest signals in the same experiments were used as negative controls.

[287] In order to harvest primary neural cultures harvested from R255X Rett model mice postnatal day 0-2 mouse pups were taken from R255X heterozygous females (the Jackson Laboratory, stock #012602) crossed to wild type male mice of the same mouse strain. Pups were euthanized to collect brain and skin samples. The skin samples were used for genotyping. Brains were isolated and the cerebellum was removed. The rest of the brain was digested in papain, washed with Lo Ovomucoid and DNase, dissociated, and filtered through a 70-micron filter. Cell cultures were infected the next day with lentiviruses expressing the selected engineered tRNAs,

as described herein. Cells were harvested one week after dissection (6 days post-transfection). Protein purification process was performed on the cells using mechanical dissociation in RIPA buffer containing a protease inhibitor (ThermoFisher 78444). MeCP2 protein levels were measured by immunoblotting using an antibody to MeCP2's C-terminus (Thermofisher 49-1049). Detection of MeCP2 protein was used to examine engineered tRNA readthrough of the R255X. **FIG. 15A** illustrates a representative western blot example showing a lack of readthrough by the engineered tRNA comprising the sequence of SEQ ID NO: 19 (negative control) and showing successful readthrough at varying levels by the engineered tRNAs respectively comprising the sequences of SEQ ID NOS: 20, 3, and 7. **FIG. 15B** shows a histogram of the MeCP2 ECl signal normalized by the Gapdh signal in each sample, and then to wild type controls. Each column represents an engineered tRNA readthrough and each data point (dark circles) within each column represents data collected from a different mouse. Data represents mean +/- SEM (error bars).

[288] In another study, primary neural cultures as described herein were treated with four engineered tRNAs and variants thereof (e.g. SEQ ID NOS: 8, 39, 45, and 7). Flow cytometry results are shown in **FIGs. 16A-16F**. Neuronal cultures were infected 5 days after isolation and analyzed by flow cytometry one week after isolation (48 hours after infection). The viral vector design can be shown in **FIG. 14B** and was delivered using a lentivirus. Single live cells were selected. Then further selection (flow cytometry gating) were made to gate for neurons (NeuN+ cells) which also had an mCherry signal (mCherry+) indicating presence of an engineered tRNA (tRNA+ cells) and finally the MeCP2 antibody signal. The selected populations are shown using a trapezoid for different treatment groups in **FIGs. 16A-16F**. Untreated R255X cells (**FIG. 16A**) did not have an mCherry+ cells as they were not transduced by the mCherry-expressing lentivirus. R255X cells treated with the engineered tRNA SEQ ID NO: 8 showed a negligible MeCP2+ population (**FIG. 16B**). R255X cells treated with the engineered tRNA variant (e.g. SEQ ID NO: 39) (**FIG. 16C**), the engineered tRNA variant SEQ ID NO: 45 (**FIG. 16D**), or the engineered tRNA SEQ ID NO: 7 (**FIG. 16E**) show a respective increase in the MeCP2 positive population. In fact, the MeCP2+ population distribution in cells treated with the engineered tRNA SEQ ID NO: 7 was comparable to a wild type (WT) population infected with the engineered tRNA SEQ ID NO: 8 which was used as a negative control (**FIG. 16F**). The WT control group was used to confirm the gating strategy in the flow cytometry process.

[289] The flow cytometry used herein to measure MeCP2 expression in neurons was further validated using cell cultures from R255X heterozygous females (the Jackson Laboratory, stock #012602) crossed to wild type male mice of the same mouse strain. Cultures were generated as described herein. **FIGs. 17A-17D** illustrate MeCP2 signal results using antibody D4F3. The Cell Signaling MeCP2 antibody D4F3 demonstrates strong expression in wild type (WT) neural cultures (**FIG. 17A**), approximately half of wild type expression in R255X heterozygous females (**FIG. 17B**), and no appreciable expression in R255X males (**FIG. 17C**), or unstained samples (**FIG. 17D**). **FIGs. 17E-17H** illustrate MeCP2 signal results using antibody Sigma NeuN antibody MAB377. The Sigma NeuN antibody MAB377 did not produce a signal in unstained control LUHMES (LUH) cells (**FIG. 17E**) or unstained primary cultures (**FIG. 17H**). However, it stained almost all differentiated LUHMES cells (**FIG. 17F**), which acted as a positive control given the complete differentiation that this cell line undergoes after treatment with doxycycline to stop a v-myc transgene. In primary neural cultures (**FIG. 17G**), this NeuN antibody stained about 60% of all cells, corresponding to a predicted ratio of neurons **1701** to other neural cell types **1702**, such as astrocytes and oligodendrocytes.

[290] In another experiment, primary neural cultures were isolated from R255X Rett model mice as described herein. 5 days after isolation, the neural cultures were infected with a lentivirus carrying a construct (**FIG. 14B**) comprising one of 9 selected engineered tRNAs or engineered tRNAs variant sequences: SEQ ID NOS: 39, 28, 34, 40, 7, 45, 6, 32 or 3. The cell cultures were analyzed 2 days post-infection as described in **FIGs. 17A-17H**. A histogram depicting the percentage of neurons (NeuN+) transduced by the lentivirus (mCherry+) that expressed MeCP2 can be illustrated in **FIG. 18A**. Almost 60% of the neurons transduced by a lentivirus carrying the engineered tRNA SEQ ID NO: 3 showed MeCP2 signal. This level of MeCP2 expression can be comparable to the wild type cells, in which MeCP2 was expressed in approximately 70% of transduced neurons. **FIG. 18B** shows a total percent of PTCs that were suppressed in cells 2 days after transduction. The mean fluorescence intensity (MFI) of the MeCP2 in cells transduced with a lentivirus carrying a construct as described herein was normalized to the MeCP2 MFI in neural cultures isolated from wild type mice. The engineered tRNA comprising the sequence of SEQ ID NO: 6 could suppress about 65% of the PTCs (65% MeCP2 production in cells).

Example 8. Vector design

[291] Seven different vectors were designed to examine if (I) an exogenous promoter enhances tRNA production and thus efficacy (II) copy number enhances tRNA production and thus efficacy. **FIGs. 19A-19G** shows the seven different constructs carrying one, two or three copies of the engineered tRNA SEQ ID NO: 3. The constructs comprised a mU6, a hU6, or a combination thereof. HEK293s were transfected with an engineered tRNA comprising the sequence of SEQ ID NO: 3 (300 ng)) in 1 to 6 copies (**FIG. 19A – FIG. 19G** show example schematics of constructs with 1 to 3 copies of the engineered tRNA suppressors), with and without an upstream hU6. The constructs also comprised a GFP gene (ST21 backbone) broken by either R74X or R97X premature stop codons (300 ng), where X represents the premature stop codon, similar to the genes shown in **FIG. 11A**. Transfection was achieved using the TransIT293 reagent (Mirus Bio MIR2705) according to manufacturer instructions in a 3:1 ratio with DNA. 50K cells were plated per well. Two biological replicates were performed. Using flow cytometry, cells were gated to remove debris, doublets, and dead cells (Zombie Violet positive). Then, live cells were further gated by mCherry to select the population of live cells that had received the engineered tRNA, and then by GFP to select the population of the engineered tRNA-containing cells (tRNA+) that exhibited readthrough of the engineered broken GFP gene. **FIG. 20A** shows the percent of tRNA+ cells that also express GFP, indicating that readthrough can be occurring. The results showed that the upstream hU6 promoter did not provide an appreciable benefit over the tRNA internal promoters. **FIG. 20B** shows the total percent of PTCs that were suppressed by normalizing GFP mean fluorescence intensity (MFI) in cells transfected with the broken GFP and the engineered tRNA to a GFP MFI in cells transfected with unbroken GFP (control cells). As shown in **FIG. 20B** higher tRNA copy numbers resulted in more PTC suppression.

Example 9. Treating Rett Syndrome

[292] An Arg-tRNA isodecoder identified using the GFP screen can be cloned into an AAV vector. The AAV vector can be transfected into a mammalian cell line containing an AAV helper vector to produce AAV viral particles containing a transgene coding for the Arg-tRNA isodecoder. Examples of AAV vectors include but are not limited to an AAV 2/5 vector, an AAV 2/6 vector, an AAV 2/7 vector, an AAV2/8 vector, or an AAV 2/9 vector, or any AAV vector described herein.

[293] Varying doses of the AAV are administered in subjects suffering Rett Syndrome due to an R168X, R225X, R270X, R294X, R198X, R186X and R453X mutation in an mRNA encoding an MeCP2 polypeptide, where X represents a UGA opal stop codon. Subjects are monitored for improvements in the pathology of Rett Syndrome over a treatment course. Improvements in the pathology are due to suppression of the premature stop codon by the Arg-tRNA isodecoder, resulting in readthrough of the stop codon and insertion of an arginine into the MeCP2 polypeptide in response to the premature stop codon.

Example 10. Treating Deafness

[294] The AAV vector prepared as described above can be used to treat subjects suffering from deafness due to an R1933X mutation in an mRNA encoding a MYH9 polypeptide, an R845X mutation in an mRNA encoding a COL11A2 polypeptide, or an R666X mutation in an mRNA encoding a MYO7A polypeptide, where X represents a UGA opal stop codon. Subjects are monitored for improvements in the pathology of deafness over a treatment course using audiometry testing. Improvements in the pathology are due to suppression of the premature stop codon by the Arg-tRNA isodecoder, resulting in readthrough of the stop codon and insertion of an arginine into the MYH9, COL11A2, or MYO7A polypeptides in response to the premature stop codon.

Example 11. Self-complementary vector designs

[295] Data shown in **FIG. 24 to FIG. 28B** were collected as follows: Broken GFP constructs comprising a R74X (STX488) or R97X premature stop codon (STX489) were stably integrated into K562 cells using the piggybac system at 1:5 and 1:2.5 transposase to transposon ratios two generate two independent piggybac cell lines per broken Arginine. Cells containing the piggybac payload were selected using 5ug/mL puromycin for at least 8 days before use. Constructs comprising varying copy numbers of an engineered tRNA comprising the sequence of SEQ ID NO: 3, were cloned into single strand or self-complementary backbones. These constructs were packaged into AAV2/9. **Table 5** describes the constructs. K562 cells (50K per well in a 96 well format) with the piggybac integrations of the broken GFP constructs were infected with 1 million vg/cell of virus. Cells were analyzed by flow cytometry 48 hours later. Each data point is a biological replicate (n=2-3). Two of the replicates are from the 1:5 integration and the third is from the 1:2.5 integration. Data represent mean +/- SEM.

Table 5. Constructs used in FIGs. 24-28B

Plasmid Name	Vector Backbone Name:	Insert DNA Name:	Promoter info (for qPCR):	PolyA info (for qPCR):	Size of transgene cassette from ITR to ITR:
pssAAV-wtITR2-0xCCT2-CMVmChSV40-lambda-strw2-wtITR2	pUC57	STX588	CMV	SV40	4309
pssAAV-wtITR2-1xCCT2-CMVmChSV40-lambda-strw2-wtITR2	pUC57	STX626	CMV	SV40	4435
pssAAV-wtITR2-3xCCT2-CMVmChSV40-lambda-strw2-wtITR2	pUC57	STX627	CMV	SV40	4593
pssAAV-wtITR2-6xCCT2-CMVmChSV40-lambda-strw2-wtITR2	pUC57	STX593	CMV	SV40	4824
pscAAV-scITR-0xCCT2-CMVmChSV40-lambda-strw2-wtITR2	pUC57	STX586	CMV	SV40	1922/3747

pscAAV-scITR-1xCCT2-CMVmChSV40-lambda-strw2-wtITR2	pUC57	STX587	CMV	SV40	2004/3911
pscAAV-scITR-3xCCT2-CMVmChSV40-lambda-strw2-wtITR2	pUC57	STX589	CMV	SV40	2162/4227
pscAAV-scITR-6xCCT2-CMVmChSV40-lambda-strw2-wtITR2	pUC57	STX590	CMV	SV40	2393/4689

[296] **FIG. 24** shows a cartoon illustrating the gating strategy used for **FIG. 25** to **FIG. 28B**. The representative sample displayed is a self-complementary stuffer sequence (sc 0x). Cells were gated to remove debris, doublets, and dead cells (Zombie Violet positive). Live cells were further gated by mCherry to select the population of live cells that had received the Arg tRNA suppressor molecules, and then by GFP to select the population of Arg tRNA suppressor molecule containing cells (tRNA+) that exhibited readthrough of the engineered broken GFP gene.

[297] **FIG. 25** shows representative flow plots for self-complementary AAV2/9 packaging 0, 1, 3, or 6 copies of an engineered tRNA comprising the sequence of SEQ ID NO: 3. The y-axis depicts the percent of tRNA+ cells that also express GFP (indicating that readthrough is occurring). The x-axis depicts side scatter. There was no readthrough of the broken GFP construct (R97X is shown here) upon transduction of cells with AAV2/9 expressing 0x (stuffer sequence only) or 1x copies of the engineered tRNA comprising the sequence of SEQ ID NO: 3. In contrast, there was approximately 60-70% readthrough upon transduction with AAV2/9 expressing 3x or 6x copies of the engineered tRNA comprising the sequence of SEQ ID NO: 3.

[298] **FIG. 26** shows transduction of K562 cells with the piggybac integrations of the broken GFP constructs with 1 million vg/cell of virus did not alter cell viability. Here, cells were gated to remove debris, doublets, and dead cells (Zombie Violet positive). Viability was approximately 90-100% of cells in noninfected and infected cells.

[299] **FIG. 27A** and **FIG. 27B** show transduction efficiencies of single strand and self-complementary AAVs with varying copy numbers of tRNA. As shown in **FIG. 27A**, the percentage of live cells that expressed mCherry, indicated that the cells have been transduced by the specified AAV. **FIG. 27B** shows mean fluorescent indexes of mCherry in cells that were transduced (mCherry+) and exhibited PTC suppression (GFP+). Despite normalization of viral load in each of these samples (1 million vg/cell), the 1x AAV transduced more cells and at a higher level, than the 3x or 6x viruses. The single strand backbone provided almost no transduction efficiency.

[300] **FIG. 28A** and **FIG. 28B** show plots of readthrough of the premature stop codons with each group tested. In the plot of **FIG. 28A**, the y-axis represents that percentage of cells that received the specified tRNA that exhibited PTC suppression. The 3x and 6x versions of the engineered tRNA comprising the sequence of SEQ ID NO: 3 generated readthrough in 50 to 85% of transduced cells expressing R97X and readthrough in 90 to 100% of transduced cells expressing R74X. In the plot of **FIG. 28B**, the y-axis shows the total percent of PTCs that were suppressed by normalizing GFP mean fluorescence intensity (MFI) in cells transduced with the engineered Arg tRNA suppressor molecules to GFP MFI in cells transfected with unbroken GFP (control cells). The self-complementary version of 6x the engineered tRNA comprising the sequence of SEQ ID NO: 3 demonstrated superior readthrough to the single stranded version. However, the self-complementary version of 3x the engineered tRNA comprising the sequence of SEQ ID NO: 3 performed better than the self-complementary version of 6x the engineered tRNA comprising the sequence of SEQ ID NO: 3. Each data point is a biological replicate (n=2-3). Data represent mean +/- SEM.

[301] AAV2/9 vectors were designed to carry 6 (**FIG. 21A**), 3 (**FIG. 21B**), or 1 (**FIG. 21C**) copies of the engineered tRNA comprising the sequence of SEQ ID NO: 3. The AAV vectors were made from single strand backbone (FIG. 22A) or self-complementary backbone (FIG. 22B). Single strand backbones included a large amount of stuffer DNA 2201, 2202, or 2203 to fill the AAV vector. Constructs included a CMV-promoter driving expression of an mCherry tag. To

study the efficiency of self-complementary backbone design AAV9 vectors expressing 0, 3, or 6 copies of the engineered tRNA comprising the sequence of SEQ ID NO: 3 in a self-complementary backbone will be injected unilaterally into the lateral ventricles (ICV) of male R255X mice 0-2 or 5-8 days old pups (See **Table 6** for sample sizes) at a titer of 3*10^10 vg/brain. Control mice include non-injected R255X mice including 2 mice (n=2) and injected WT mice (0x construct) with 4 mice (n=4). Brains will be microdissected into 3 regions (hippocampus, cortex, and rest of the brain) and processed for flow cytometry to assess expression of full-length MeCP2 in transduced cells (mCherry+).

Table 6. tRNA copy number study design

Genotype	Sex	Injection	AAV	Age	Sample size
R255X	M	Unilateral	scAAV9-0x	P0-2 P5-8	2 2
			scAAV9- CCT2-3x	P0-2 P5-8	3 3
			scAAV9- CCT2 -6x	P5-8	2
			No injection	P5-8	2
WT	M	Unilateral	scAAV9-0x	P0-2 P5-8	2 2

Example 12. Vector designs with spacer sequences

[302] The effect of placing an engineered tRNA or a variant thereof in between inverted terminal repeats (ITRs) using different length spacers in a vector construct on readthrough efficiency of the engineered tRNA was tested. Examples of AAV backbones with engineered tRNA or variants thereof placed at different distances from the ITRs are schematically shown in **FIG. 23A**. The vector comprised an AAV vector with three copies of the engineered tRNA comprising the sequence of SEQ ID NO: 3. The three copies of the engineered tRNA or variants thereof were separated by 6, 50, 100, 200, or 500 nucleotide spacer sequences. The spacer sequence can comprise a 6 nucleotide linker sequence of AACAAA or a termination sequence of TTTTTT. The spacer sequence can include a stuffer sequence disclosed herein. The linker sequence is based on a bi-cistronic tRNA-sncRNA found in rice. An example of the study design, described herein, is provided in **Table 6**.

[303] **FIG. 29A** and **29B** show the results of plasmid transfection in K562 cells with vector designs comprising varying spacer lengths. Broken GFP constructs comprising a R74X (STX488) and or R97X premature stop codon (STX489) were stably integrated into K562 cells using the piggybac system at 1:5 and 1:2.5 transposase to transposon ratios two generate two independent piggybac cell lines per broken Arginine. Cells containing the piggybac payload were selected using 5ug/mL puromycin for at least 8 days before use. 2x constructs comprising 2 copies of the SEQ ID NO: 3 engineered tRNA and a 6 base pair spacer, or 3x constructs comprising 3 copies of the SEQ ID NO: 3 engineered tRNA and 50 (STX617), 100 (STX618), 200 (STX619), or 500 (STX620) base pair spacers of varying spacer lengths were cloned into self-complementary backbones. These constructs were nucleofected into the aforementioned K562 cells (SF buffer, FF120 program, 1 μg DNA per well, 200K cells per well). mCherry was not included in this backbone due to size constraints with the 500 bp spacer construct. Cells were analyzed by flow cytometry 72 hours later. Cells were gated to remove debris, doublets, and dead cells (Zombie Violet positive). Live cells were further gated by GFP to select the population of cells that exhibited readthrough of the engineered broken GFP gene. Two of the replicates are from the 1:5 integration and the third is from the 1:2.5 integration. Each data point is a biological replicate (n=3). Data represent mean +/- SEM.

[304] In **FIG. 29A**, the y-axis represents that percentage of cells that exhibited PTC suppression. The 100 and 200 bp spacer versions of the engineered tRNA comprising the sequence of SEQ ID NO: 3 generated readthrough in 60 to 95% of transduced cells expressing either broken Arginine residue. In contrast, the SEQ ID NO: 3 constructs with 50 bp and 500 bp spacers exhibited reduced efficiency for the R97X line. In **FIG. 29B**, the y-axis shows the total percent of PTCs that were suppressed by normalizing GFP mean fluorescence intensity (MFI) in cells transfected with the engineered Arg tRNA suppressor molecules to GFP MFI in cells transfected with unbroken GFP (control cells). Again, the SEQ ID NO: 3 constructs with 100 or 200 bp spacers exhibited the highest GFP intensity.

[305] Additionally, the effect of the orientation of the engineered tRNA comprising the sequence of SEQ ID NO: 3 in the AAV construct on readthrough efficiency of the engineered tRNA was tested. Examples of engineered tRNA or variants thereof placed in an AVAV backbone in different directions are shown in **FIG. 23B-23C**. **FIG. 30A** and **30B** include data for constructs with engineered tRNAs at various positions or orientations.

[306] 1x SEQ ID NO: 3 constructs with different locations relative to the ITRs were cloned into self-complementary backbones (**Table 7**). These constructs were packaged into AAV2/9. Broken GFP constructs comprising a R74X (STX488) or R97X premature stop codon (STX489) were stably integrated into K562 cells using the piggybac system at a 1:5 transposase to transposon ratio. K562 cells (50K per well in a 96 well format) with the piggybac integrations of the broken GFP constructs were infected with 1 million vg/cell of virus. Cells were analyzed by flow cytometry 48 hours later. Cells were gated to remove debris, doublets, and dead cells (Zombie Violet positive). Live cells were further gated by GFP to select the population of cells that exhibited readthrough of the engineered broken GFP gene. Each data point is a biological replicate (n=3). Data represent mean +/- SEM. Experimental groups were compared to unbroken GFP (labeled ST21; positive control) and no transduction (negative control).

Table7. Constructs used in FIG. 30A and 30B

Plasmid Name	Vector Backbone Name:	Insert DNA Name:	Promoter info (for qPCR):	PolyA info (for qPCR):	Size of transgene cassette from ITR to ITR:
pscAAV-wtITR2-CCT2-lambda-scITR	pUC57	STX0631	None	None	2273/4456
pscAAV-wtITR2-lambda-CCT2-lambda-scITR	pUC57	STX0632	None	None	2272/4454
pscAAV-wtITR2-lambda-CCT2-scITR	pUC57	STX0633	None	None	2297/4504

Example 13. In Vivo Characterization of Engineered tRNAs

[307] An in vivo experiment in mice is designed to further determine an efficiency of the engineered tRNA or the variants thereof to perform stop codon readthrough, monitor toxicity, and readthrough efficiency in different cell types and/or different brain regions. Male wild type (WT), R255X, and R168X mice will be injected bilaterally in the lateral ventricles (ICV) at 5-8

days old with an AAV (e.g. AAV2/9) expressing an engineered tRNA or variants thereof comprising the sequence of SEQ ID NOS: 3, 6, 7, 32 or 45, at a dose of $3x10^{10}$ vector genome (vg)/brain. Mice will be tested for performance on the elevated plus maze (EPM) and nesting tests and will be euthanized 3 weeks post injection. One brain hemisphere will be microdissected into the hippocampus, cortex, and the rest of the brain and dissociated for assessments of full-length MeCP2 by flow cytometry. The other hemisphere can be used for immunohistochemistry (IHC), western blot, or mRNA analyses. mCherry will be present in the AAV. This cohort will demonstrate the extent of MeCP2 readthrough in each brain region and neural cell types, toxicity, and determining the extent of premature stop codon readthrough. An example of the study design, described herein, is provided in **Table 8**.

Table 8. In vivo characterization of engineered tRNAs study design

Genotype	Sex	Treatment	Dose	Sample size
WT R168X R255X	M	No injection	NA	3
		Injection of stuffer sequence only viral vector	3*10^10 vg/brain	5
		Injection of AAV-tRNA SEQ ID NO: 3		5
		Injection of AAV-tRNA SEQ ID NO: 6		5
		Injection of AAV-tRNA SEQ ID NO: 7		5
		Injection of AAV-tRNA SEQ ID NO: 32		5
		Injection of AAV-tRNA SEQ ID NO: 45		5

Example 14. In Vivo Efficacy Study of Engineered tRNAs

[308] An in vivo experiment in mice is designed to analyze the efficacy of selected engineered tRNA or variants thereof. Two engineered tRNA or variants thereof from example 9 that showed the highest performance will be selected. Male WT, R255X, and R168X mice will be injected unilaterally in the lateral ventricles (ICV) at 0-2 days old with an AAV (e.g. AAV2/9) expressing the top two engineered tRNA or variants thereof identified from example 9 at a dose of $3x10^{10}$ vector genome (vg)/hemisphere. Mice will undergo a full behavioral analysis including nesting, open field test (OFT), elevated plus maze (EPM), conditioned and contextual fear

conditioning, and sensorimotor tests. Mice will either be euthanized 2 months after injection or lifespan analyses can be performed. One brain hemisphere will be microdissected and dissociated for assessments of full-length MeCP2 by flow cytometry. The other hemisphere can be used for immunohistochemistry (IHC), western blot, and/or mRNA analyses. mCherry will not be present in the AAV. This experiment will demonstrate if the extent of MeCP2 readthrough in each brain region and neural cell type is sufficient to affect behavioral outcomes. We will also monitor any signs of toxicity by collecting and analyzing samples from central spinal fluid, blood plasma, blood serum, liver, kidney, lung, or heart. An example of the study design, described herein, is provided in **Table 9.**

Table 9. In vivo efficacy study of engineered tRNAs study design

Genotype	Sex	Treatment	Dose	Sample size
WT	M	No injection		10
	M	Injection of stuffer sequence only viral vector	3*10^10 vg/hemisphere	20
	M	Injection of AAV-tRNA suppressor A		20
	M	Injection of AAV-tRNA suppressor B		20
R168X R255X	M	No injection		10
	M	Injection of stuffer sequence only viral vector	3*10^10 vg/hemisphere	30
	M	Injection of AAV-tRNA suppressor A		30
	M	Injection of AAV-tRNA suppressor B		30

Example 15. Exemplary plasmids

[309] Some non-limiting examples of nucleic acid constructs that can be used in the methods and compositions described herein are included in **Table 10** and **FIGs. 47A-47E**. Individual features of these constructs can be used alone or in combination with features of other constructs or embodiments. A nucleic acid construct comprising pscAAV-scITR-0x-CMVmChSV40-lambda-strw2-wtITR2 can be adapted to encode an engineered tRNA or engineered tRNA variant described herein.

Table 10. Vector maps and plasmid sequences

pscAAV-scITR-3xCCT2-CMVmChSV40-lambda-strw2-wtITR2	**pStw**	**STX604**	**FIG. 47A**	**SEQ ID NO: 52**
pscAAV-scITR-3xTCT1-CMVmChSV40-lambda-strw2-wtITR2	**pStw**	**STX605**	**FIG. 47B**	**SEQ ID NO: 53**
pscAAV-scITR-3xTCT1.11-CMVmChSV40-lambda-strw2-wtITR2	**pStw**	**STX606**	**FIG. 47C**	**SEQ ID NO: 54**
pscAAV-scITR-3xCCT2.10-CMVmChSV40-lambda-strw2-wtITR2	**pStw**	**STX607**	**FIG. 47D**	**SEQ ID NO: 55**
pscAAV-scITR-3xCCT4-CMVmChSV40-lambda-strw2-wtITR2	**pStw**	**STX608**	**FIG. 47E**	**SEQ ID NO: 56**

Example 16. Spacer sequence

[310] Some embodiments include a stuffer sequence. Non-limiting examples of polynucleotide constructs that include a stuffer sequence are shown in **48A-48B**. Individual features of these constructs can be used alone or in combination with features of other constructs or embodiments. The nucleic acid constructs of some such embodiments can to encode an engineered tRNA or engineered tRNA variant described herein. **FIGS. 48A-48B** can show how a filler sequence (e.g. SEQ ID NO: 57) can be oriented within an AAV construct, and include a 6X-100bp spacer map (e.g. for the engineered tRNA comprising the sequence of SEQ ID NO: 3). An orientation used in a construct described herein can be similar in orientation to those shown in the figures, or can be modified with various isodecoder sequences or filler sequences.

[311] While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein can be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

CLAIMS

WHAT IS CLAIMED IS:

1. A composition comprising:

an engineered tRNA variant comprising a mutation in a T-loop, a T-stem, a D-loop, a D-stem, a variable loop, an anticodon stem, or an anticodon loop relative to the nucleic acid sequence of any one of SEQ ID NOs: 3-22 or 103-122,

wherein upon administration to a subject, the engineered tRNA variant is capable of restoring production of at least 10% of a substantially full length polypeptide by suppression of a premature stop codon in a target mRNA encoding for the substantially full length polypeptide, relative to a comparable polypeptide produced using a comparable mRNA that lacks the premature stop codon, as determined by:

a) transfecting a first vector encoding the engineered tRNA or variant thereof and a second vector encoding a screening mRNA encoding a first green fluorescent protein into a first human cell, wherein the screening mRNA encoding the first green fluorescent protein comprises the premature stop codon;

b) transfecting a third vector encoding a comparable screening mRNA encoding a second green fluorescent protein into a second human cell, wherein the comparable screening mRNA does not comprise the premature stop codon; and

c) comparing an amount of fluorescence emitted from the first human cell and the second human cell.

2. The composition of claim 1, wherein the engineered tRNA variant has at least 70% sequence identity to any one of SEQ ID NOs: 23-48 or 123-148.

3. The composition of claim 1 or 2, wherein the engineered tRNA variant comprises a sequence that is at least 70% identical to SEQ ID NO: 6 or 106, and has a substitution at position 2, 4, 6, 12, 23, 27, 28, 31, 39, 40, 42, 43, 44, 46, 49, 50, 64, 65, 67, 69, or 71, of SEQ ID NO: 6 or 106.

4. The composition of claim 3, wherein the substitution at position 2 is to a C, the substitution at position 4 is to a C, the substitution at position 6 is to a T, the substitution at position 6 is to an A, the substitution at position 12 is to a C, the substitution at position 23 is to a G, the substitution at position 27 is to a C, the substitution at position 28 is to a C, the substitution at position 31 is to a C, the substitution at position 39 is to a G, the substitution at

position 40 is to a C, the substitution at position 42 is to a G, the substitution at position 43 is to a G, the substitution at position 44 is to a G, the substitution at position 46 is to an A, the substitution at position 49 is to a G, the substitution at position 50 is to a T, the substitution at position 64 is to an A, the substitution at position 65 is to a C, the substitution at position 67 is to an A, the substitution at position 67 is to a T, the substitution at position 69 is to a G, the substitution at position 71 is to a C, or the substitution at position 71 is to a G.

5. The composition of claim 3 or 4, wherein the sequence of the engineered tRNA variant is identical to SEQ ID NO: 6 or 106, except for the substitution.

6. The composition of any one of claims 3-5, wherein the engineered tRNA variant exhibits an increased stability *in vivo*, as compared with a comparable tRNA comprising the sequence provided in SEQ ID NO: 6 or 106, as determined by a proxy measurement, a half life measurement, an amino acid charging efficiency measurement, or a measurement of binding to a synthetase or ribosomal machinery.

7. The composition of any one of claims 1-6, wherein the engineered tRNA variant comprises the sequence of SEQ ID NO: 45 or 145.

8. The composition of claim 1 or 2, wherein the engineered tRNA variant comprises a sequence that is at least 70% identical to any one of SEQ ID NO: 3 or 103, and has a substitution at position 2, 6, 13, 15, 22, 28, 31, 37, 39, 42, 44, 50, 64, 67, 71, or 72, of SEQ ID NO: 3 or 103.

9. The composition of claim 8, wherein the substitution at position 2 is to a G, the substitution at position 6 is to a G, the substitution at position 13 is to a C, the substitution at position 15 is to a G, the substitution at position 22 is to a G, the substitution at position 28 is to a C, the substitution at position 31 is to a A, the substitution at position 37 is to a G, the substitution at position 39 is to a T, the substitution at position 42 is to a G, the substitution at position 44 is to an A, the substitution at position 50 is to a C, the substitution at position 64 is to a G, the substitution at position 67 is to a C, the substitution at position 71 is to a C, or the substitution at position 72 is to a C.

10. The composition of claim 8 or 9, wherein the sequence of the engineered tRNA variant is identical to SEQ ID NO: 3 or 103, except for the substitution.

11. The composition of any one of claims 8-10, wherein the engineered tRNA variant exhibits an increased stability *in vivo*, as compared with a comparable tRNA comprising the

sequence provided in SEQ ID NO: 3 or 103, as determined by a proxy measurement, a half life measurement, an amino acid charging efficiency measurement, or a measurement of binding to a synthetase or ribosomal machinery.

12. The composition of any one of claims 1, 2 or 8-11, wherein the engineered tRNA variant comprises the sequence of SEQ ID NO: 32 or 132.

13. The composition of claim 1 or 2, wherein the engineered tRNA variant comprises a sequence that is at least 70% identical to SEQ ID NO: 5 or 105, and has a substitution at position 73 of SEQ ID NO: 5 or 105.

14. The composition of claim 13, wherein the substitution at position 73 is to a G.

15. The composition of claim 13 or 14, wherein the sequence of the engineered tRNA variant is identical to SEQ ID NO: 5 or 105, except for the substitution.

16. The composition of any one of claims 13-15, wherein the engineered tRNA variant exhibits an increased stability *in vivo*, as compared with a comparable tRNA comprising the sequence provided in SEQ ID NO: 5 or 105, as determined by a proxy measurement, a half life measurement, an amino acid charging efficiency measurement, or a measurement of binding to a synthetase or ribosomal machinery.

17. The composition of any one of claims 1-16, wherein the engineered tRNA variant is acylated with an amino acid comprising lysine, arginine, histidine, glycine, alanine, valine, leucine, isoleucine, methionine, phenylalanine, tryptophan, proline, serine, threonine, cysteine, tyrosine, asparagine, glutamine, aspartate, glutamate, pyrolysine, or selenocysteine, or is acylated with a non-canonical amino acid.

18. The composition of any one of claims 1-17, wherein the premature stop codon is an opal stop codon, an ochre stop codon, or an amber stop codon.

19. The composition of any one of claims 1-18, wherein the polypeptide comprises an MeCP2 polypeptide, a FoxG1 polypeptide, a CDKL5 polypeptide, a MYH9 polypeptide, a COL11A2 polypeptide, or a MYO7A polypeptide.

20. The composition of any one of claims 1-19, wherein the engineered tRNA variant is capable of restoring production of at least 20% of the substantially full-length polypeptide.

21. The composition of any one of claims 1-19, wherein the engineered tRNA variant is capable of restoring production of at least 40% of the substantially full-length polypeptide.

22. The composition of any one of claims 1-19, wherein the engineered tRNA variant is capable of restoring production of at least 60% of the substantially full-length polypeptide.

23. The composition of any one of claims 1-19, wherein the engineered tRNA variant is capable of restoring production of at least 80% of the substantially full-length polypeptide.

24. The composition of any one of claims 1-23, wherein the composition comprises a polynucleotide encoding the engineered tRNA variant.

25. The composition of embodiment 24, wherein the polynucleotide is in a viral vector.

26. The composition of claim 25, wherein the viral vector is an adenoviral vector, an adeno-associated viral (AAV) vector, or a lentiviral vector.

27. The method of 26, wherein the AAV vector comprises an AAV 2/5 vector, an AAV 2/6 vector, an AAV 2/7 vector, an AAV2/8 vector, or an AAV 2/9 vector.

28. The composition of any one of claims 26-27, wherein the AAV comprises a capsid from AAV5.

29. The composition of any one of claims 26-28, wherein the polynucleotide comprises an inverted terminal repeat (ITR) sequence from AAV2.

30. The composition of any one of claims 26-29, wherein the polynucleotide comprises an inverted terminal repeat with a mutated terminal resolution site and lacking terminal nucleotides.

31. The composition of claim 30, wherein the terminal nucleotides comprise at least a portion of the A region, at least a portion of the D region, or both.

32. The composition of any one of claims 27-31, wherein the polynucleotide is self-complementary.

33. The composition of any one of claims 25-32, wherein the polynucleotide encodes two or more, three or more, four or more, five or more, or six or more copies of the same engineered tRNA variant.

34. The composition of any one of claims 25-33, wherein the polynucleotide encodes two or more, three or more, four or more, five or more, or six or more copies of different engineered tRNA variants.

35. The composition of any one of claims 25-34, wherein the polynucleotide comprises a stuffer sequence comprising at least about 50 nucleotides, at least about 100 nucleotides, at least about 150 nucleotides, or at least about 200 nucleotides.

36. The composition of claim 35, wherein the stuffer sequence is 3' or 5' of one or more copies of the engineered tRNA variant within the polynucleotide.

37. The composition of claim 35 or 36, wherein the stuffer sequence separates two or more copies of the engineered tRNA variant within the polynucleotide.

38. The composition of any one of claims 24-37, wherein the engineered tRNA variant or the polynucleotide encoding the engineered tRNA is present in a delivery system.

39. The composition of claim 38, wherein the delivery system comprises a liposome, a charged polymer, an uncharged polymer, a nanoparticle, a surfactant, a penetrating enhancer, a gene transfer agent, a phospholipid, a micelle, a synthetic vector, a macromolecule, a dendrimer, a biopolymer, a viral particle, or any combination thereof.

40. A pharmaceutical composition comprising the composition of any one of claims 1-39 and a pharmaceutically acceptable excipient, carrier or diluent.

41. The pharmaceutical composition of claim 40 in a dose unit form.

42. A kit comprising the composition of any one of claims 1-39 or the pharmaceutical composition of claim 40 or 41, and a packaging or container.

43. A method of making the kit of claim 42, comprising contacting the composition or pharmaceutical composition with the packaging or container.

44. A method of treatment or prevention of a disease or disorder, comprising:

administering to a subject in need thereof the composition of any one of claims 1-40.

45. A method of treating or preventing a disease or condition in a subject in need thereof, comprising:

administering to the subject an engineered tRNA or variant thereof or a polynucleotide encoding the engineered tRNA or variant thereof; and

producing a substantially full-length polypeptide *in vivo* at an efficiency of at least about 10%, relative to a comparable polypeptide produced using an mRNA that lacks a premature stop codon,

wherein the engineered tRNA or the variant thereof is capable of reading through the premature stop codon in a target mRNA encoding for the substantially full-length polypeptide.

46. A method of treating or preventing a disease or condition in a subject in need thereof, comprising:

administering to the subject an engineered tRNA or variant thereof or a polynucleotide encoding the engineered tRNA or variant thereof, thereby at least partially treating the disease or condition in the subject;

wherein the engineered tRNA or variant thereof recognizes a premature stop codon in a target mRNA encoding a polypeptide, wherein the engineered tRNA or variant thereof during translation of the target mRNA at least partially transforms interpretation of the premature stop codon into a sense codon and produces a substantially full-length polypeptide *in vivo* at an efficiency of at least about 10%, relative to a comparable polypeptide produced using a comparable mRNA that lacks the premature stop codon, as determined by:

a) transfecting a first vector encoding the engineered tRNA or variant thereof and a second vector encoding a screening mRNA encoding a first marker into a first human cell, wherein the screening mRNA encoding the first marker comprises the premature stop codon;
b) transfecting a third vector encoding a comparable screening mRNA encoding a second marker into a second human cell, wherein the comparable screening mRNA does not comprise the premature stop codon; and
c) comparing an amount of a detectable signal emitted from the first human cell and the second human cell.

47. The composition of claim 45 or 46, wherein the engineered tRNA or variant thereof has at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% sequence identity to any one of SEQ ID NOs: 003-48 or 103-148.

48. The method of any one of claims 45-47, wherein the engineered tRNA or variant thereof comprises any one of SEQ ID NOS: 3-48 or 103-148.

49. The method of claim 45 or 46, wherein the engineered tRNA or variant thereof comprises any one of SEQ ID NOS: 3, 6, 7, 32, 45, 103, 106, 107, 132, or 145.

50. The method of any one of claims 45-49, wherein administering to the subject an engineered tRNA or variant thereof comprises administering a virus comprising a polynucleotide encoding the engineered tRNA or variant thereof.

51. The method of claim 50, wherein the virus comprises an adenovirus, an adeno-associated virus (AAV), or lentivirus.

52. The method of claim 50 or 51, wherein the virus comprises AAV 2/5, AAV 2/6, AAV 2/7, AAV2/8, or AAV 2/9.

53. The method of any one of claims 50-52, wherein the virus comprises an AAV5 capsid.

54. The method of any one of claims 50-53, wherein the polynucleotide encoding the engineered tRNA or variant thereof comprises an AAV2 inverted terminal repeat (ITR) sequence.

55. The method of any one of claims 50-54, wherein the polynucleotide encoding the engineered tRNA or variant thereof comprises an inverted terminal repeat with a mutated terminal resolution site and lacking terminal nucleotides.

56. The method of claim 55, wherein the terminal nucleotides comprise at least a portion of the A region, at least a portion of the D region, or both.

57. The method of any one of claims 50-56, wherein the polynucleotide encoding the engineered tRNA or variant thereof is self complementary.

58. The method of any one of claims 50-57, wherein the polynucleotide encoding the engineered tRNA or variant thereof encodes an additional one or more, two or more, three or more, four or more, five or more, or six or more copies of the engineered tRNA or variant thereof.

59. The method of any one of claims 50-58, wherein the polynucleotide encoding the engineered tRNA or variant thereof encodes two or more, three or more, four or more, five or more, or six or more copies of a second engineered tRNA or variant thereof that is different from the engineered tRNA or variant thereof.

60. The method of any one of claims 50-59, wherein the polynucleotide encoding the engineered tRNA or variant thereof comprises a stuffer sequence comprising at least about 50 nucleotides, at least about 100 nucleotides, at least about 150 nucleotides, or at least about 200 nucleotides.

61. The method of claim 60, wherein the stuffer sequence is 3' or 5' of one or more copies of the engineered tRNA or variant thereof within the polynucleotide.

62. The method of claim 60 or 61, wherein the stuffer sequence separates two or more copies of the engineered tRNA or variant thereof within the polynucleotide.

63. The method of any one of claims 45-62, wherein the engineered tRNA or variant thereof or the polynucleotide encoding the engineered tRNA or variant thereof, is in a delivery system.

64. The method of claim 63, wherein the delivery system comprises a liposome, a charged polymer, an uncharged polymer, a nanoparticle, a surfactant, a penetrating enhancer, a gene transfer agent, a phospholipid, a micelle, a synthetic vector, a macromolecule, a dendrimer, a biopolymer, a viral particle, or any combination thereof.

65. The method of any one of claims 50-64, wherein the engineered tRNA or variant thereof or the polynucleotide encoding the engineered tRNA or variant thereof, is in a pharmaceutical composition comprising a pharmaceutically acceptable excipient, carrier or diluent.

66. The method of claim 65, wherein the pharmaceutical composition is in a dose unit form.

67. The method of any one of claims 44-66, wherein the subject has a disease or condition related to the premature stop codon in the target mRNA.

68. The method of any one of claims 44-67, wherein the polypeptide is produced at an efficiency of at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, or at least about 90%, relative to a comparable polypeptide produced using a comparable mRNA that lacks the premature stop codon, thereby at least partially treating the disease or condition in the subject.

69. The method of claim 67 or 68, wherein the disease or condition comprises Rett Syndrome, cystic fibrosis, retinitis pigmentosa, or deafness.

70. The method of claim 69, wherein the deafness comprises autosomal dominant 17 deafness, autosomal dominant 13 deafness, or autosomal dominant 11 deafness.

71. The method of any one of claims 44-70, wherein the subject is a human.

72. The method of any one of claims 44-70, wherein the subject is a non-human animal.

73. The method of any one of claims 44-72, wherein the administration is oral, rectal, parenteral, intravenous, intra-arterial, intrathecal, intraocular, otic, intracerebroventricular, intra cisterna magna, intracerebroventricular, or intraperitoneal.

74. The method of any one of claims 67-73, wherein the disease or condition is Rett Syndrome and at least one symptom of Rett Syndrome is alleviated.

75. The method of claim 74, wherein the at least one symptom of Rett Syndrome comprises slowed growth, slowed brain growth, microcephaly, a decrease or loss of movement or coordination, reduced hand control, decreased walking ability, rigid or spastic movement, a decreased ability to speak, decreased eye, disinterestedness, repetitive hand movement, unusual eye movements, difficulty breathing, irritability, fear, anxiety, a cognitive defect, seizures, an abnormal electroencephalogram, scoliosis, irregular heartbeat, or an abnormal sleep pattern.

76. The method of any one of claims 44-75, wherein from 1×10^{12} to 1×10^{15} viral genomes are administered.

77. The method of any one of claims 44-76, wherein the subject is around 10 to 30 years of age.

78. The method of any one of claims 44-77, wherein the composition decreases or inhibits nonsense-mediated mRNA decay (NMD) of the target mRNA.

79. The method of any one of claims 44-78, wherein the composition increases an amount of the target mRNA in the subject, relative to a baseline target mRNA measurement.

80. The composition or method of any one of claims 1-79, wherein the polypeptide comprises an MeCP2 polypeptide.

81. The composition or method of claim 80, wherein the premature stop codon results from a mutation encoding for an R at amino acid position 168, 255, 270, 294, 198, 186, 453, 8 (e.g., in isoform 2), 9 (e.g., in isoform 1), 84, 85, 89, 91, 106, 111, 115, 133, 162, 167, 188, 190, 211, 250, 253, 268, 306, 309, 344, 354, 420, 458, 468, 471, 478, or 484, of the MeCP2 polypeptide.

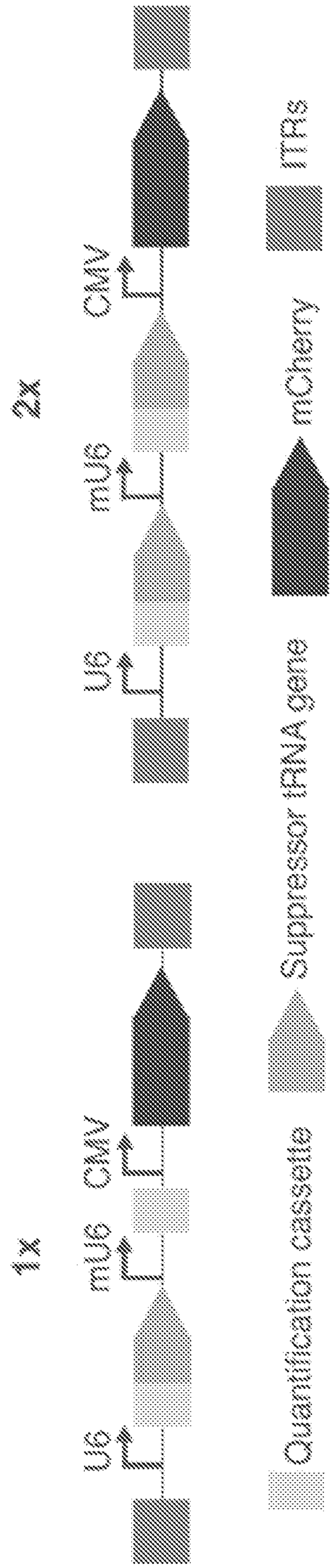

2x
U6
mU6
CMV
mRNA gene
mCherry
1x
U6
mU6
CMV
tion cassette
Suppressor t
Quantifica
ITR

FIG. 1

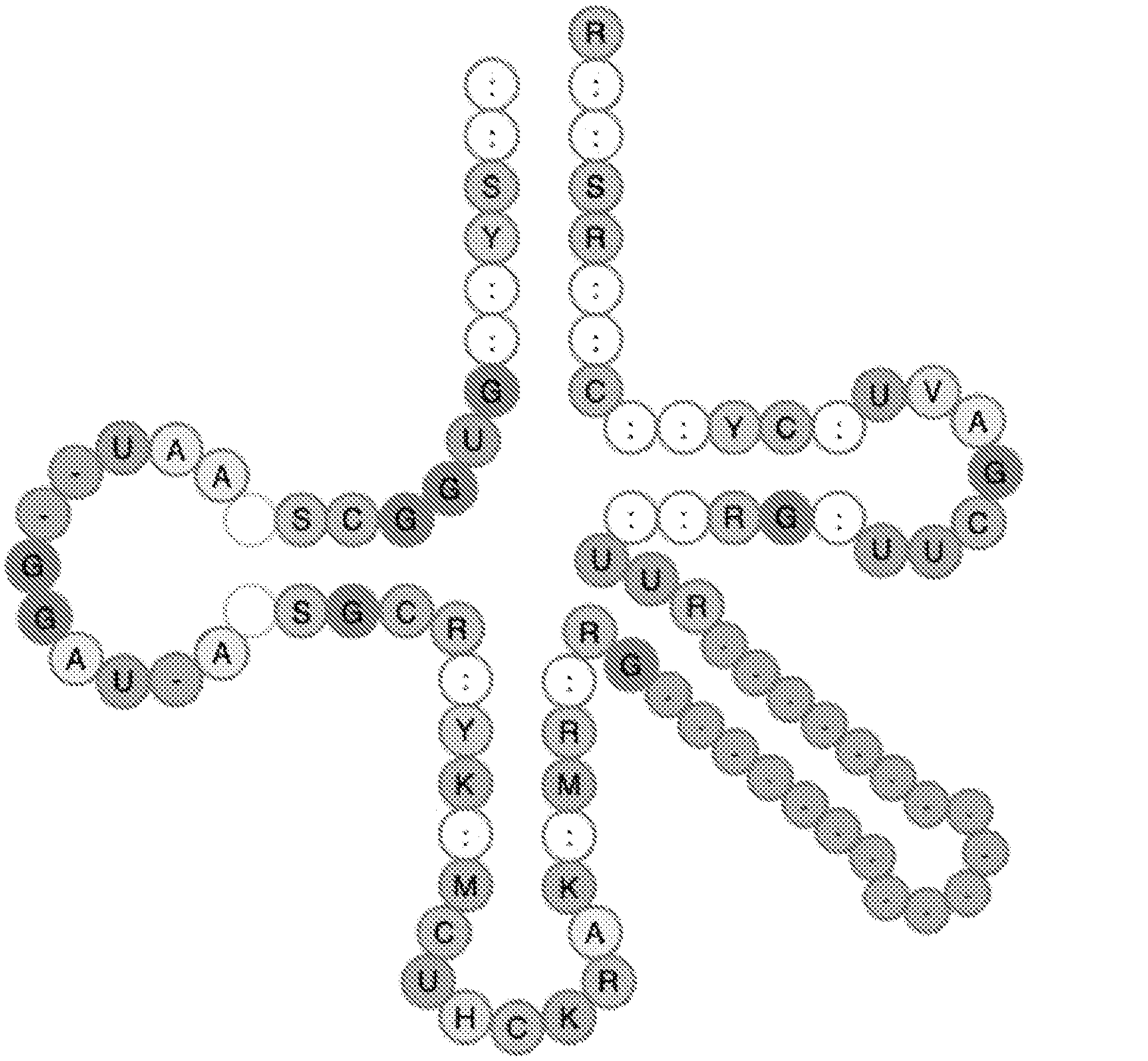

Feature colors
A
C
G
U
Purine
Pyrimidine
Weak (A/U)
Strong (C/G)
Amino (A/C)
Keto (G/U)
B (C/G/U)
D (A/G/U)
H (A/C/U)
V (A/C/G)
A:U
U:A
G:C
C:G
G:U
U:G
A:G
G:A
C:U
U:C
A:C
C:A
A:A
C:C
G:G
U:U
Paired
High mismatch rate
Absent (- or -:-)
Malformed (N:- or -:N)

FIG. 2A

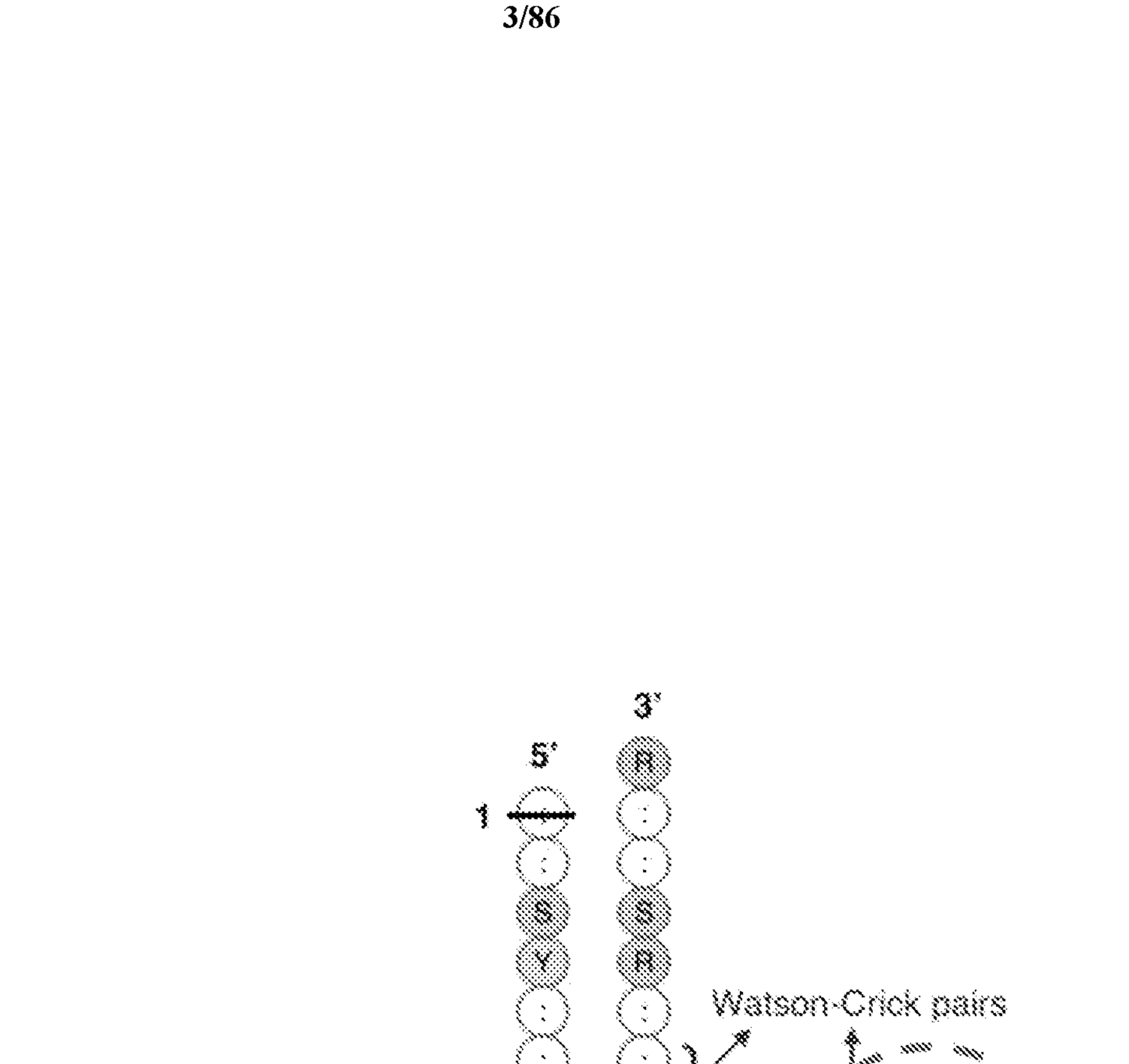

5'
3'
1
8-16: A-box
8
16
12
18
20
23
27
29
32
34
36
38
41
46
52
57
63
Watson-Crick pairs

FIG. 2B

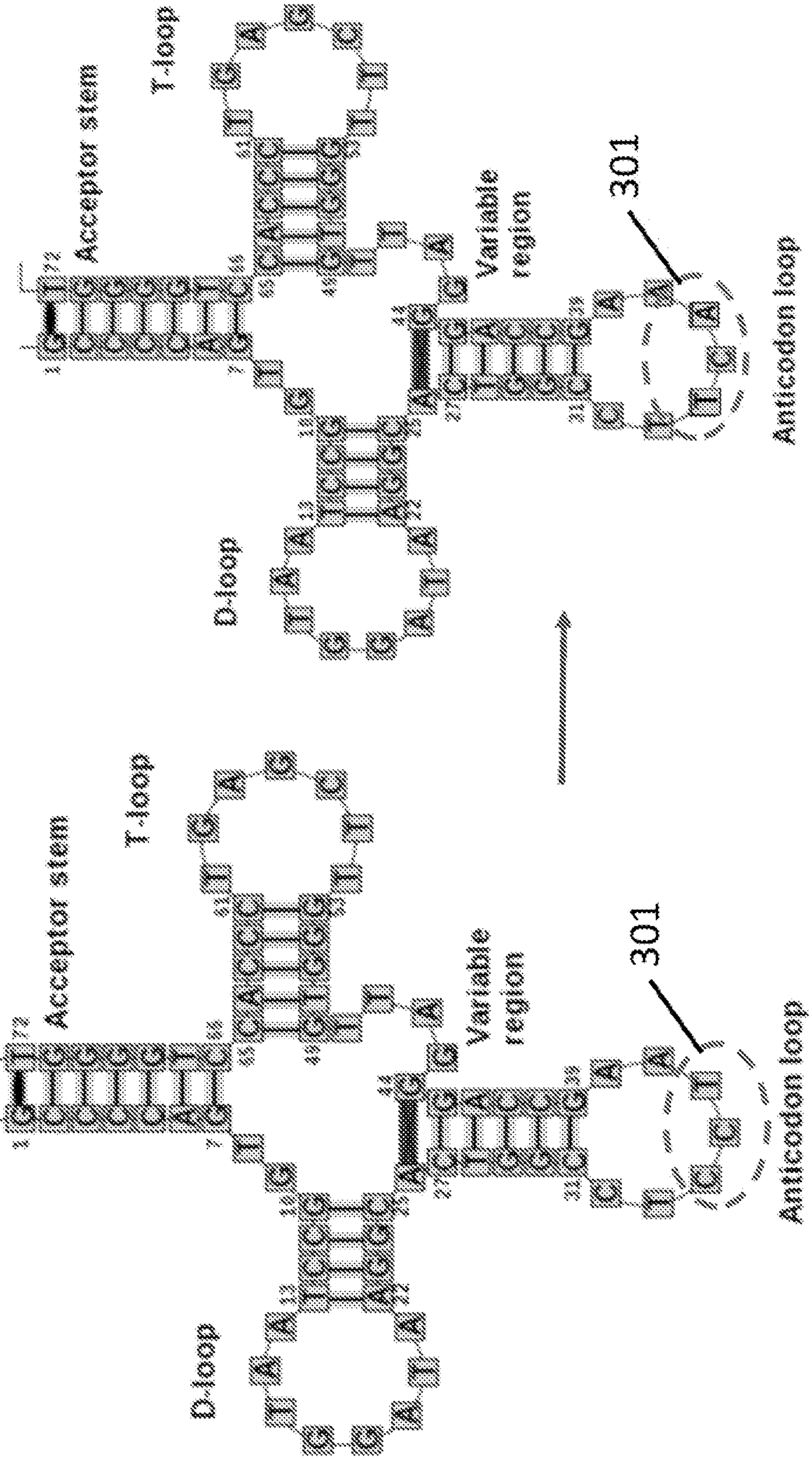

Acceptor stem
T-loop
D-loop
Variable region
Anticodon loop
301

FIG. 3A

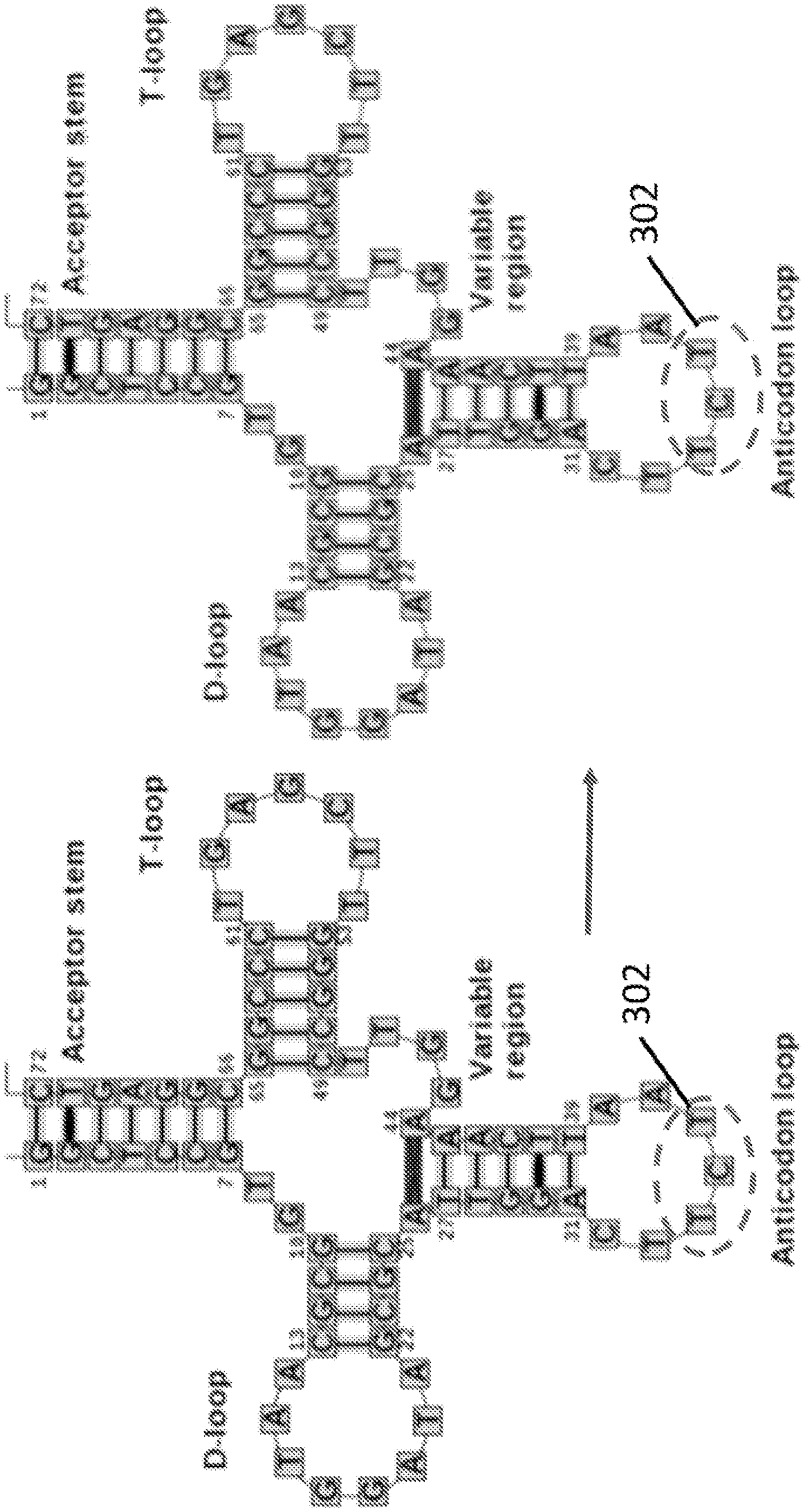

Acceptor stem
T-loop
Variable region
302
Anticodon loop
D-loop

FIG. 3

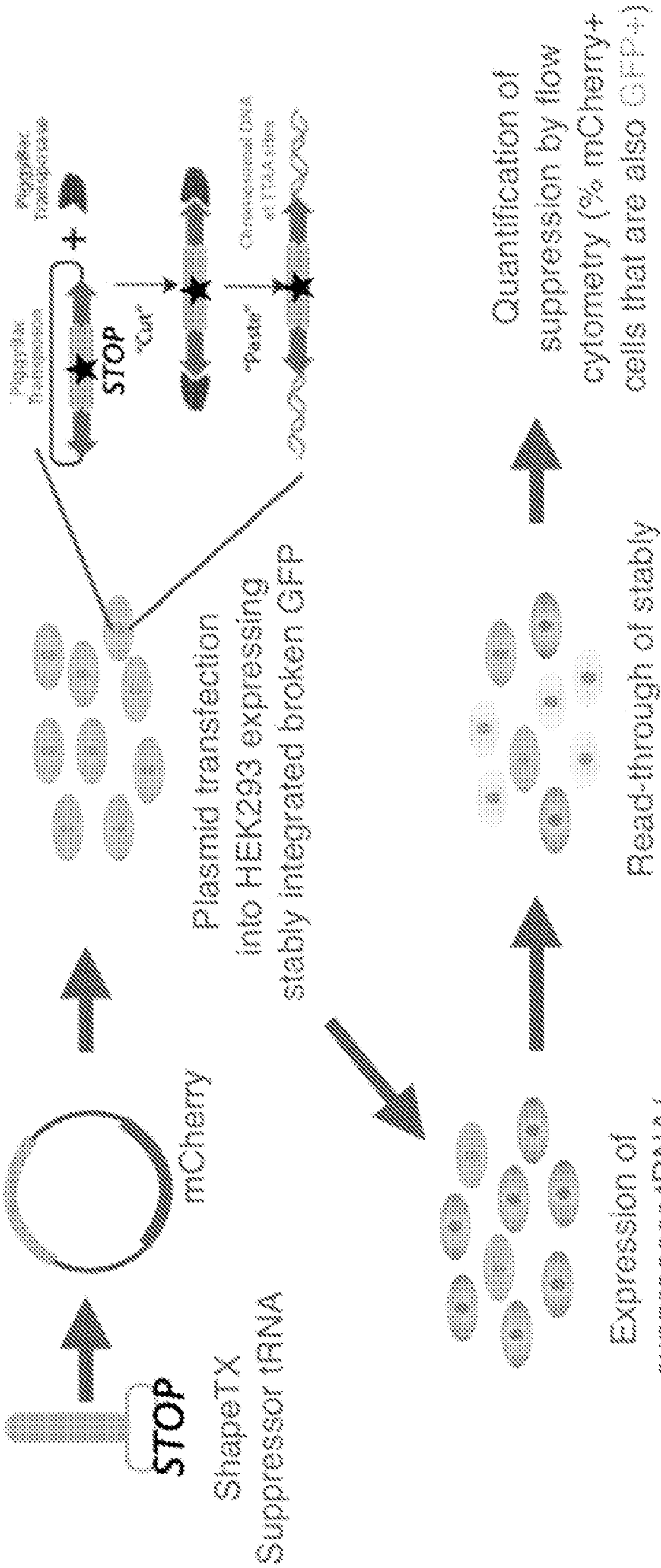

Plasmid transfection into HEK293 expressing stably integrated broken GFP
Suppressor tRNA
mCherry
Shape TX
STOP
Expression of suppressor tRNA/ mCherry
Read-through of stably integrated broken GFP producing green fluorescence
Quantification of suppression by flow cytometry (% mCherry+ cells that are also GFP+)

FIG. 4

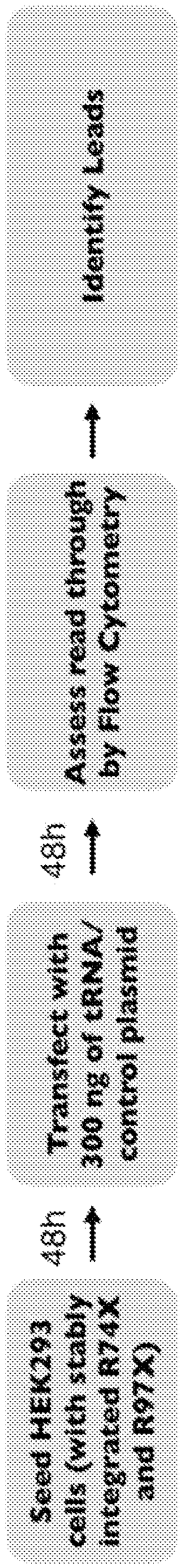

Seed HEK293 cells (with stably integrated R74X and R97X)
48h
Transfect with 300 ng of tRNA/ control plasmid
48h
Assess read through by Flow Cytometry
Identify Leads

FIG. 5A

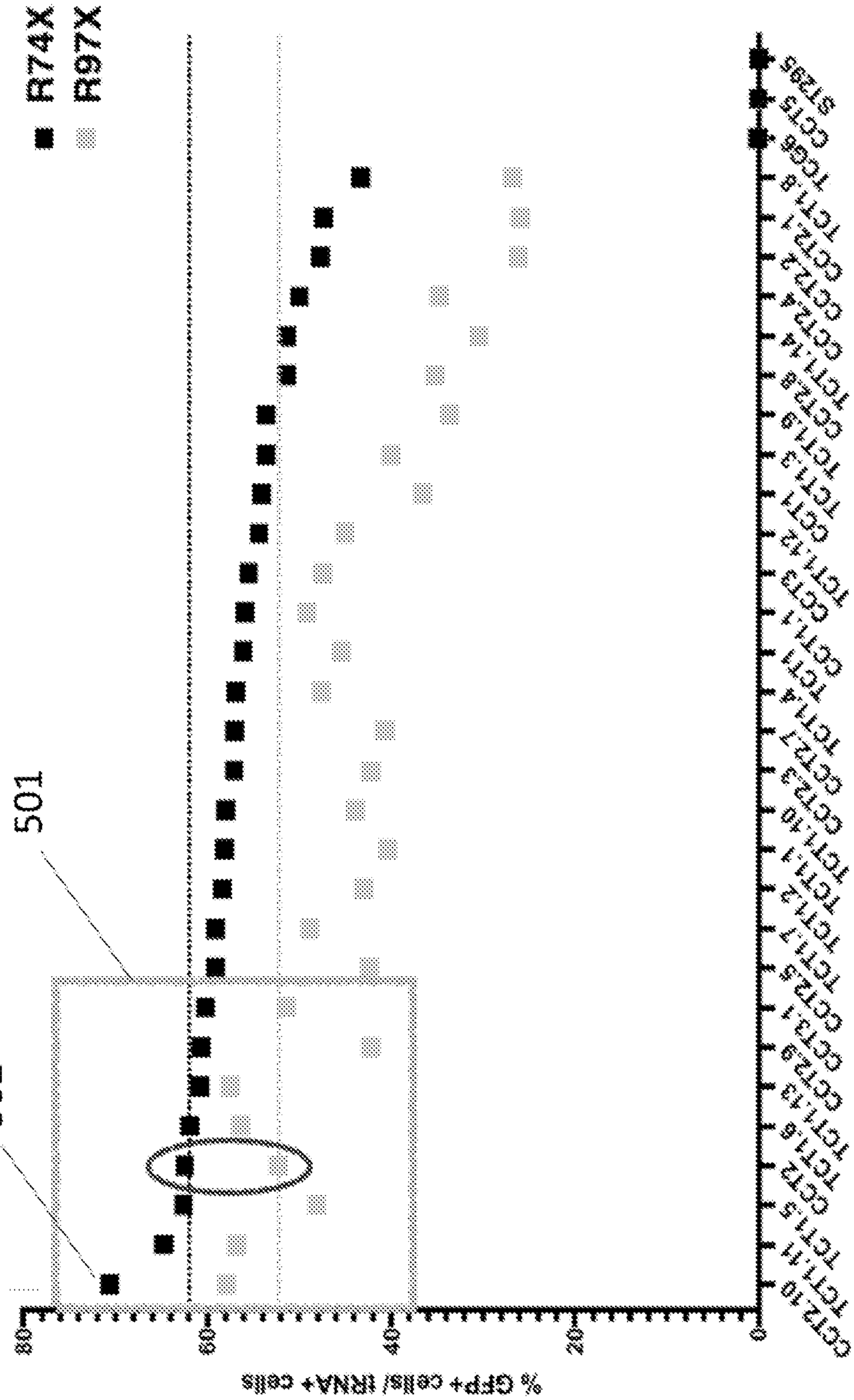

R74X
R97X
501
502
80
60
40
20
0
% GFP+ cells/ tRNA+ cells
Arg Suppressor tRNAs
CCT2.10 TCT1.11 TCT1.15 TCT1.6 TCT1.13 TCT1.9 TCT1.3 TCT1.12 CCT1.1 CCT2.9 CCT3.1 TCT2.5 TCT1.7 TCT1.2 TCT1.4 TCT1.10 CCT2.3 CCT2.7 TCT1.4 CCT2.4 TCT1.14 TCT2.8 TCT1.9 TCT1.3 CCT1.2 TCT1.1 CCT3 CCT2 TCT1.5 TCT1.8 TCT2.4 CCT2.1 TCT1.12 CCT1.3 TCT1.13 TCT1.6 CCT1.8 TCT2.1 CCT2.5 TCT1.11 TCT1.15 CCT2.10

FIG. 5B

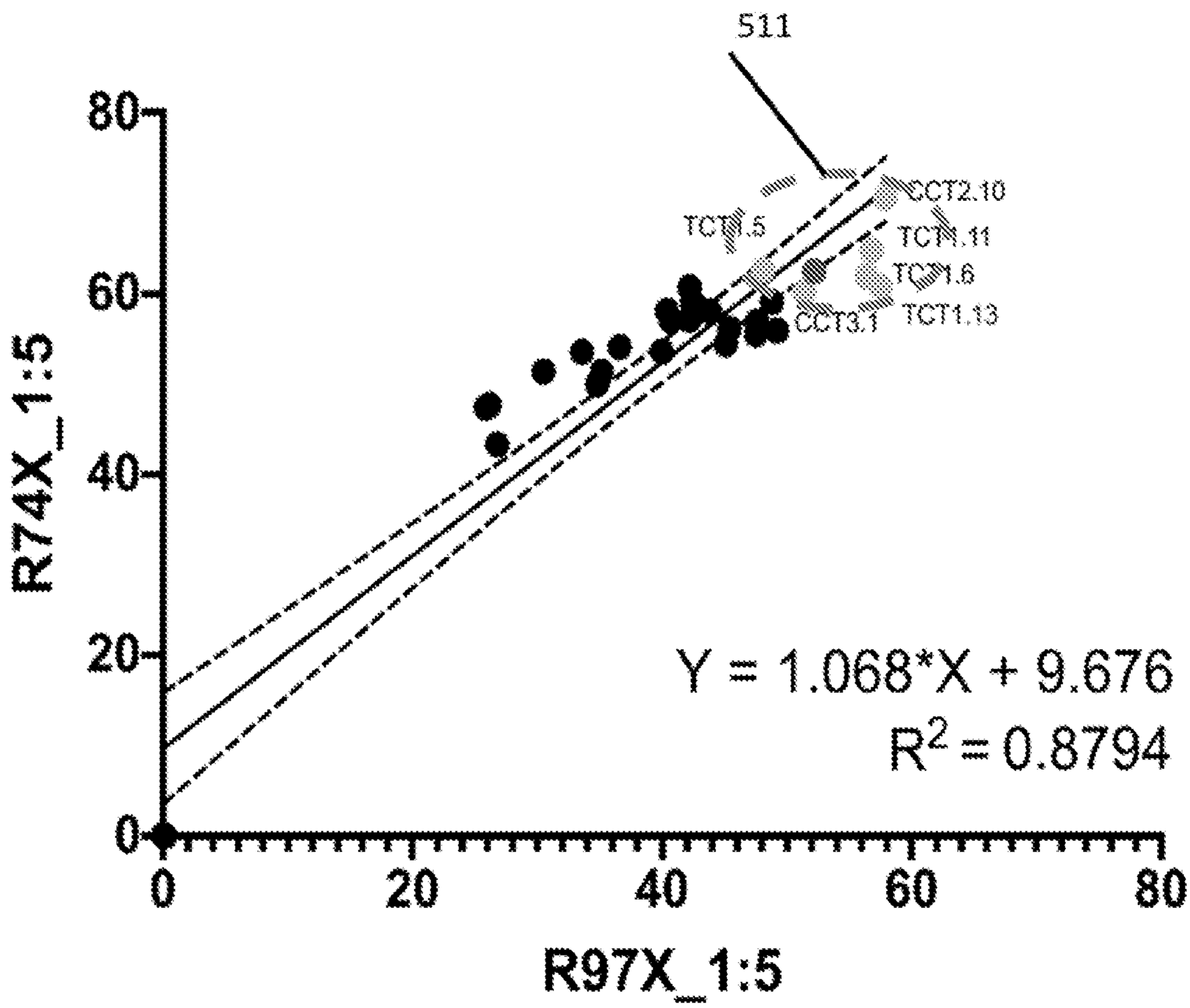

511
80
60
40
20
0
R74X_1:5
TCT1.5
CCT2.10
TCT1.11
TCT1.6
CCT3.1
TCT1.13
Y = 1.068*X + 9.676
R2 = 0.8794
0
20
40
60
80
R97X_1:5

FIG. 5C

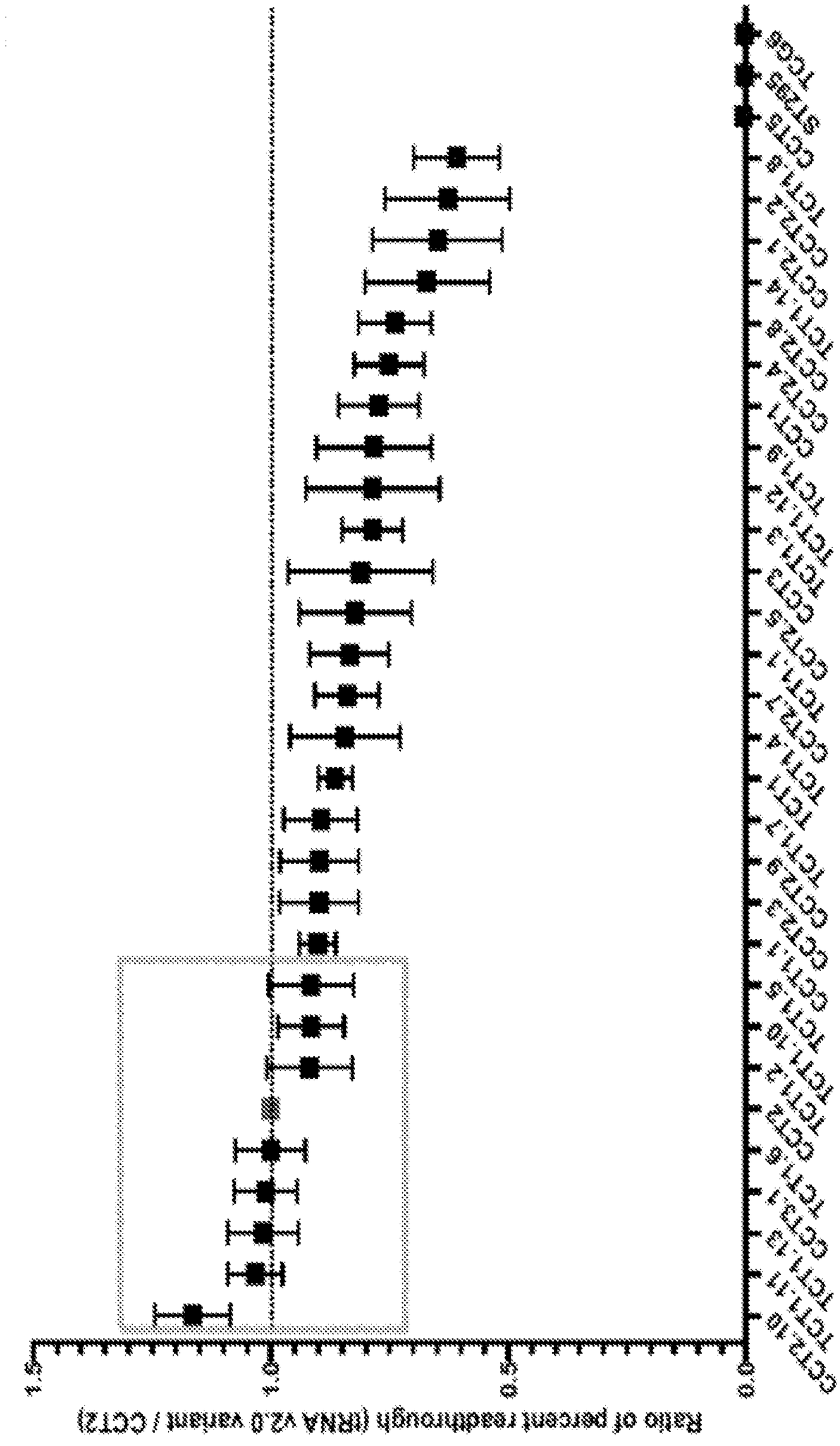

Arg Suppressor tRNAs
Ratio of percent readthrough (tRNA v2.0 variant / CCT2)
-2.5
-2.0
-1.5
-1.0
-0.5
0.0

FIG. 6

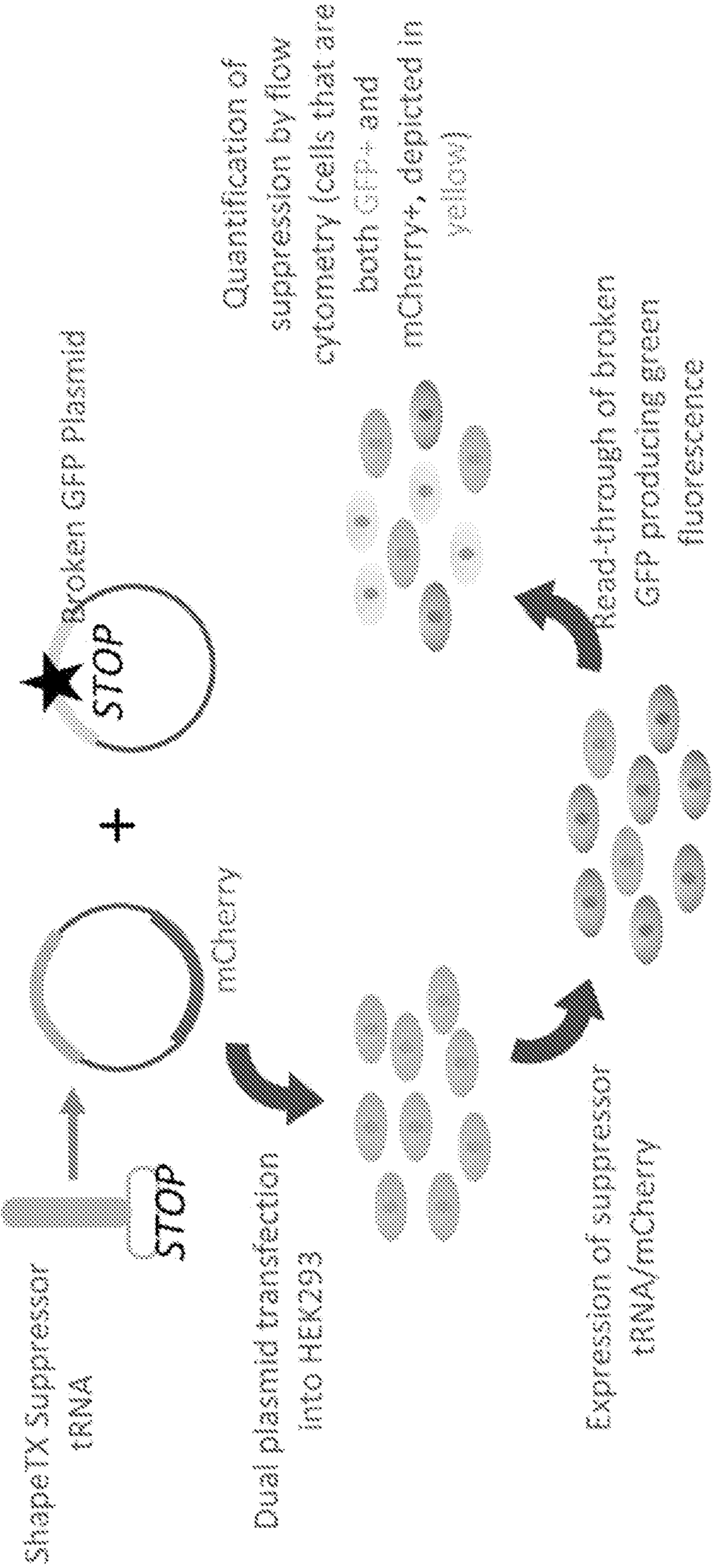

Shape TX Suppressor tRNA
STOP
Broken GFP Plasmid
+
Dual plasmid transfection into HEK293
mCherry
Expression of suppressor tRNA/mCherry
Read-through of broken GFP producing green fluorescence
Quantification of suppression by flow cytometry of cells that are both GFP+ and mCherry+, depicted in yellow

FIG. 7

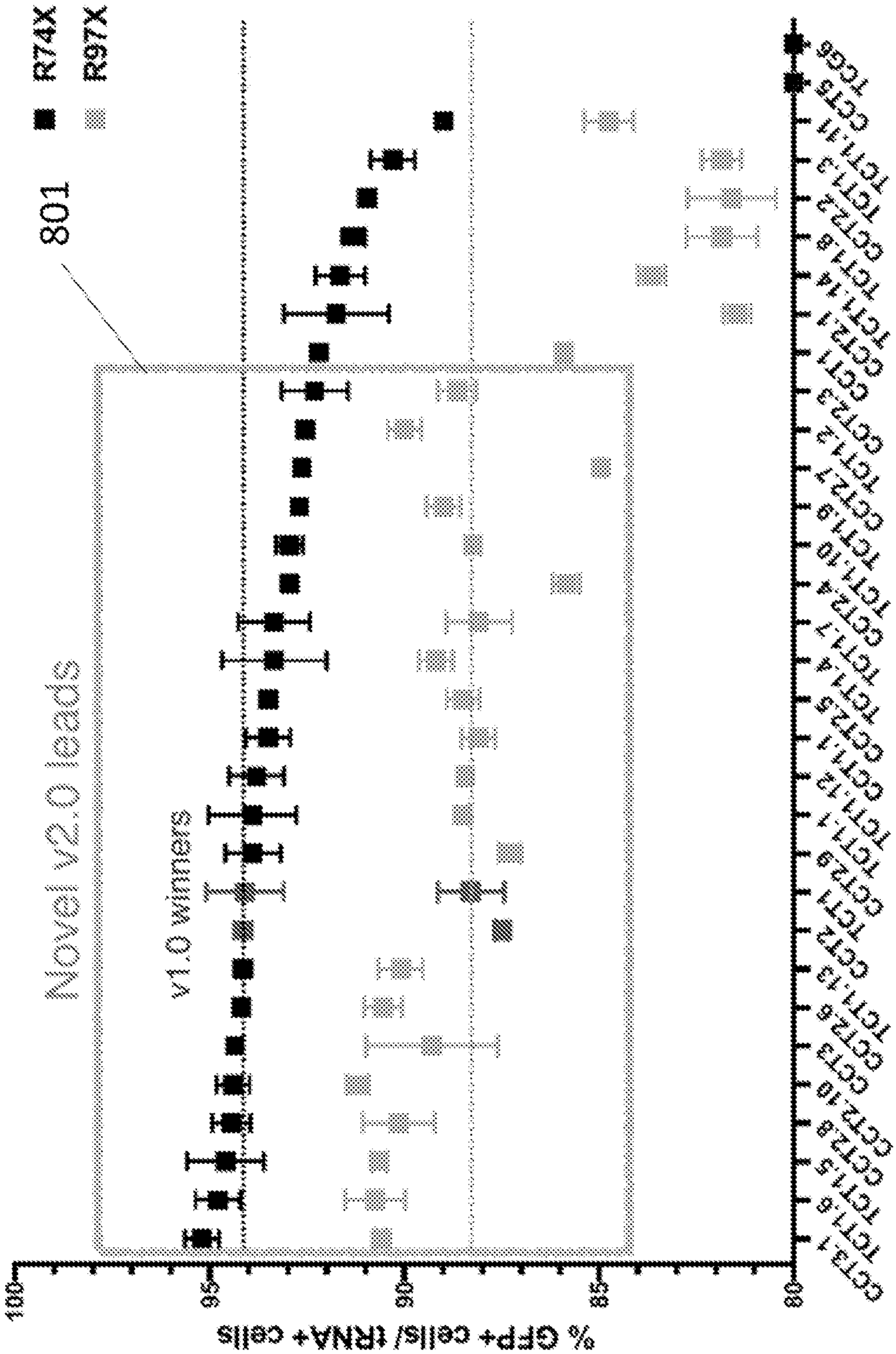

R74X
R97X
801
Dual plasmid system_% of tRNA+ cells with read-through
Novel v2.0 leads
v1.0 winners
% GFP+ cells/ tRNA+ cells
100
95
90
85
80
Arg Suppressor tRNAs

FIG. 8A

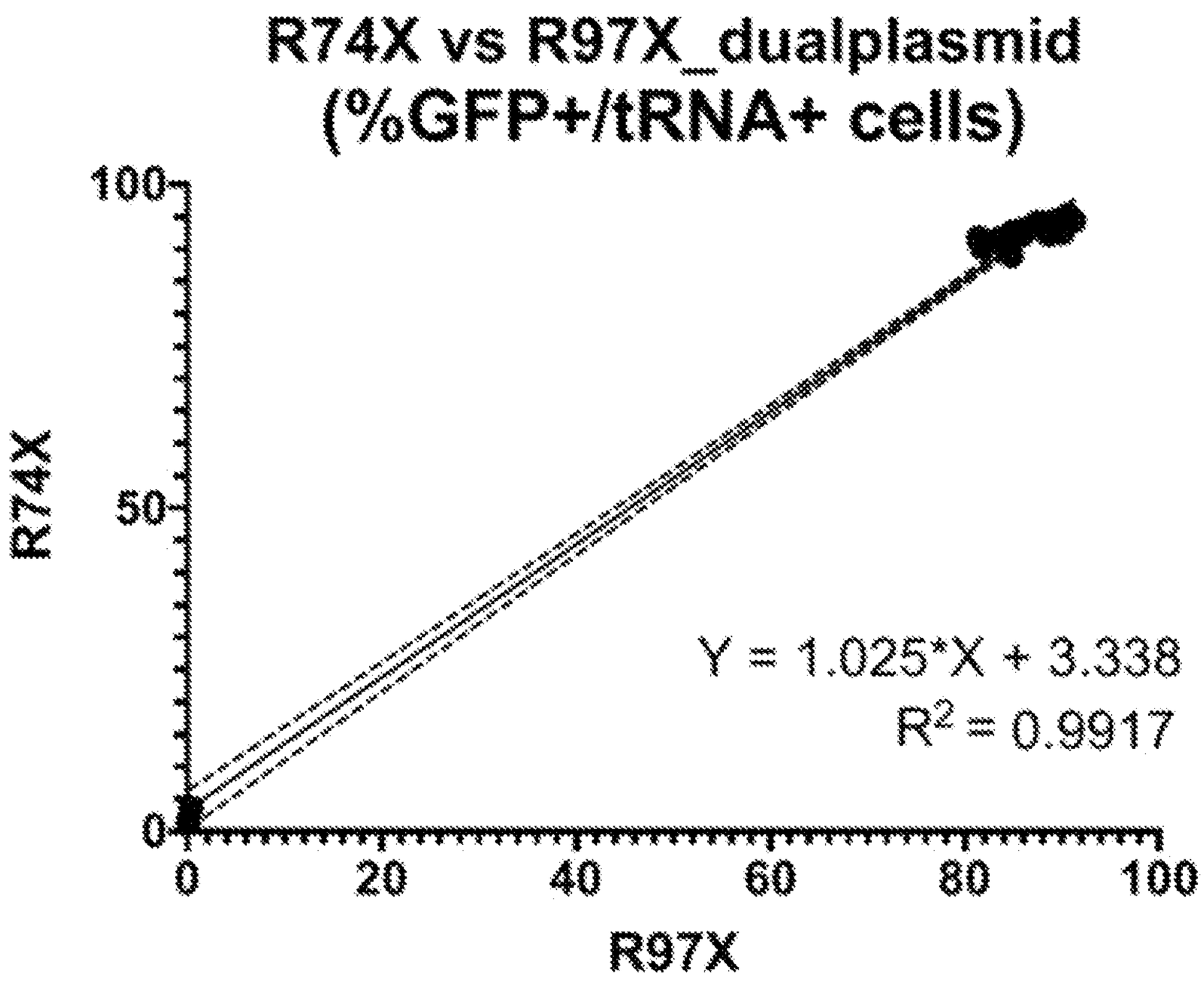

R74X vs R97X_dualplasmid
(%GFP+/tRNA+ cells)
R74X
R97X
100
50
0
0
20
40
60
80
100
Y = 1.025*X + 3.338
R2 = 0.9917

FIG. 8B

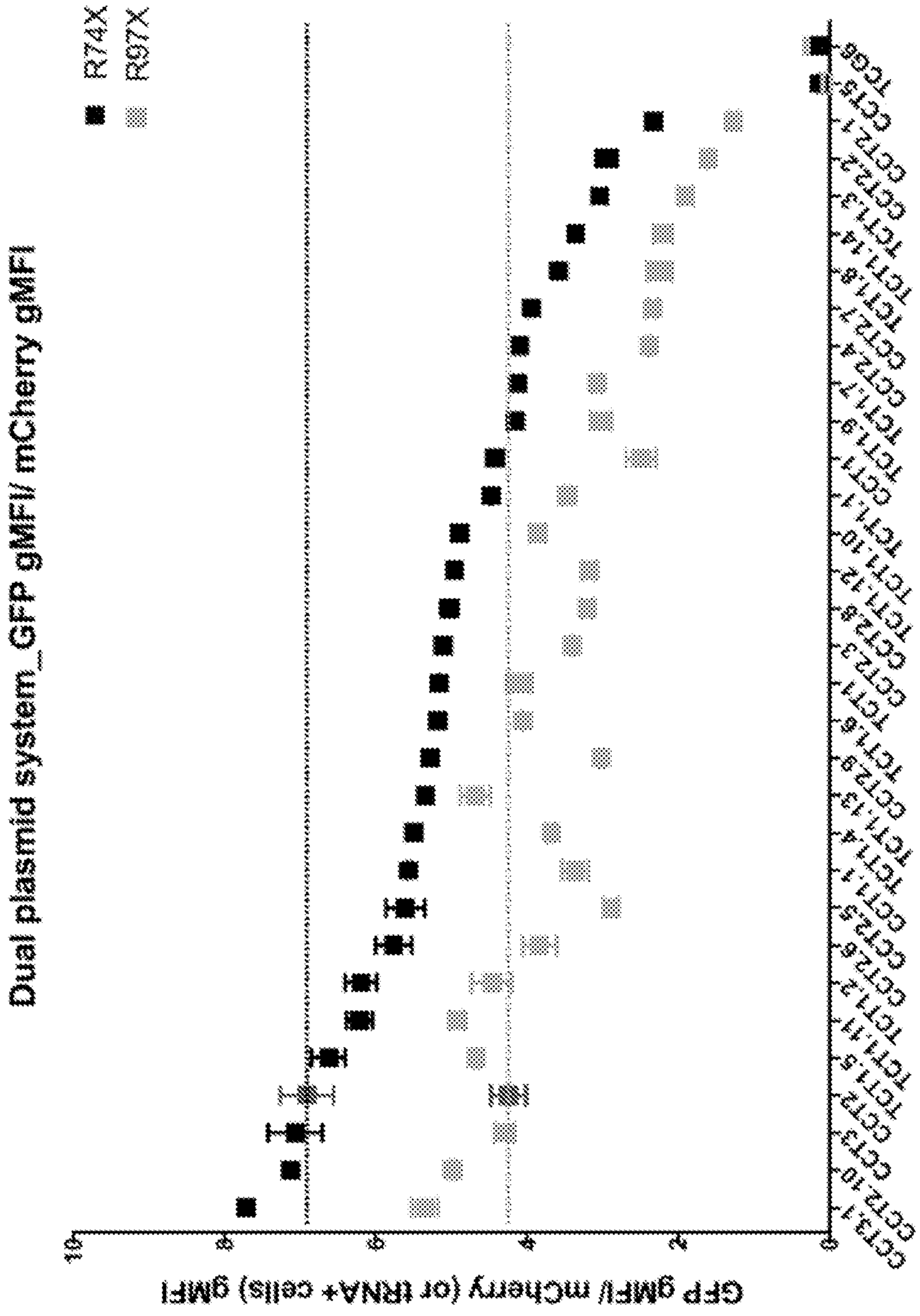

R74X
R97X
Dual plasmid system_GFP gMFI/ mCherry gMFI
GFP gMFI/ mCherry (or tRNA+ cells) gMFI
Arg suppressor tRNAs

FIG. 9A

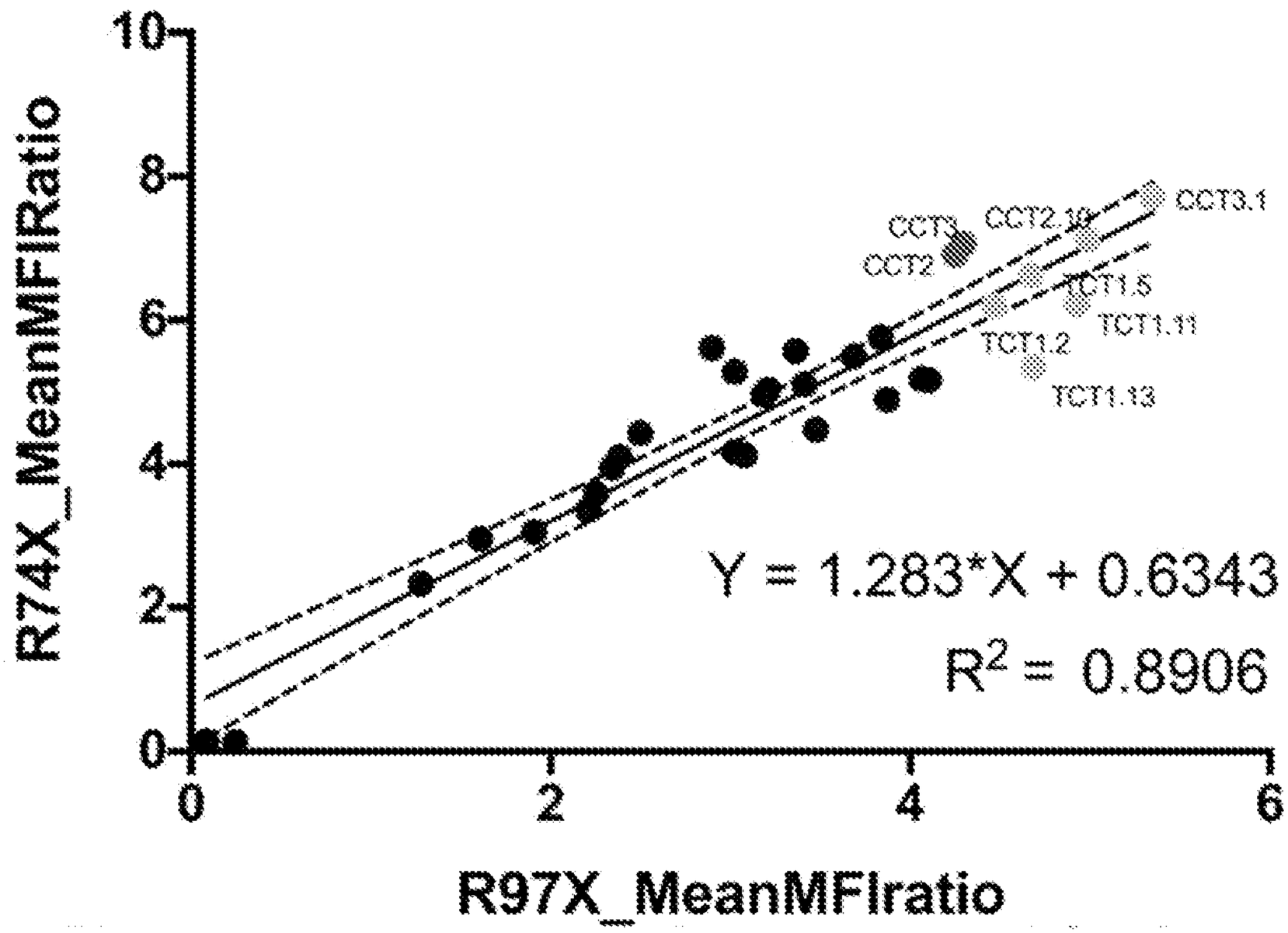

R74X_MeanMFIRatio
10
8
6
4
2
0
0
2
4
6
R97X_MeanMFIratio
CCT3
CCT2.10
CCT3.1
CCT2
TCT1.5
TCT1.11
TCT1.2
TCT1.13
Y = 1.283*X + 0.6343
R2 = 0.8906

FIG. 9B

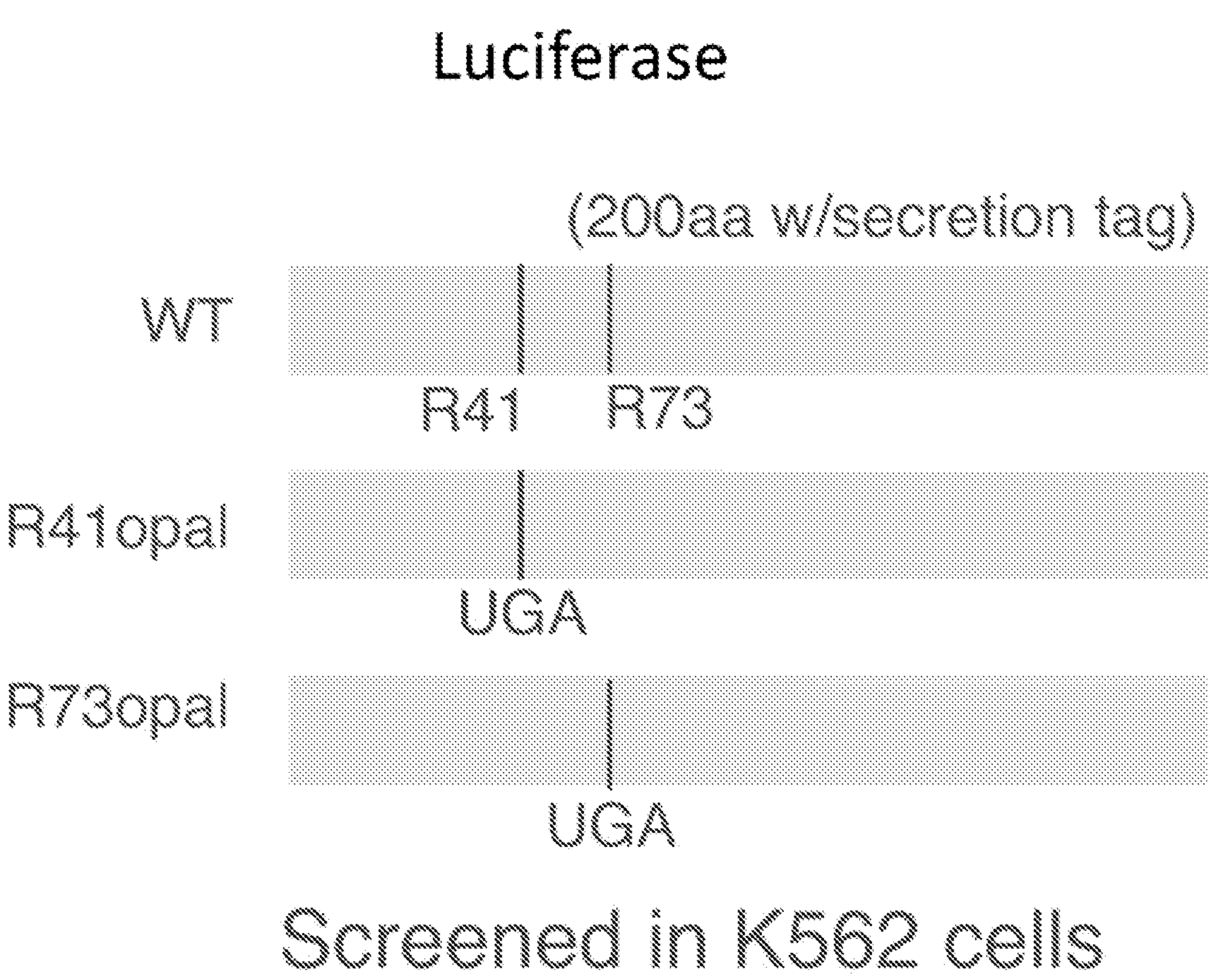

Luciferase
(200aa w/secretion tag)
WT
R41
R73
R41opal
UGA
R73opal
UGA
Screened in K562 cells

FIG. 10A

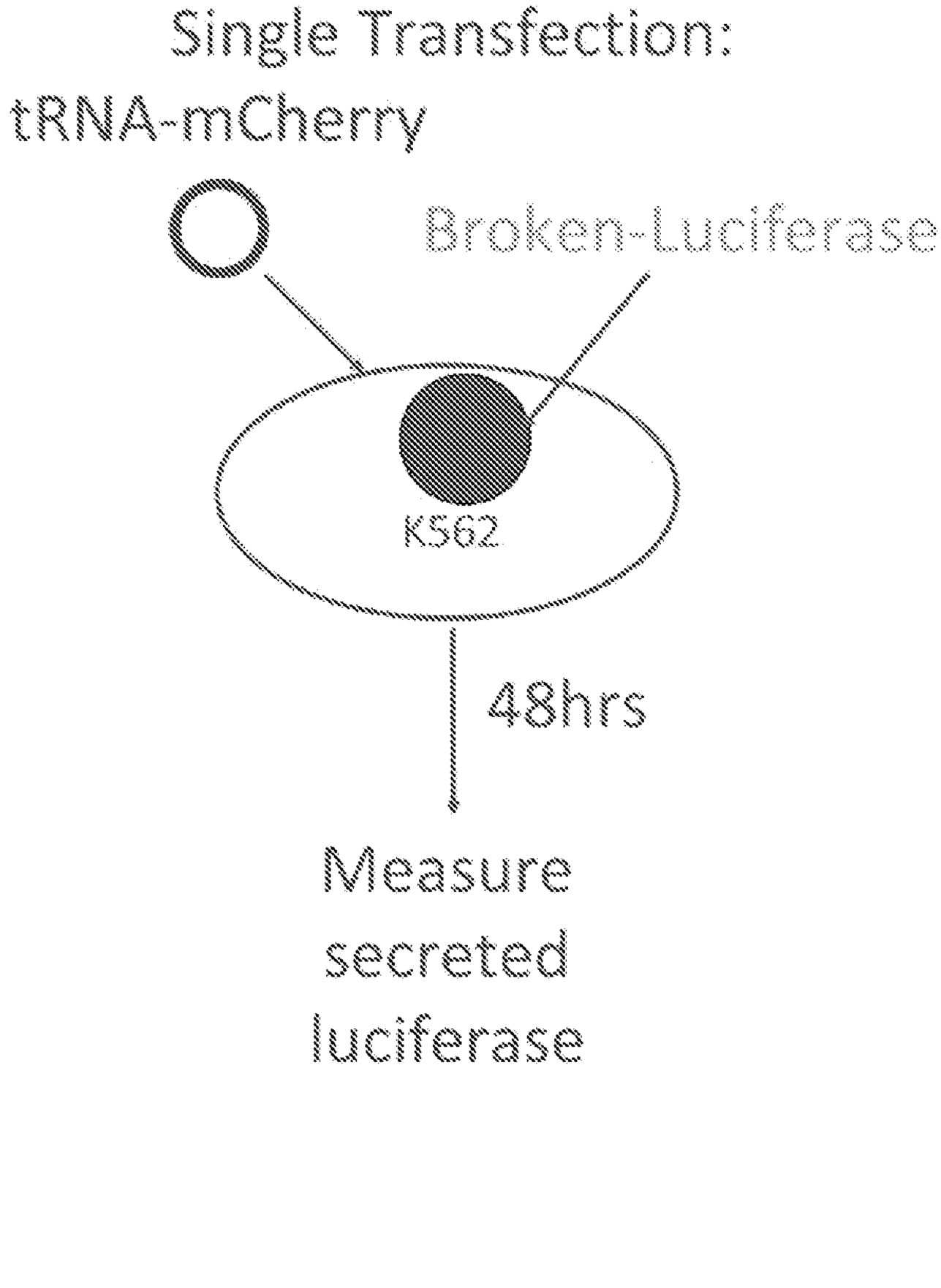

Single Transfection:
tRNA-mCherry
Broken-Luciferase
K562
48hrs
Measure
secreted
luciferase

FIG. 10B

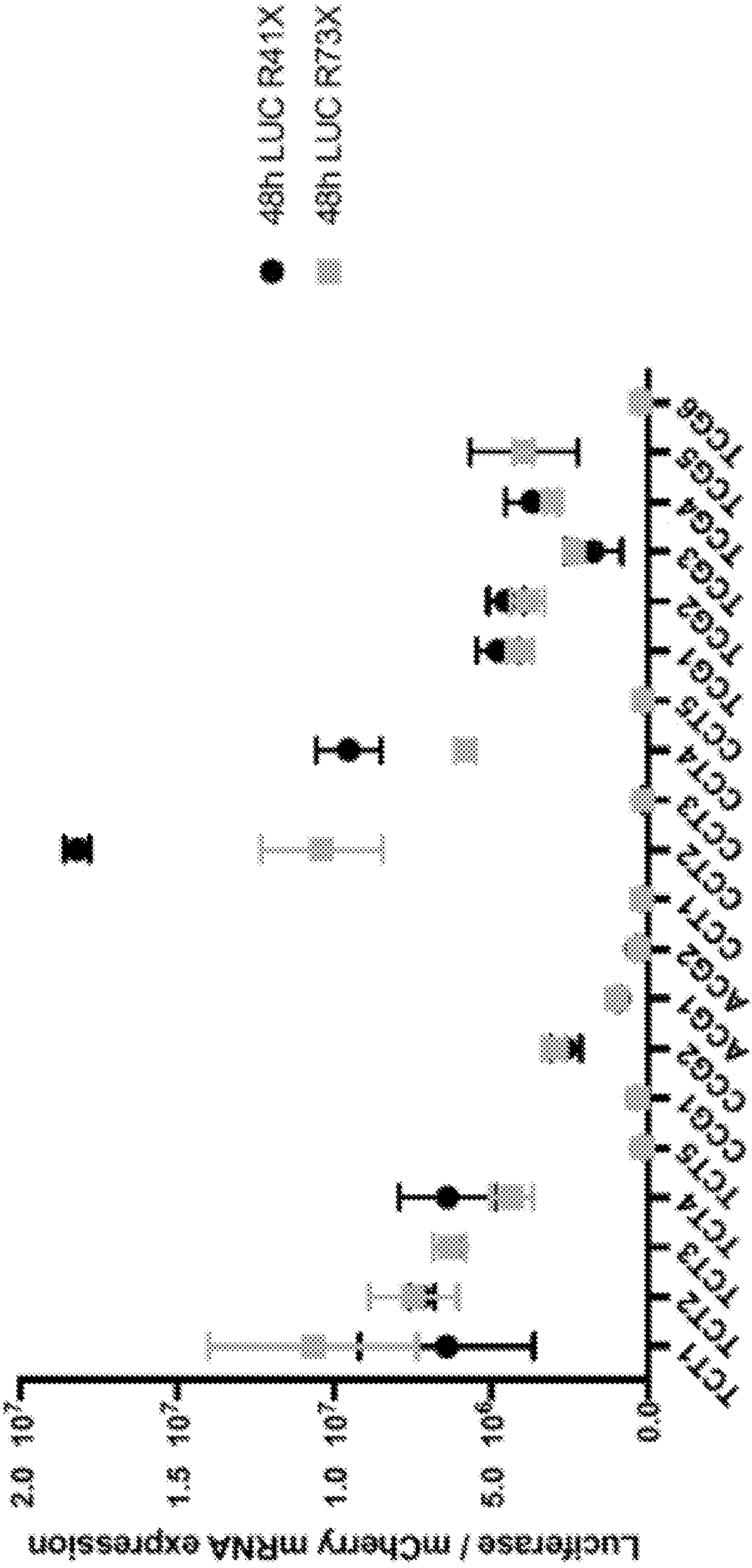

48h LUC R41X
48h LUC R73X
Arginine isodecoders with opal anticodon
TCTA TCTT TCTC TCTG CCTA CCTT CCTC CCTG ACGA ACGT ACGC ACGG TCGA TCGT TCGC TCGG TCTA TCTT TCTC TCTG
Mean +/- SEM; 2 biological replicates per data point
Luciferase / mCherry mRNA expression
2.0 10^7
1.5 10^7
1.0 10^7
5.0 10^6
0.0

FIG. 10C

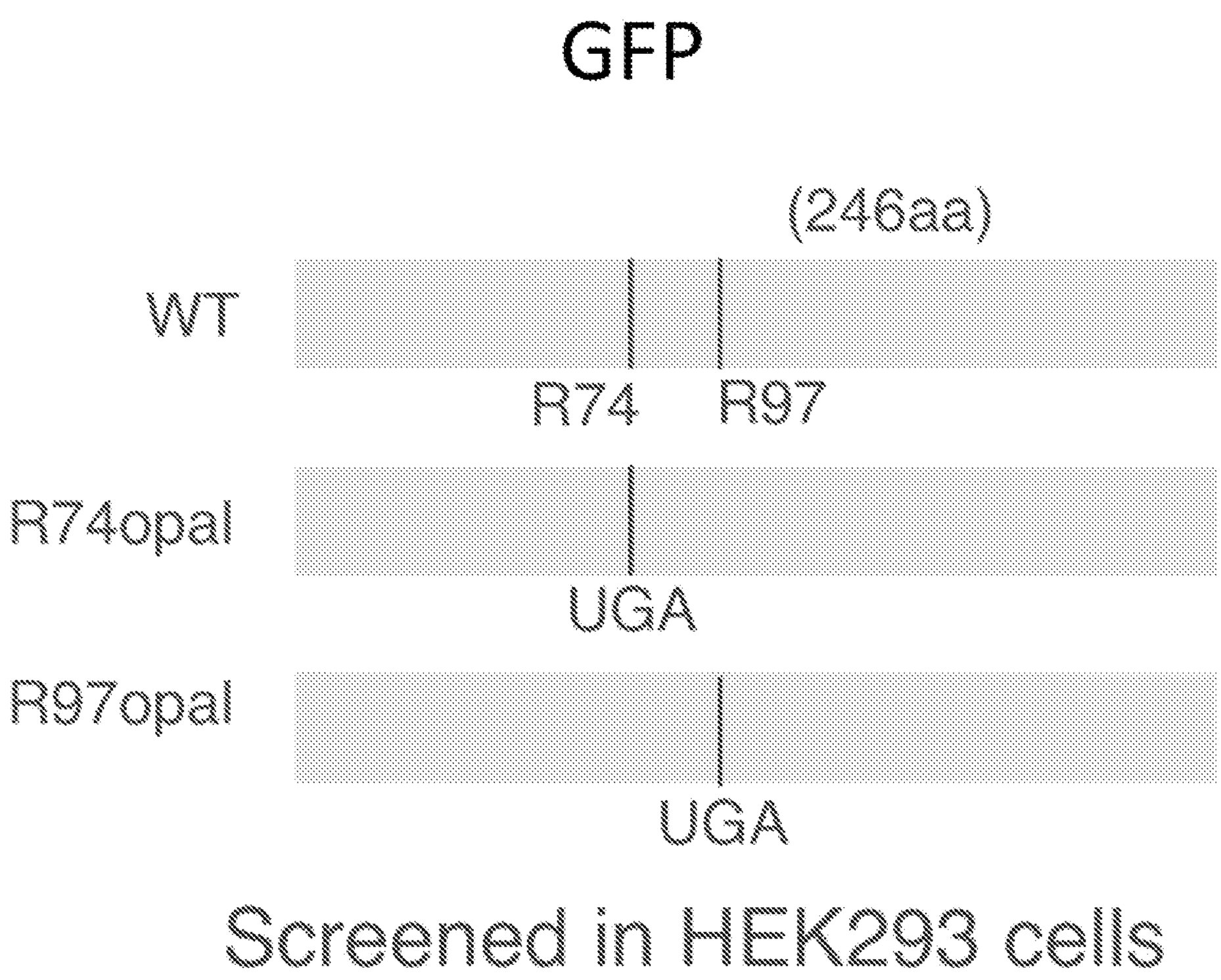

GFP
(246aa)
WT
R74
R97
R74opal
UGA
R97opal
UGA
Screened in HEK293 cells

FIG. 11A

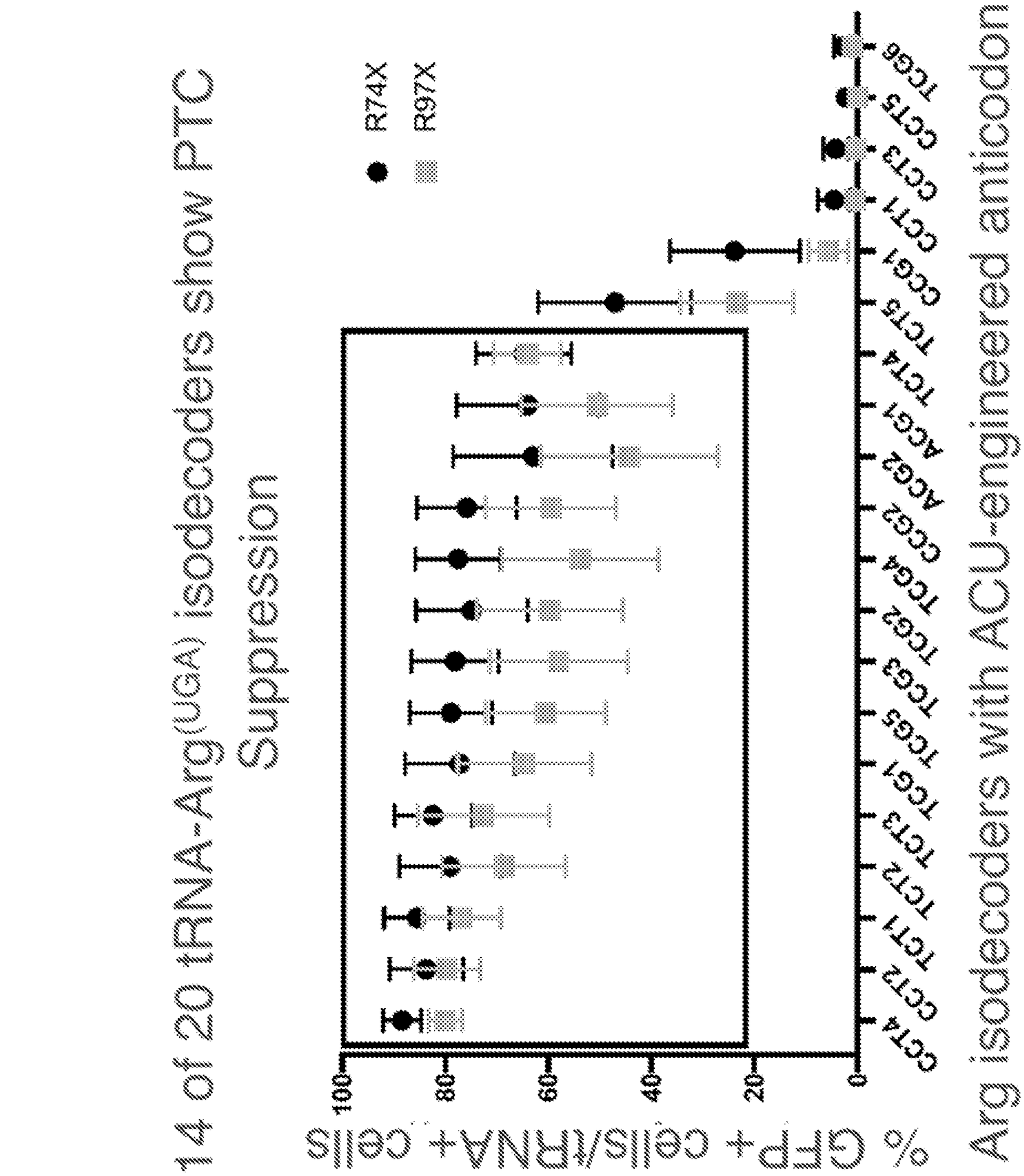

% GFP+ cells/tRNA+ cells
Activity of 20 tRNA-Arg(UGA) isodecoders with ACU-engineered anticodon
Suppression of 20 tRNA-Arg(UGA) isodecoders show PTC suppression
R74X
R97X
0
20
40
60
80
100
CCT4 CCT2 TCT1 TCT2 TCT3 TCG1 TCG5 TCG3 TCG2 TCG4 CCG4
ACG2 ACG1 TCT4 TCT5 CCG1 CCT1 CCT3 CCT5 TCG6

FIG. 11B

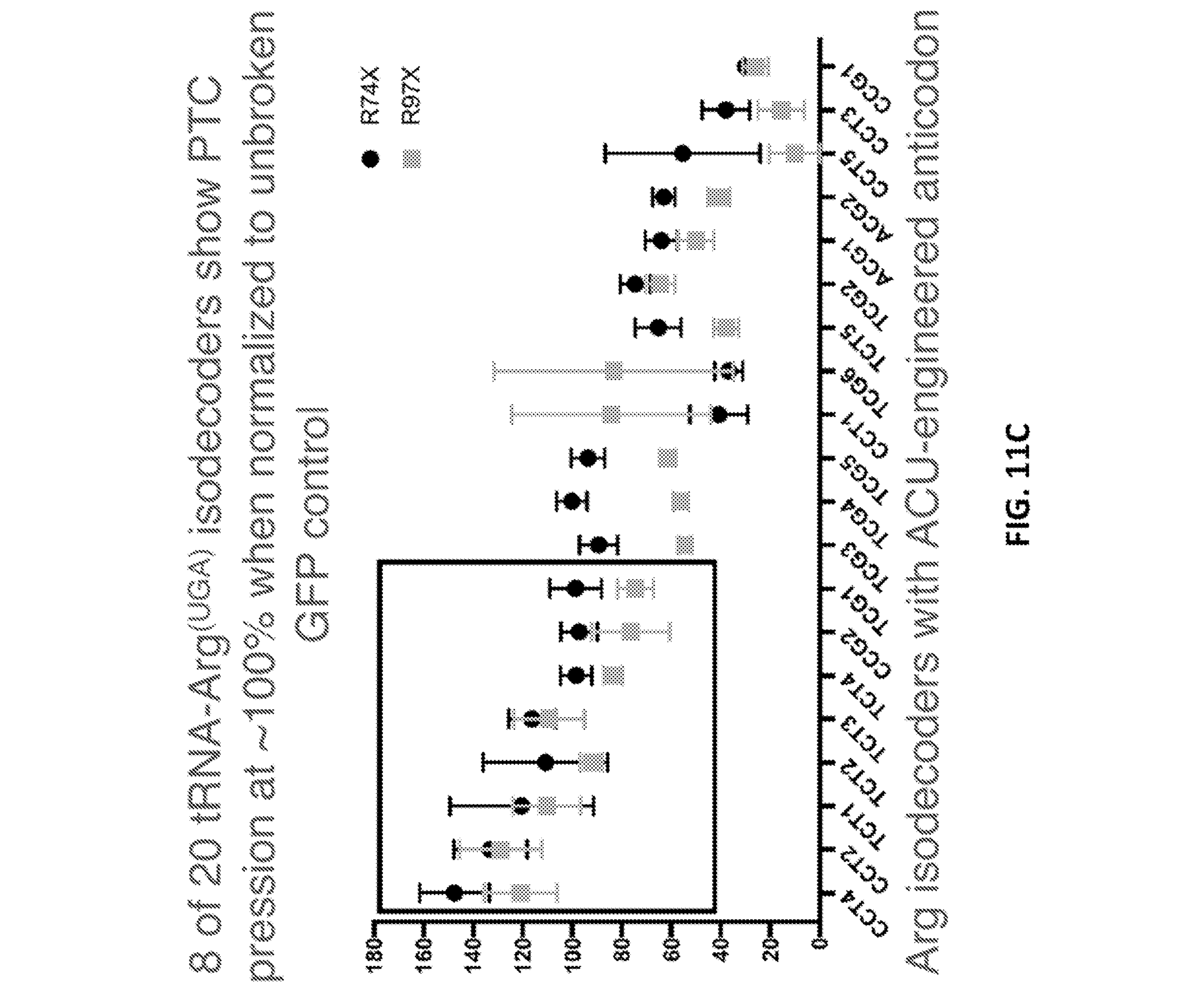

% of PTC Suppression
(GFP MFI normalized to unbroken GFP)
GFP control
R74X
R97X
Arg isodecoders with ACU-engineered anticodons
CCA CCG CCT TCA TCG TCT ACA ACG ACT TCA TCG TCT CCA CCG CCT
0
20
40
60
80
100
120
140
160
180

FIG. 11C

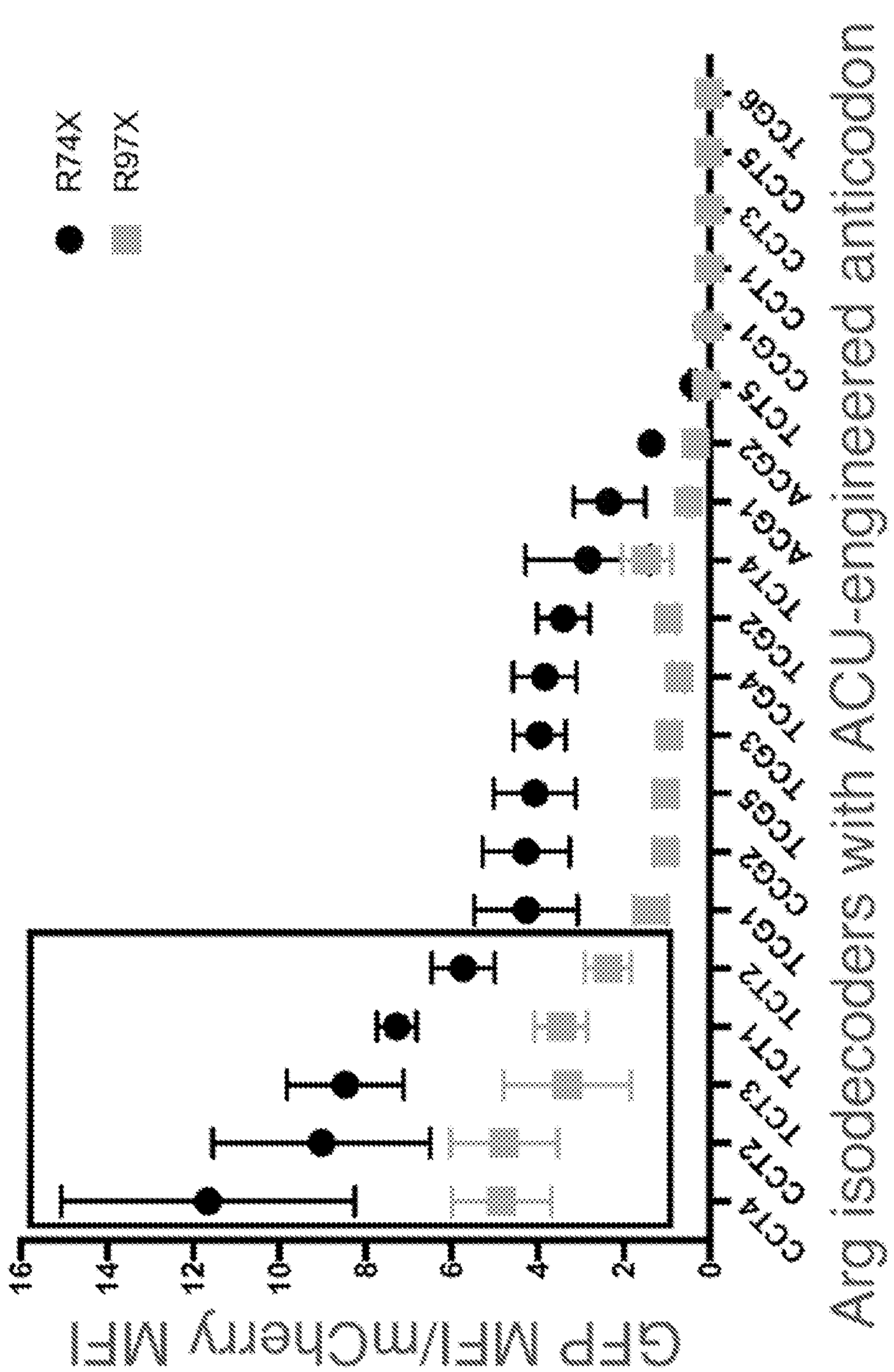

GFP MFI/mCherry MFI
0
2
4
6
8
10
12
14
16
CCA CCT2 TCT3 TCT4 TCG1 TCG2 TCG3 TCG4 ACG1 ACG2 TCT5 CCG1 CCT1 CCT3 CCT5 TCG6
Anticodon
R74X
R97X

FIG. 11D


5000
amp prom
Kan
Left ITR
U6 Promoter
1000
mU6
CMV enhancer
2000
GFP
miscellaneous
Right ITR
lac prom
3000
4000
ST0021 hU6_0GluR2 GG_mU6_CMV GFP sv40
5266 bp

FIG. 11E

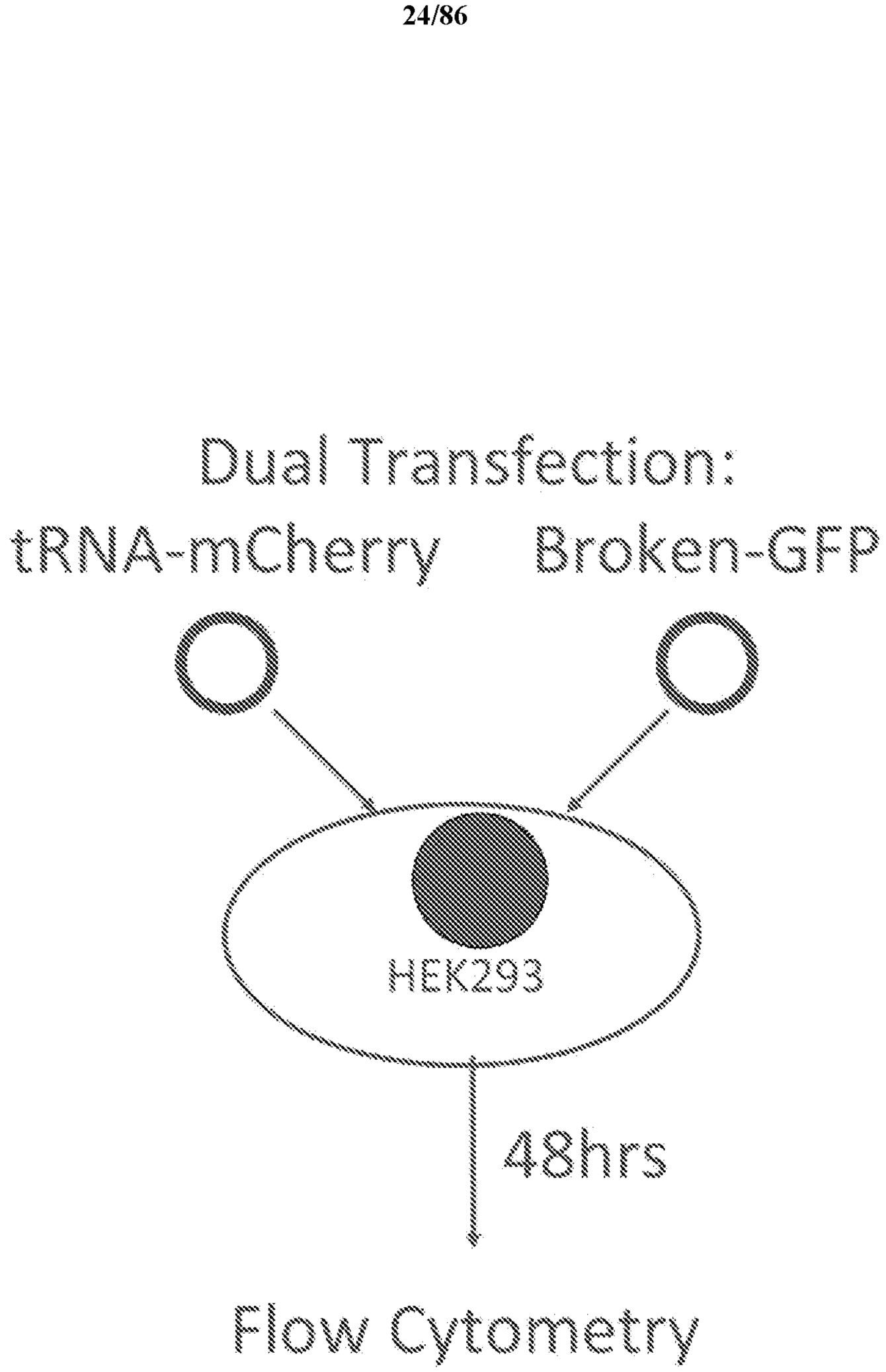

Dual Transfection:
tRNA-mCherry
Broken-GFP
HEK293
48hrs
Flow Cytometry

FIG. 11F

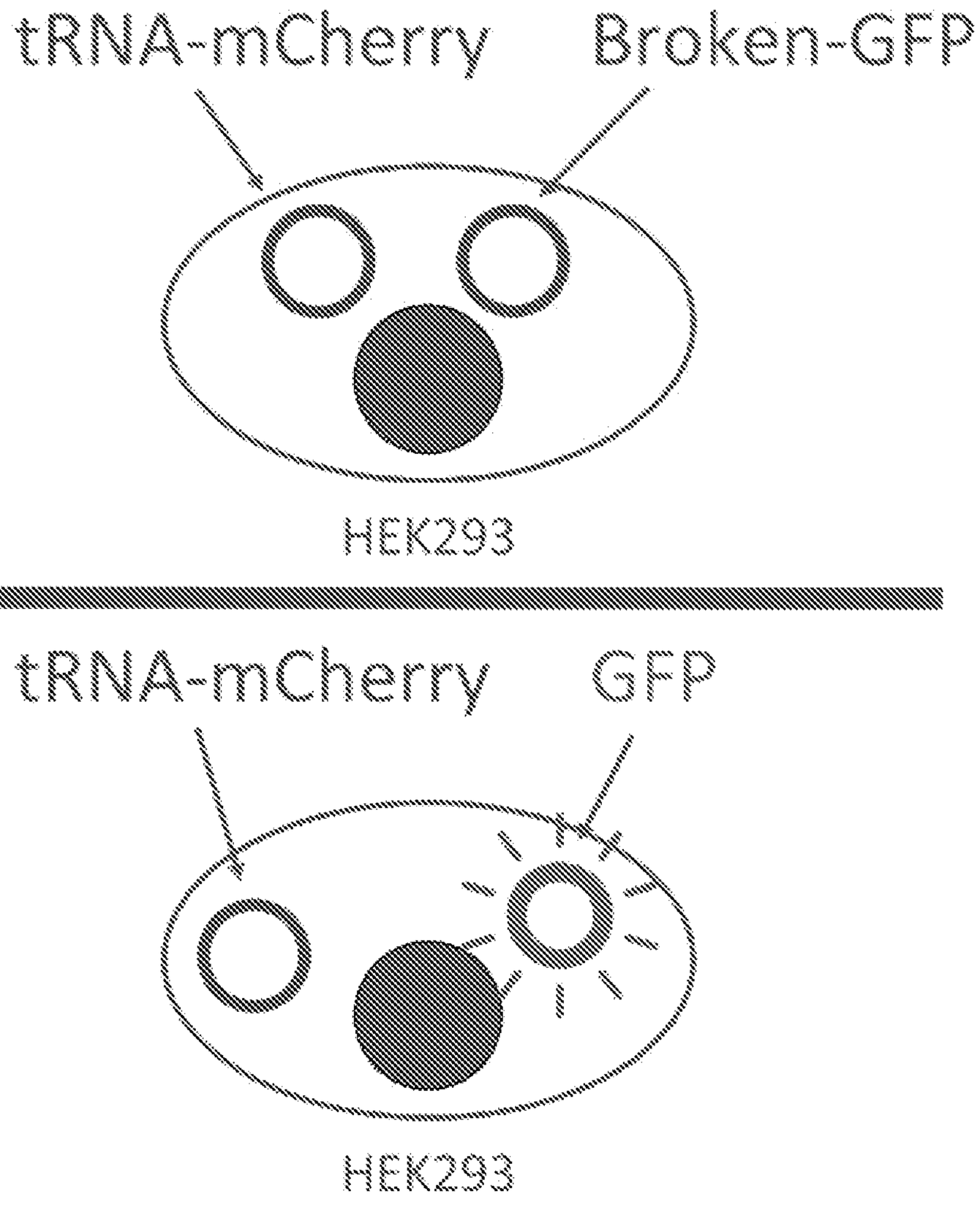


FIG. 11G

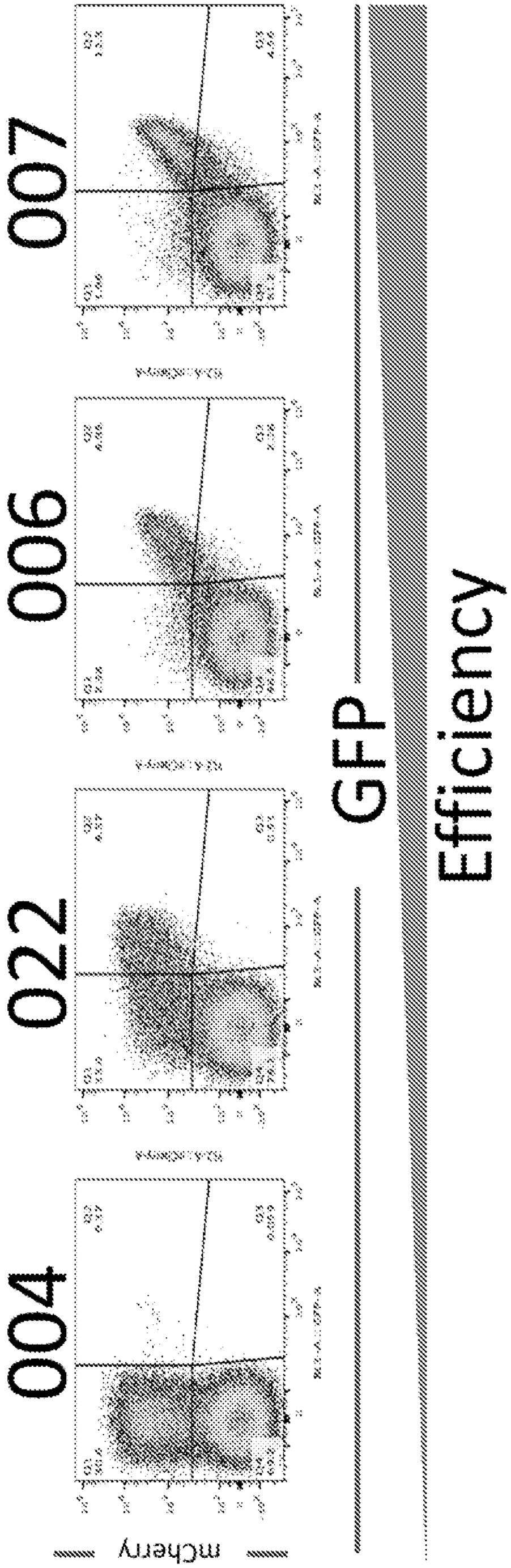


FIG. 12

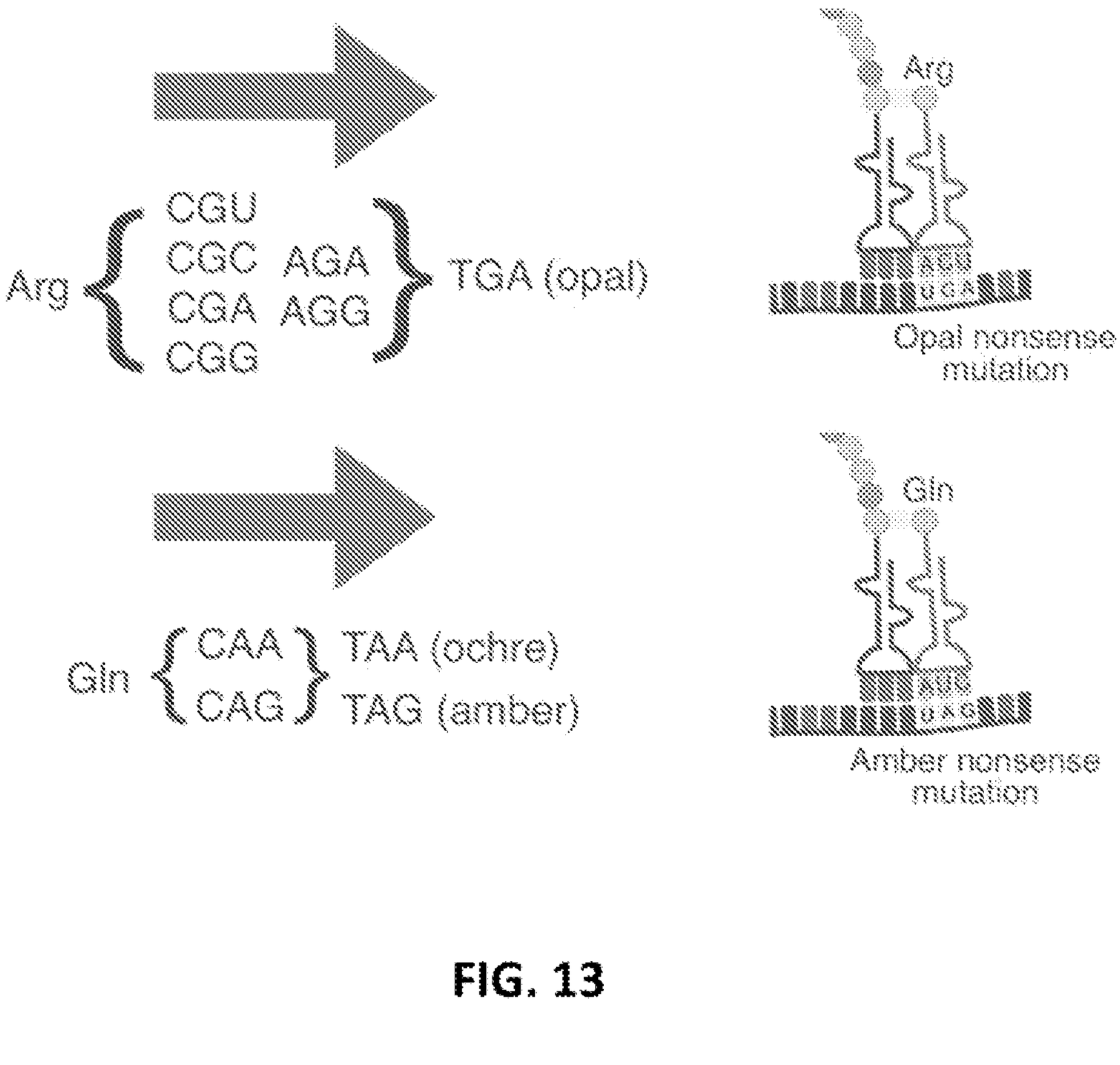

Arg
CGU
CGC
CGA
CGG
AGA
AGG
TGA (opal)
Arg
U G A
Opal nonsense mutation
Gln
CAA
CAG
TAA (ochre)
TAG (amber)
Gln
U A G
Amber nonsense mutation

FIG. 13

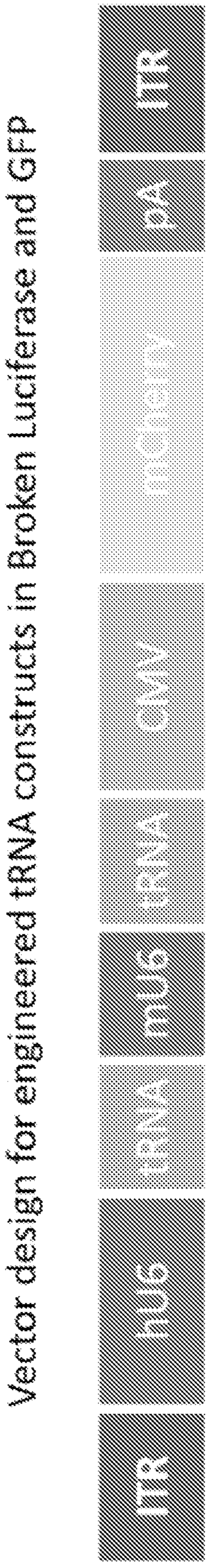


FIG. 14A

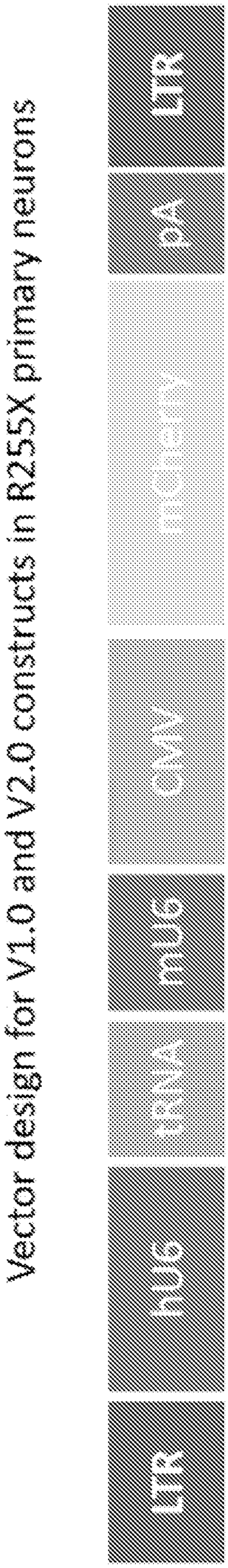


FIG. 14B

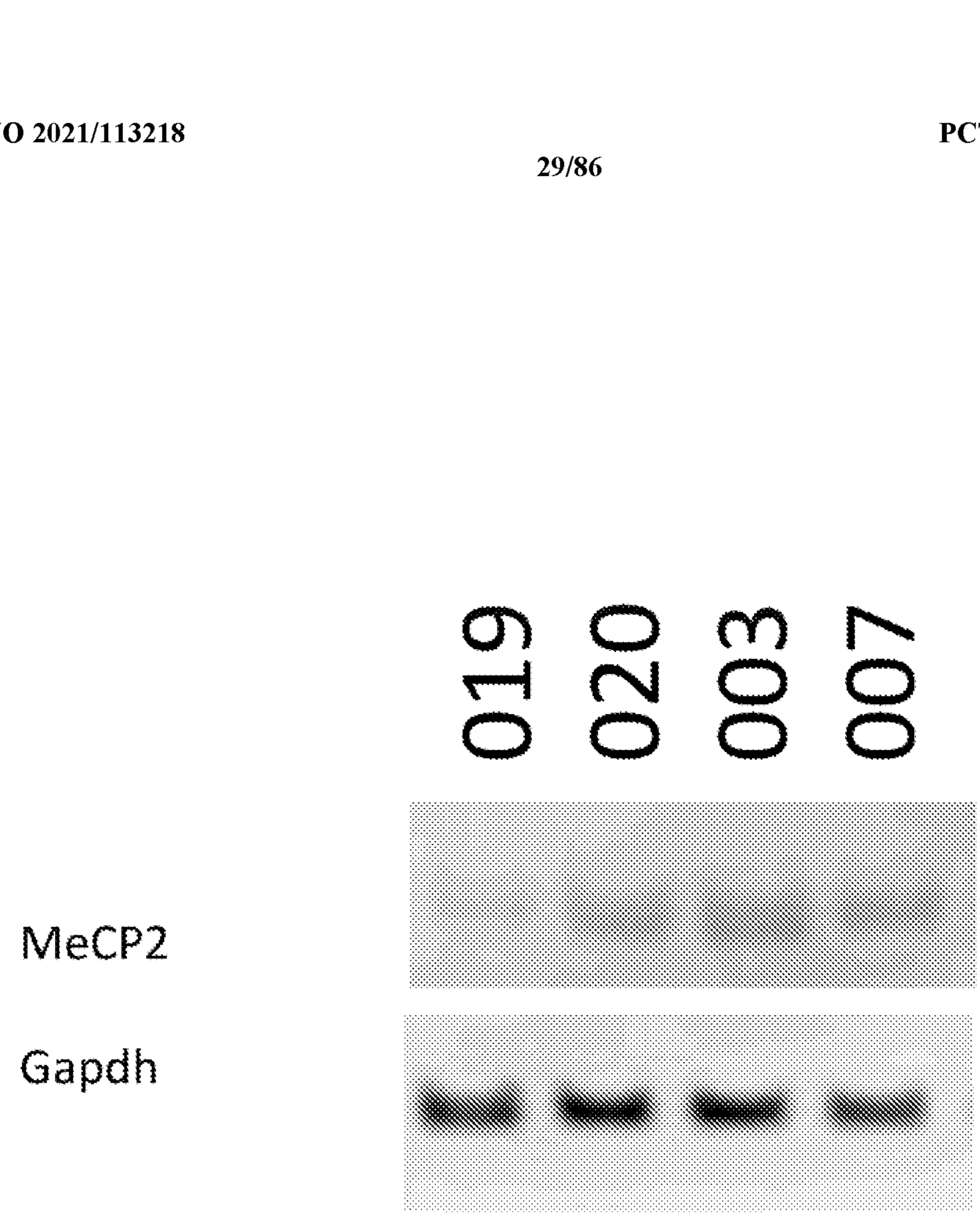


FIG. 15A

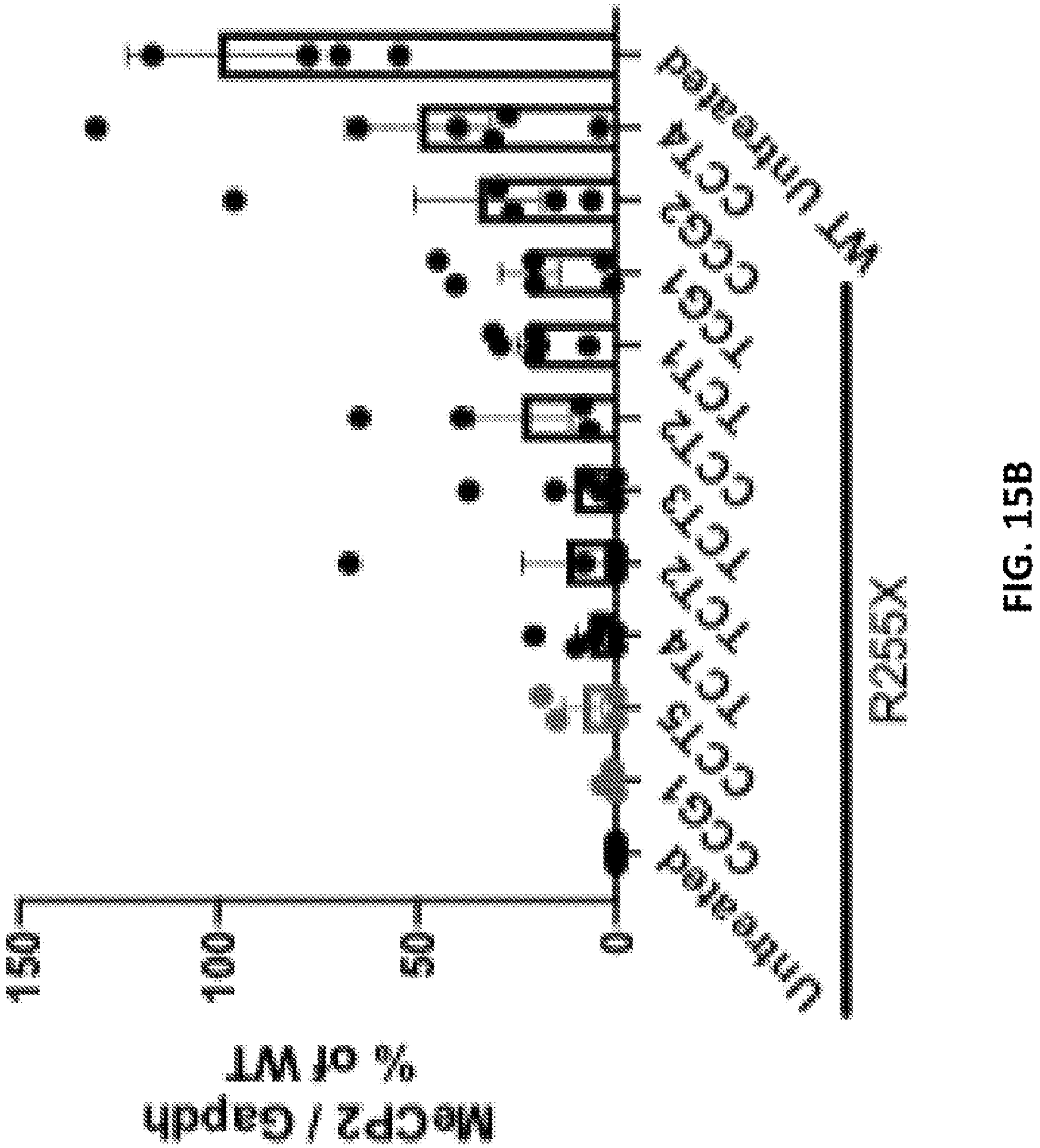

Rett Patient Mut
R133C
R133H
R294X
R270X
R255X
R168X
R106W
R306C
R306H
R168X
R255X
R270X
R294X
R133H
R133C
R306C
Patient Mut
0
0.5
1.0
1.5
% of WT
MeCP2 / Gapdh

FIG. 15B

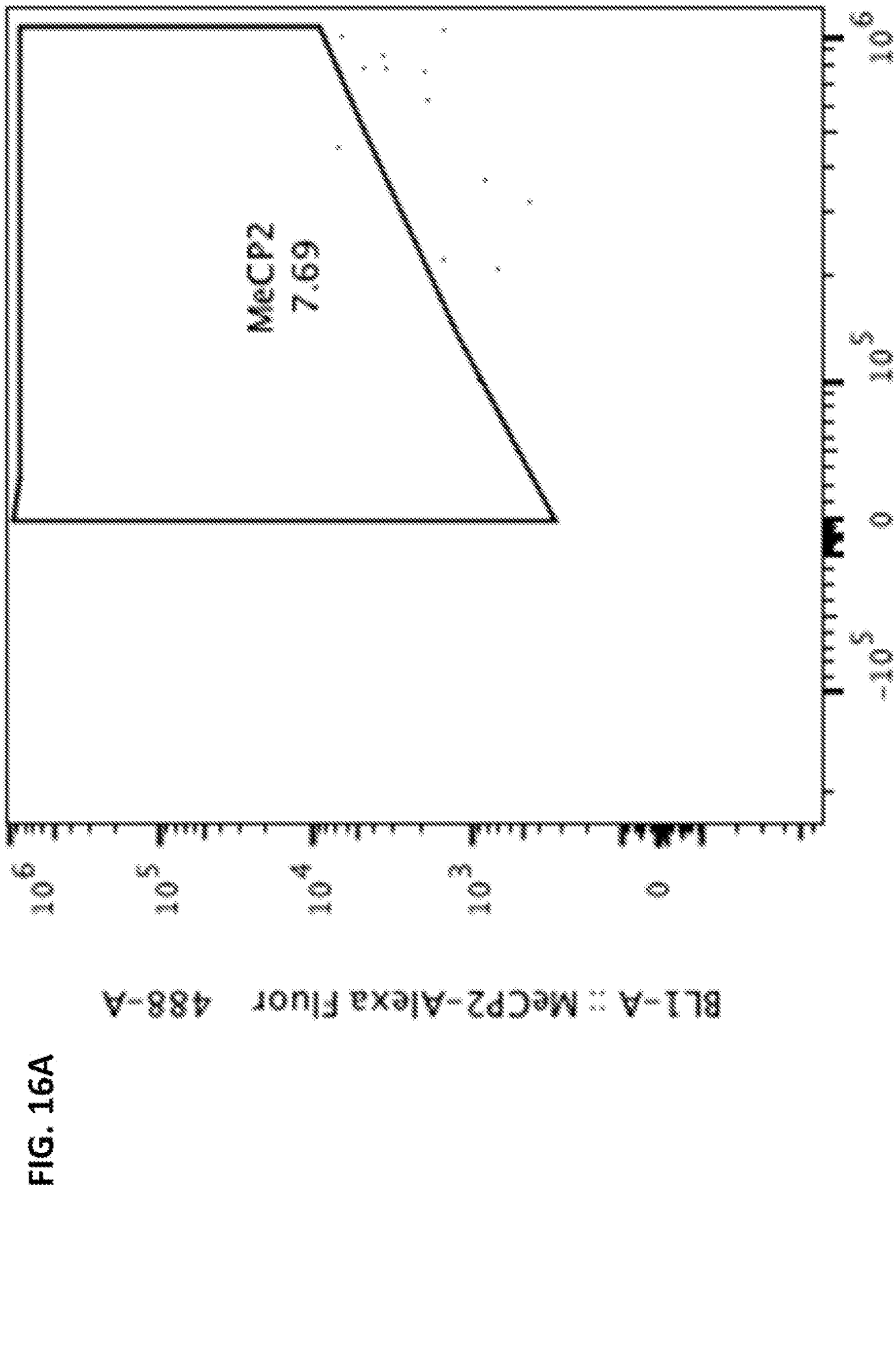
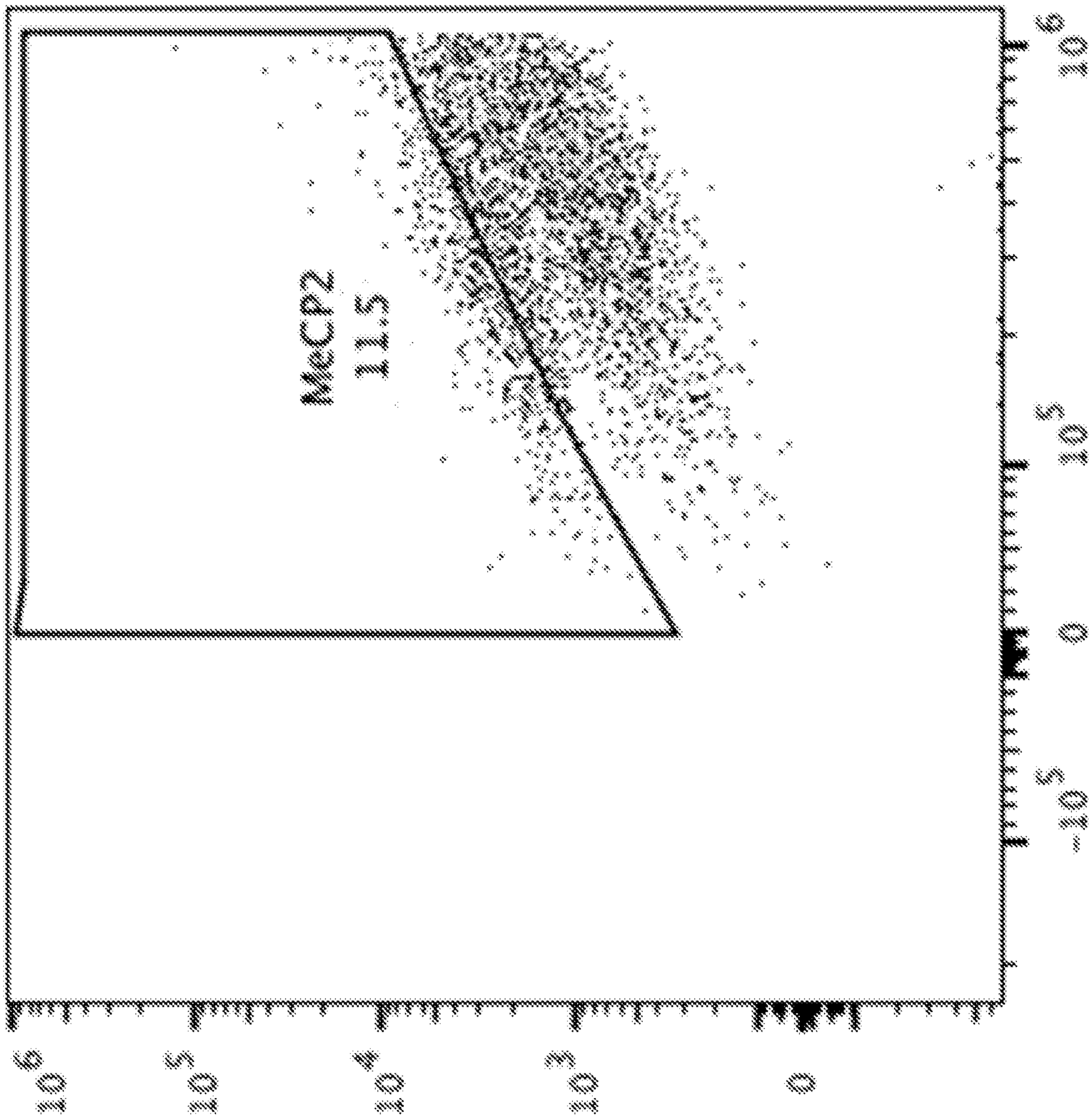

MeCP2
7.69
BL1-A :: MeCP2-Alexa Fluor 488-A
0
2e+4
4e+4
6e+4
8e+4
1e+5
0
2e+4
4e+4
6e+4
8e+4
1e+5

FIG. 16A



BL1-A :: MeCP2-Alexa Fluor 488-A

FIG. 16B

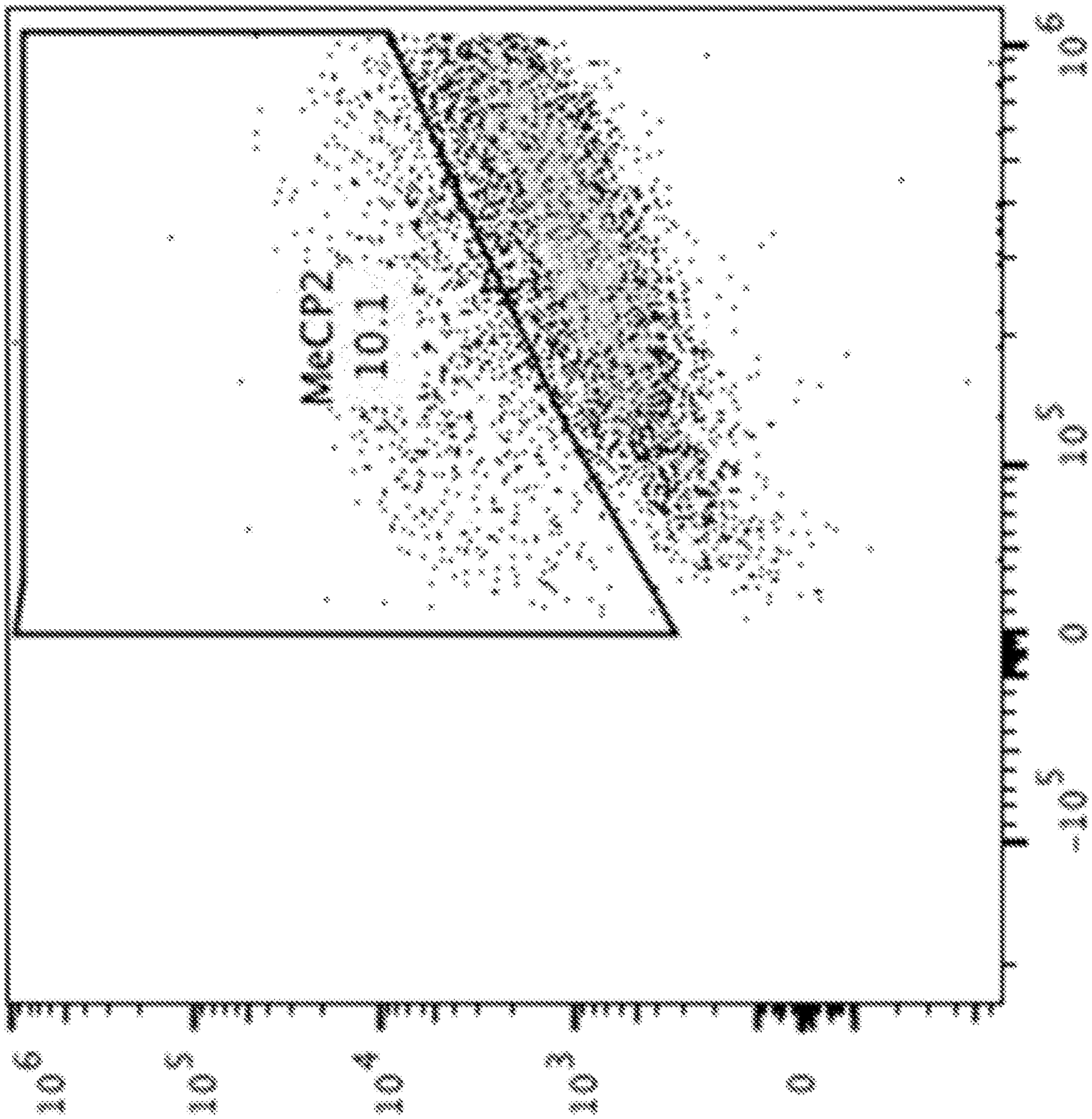

MeCP2
BL1-A :: MeCP2-Alexa Fluor 488-A
0
5e1
1e2
1.5e2
2e2
2.5e2
0
5e1
1e2
1.5e2
2e2
2.5e2

FIG. 16C

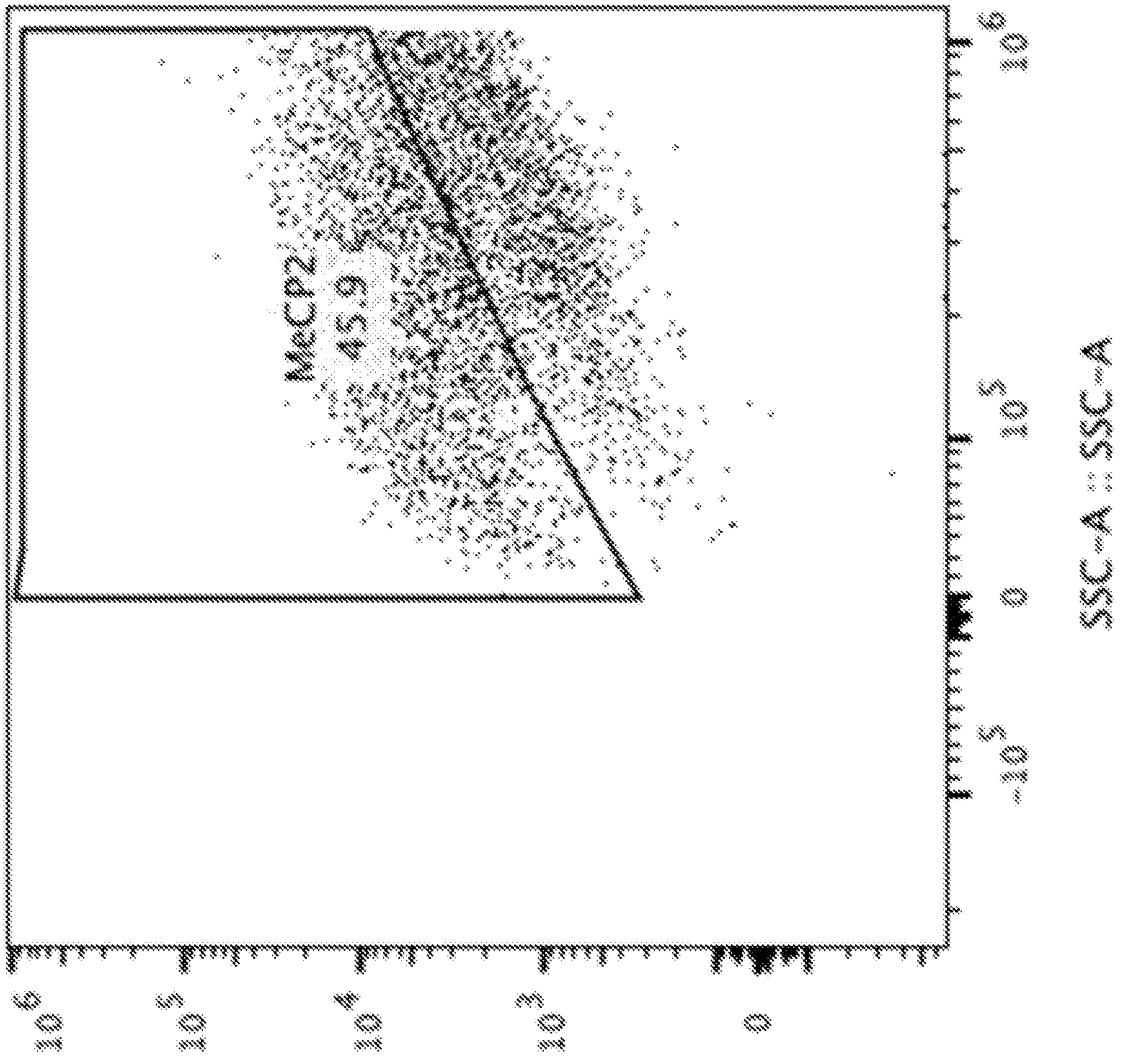


FIG. 16D

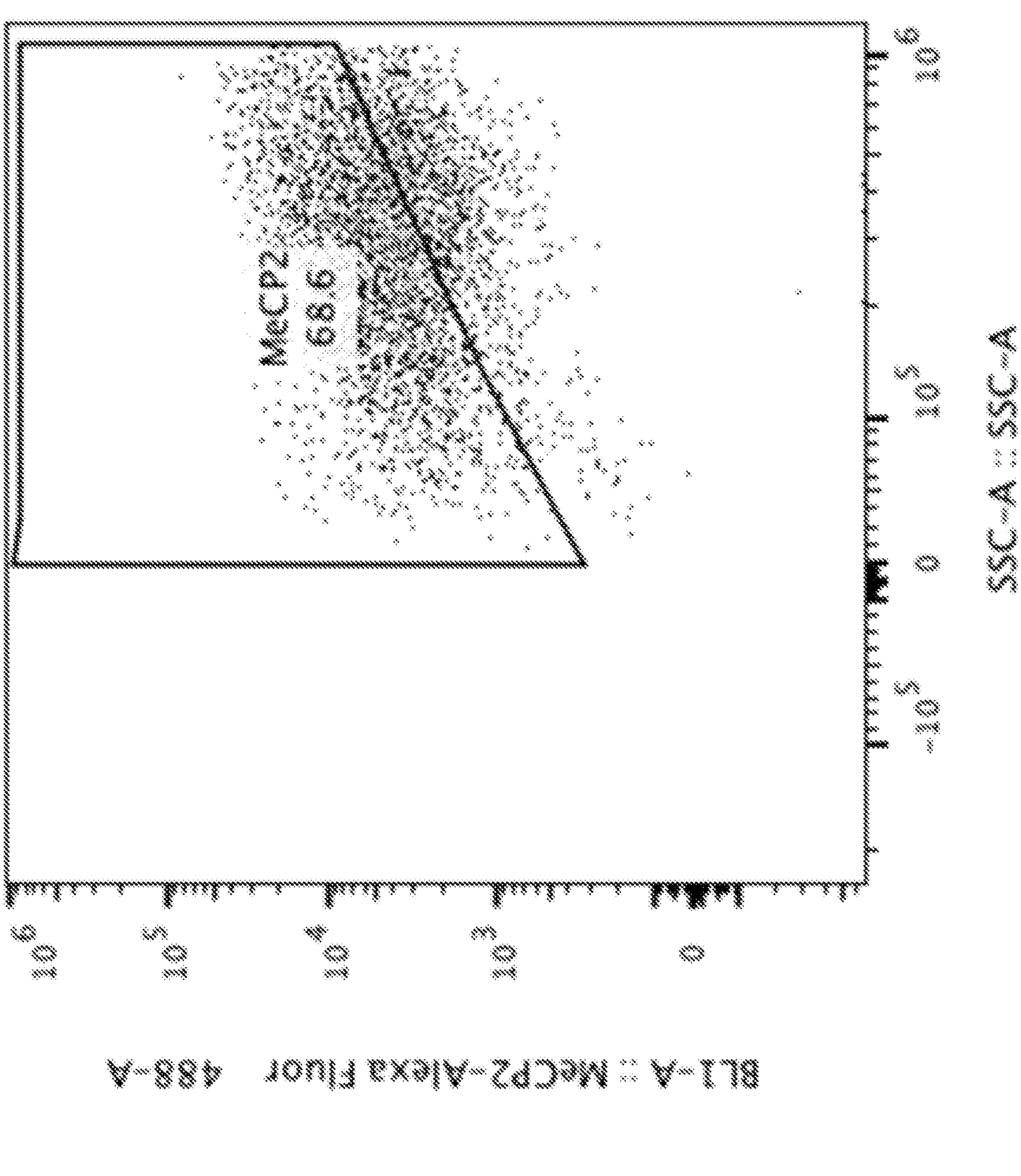

MeCP2
68.6
BL1-A :: MeCP2-Alexa Fluor 488-A
SSC-A :: SSC-A
0
5e1
1e2
0
1e3
2e3
3e3
4e3
5e3

FIG. 16E

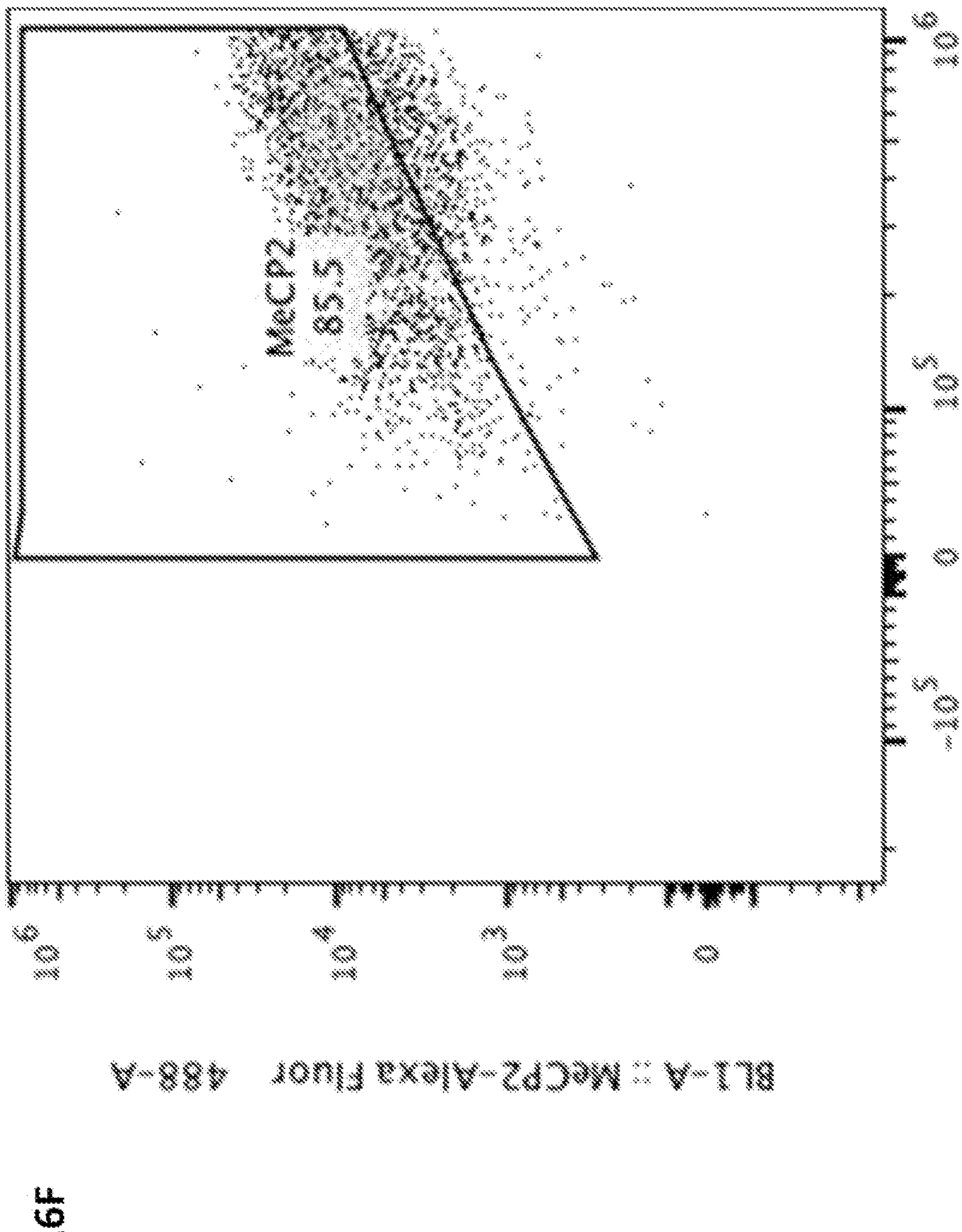

MeCP2 65 5
BL1-A :: MeCP2-Alexa fluor 488-A
SSC-A :: SSC-A
0
5e3
1e4
1.5e4
2e4
2.5e4
0
5e3
1e4
1.5e4
2e4

FIG. 16F

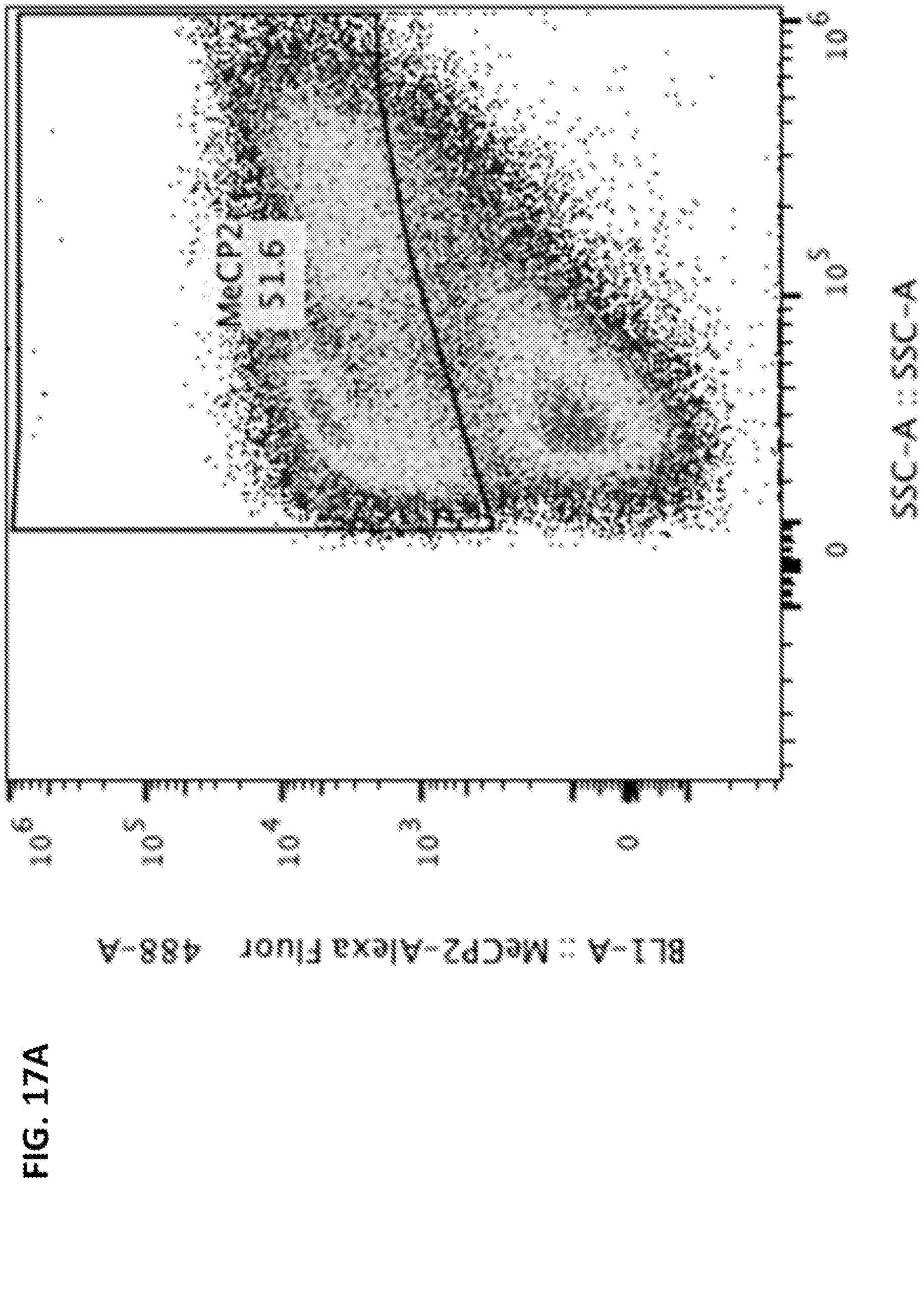

SSC-A :: SSC-A
0
10^5
2×10^5
3×10^5
4×10^5
5×10^5
MeCP2-Alexa fluor 488-A
BL1-A :: MeCP2-Alexa fluor 488-A
S19

FIG. 17A

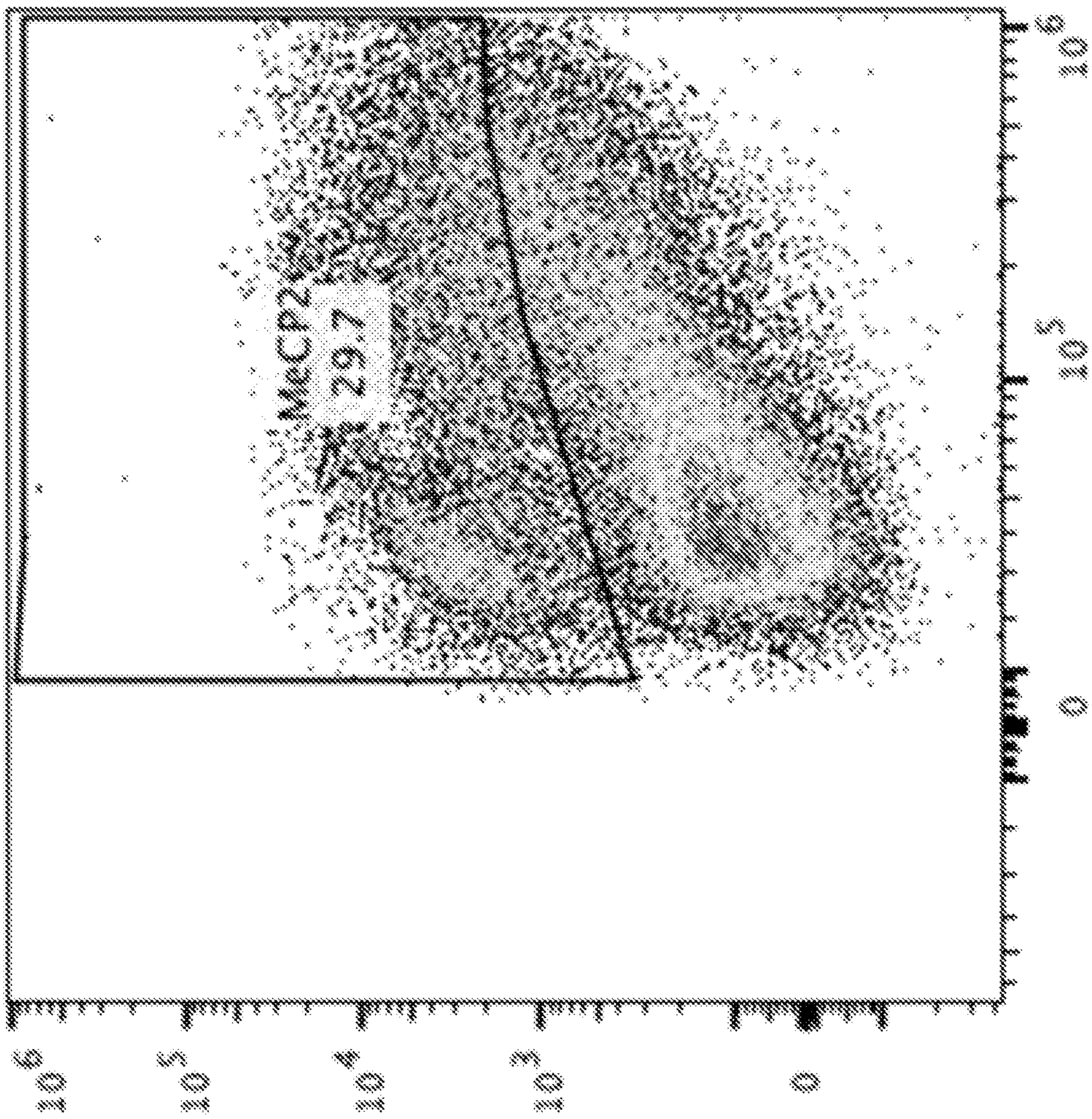

SSC-A :: SSC-A
0
5e1
1e2
BL1-A :: MeCP2-Alexa fluor 488-A
0
2e4
4e4
6e4
8e4
MeCP2 29.7

FIG. 17B

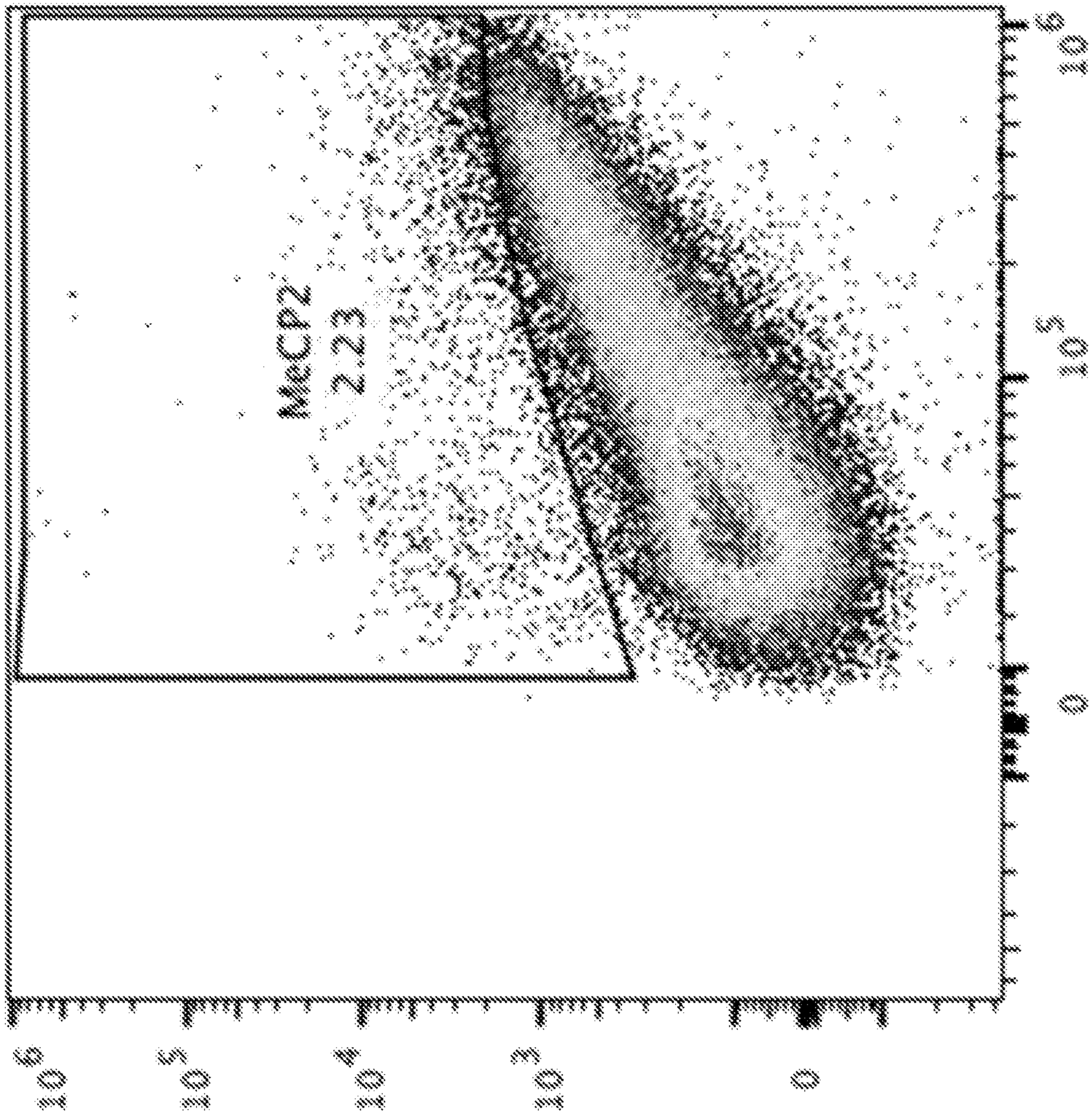

MeCP2
223
SSC-A :: SSC-A
0
5e1
1e2
1.5e2
2e2
2.5e2
BL1-A :: MeCP2-Alexa Fluor 488-A

FIG. 17C

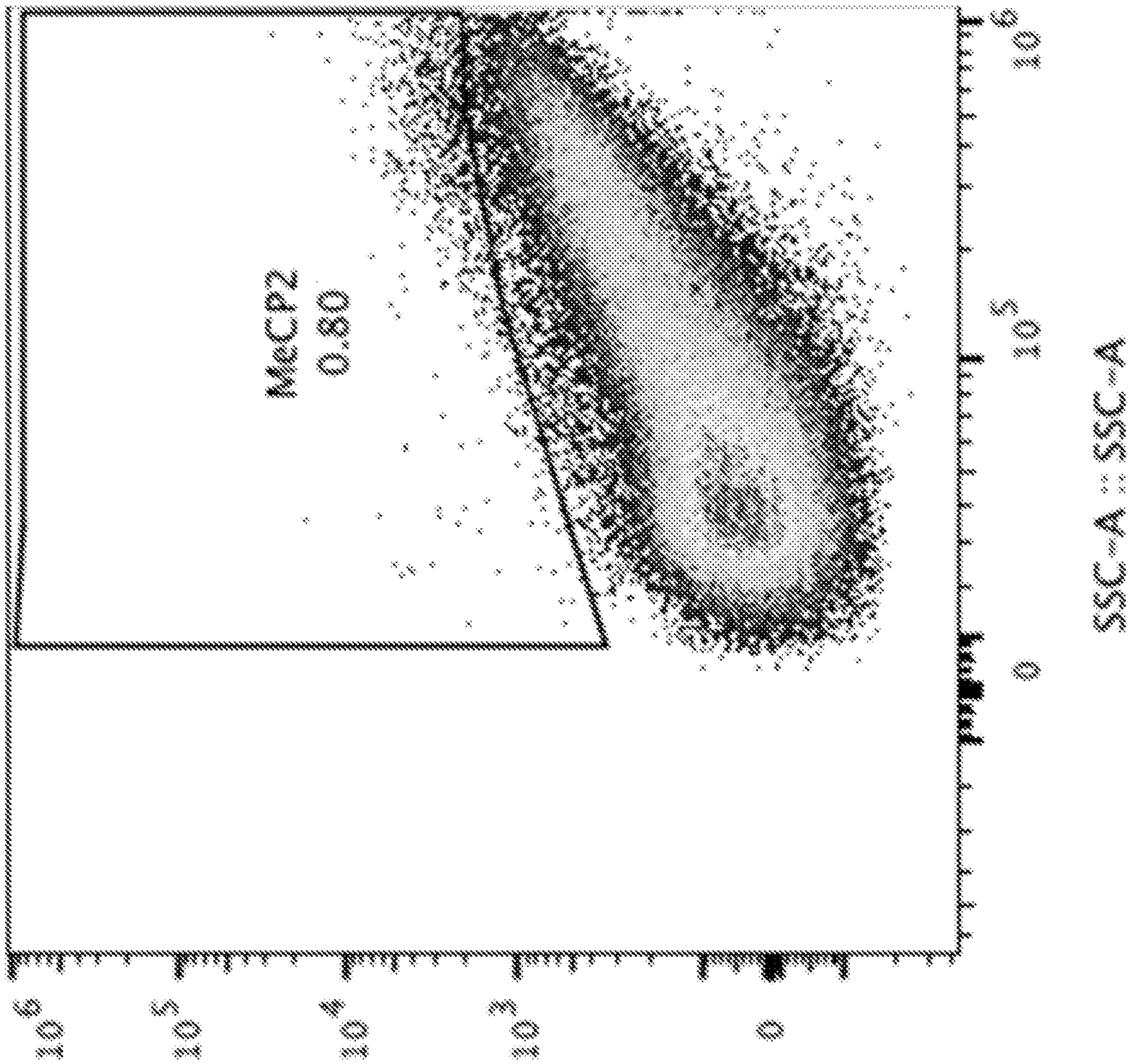

MeCP2
0.80
SSC-A :: SSC-A
0
5 0 5
BL1-A :: MeCP2-Alexa fluor 488-A
0
2 e+5
4 e+5
6 e+5
8 e+5

FIG. 17D

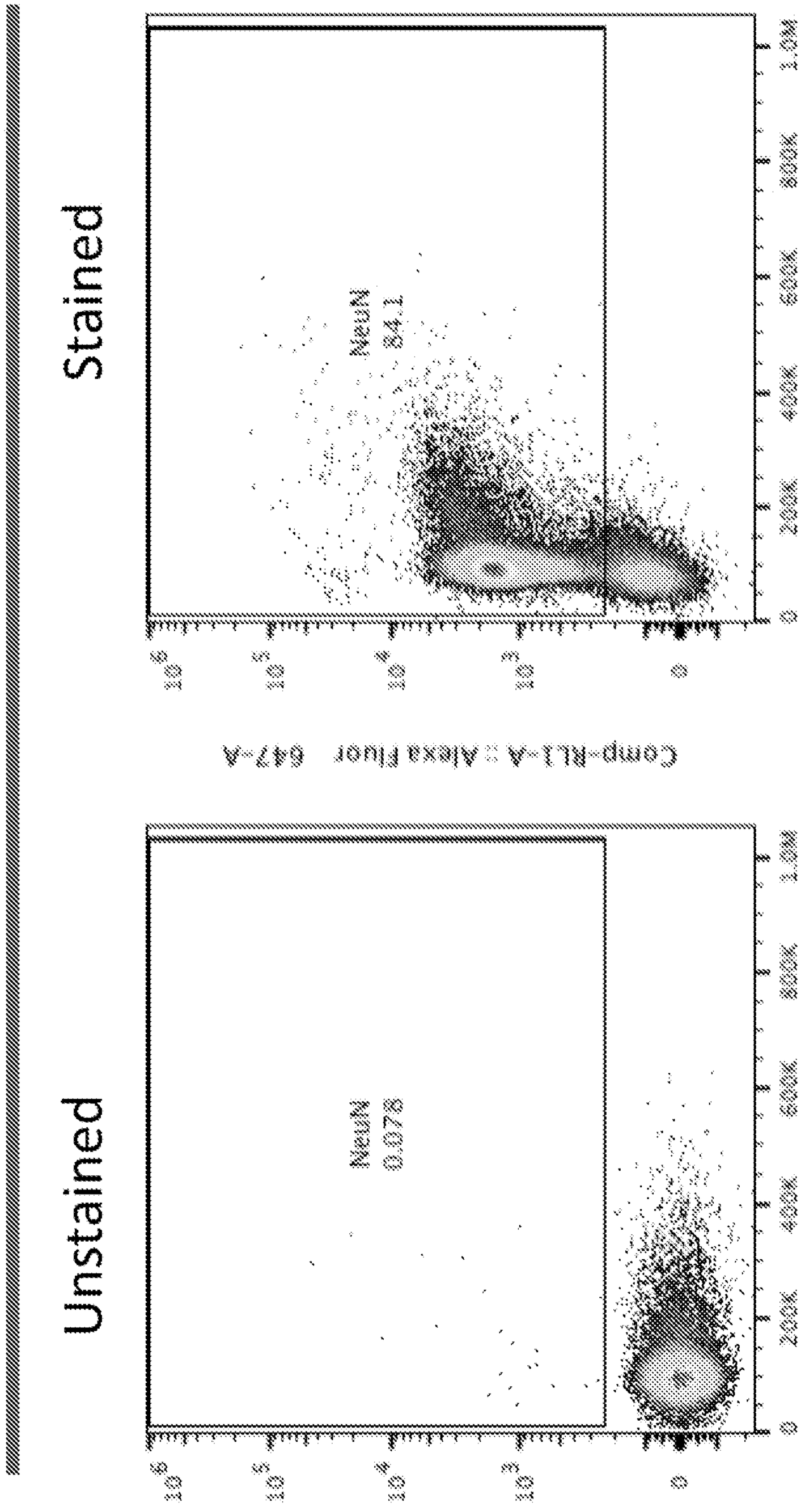


FIG. 17F

FIG. 17E

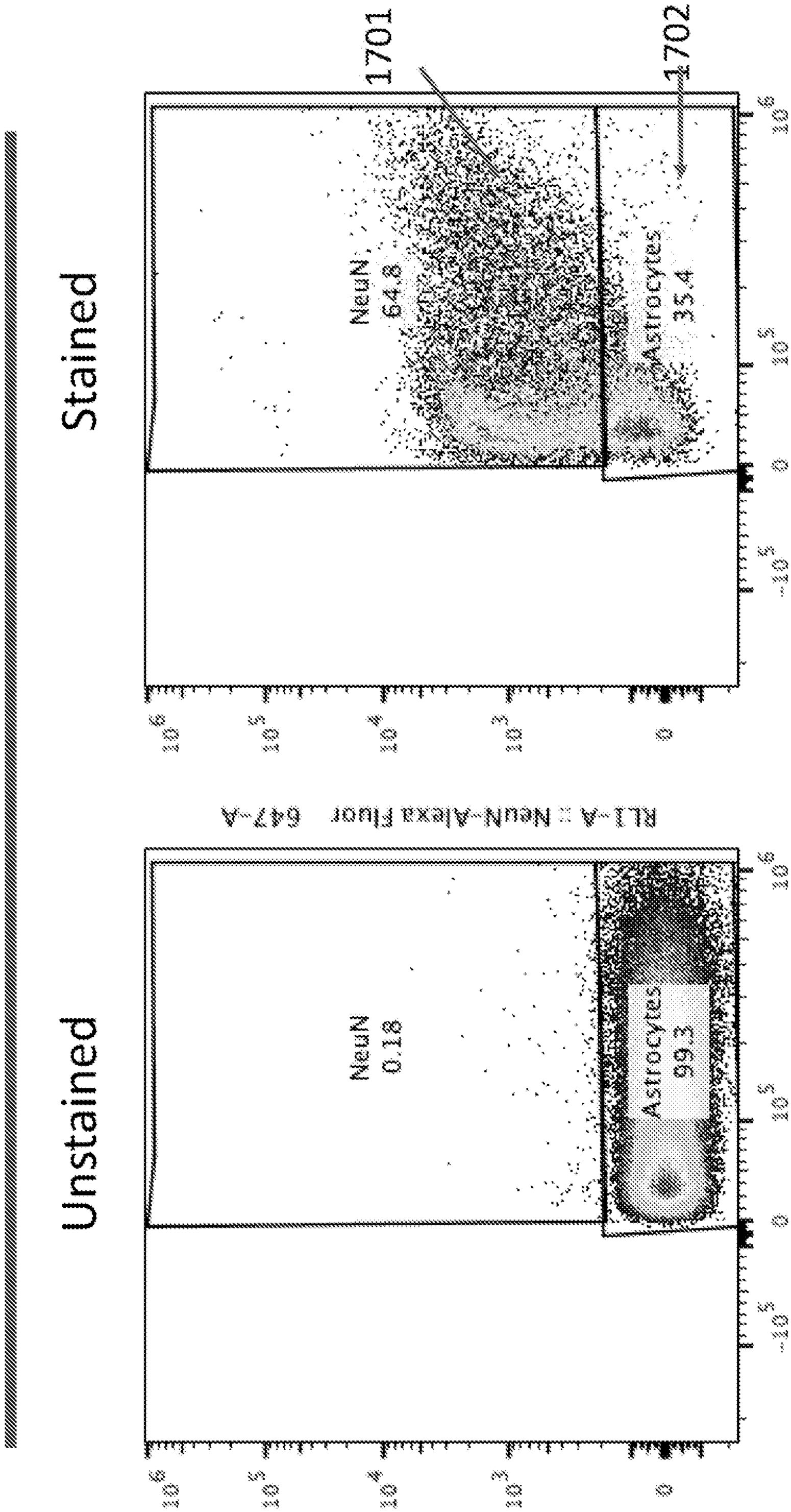


FIG. 17H

FIG. 17G

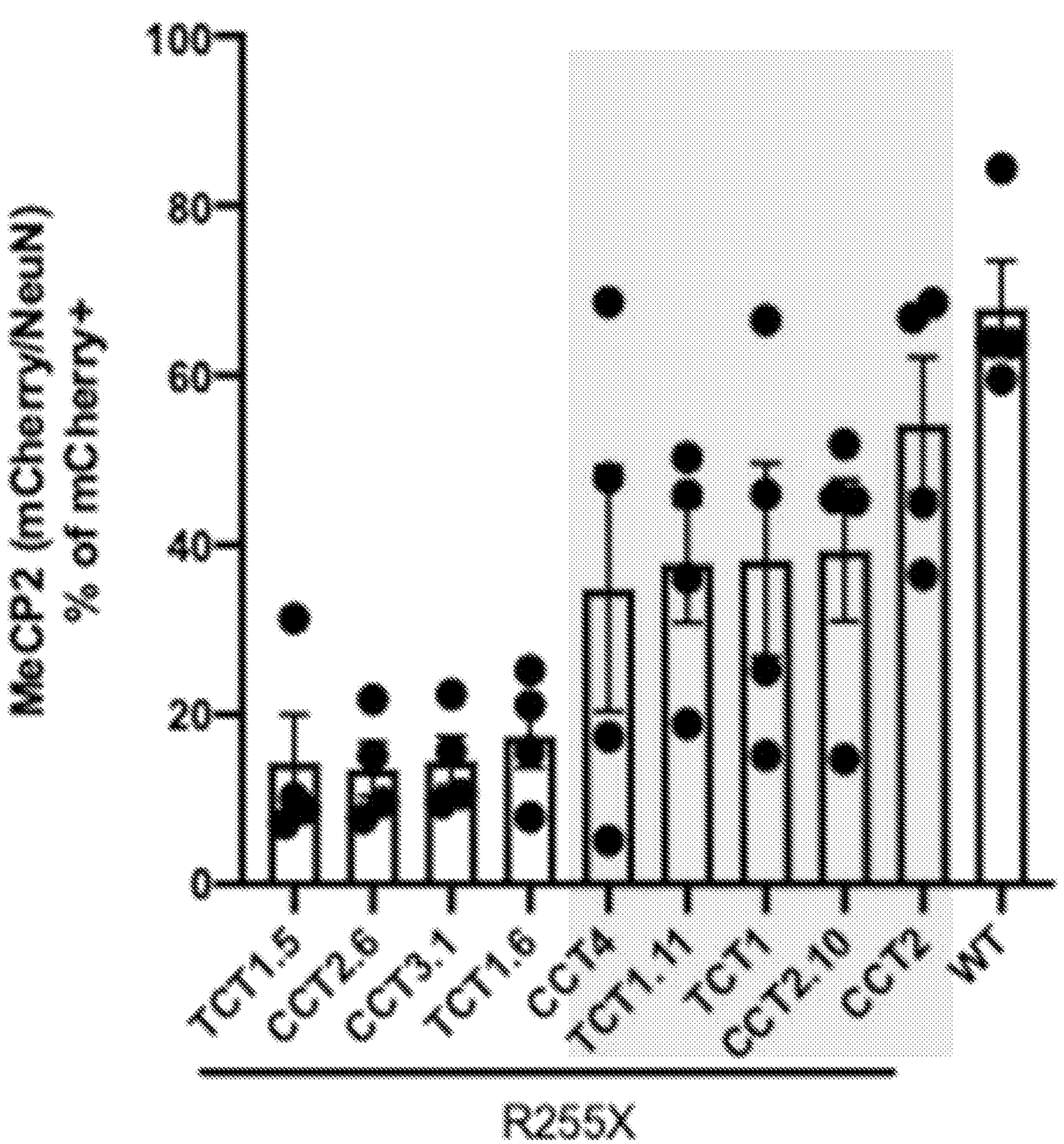

MeCP2 (mCherry/NeuN)
% of mCherry+
100
80
60
40
20
0
TCT1.5
CCT2.6
CCT3.1
TCT1.6
CCT4
TCT1.11
TCT1
CCT2.10
CCT2
WT
R255X

FIG. 18A

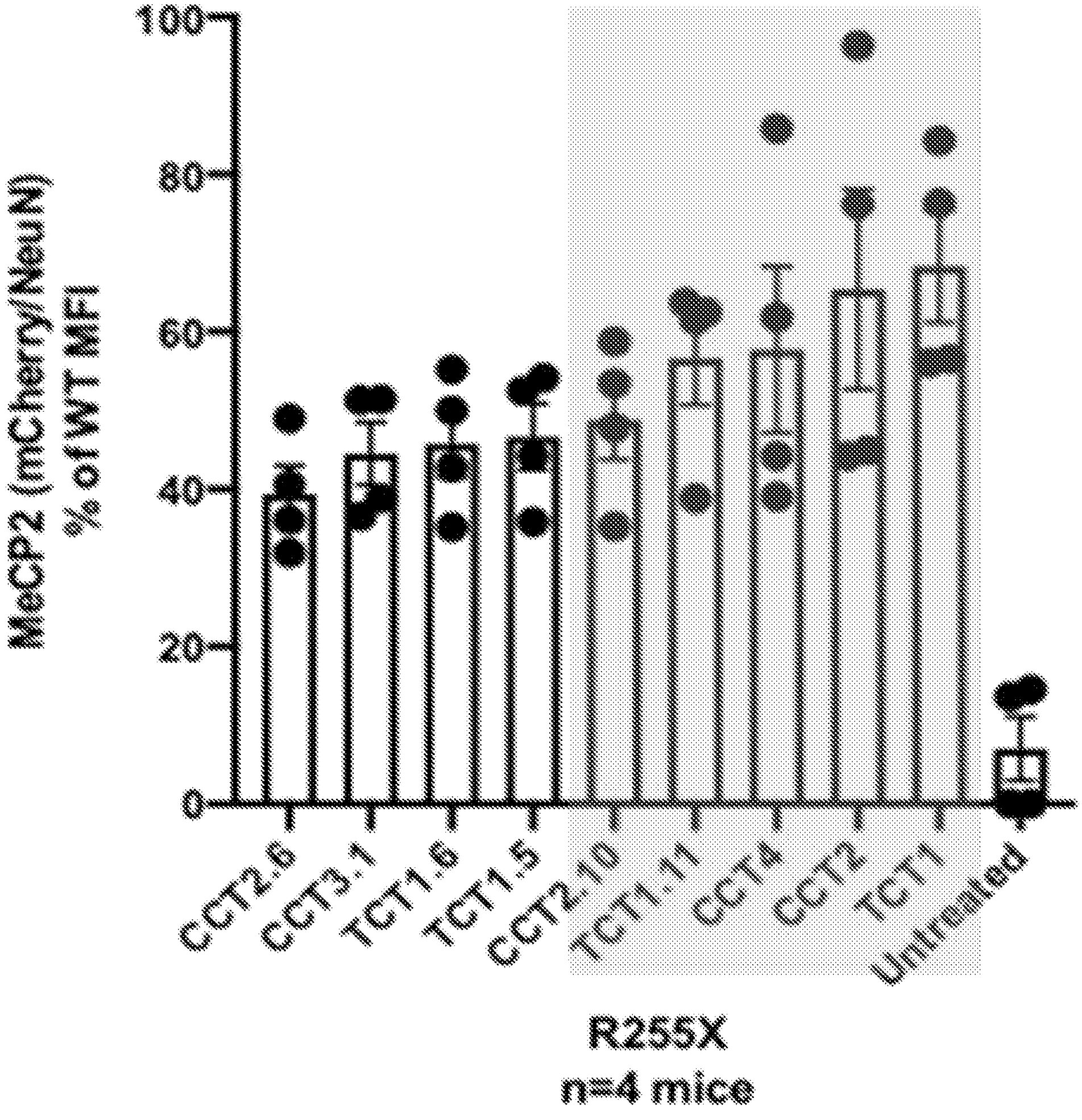

% of WT MFI
100
80
60
40
20
0
CCT2.6
CCT3.1
TCT1.6
TCT1.5
CCT2.10
TCT1.11
CCT9
CCT2
TCT1
Untreated
R255X
n=4 mice

FIG. 18B

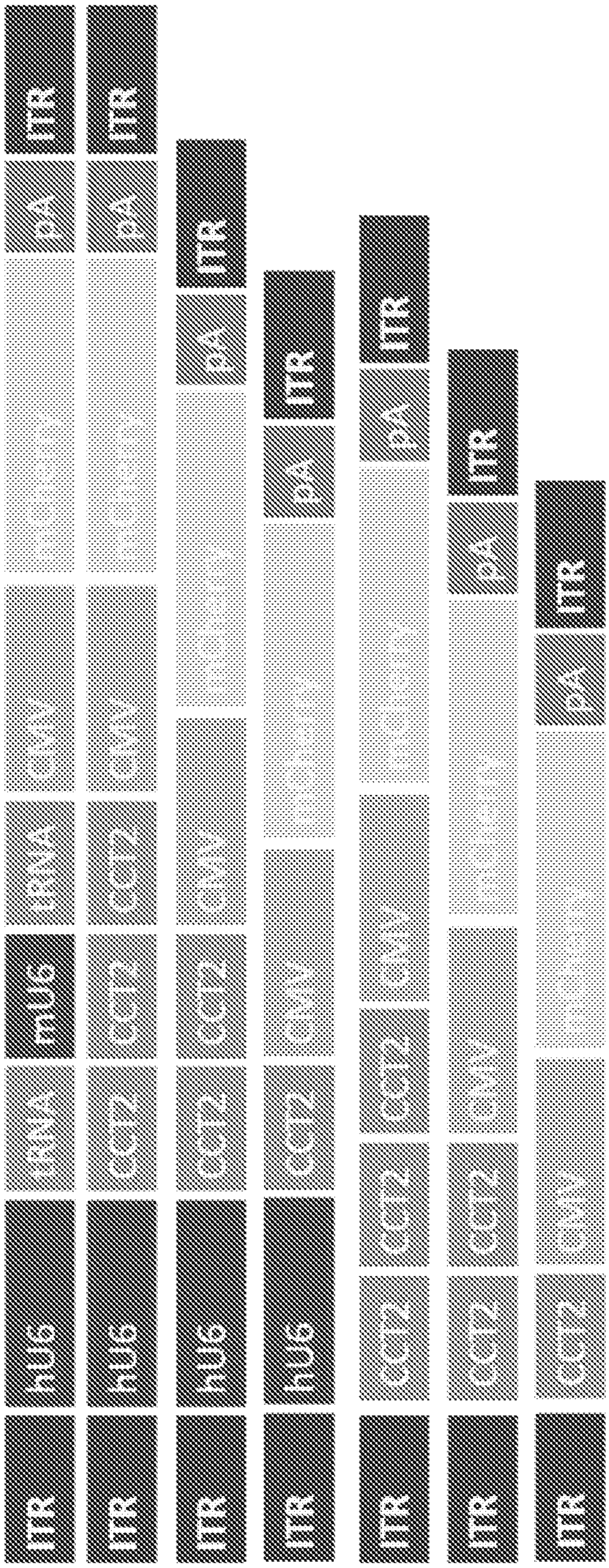


FIG. 19A
FIG. 19B
FIG. 19C
FIG. 19D
FIG. 19E
FIG. 19F
FIG. 19G

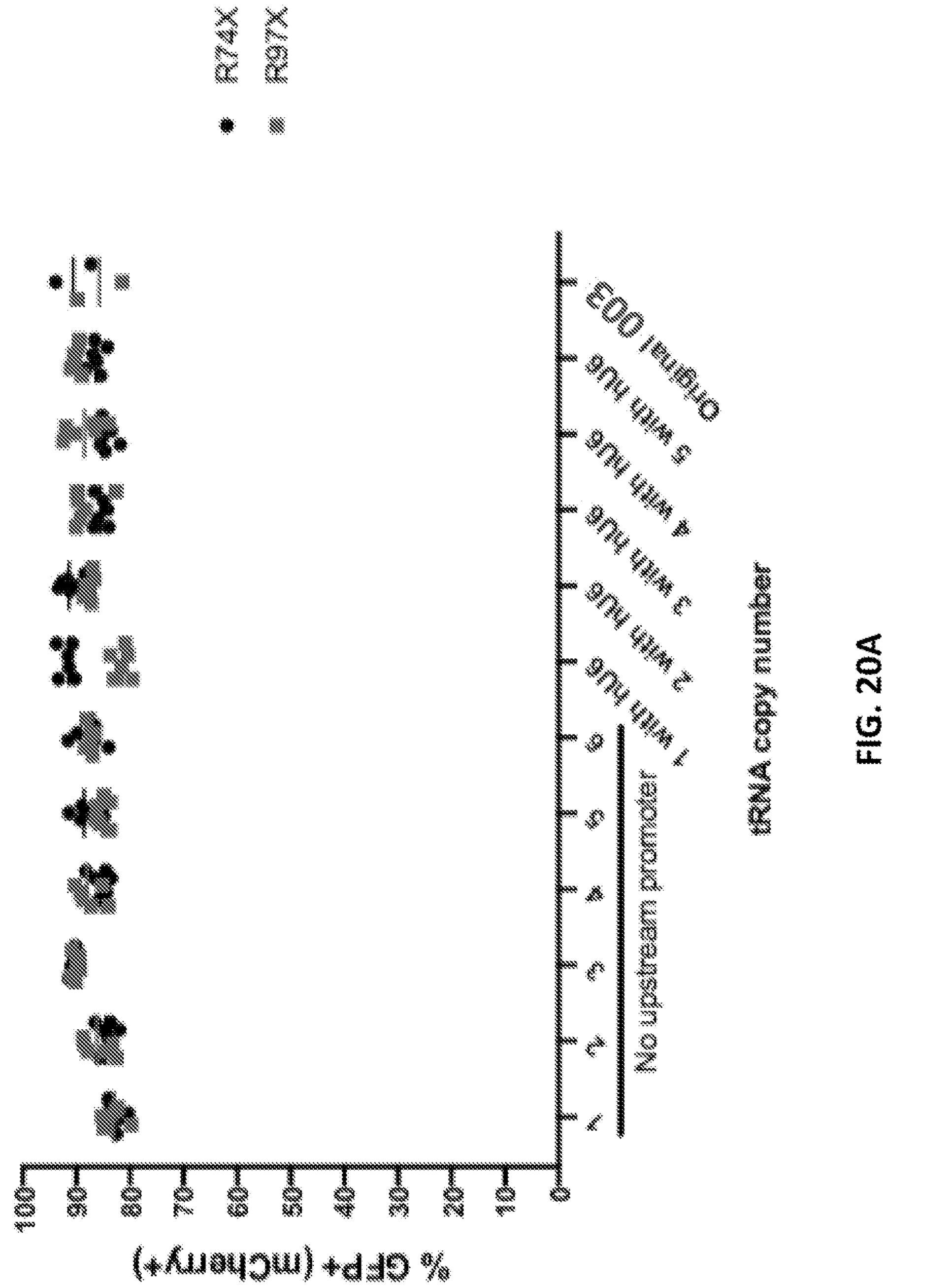

% GFP+ (mCherry+)
0
10
20
30
40
50
60
70
80
90
100
No upstream promoter
pTet
pTet + pTet
pTet + pTet + pTet
pTet + pTet + pTet + pTet
pTet + pTet + pTet + pTet + pTet
pTet + pTet + pTet + pTet + pTet + pTet
tRNA copy number

FIG. 20A

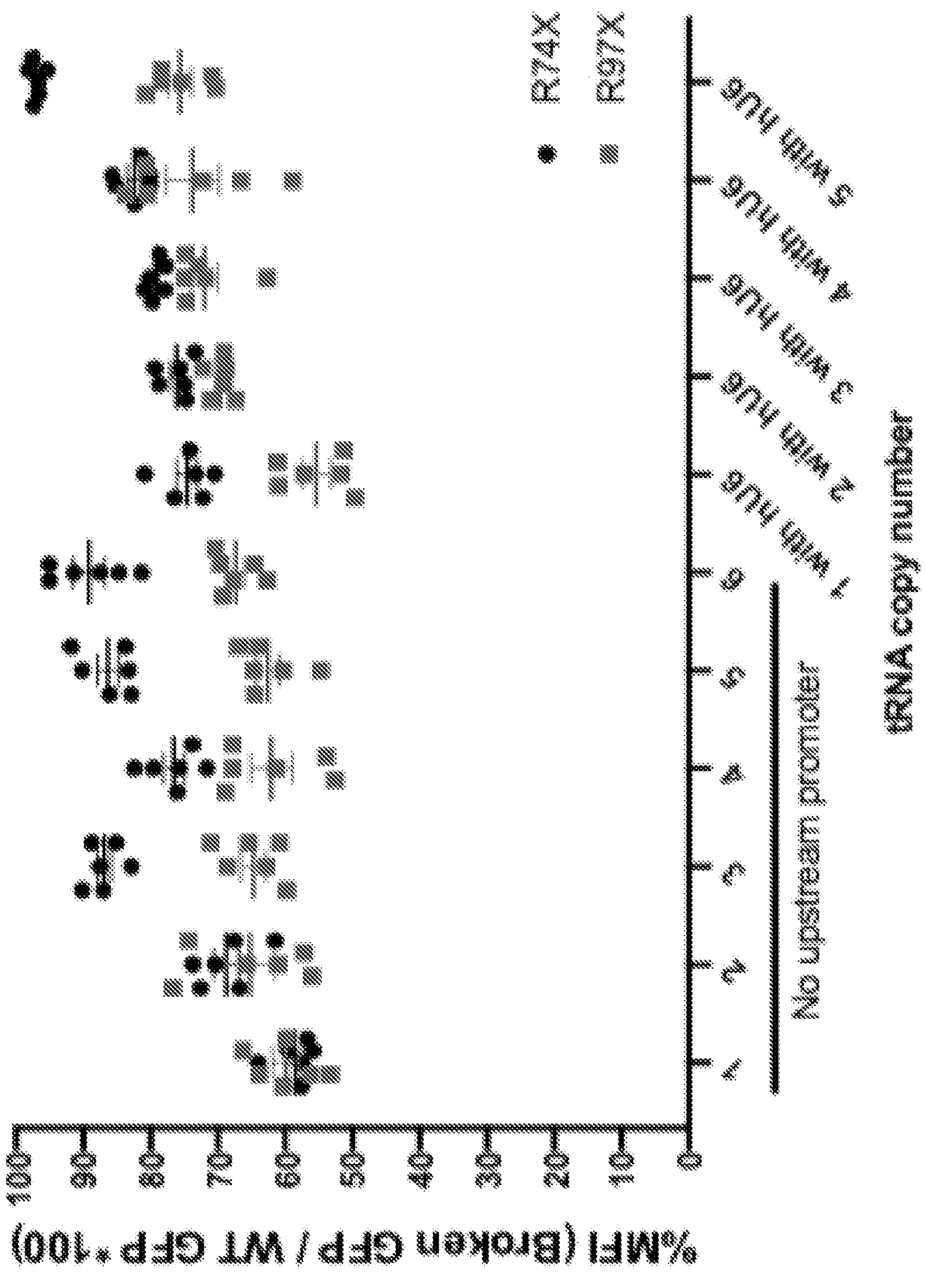

%MFI (Broken GFP / WT GFP *100)
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
No upstream promoter
1 with hU6
2 with hU6
3 with hU6
4 with hU6
5 with hU6
6 with hU6
tRNA copy number
R74X
R97X

FIG. 20B



FIG. 21A

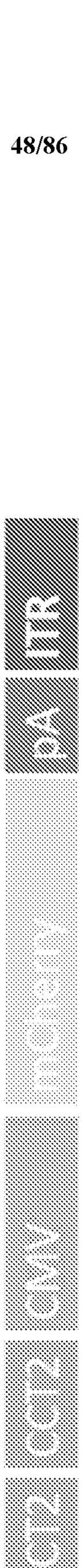


FIG. 21B

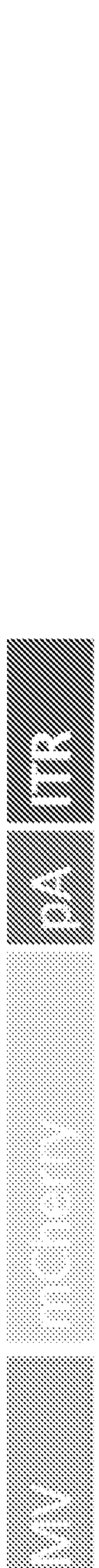


FIG. 21C

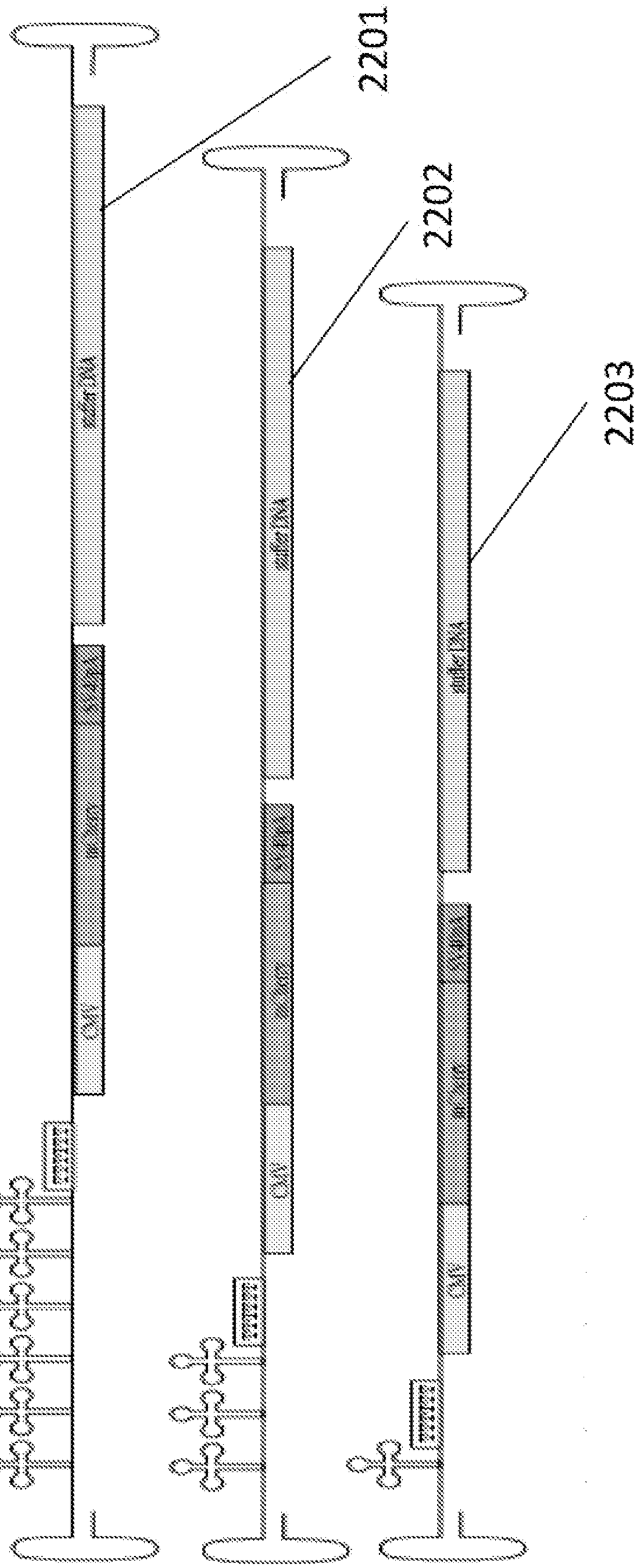


FIG. 22A

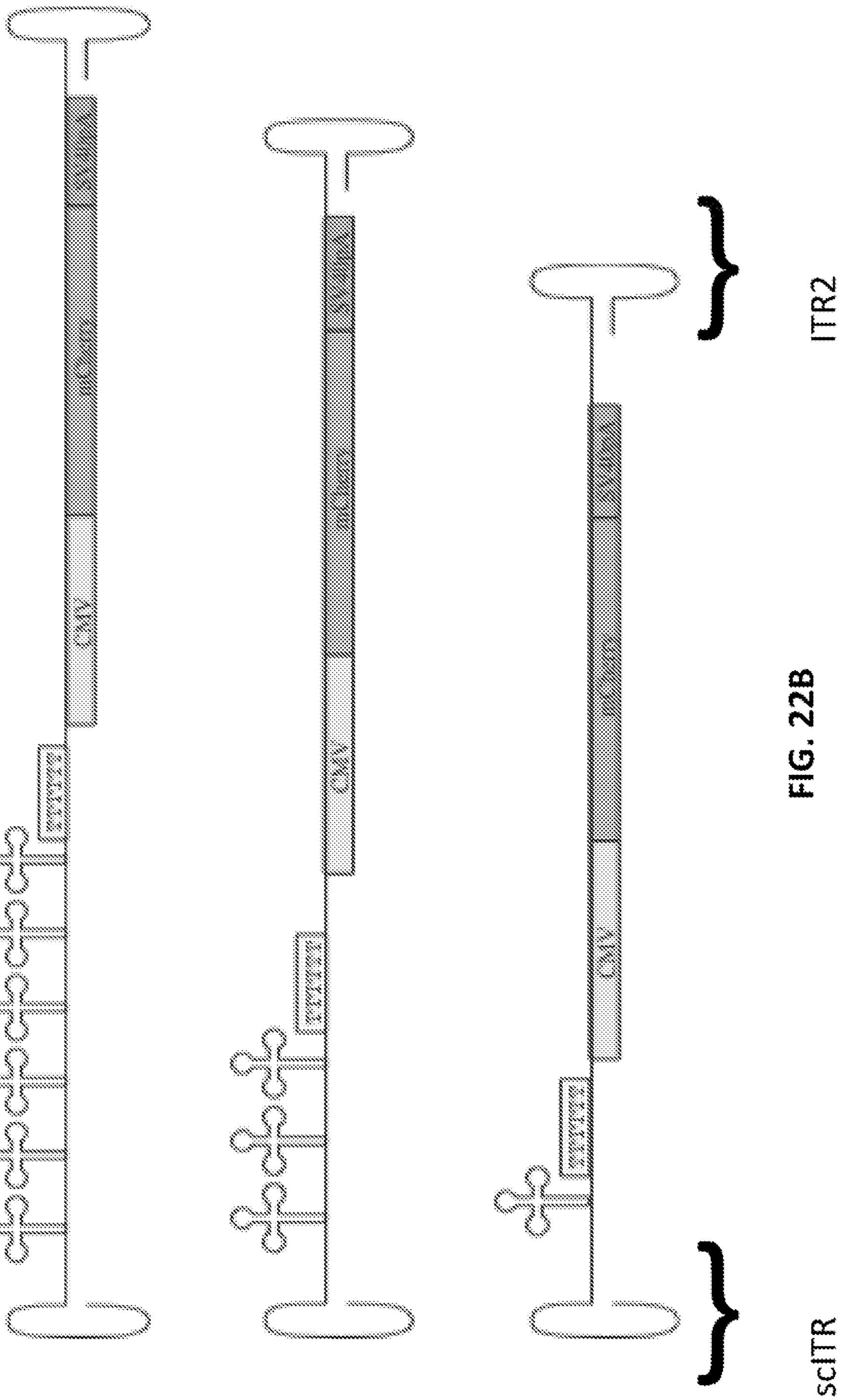


FIG. 22B

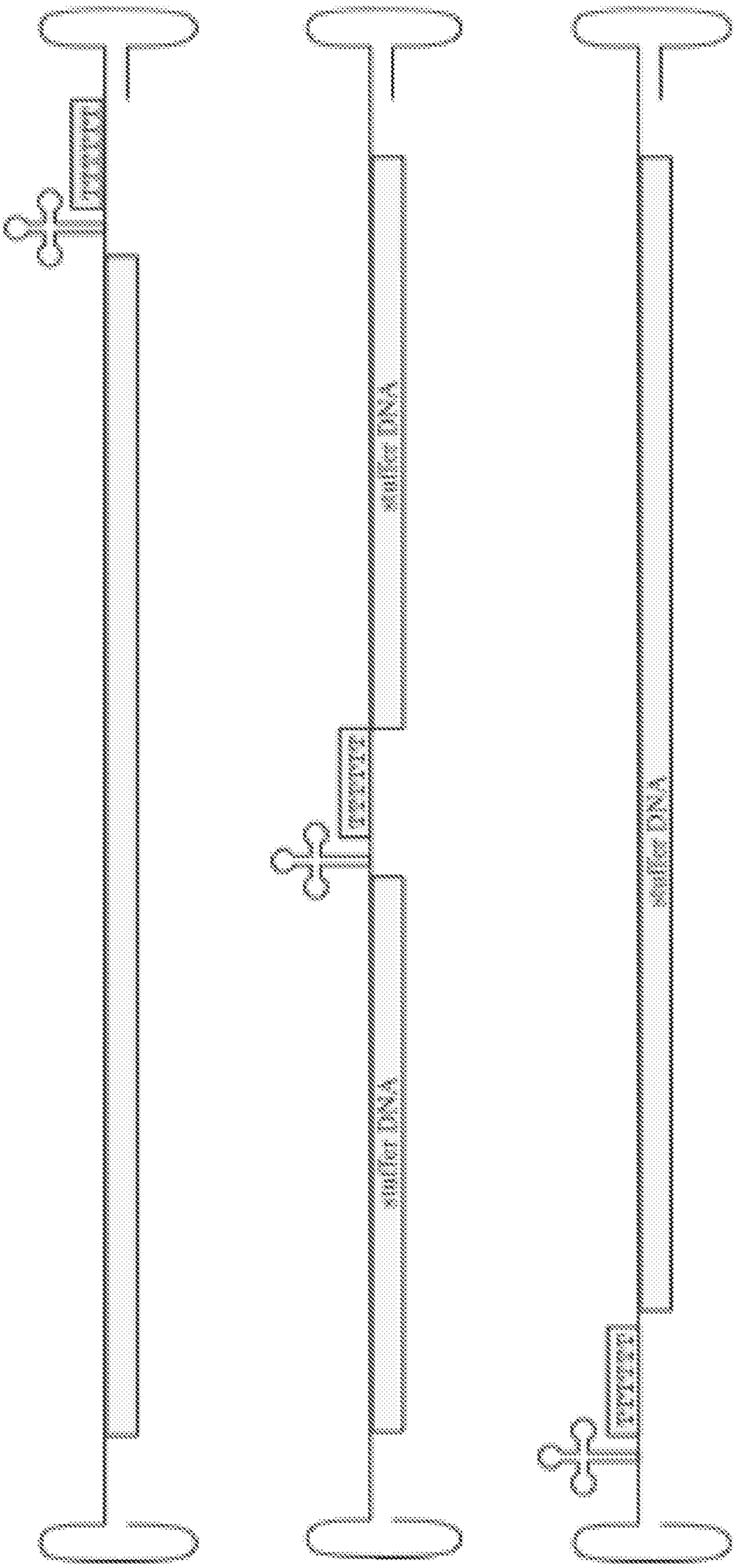


FIG. 23A

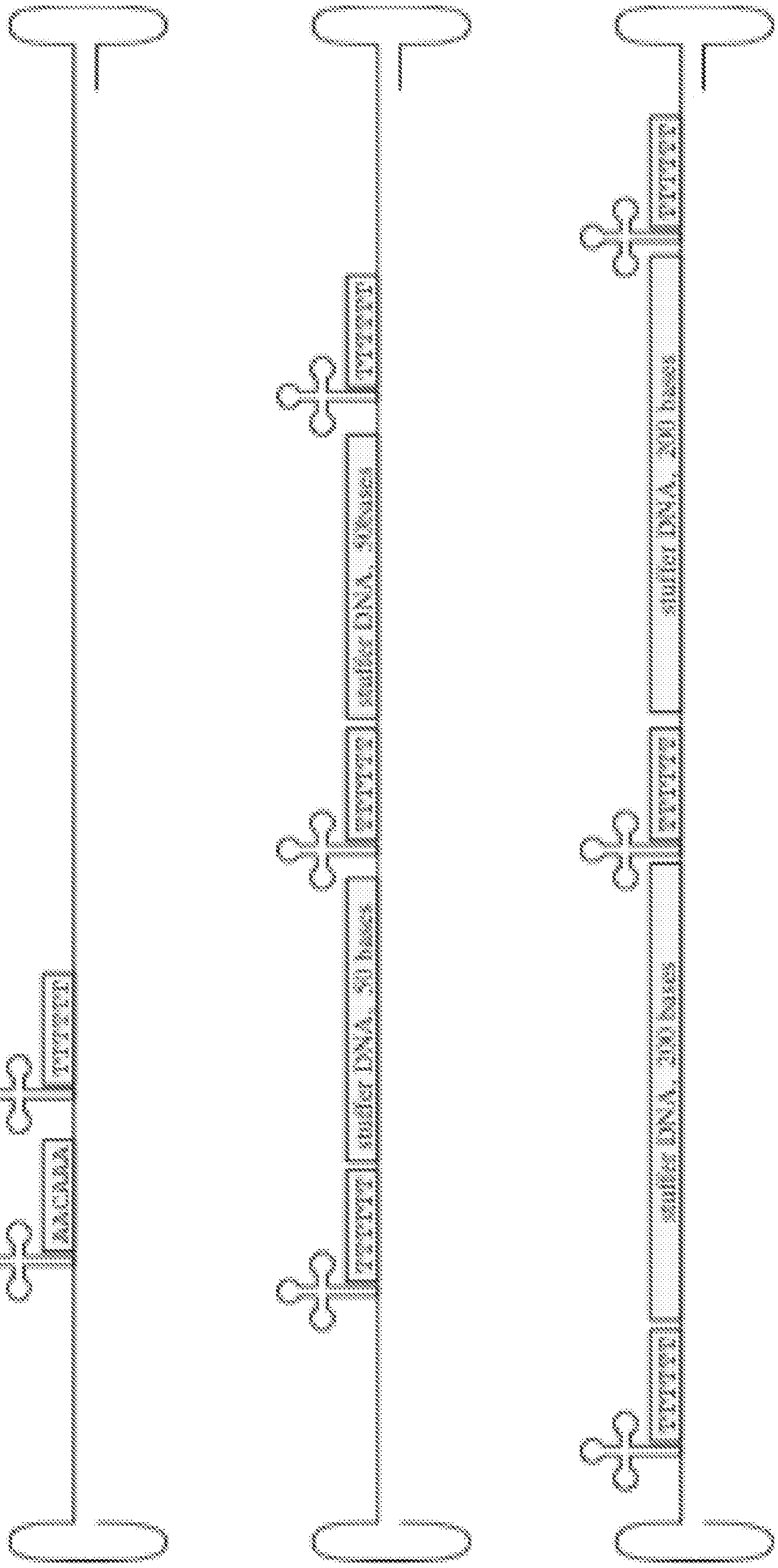


FIG. 23B

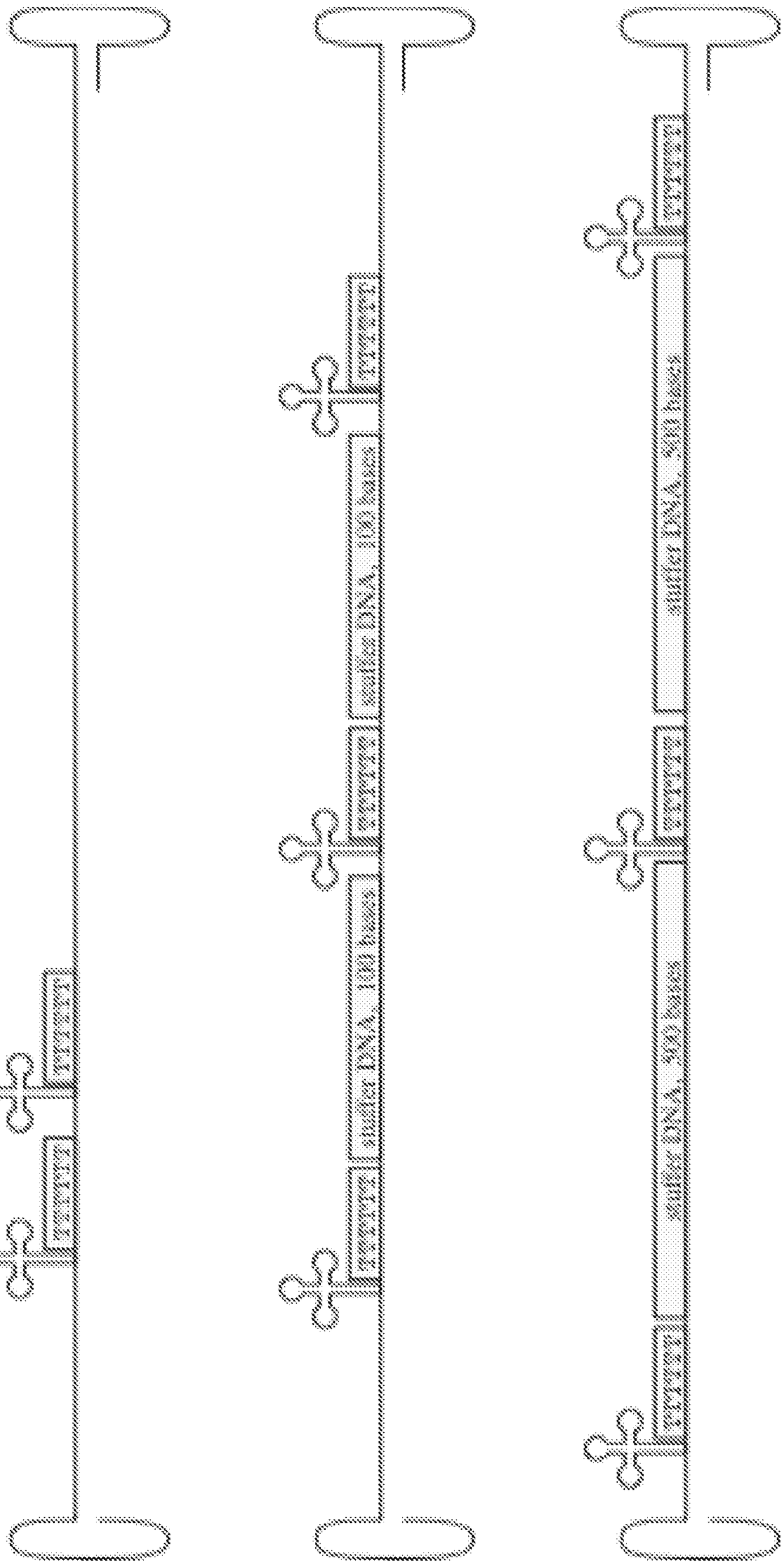


FIG. 23C

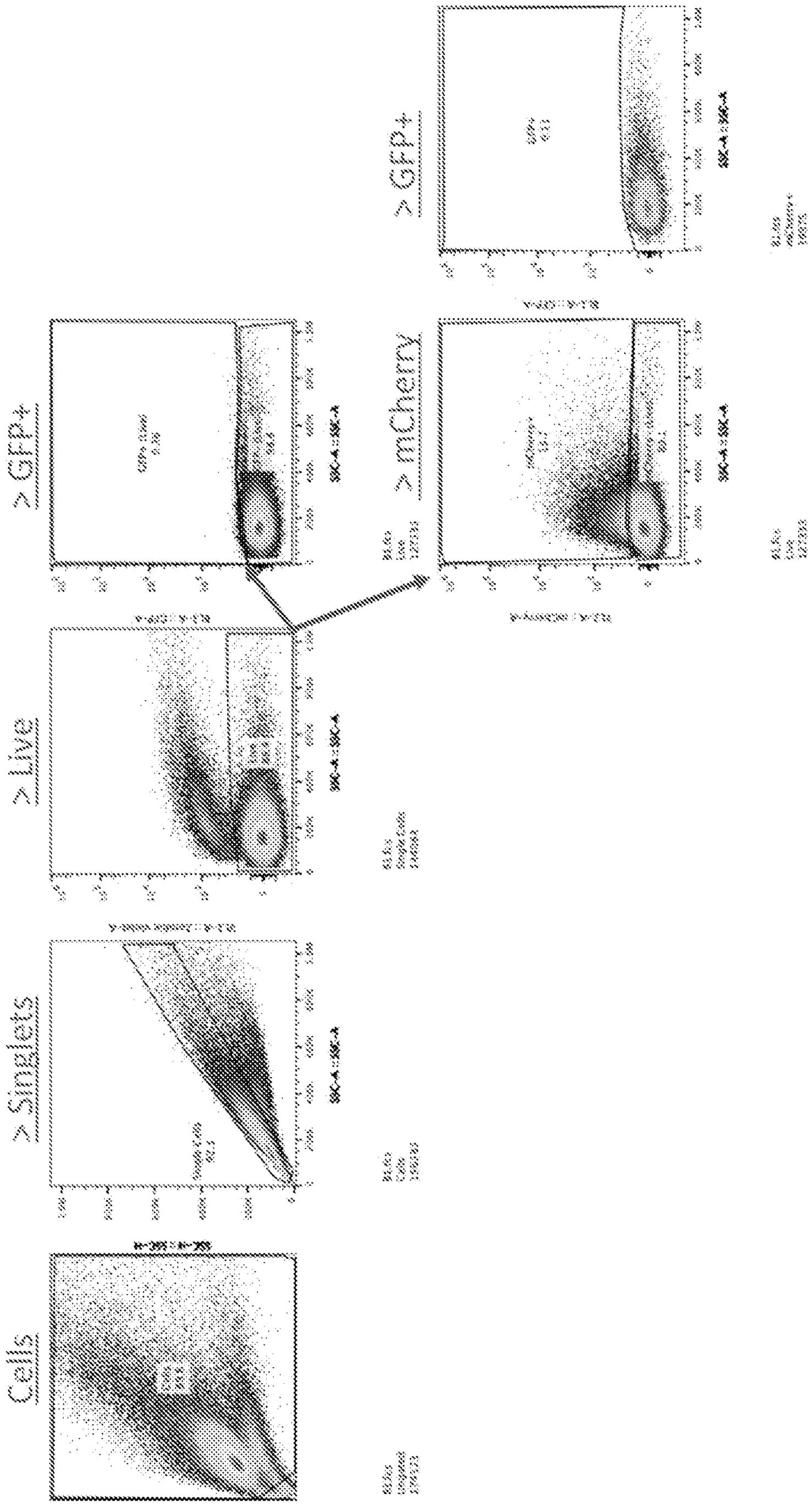
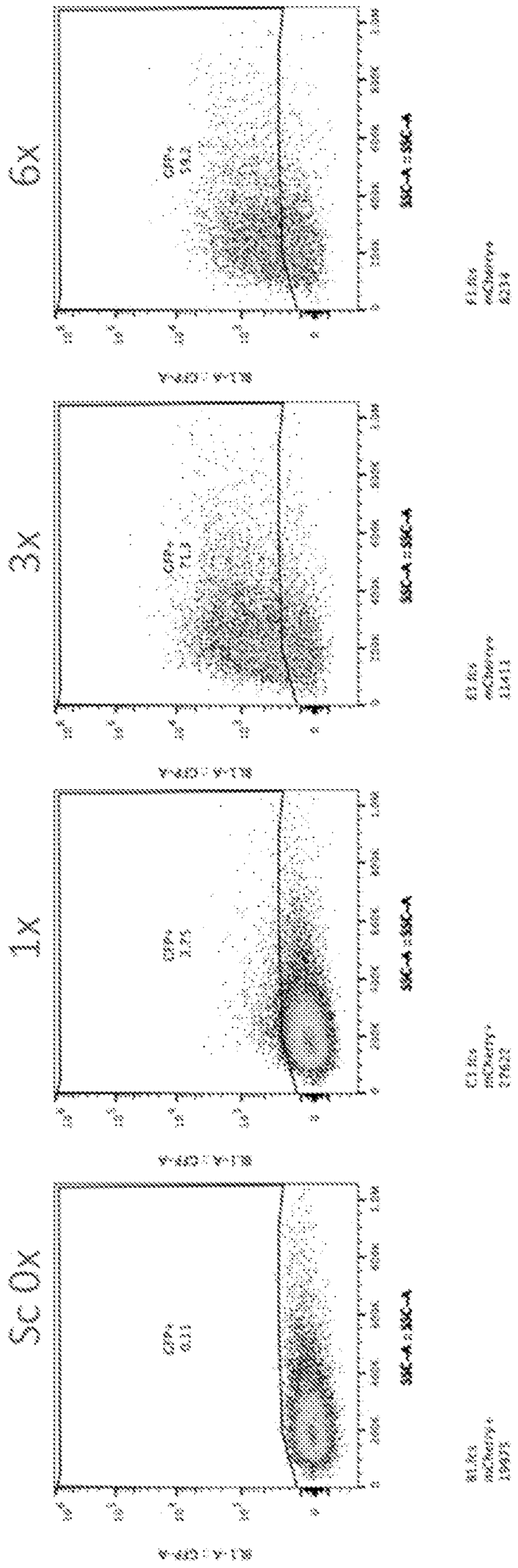


FIG. 24


mCherry+ x GFP+
Cells > Singlets > Live > mCherry+ > GFP+
Gated:
25
FIG.

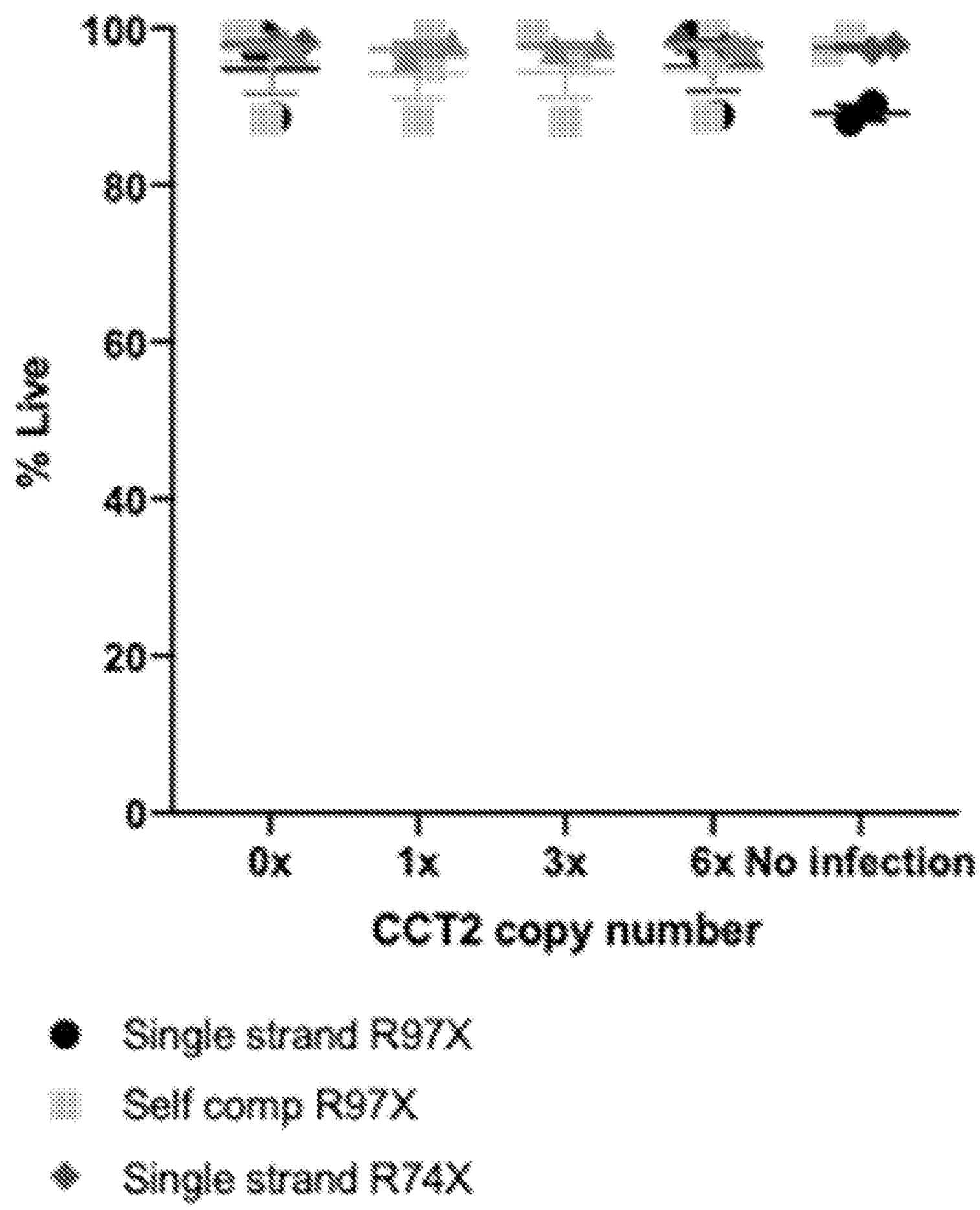

Gating: Cells > Singlets > Live
% Live
100
80
60
40
20
0
0x
1x
3x
6x No infection
CCT2 copy number
Single strand R97X
Self comp R97X
Single strand R74X
Self comp R74X

FIG. 26

FIG. 27A

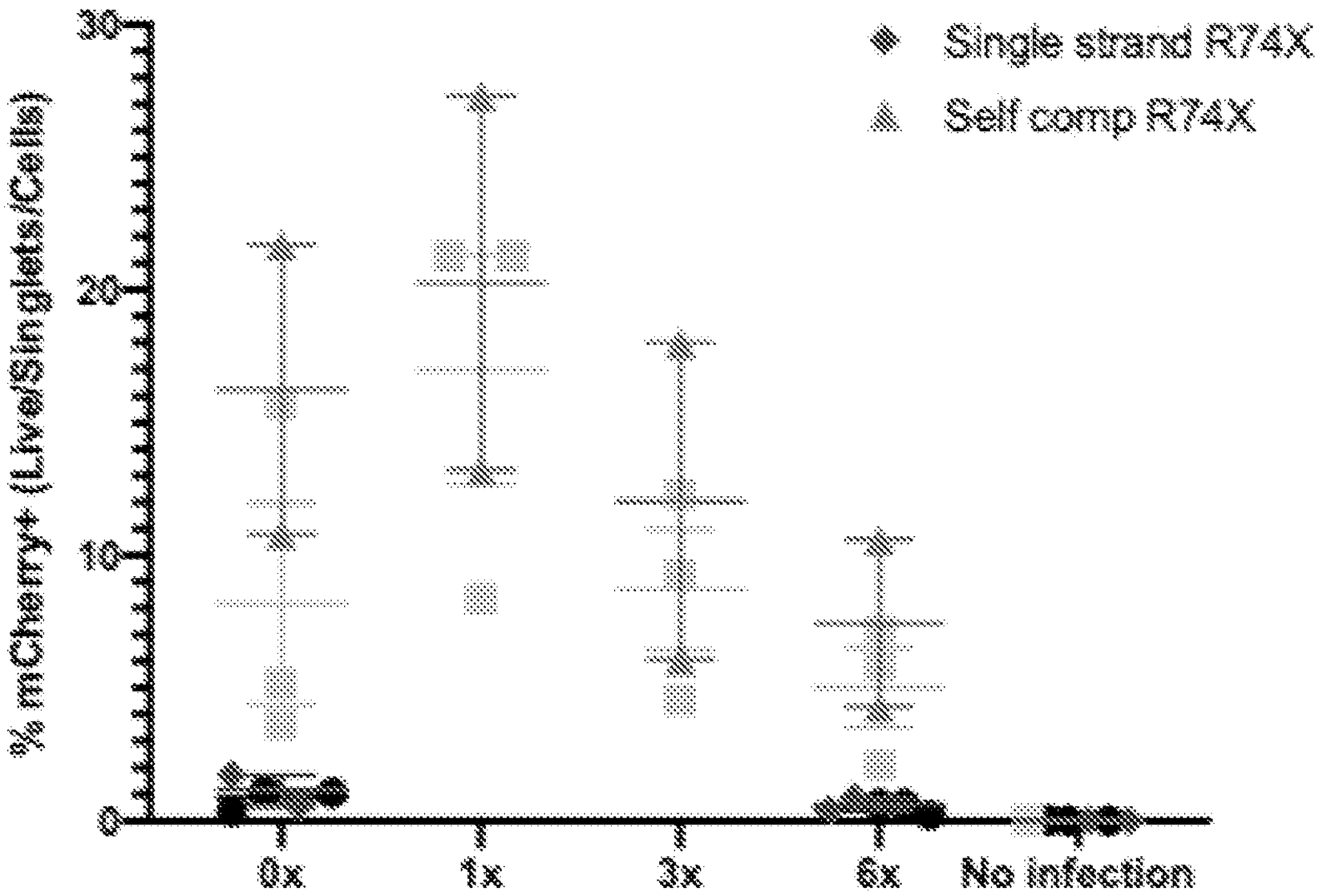


FIG. 27B

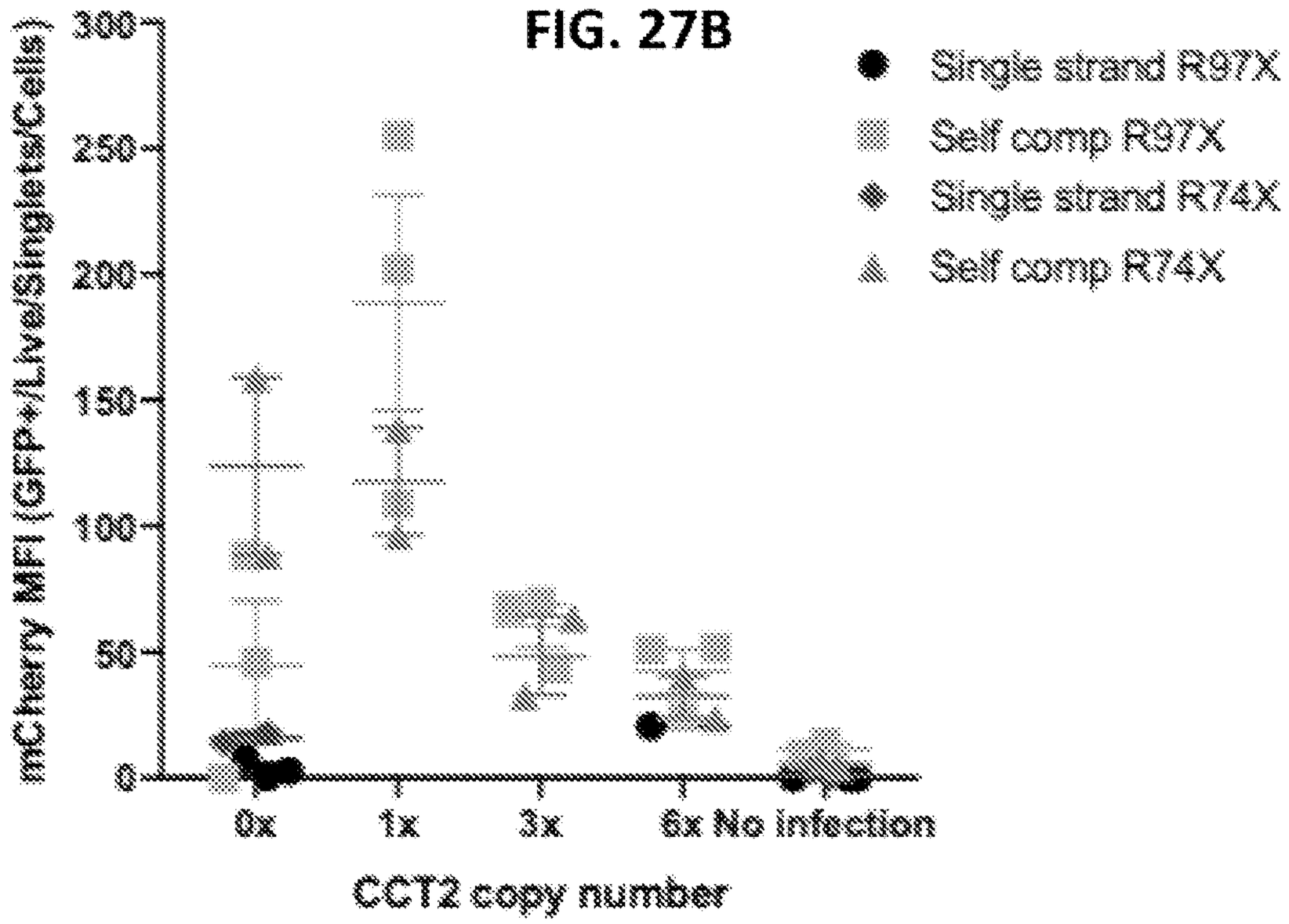


FIG. 28A

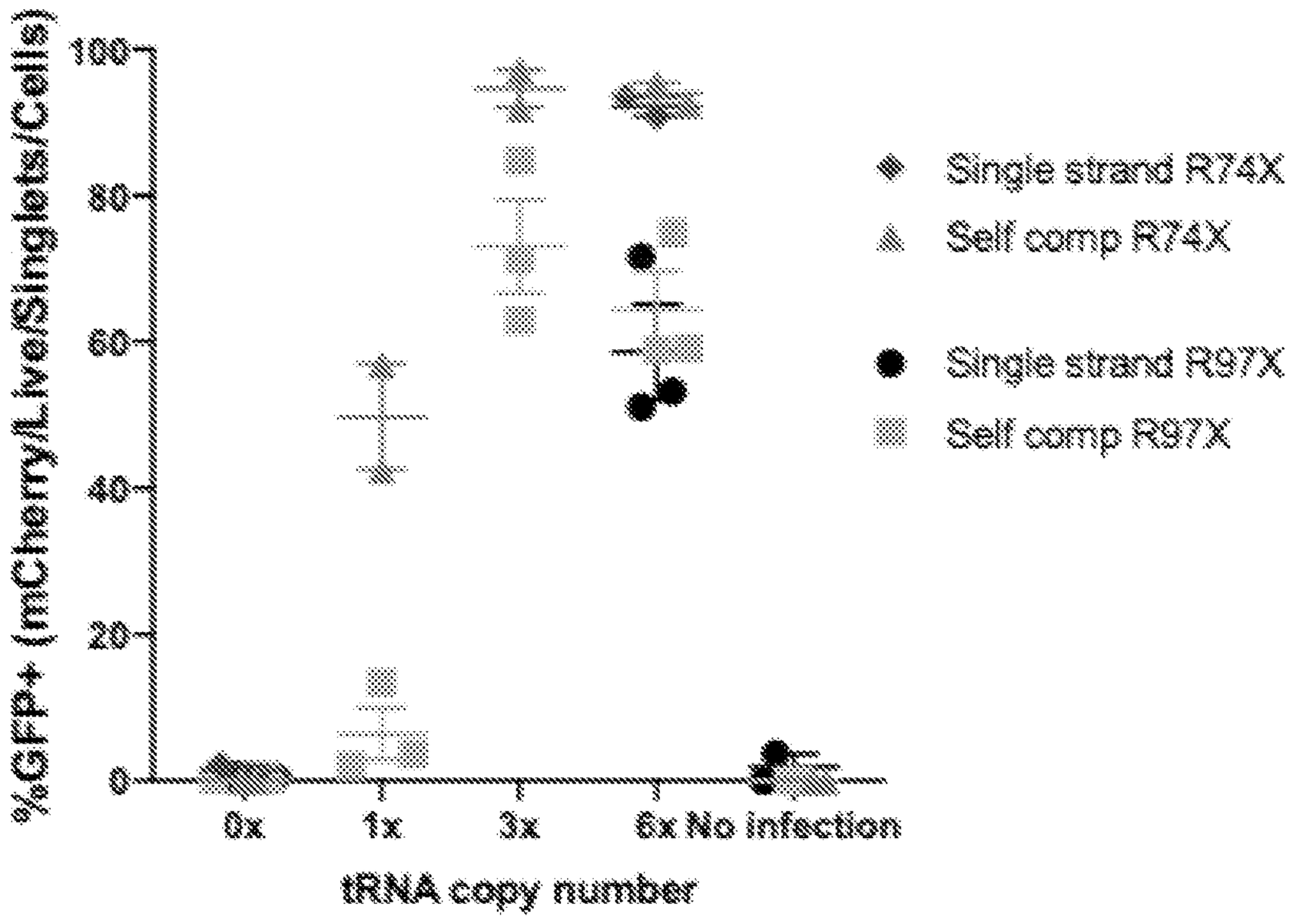


FIG. 28B

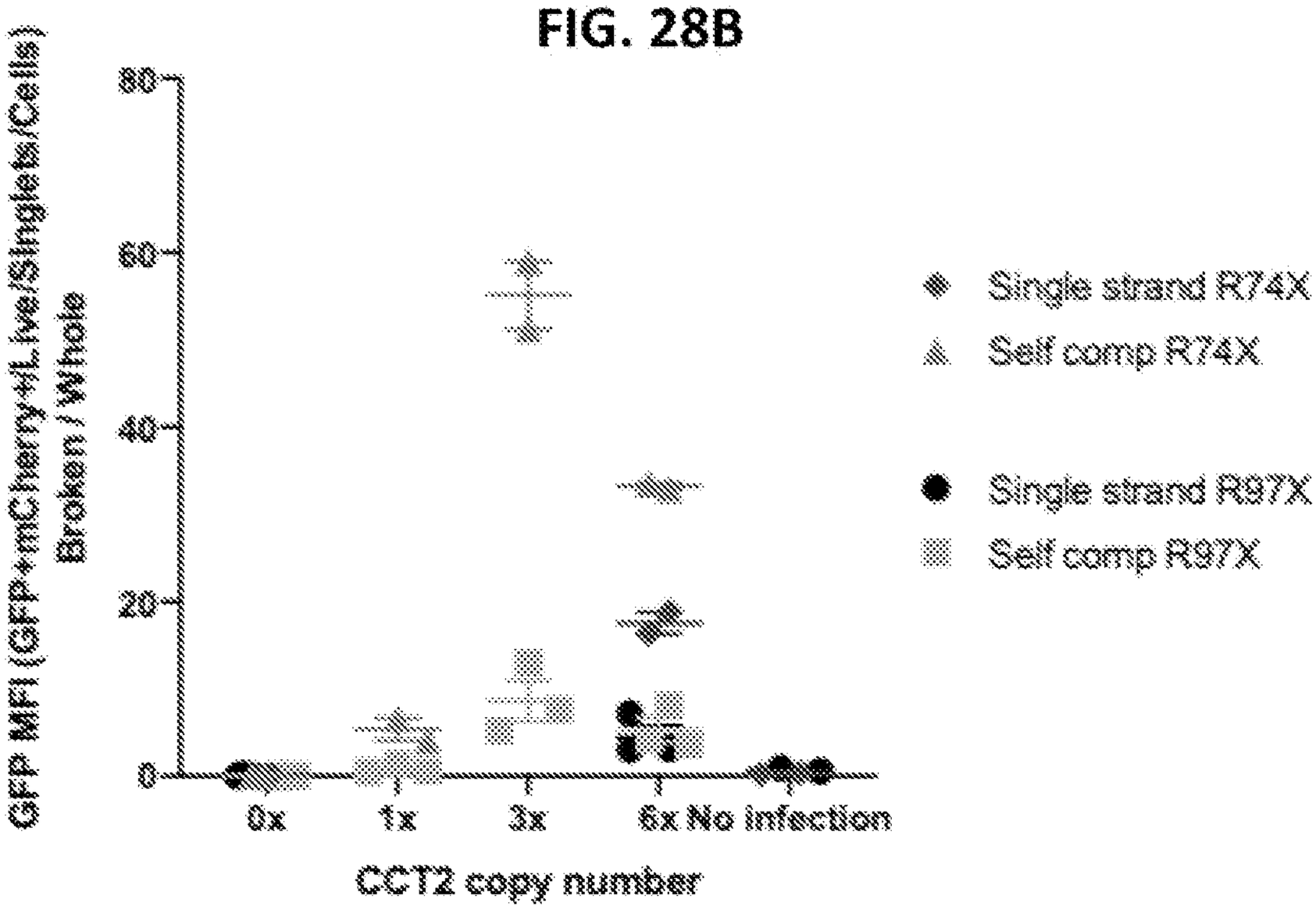
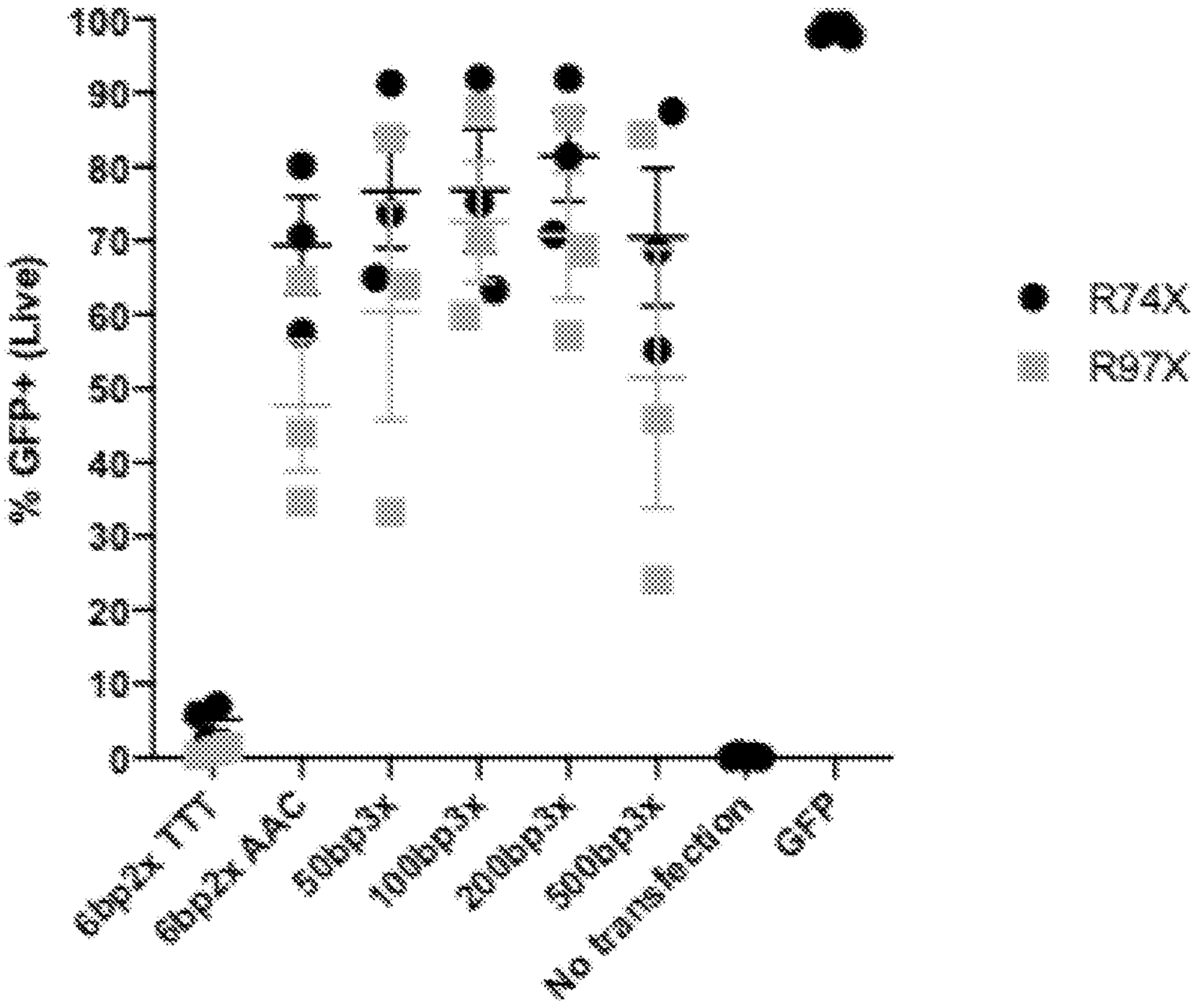


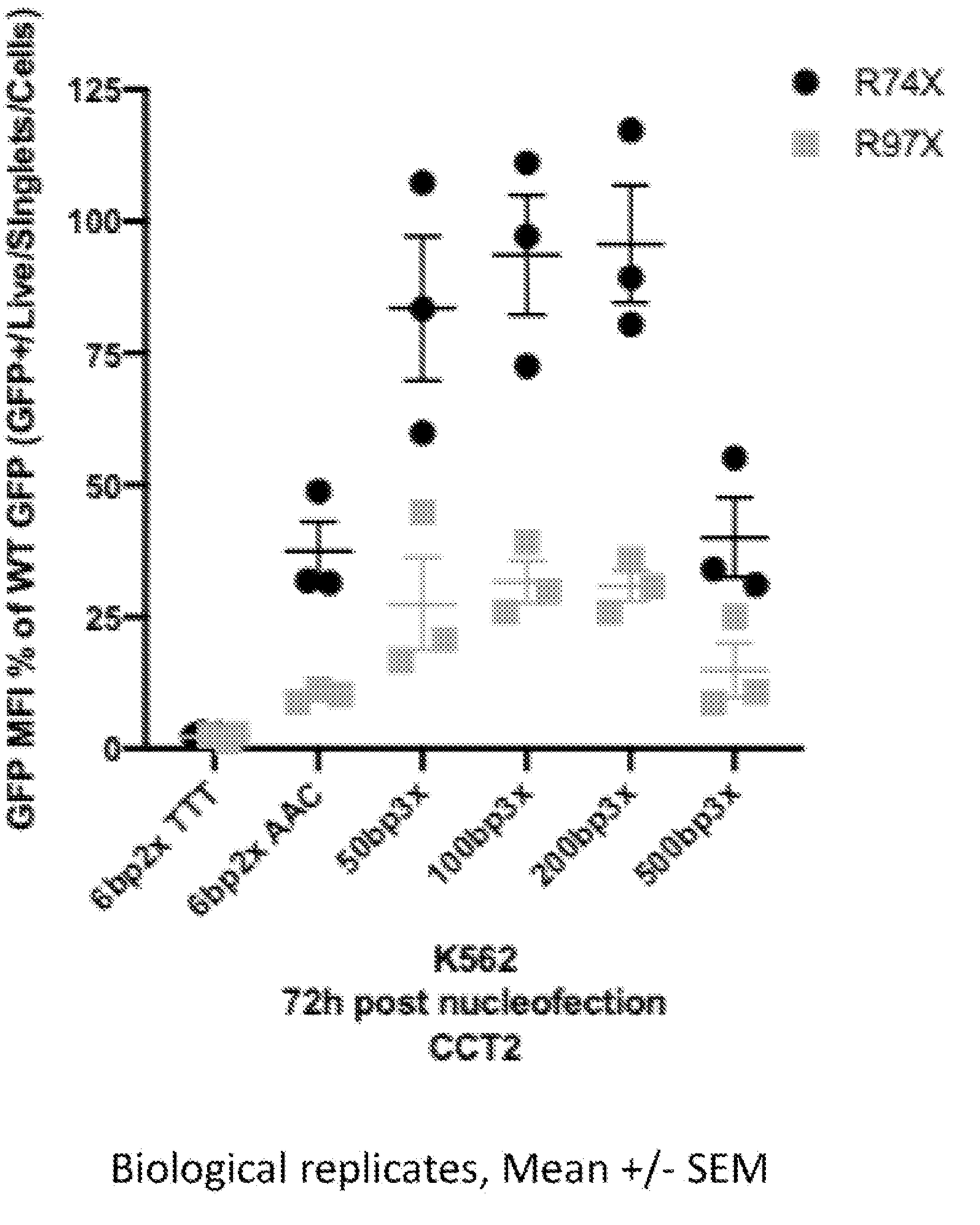
FIG. 29A



K562
72h post nucleofection
CCT2

Biological replicates, Mean +/- SEM

FIG. 29B



Biological replicates, Mean +/- SEM

FIG. 30A

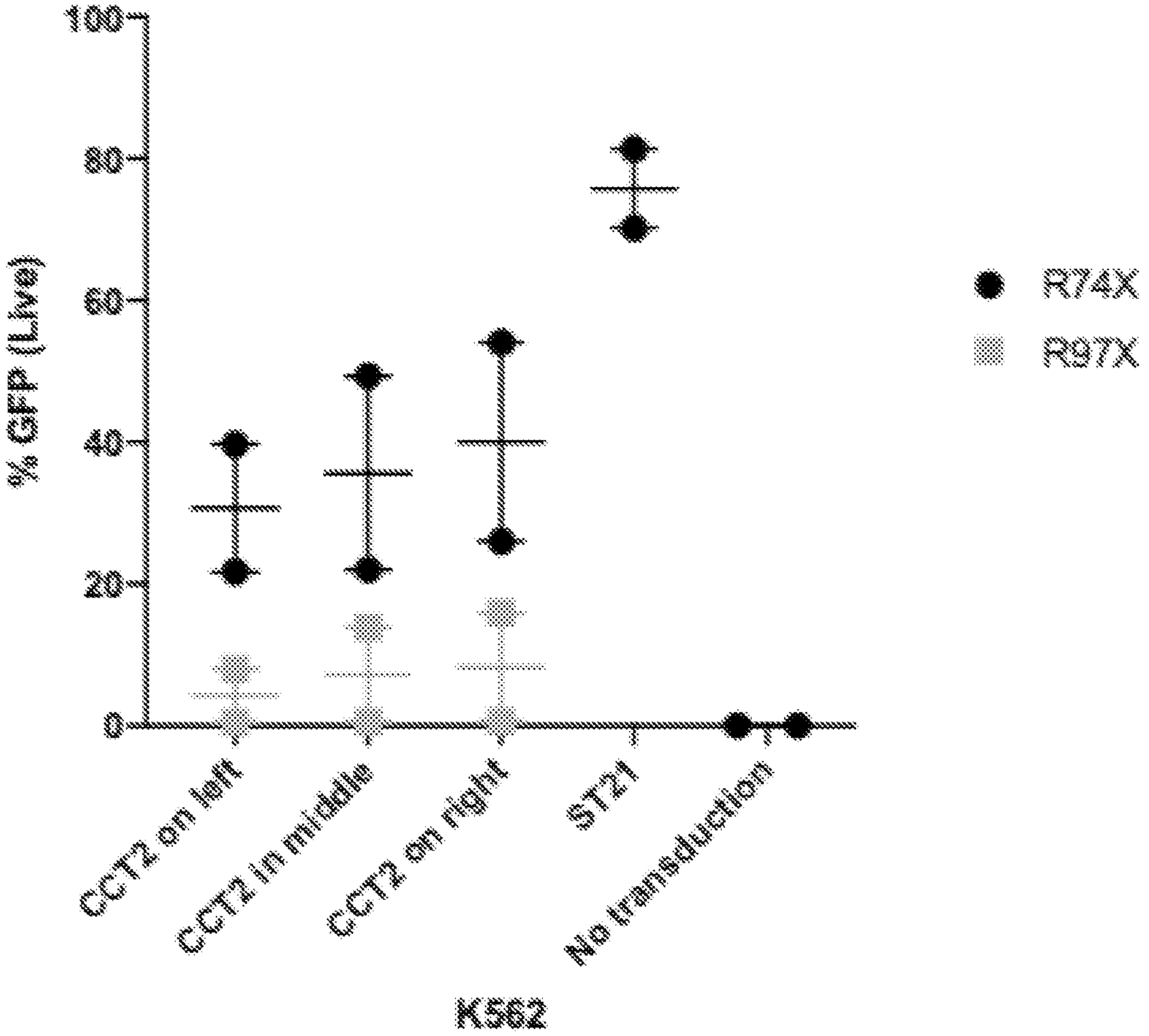
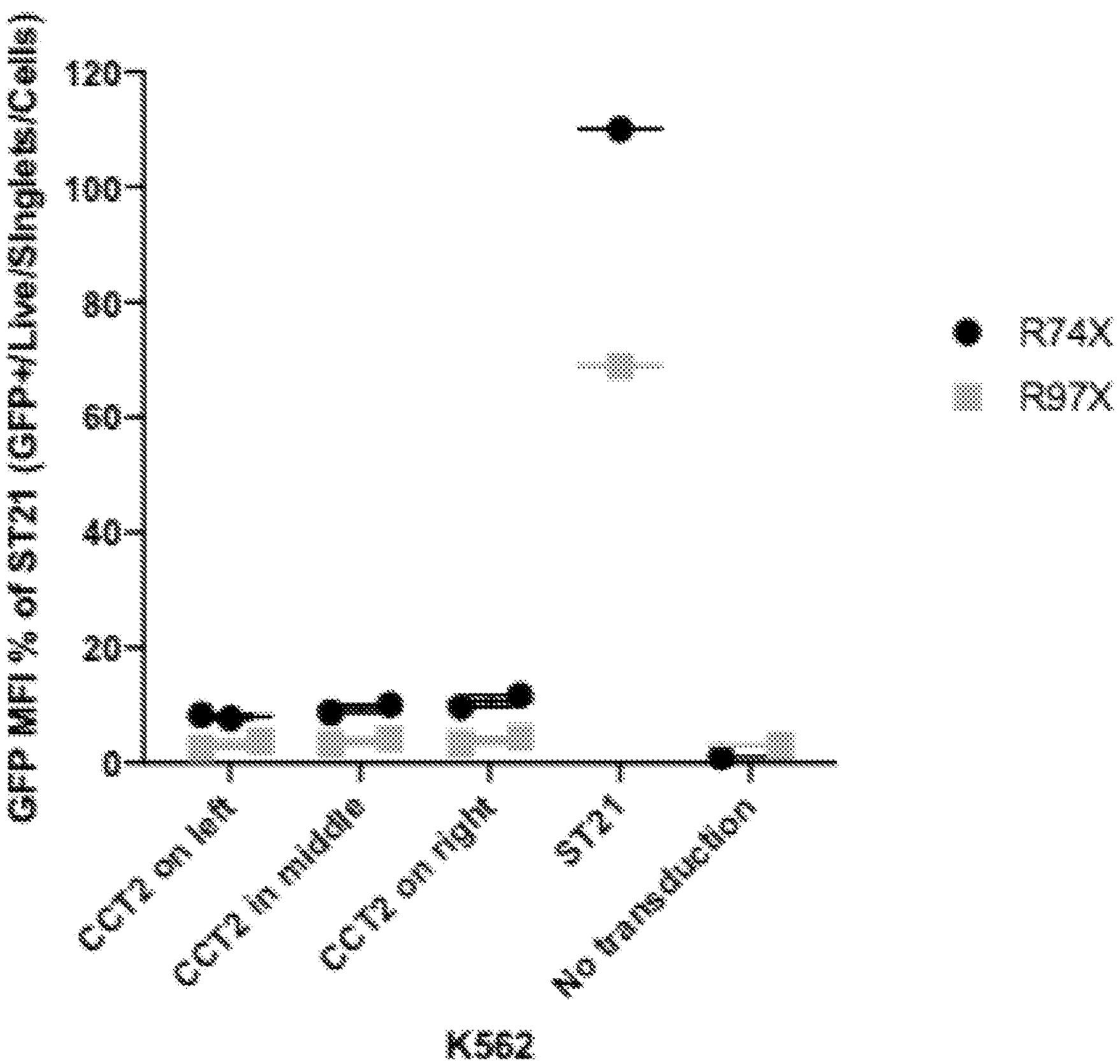

% GFP (Live)
100
80
60
40
20
0
R74X
R97X
CCT2 on left
CCT2 in middle
CCT2 on right
ST2H
No transduction
K562

FIG. 30B

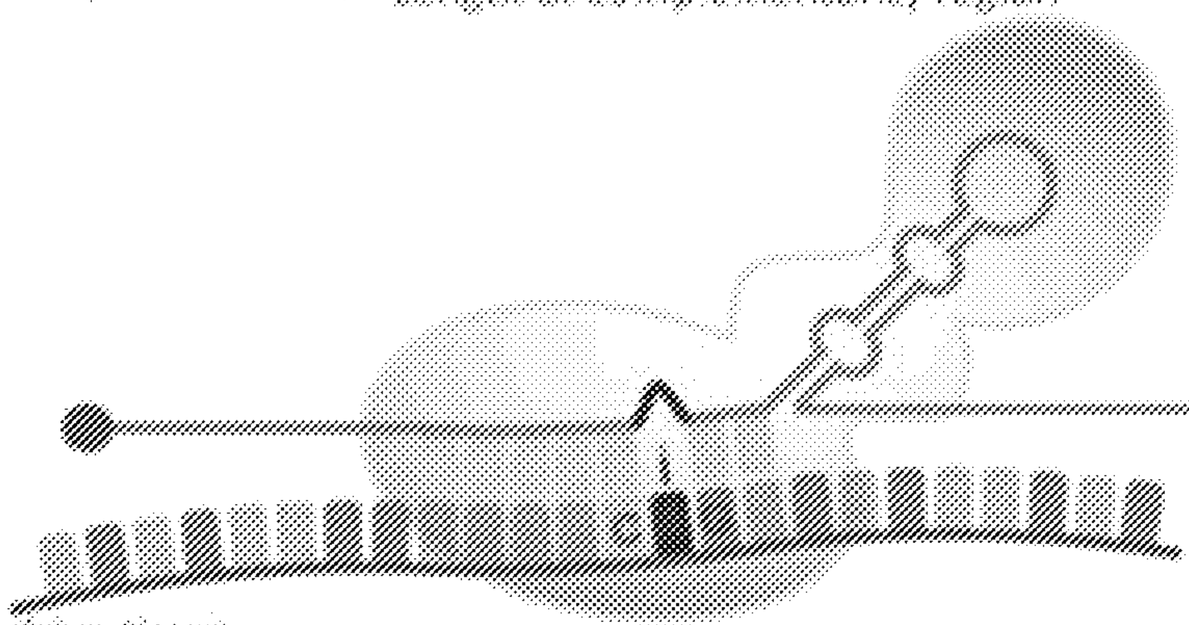

GFP MFI % of ST21 (GFP+/Live/Singlets/Cells)
0
20
40
60
80
100
120
CCT2 on left
CCT2 in middle
CCT2 on right
ST21
No transduction
K562
R74X
R97X

gRNA Stability

- Stability hairpins (e.g. U6+27)
- Create circular gRNAs
- 5' capping to resist RNA

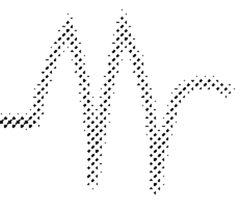
gRNA Length/Bulge Placement

- On-target editing driven by bulge placement - what is optimal?
- Length of complementarity region



gRNA dsRNA Hairpin Design

- Modify GluR2 ADAR-interacting regions
- Identify RNA structures that recruit ADAR at higher efficiency (e.g. Alu elements, synthetic RNA aptamers, mine CLIP-seq data, etc.)



gRNA Expression

- Pol-II promoter usage to drive tissue-specific expression and ADAR recruitment
- Code multiple gRNA copies

gRNA Localization

- Add cis-elements to modify sub-cellular localization
- Nuclear localization sequence from Malat1
- Nuclear retention signals from Xist, Malat1, Neat1, snoRNAs, etc.

Goal: fit-for-purpose designs to tune gRNA activity

FIG. 31

Guide length	Bulge placement	GluRD placement
Guide length range to be tested initially is 20-200nt, with intervals of 20, 30, 40, 50, 75, 100, 150, and 200.	The A/C mismatch will be placed for all lengths on either the 5' end, 3' end or in the middle.	GLUR3-recruiting domains will be placed on either the 5' end, 3' end or both ends of the guide, or not at all.

Guide Length	0_GluRD			5' 1_GluRD			3' 1_GluRD			2_GluRD		
20	20.2	20.10	20.19	20.2	20.10	20.19	20.2	20.10	20.19	20.2	20.10	20.19
30	30.3	30.15	30.28	30.3	30.15	30.28	30.3	30.15	30.28	30.3	30.15	30.28
40	40.4	40.20	40.37	40.4	40.20	40.37	40.4	40.20	40.37	40.4	40.20	40.37
50	50.5	50.25	50.46	50.5	50.25	50.46	50.5	50.25	50.46	50.5	50.25	50.46
75	75.8	75.38	75.68	75.8	75.38	75.68	75.8	75.38	75.68	75.8	75.38	75.68
100	100.10	100.50	100.91	100.10	100.50	100.91	100.10	100.50	100.91	100.10	100.50	100.91
150	150.15	150.75	150.136	150.15	150.75	150.136	150.15	150.75	150.136	150.15	150.75	150.136
200	200.20	200.100	200.181	200.20	200.100	200.181	200.20	200.100	200.181	200.20	200.100	200.181

(Length.Mismatch)

FIG. 32

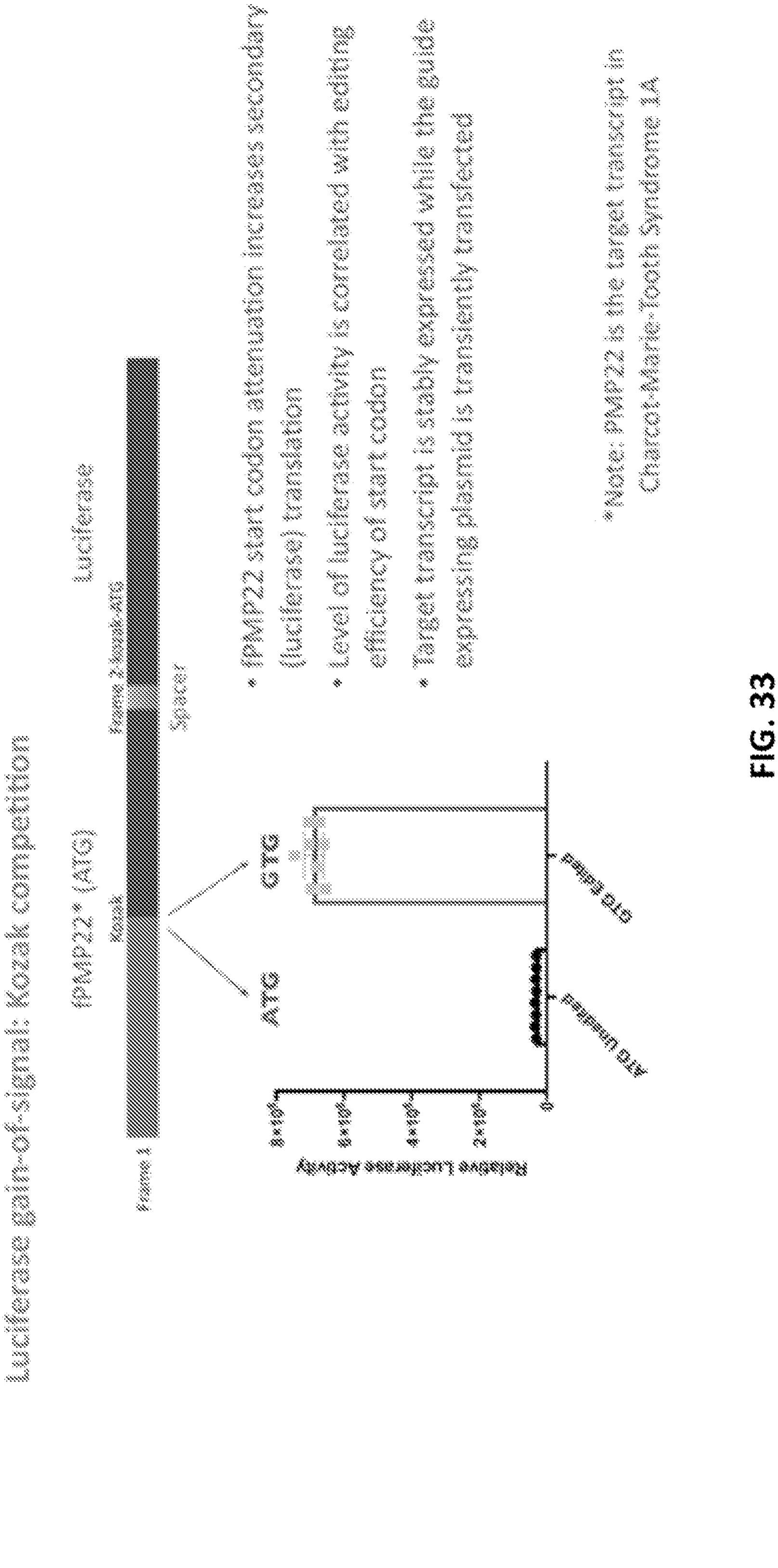

Luciferase
tPMP22 start codon attenuation increases secondary
Level of luciferase activity is correlated with ectopic
luciferase) translation
efficiency of start codon
Target transcript is stably expressed while the guide
expression plasmid is transiently transfected
*Note: PMP22 is the target transcript in
Charcot-Marie-Tooth Syndrome 1A
Luciferase 2-base-mis
Space
tPMP22 * (ATG)
Kozak
GTG
ATG
GTG Edited
ATG Unedited
Relative Luciferase Activity
8×10⁶
6×10⁶
4×10⁶
2×10⁶
0
Luciferase activity: Kozak competition
Luciferase activity: Start-of-signal competition

FIG. 33

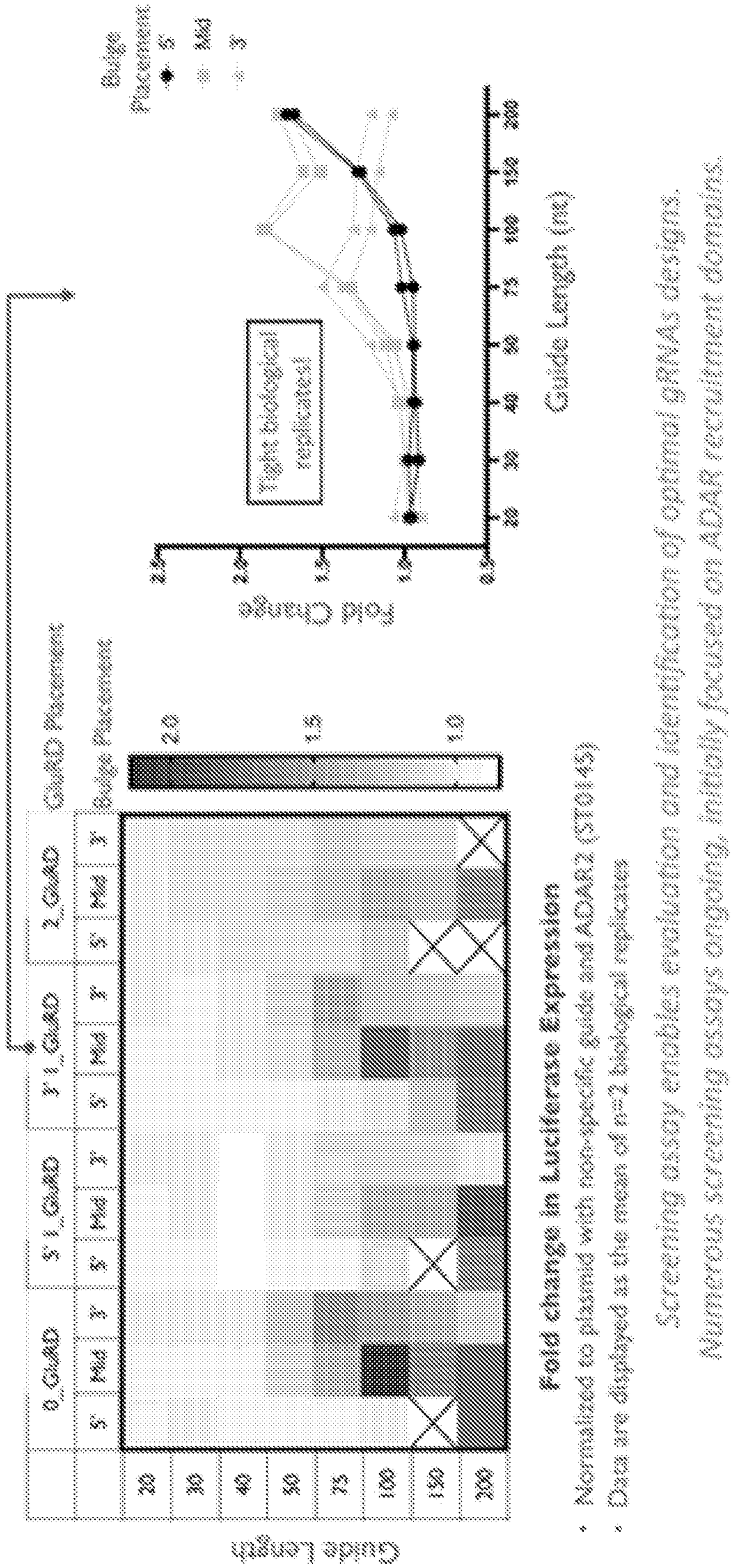

Guide Length (nt)
Fold change
Tube tubulin treatment
5'
3'
Mid
5'
3'
Guide Position
5'_GuRO
3'_GuRO
Guide length
Fold change in Luciferase Expression
Normalized to plasmid with non-specific guide and AOAH (STOP)
Data are displayed as the mean of n=2 biological replicates
Screening assays enables evaluation and identification of optimal gRNAs designed on AOAH recruitment domain
Numerous screening assays ongoing, initially focused

FIG. 34

	COMPLETED	ONGOING
SCREENING	• Luciferase gain-of-function-PMP22 • Luciferase gain-of-function-ABCA4 • Luciferase and GFP gain-of-function for tRNA-Arg^{UCA}	• Luciferase gain-of-function-SNCA • SELEX - ADAR recruitment
CELL LINE DEVELOPMENT	• ADAR1 KO (HEK293 and K562) • ADAR2 KO (HEK293 and K562) • ADAR1 KO // ADAR2 over-expression • VPR-gene activation in K562 • LUHMES human dopaminergic neuron • Differentiated myotubes (RD rhabdomyosarcoma)	• ADAR 1/2 KO (LUHMES) • ADAR2 KO // p110p150 over-expression • MECP2-specific mutations (R168X, R255X, R270X, R294X) in LUHMES cells
ANALYTICS	• ddPCR universal gRNA and target mRNA quantification • ddPCR drop-off editing quantification • ddPCR exon skipping quantification	• ddPCR multiplexing >3 • On-target/off-target transcriptome analysis • Protein quantification - aSyn, MECP2

FIG. 35

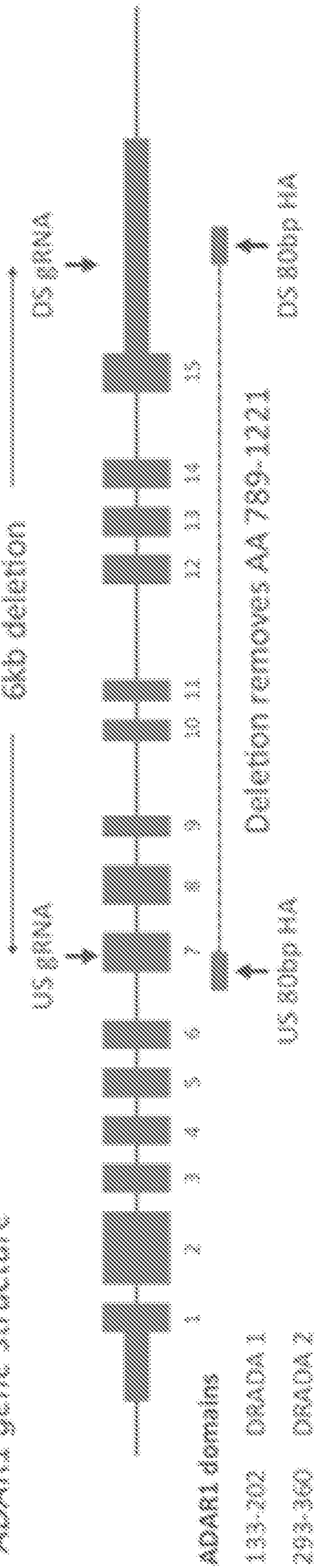

ADAR1 gene structure
1 2
ADAR1 domains
133-302 dsRBD 1
293-360 dsRBD 2
393-571 dsRBM 1
614-692 dsRBM 2
703-571 dsRBM 3
886-1221 A to I editase
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Δ6b deletion
WT
Δ6b
WT dsRNA
Δ6b dsRNA
Oligo dT
WT dsRNA
Δ6b dsRNA
Oligo dT
Anti-ADAR1
Anti-GAPDH
WT
Δ6b
WT dsRNA
Δ6b dsRNA
Oligo dT
WT dsRNA
Δ6b dsRNA
Oligo dT

FIG. 36

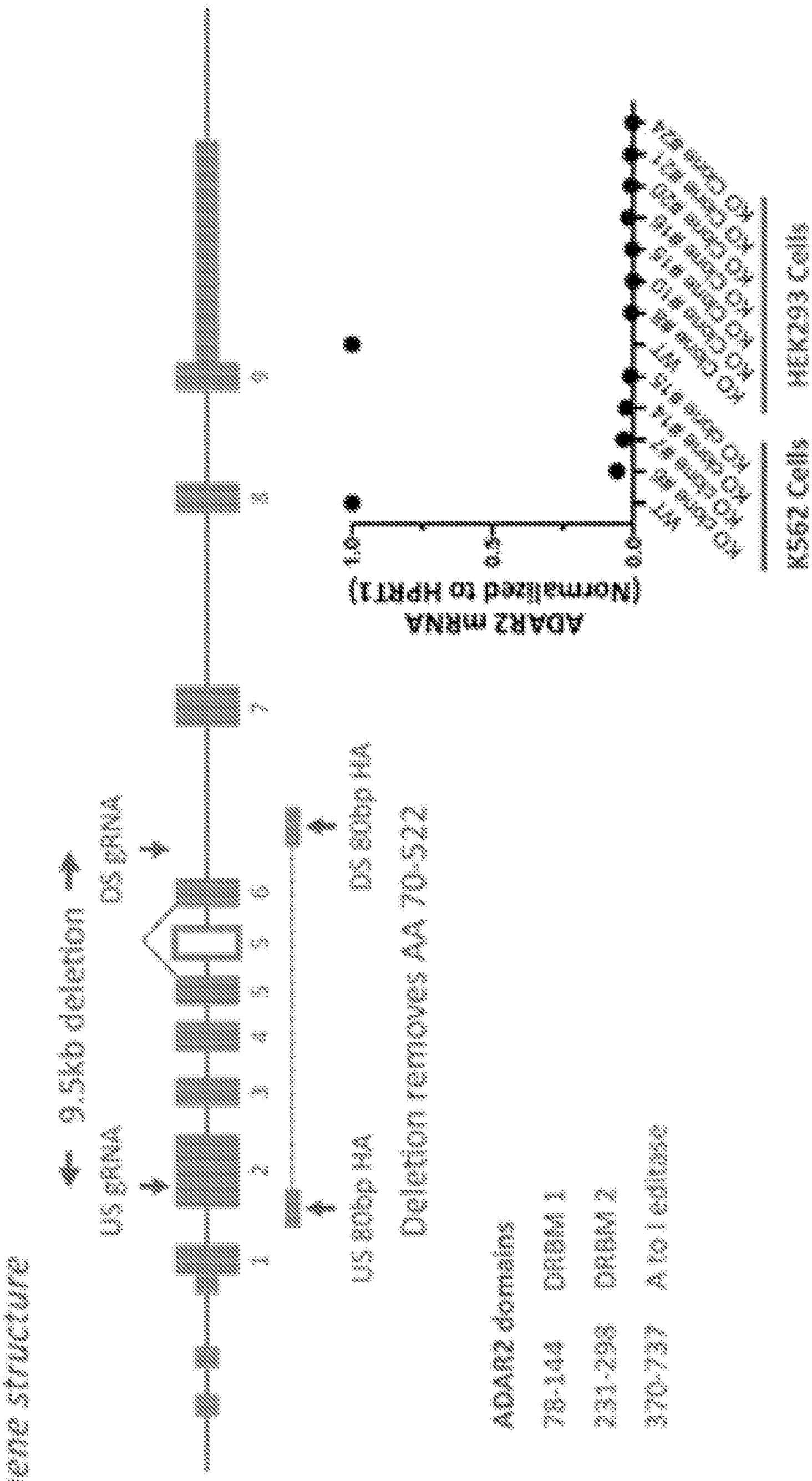

ADAR2 gene structure
9.5kb deletion
US gRNA
DS gRNA
US gRNA
DS gRNA
US 80bp HA
DS 80bp HA
Deletion removes AA 70-522
Domains
dsRBM 1
dsRBM 2
A to I editase
Amino acid
1-69
70-141
231-299
300-737
ADAR2 mRNA
(Normalized to HPRT1)
293 Cells
K562 Cells

FIG. 37

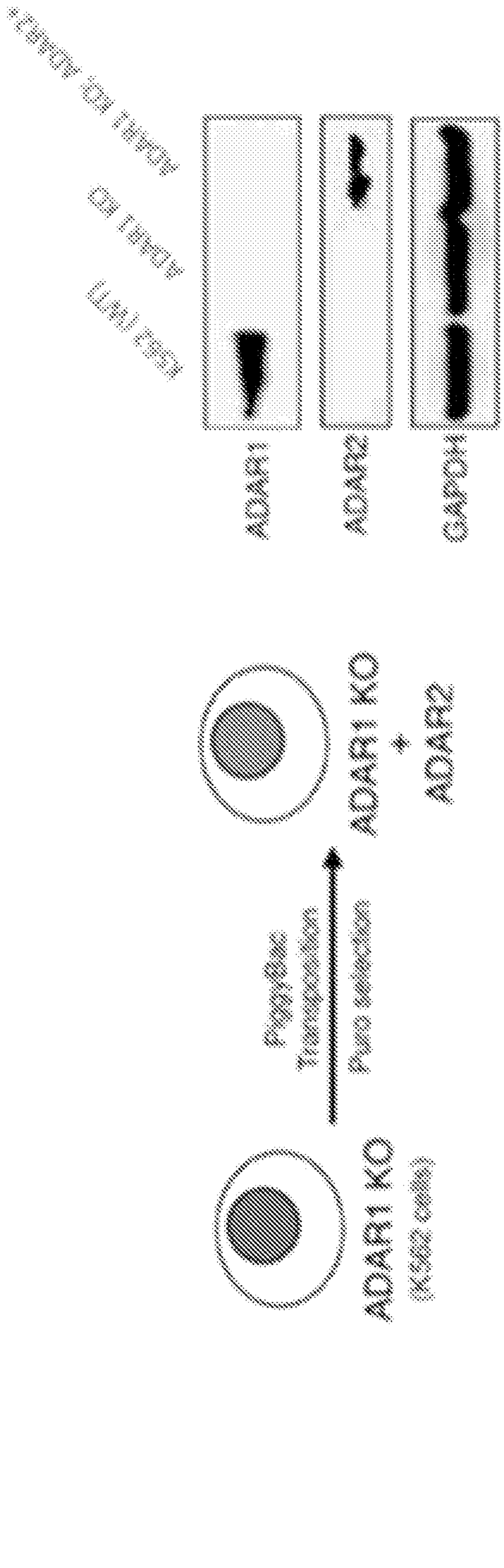

Cells
ADAR KO
ADAR KO + ADAR
Antisense oligonucleotide targeting ADAR
Incubation
Cells
ADAR KO
ADAR KO + ADAR

FIG. 38

- ddPCR divides a PCR reaction into thousands of droplets
- The droplets are then analyzed and positive and negative droplets are used to quantify the absolute amount of target in each sample
- Advantages: Higher signal-to-noise, increased precision, and absolute quantitation

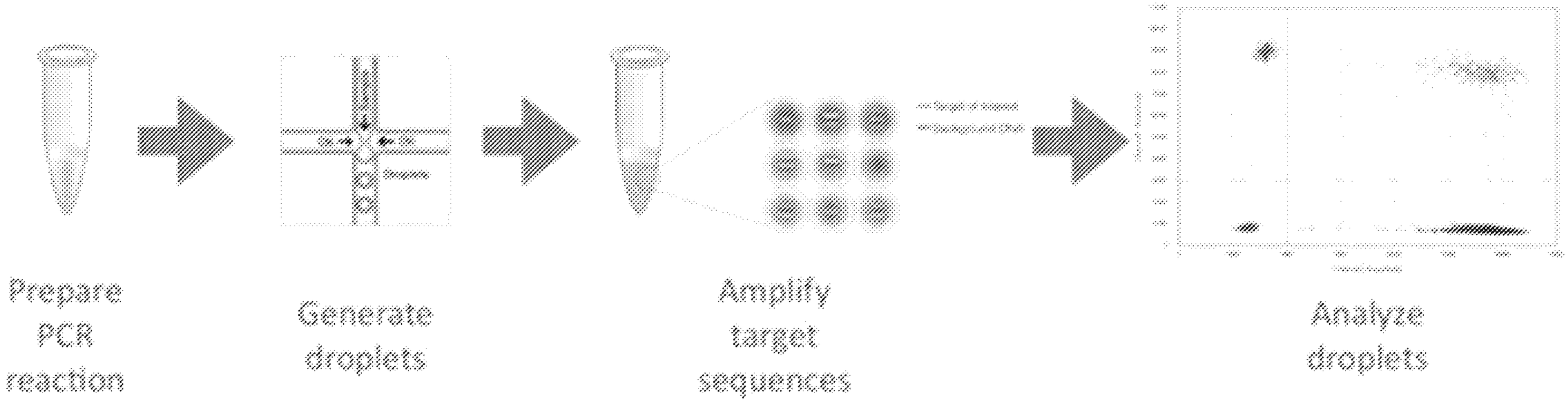


FIG. 39

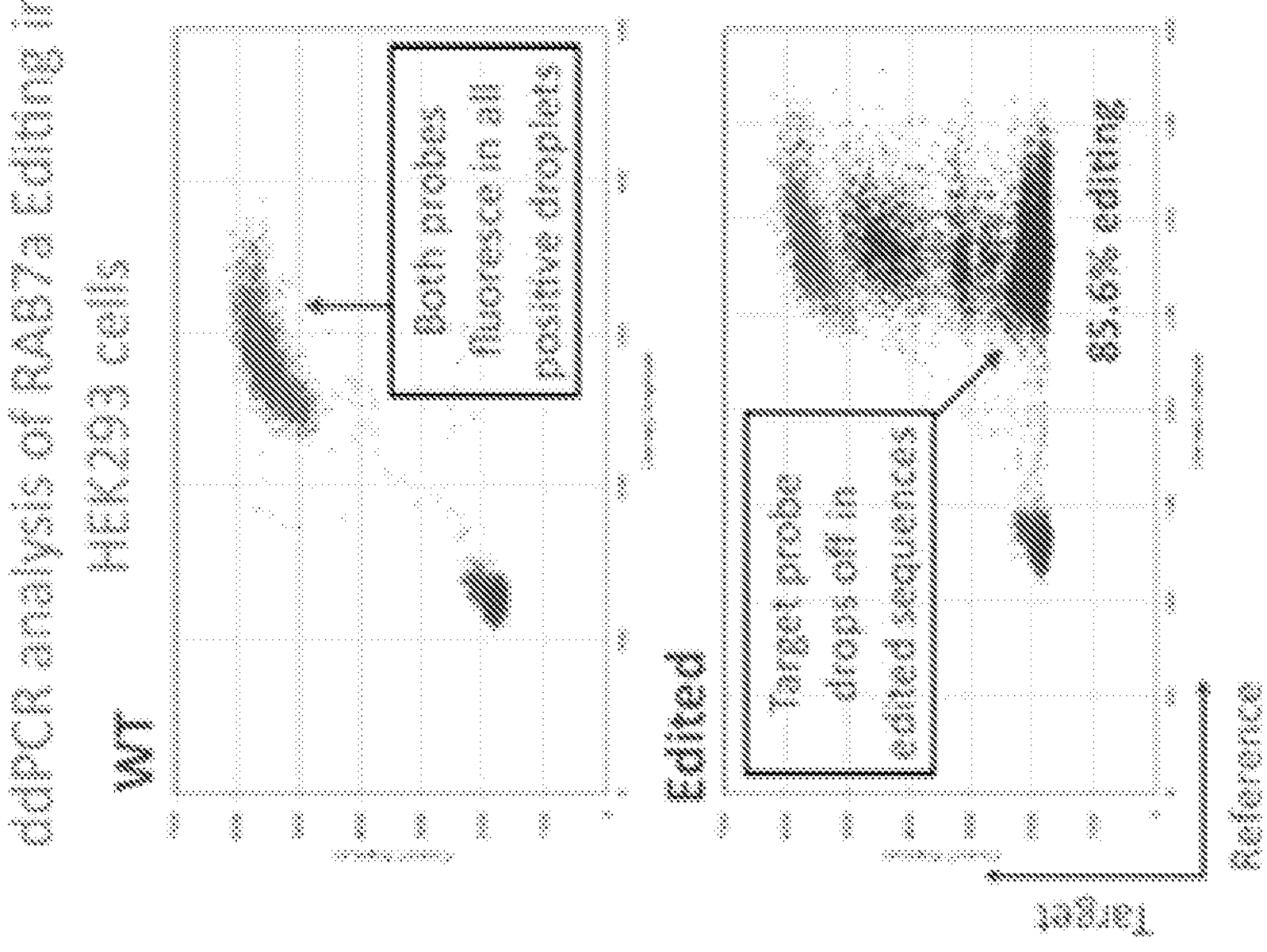
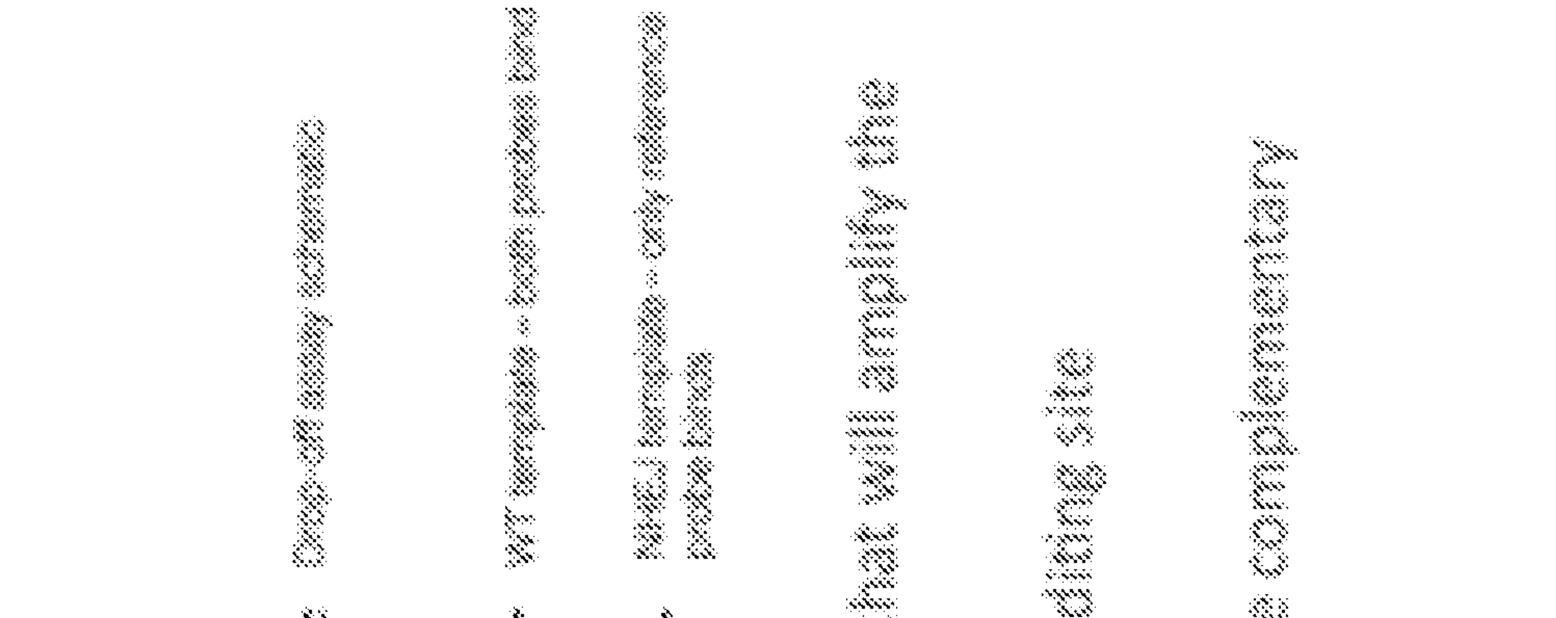

ddPCR analysis of RAB7a editing in HEK293 cells
Shift
Both probes fluoresce in all positive droplets
Edited
Target
Reference
Edited sequences
Target probe fluoresces in edited droplets
Reference probe fluoresces in all droplets
Amplitude
Target
Reference

FIG. 40


Reference probe
FAM
Droplet-off assay schematic
FAM
WT template + both probes (wild-type droplets)
FAM
Mutant template + only reference probe (edited droplets)
Design PCR primers to amplify the target region
One probe is targeted to the edited site
Second probe is placed outside complementary homology arm as a reference
Design primers to amplify the target region
One probe is targeted to the edited site
Second probe is placed outside complementary homology arm as a reference
Droplet-off probe
Quench
FAM
Target probe

Why quantify guide RNAs?

- gRNA cellular expression is a key parameter that drives editing efficiency
- Modifications to gRNA structure may alter efficiency or completion of transcription

Goal: Develop a ddPCR assay that identifies universal tags on the ends of gRNAs ("gRNA^{Q}")

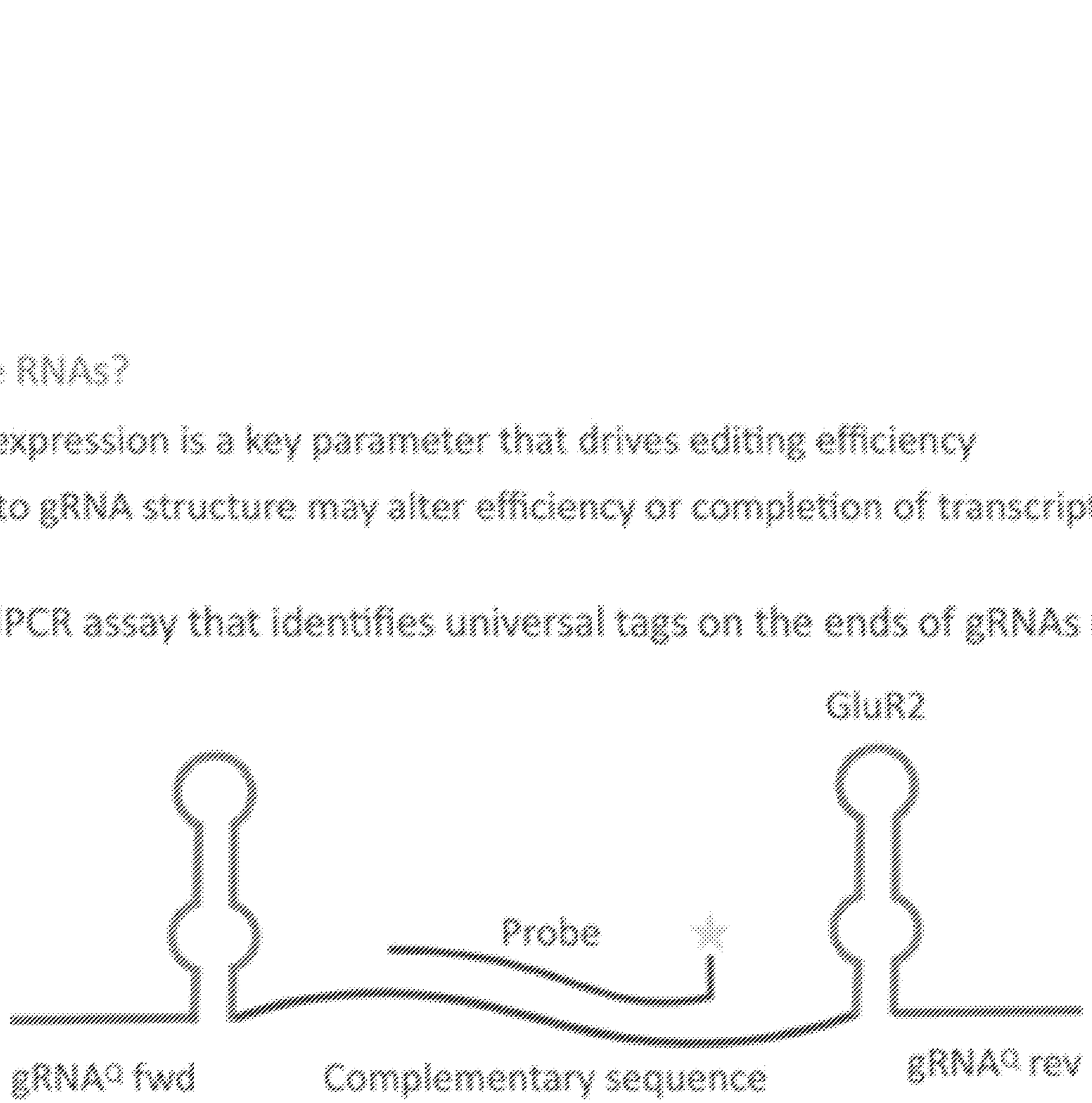


FIG. 41

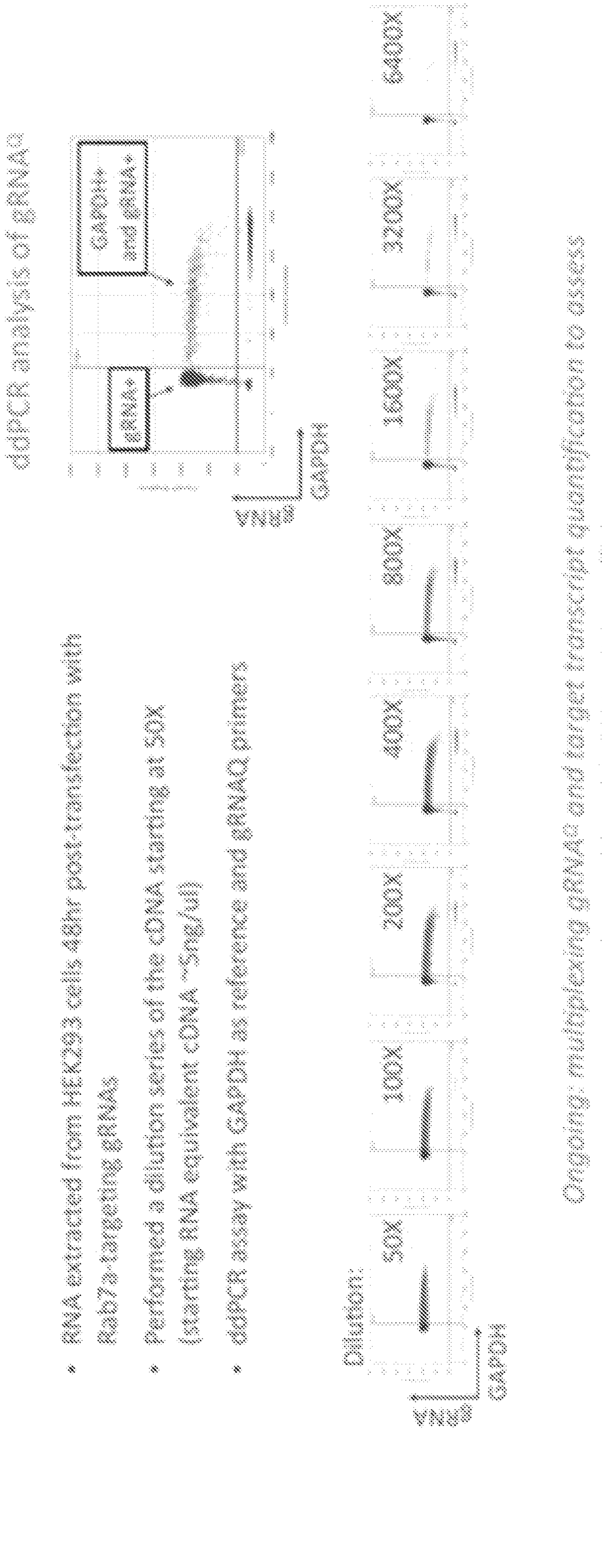
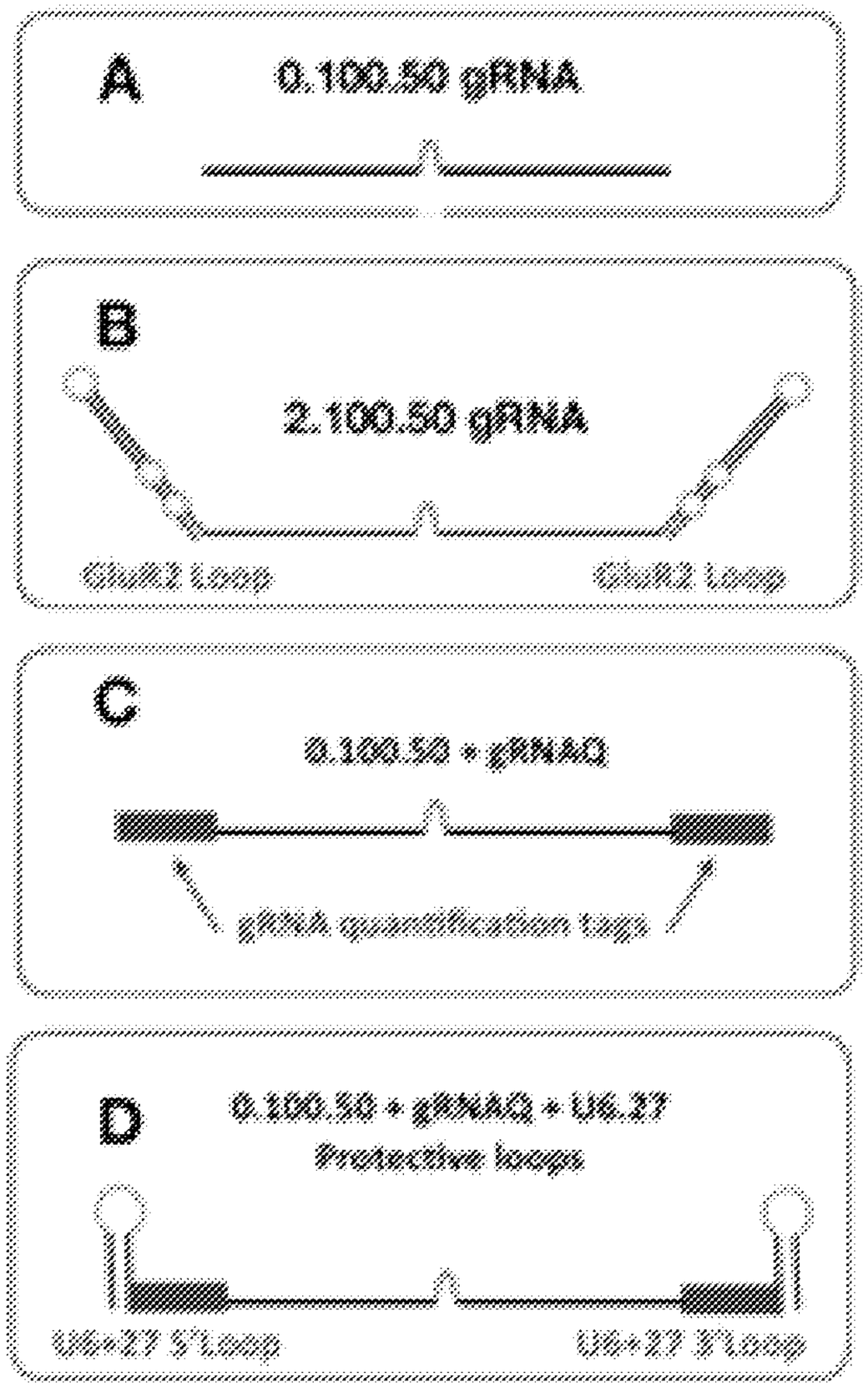
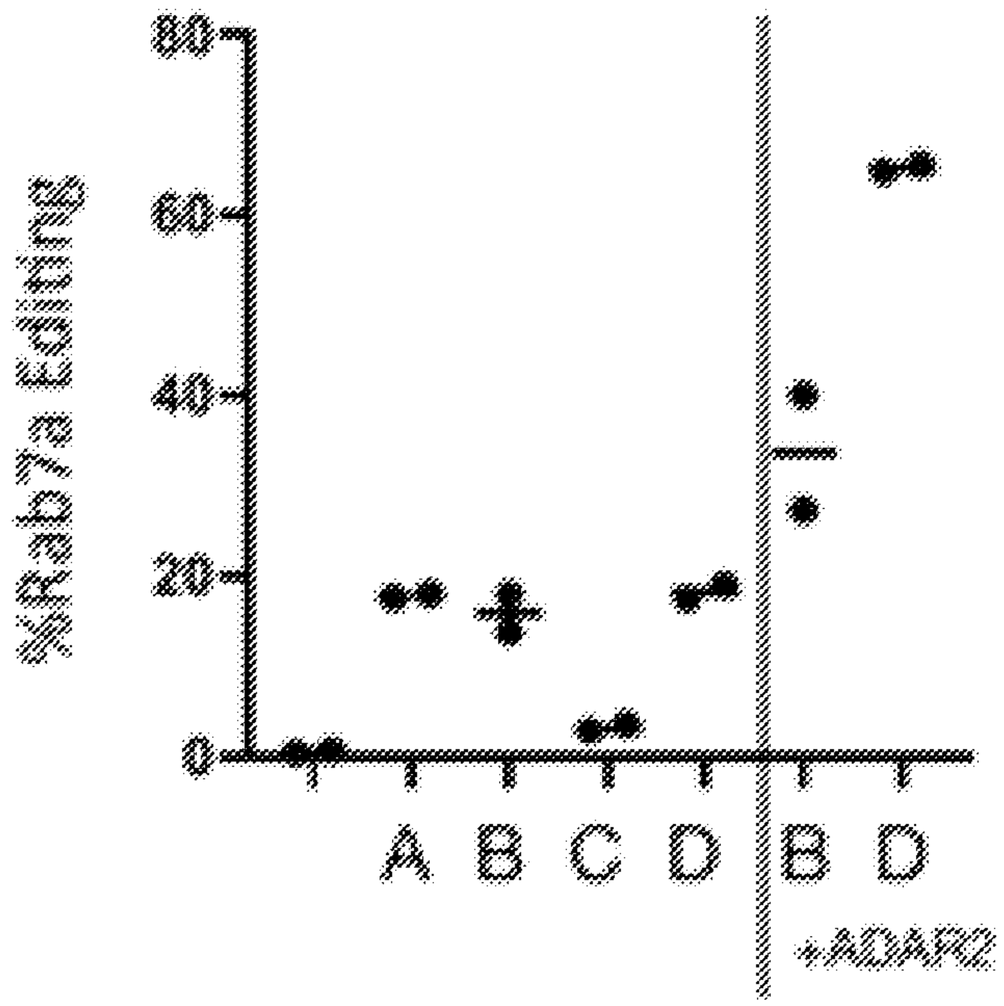

dpCas9 analysis of RNA
Cas9/sgRNA complex
sgRNA
Cas9
1x
2x
4x
100x
200x
400x
800x
Obtain 50x Cas9
RNA extracted
Reverse-transcribed cDNA template
Performed qPCR using equivalent cDNA ~5ng/ul
Cas9 protein series of the qDNA starting at 50x
Transcription with Cas9 as reference and qPCR primers
transcript quantification to assess
relationship with RNA expression
Obtain multiplexing gRNA and target
RNA extracted
Reverse-transcribed cDNA template
Performed qPCR using equivalent cDNA ~5ng/ul
gRNA series of the qDNA starting at 50x
Transcription with Cas9 as reference and qPCR primers
transcript quantification to assess
relationship with RNA expression

FIG. 42





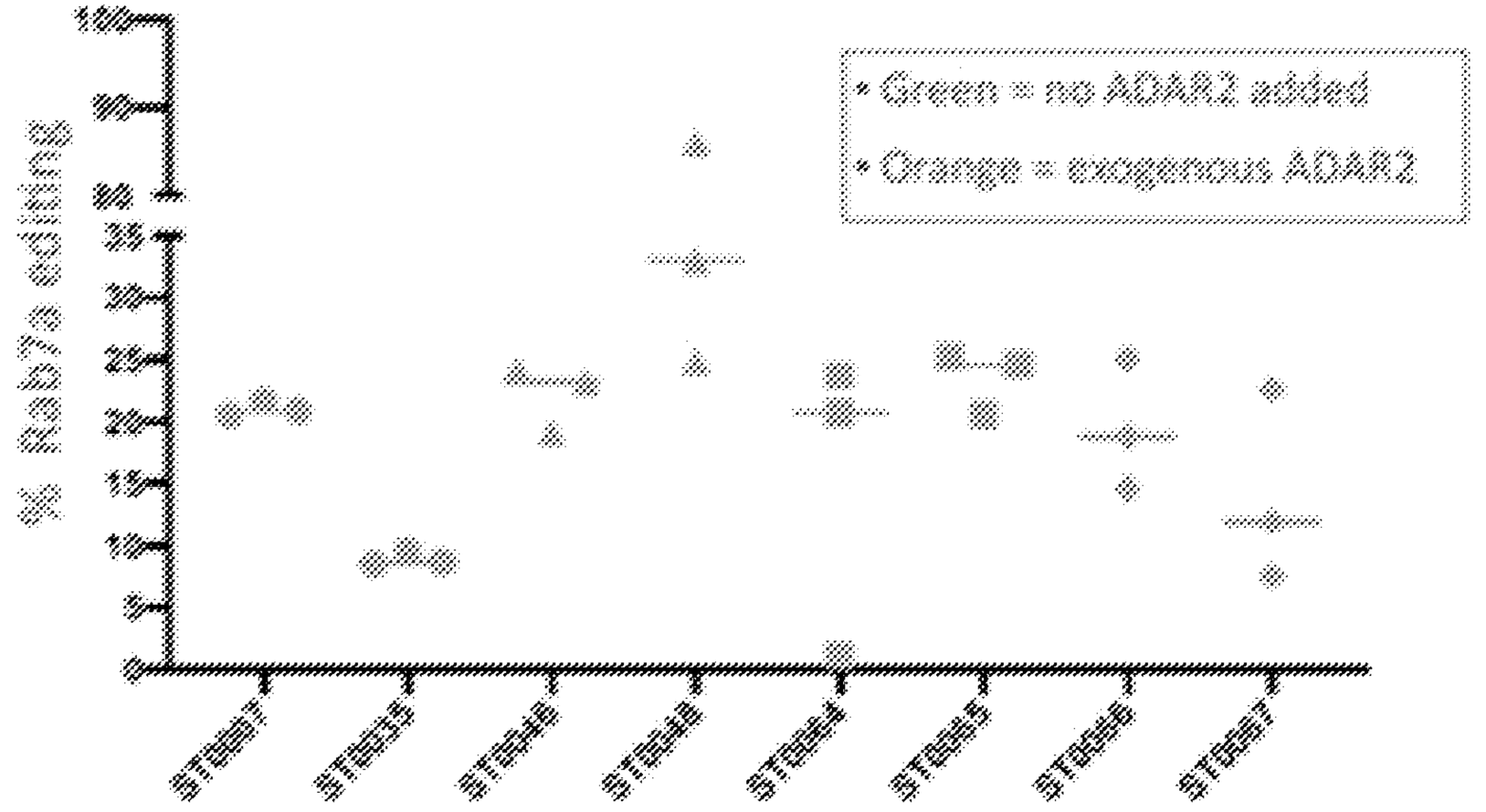
- ddPCR-analysis of Rab7a editing in HEK293 cells
- Data suggests the U6+27 loop increases editing efficiency in the presence of ADAR2

FIG. 43

Construct Designs

Construct ID	Construct Description
STO007	hU6_2_100_50_GFP
STO035	ADAR2 only, no adRNA
STO046	hU6_2_100_50_2 bulge_GFP
STO048	hU6_2_100_50_2 bulge_ADAR2
STO064	xiaCMVen-hU6_2_100_50_2 bulge_GFP
STO065	xiaCMVen-hU6_2_100_50_2 bulge_ADAR2
STO066	sgCMVen-hU6_2_100_50_2 bulge_GFP
STO067	sgCMVen-hU6_2_100_50_2 bulge_ADAR2

Rab7a editing in HEK293 cells



- Rab7a editing in HEK293T cells
- R-edit analysis of Sanger sequencing data

FIG. 44

Combining semi-rational design with high throughput screening

- ADARs recognize structure, *not sequence*, enabling significant 3D design space
  - Approach #1: Using gRNA/ADAR interaction motifs, create large gRNA mutagenesis libraries (~10^15) and SELEX to increase ADAR binding to gRNA sequences
  - Approach #2: Use Alu elements known to effectively recruit ADARs (also amenable to library screening)
  - Approach #3: mine available CLIP-seq data to identify additional natural ADAR-binding dsRNA structures

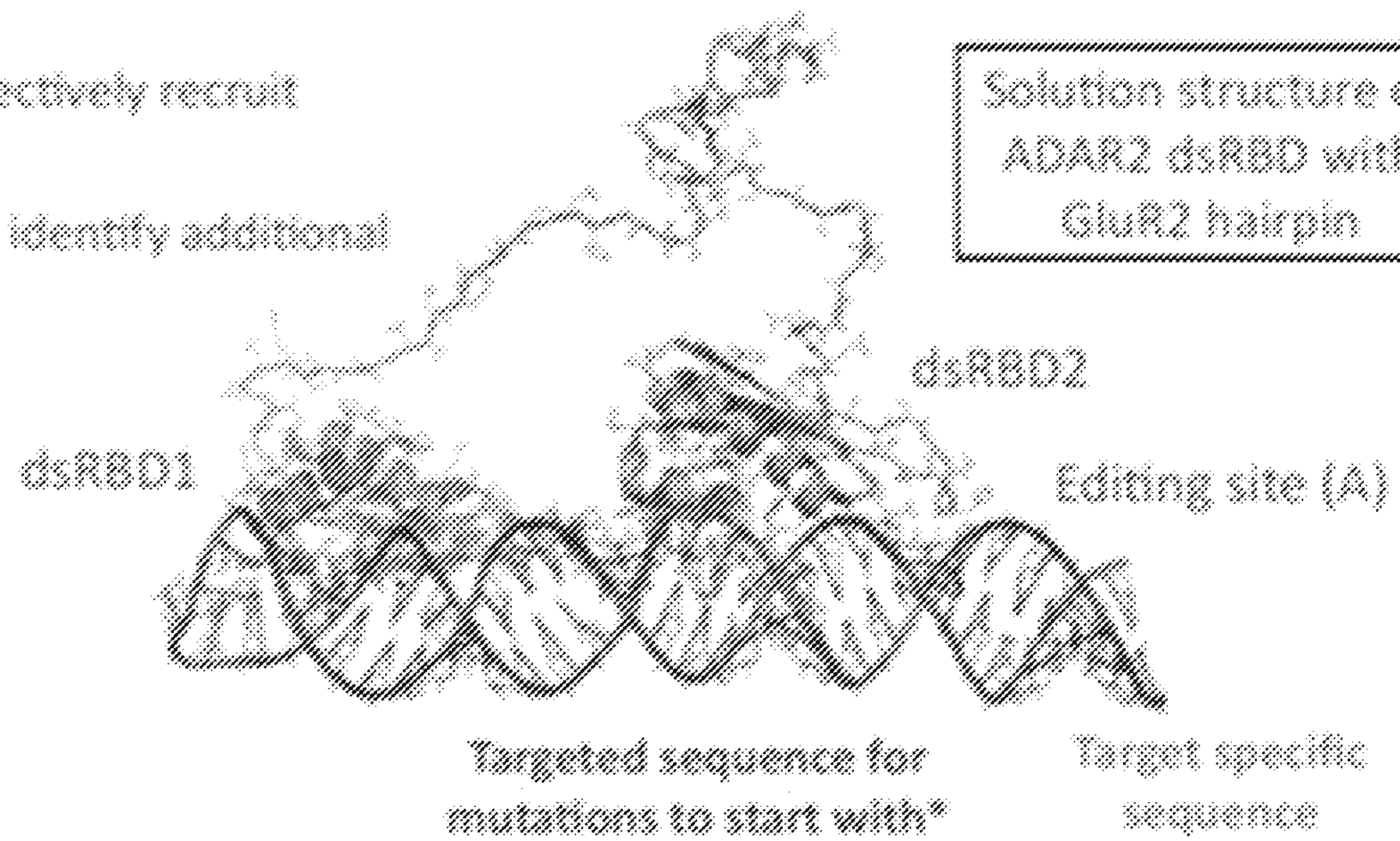
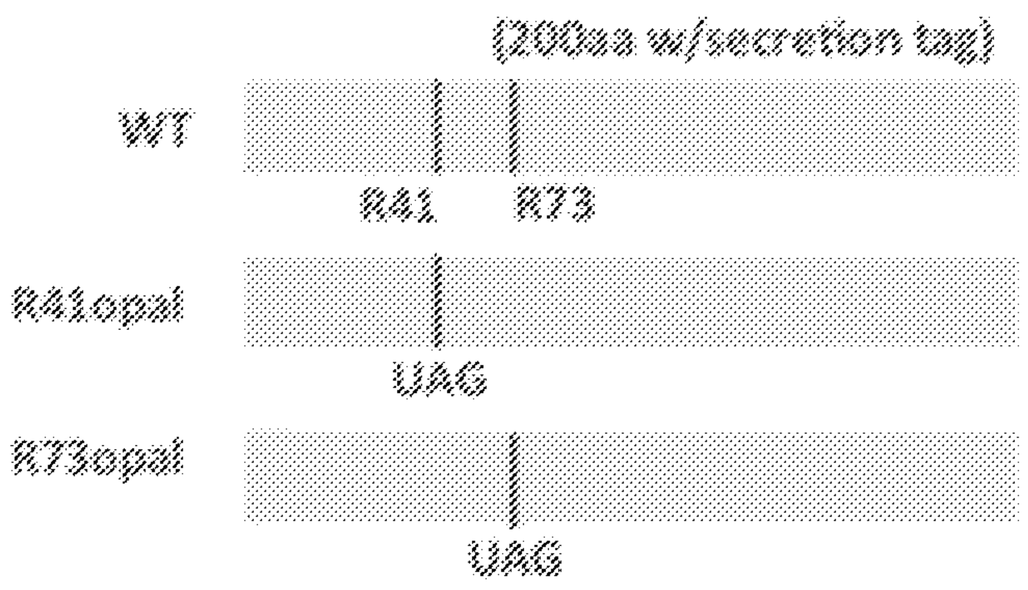


FIG. 45

*>90% of nonsense mutations in Rett syndrome are Arg>opal (CGA>UGA)**

Nano-luciferase Arg>opal screening constructs



Screening Status

- 28 natural Arg isodecoders generated with Opal-recognition anticodon loops
- Arg-tRNA^{UCA} quantification cassette developed
- Screening in HEK293 and K562 ongoing
- Note: GFP Arg>opal constructs also generated/validated

Arg>opal mutations eliminate luciferase expression

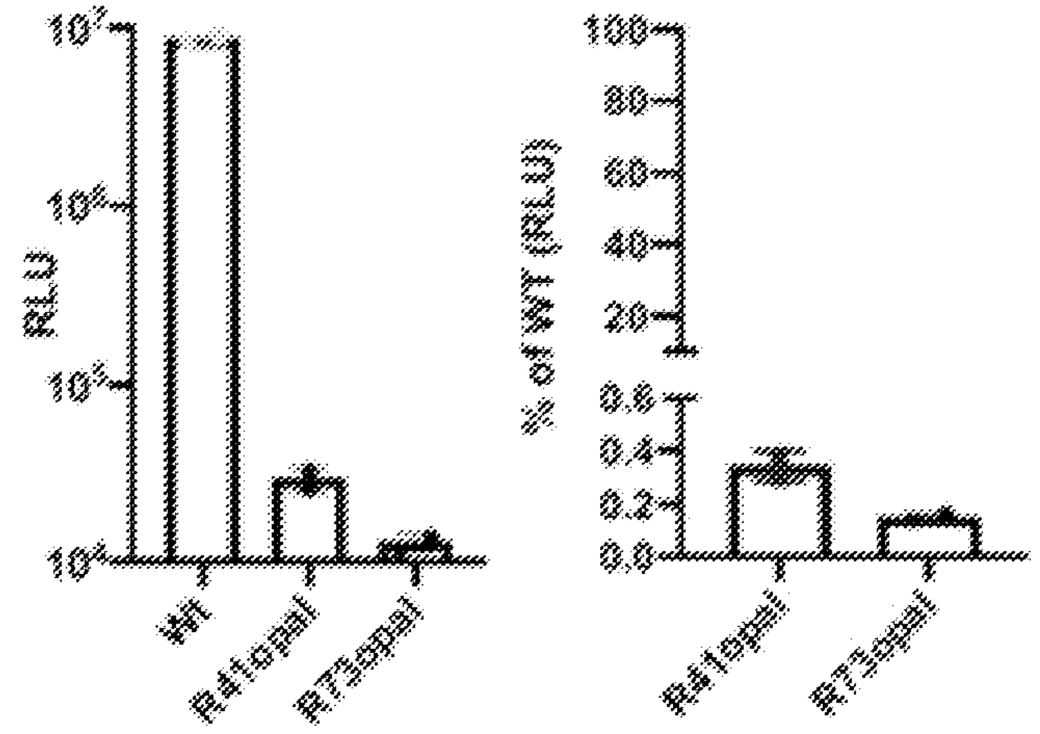


FIG. 46

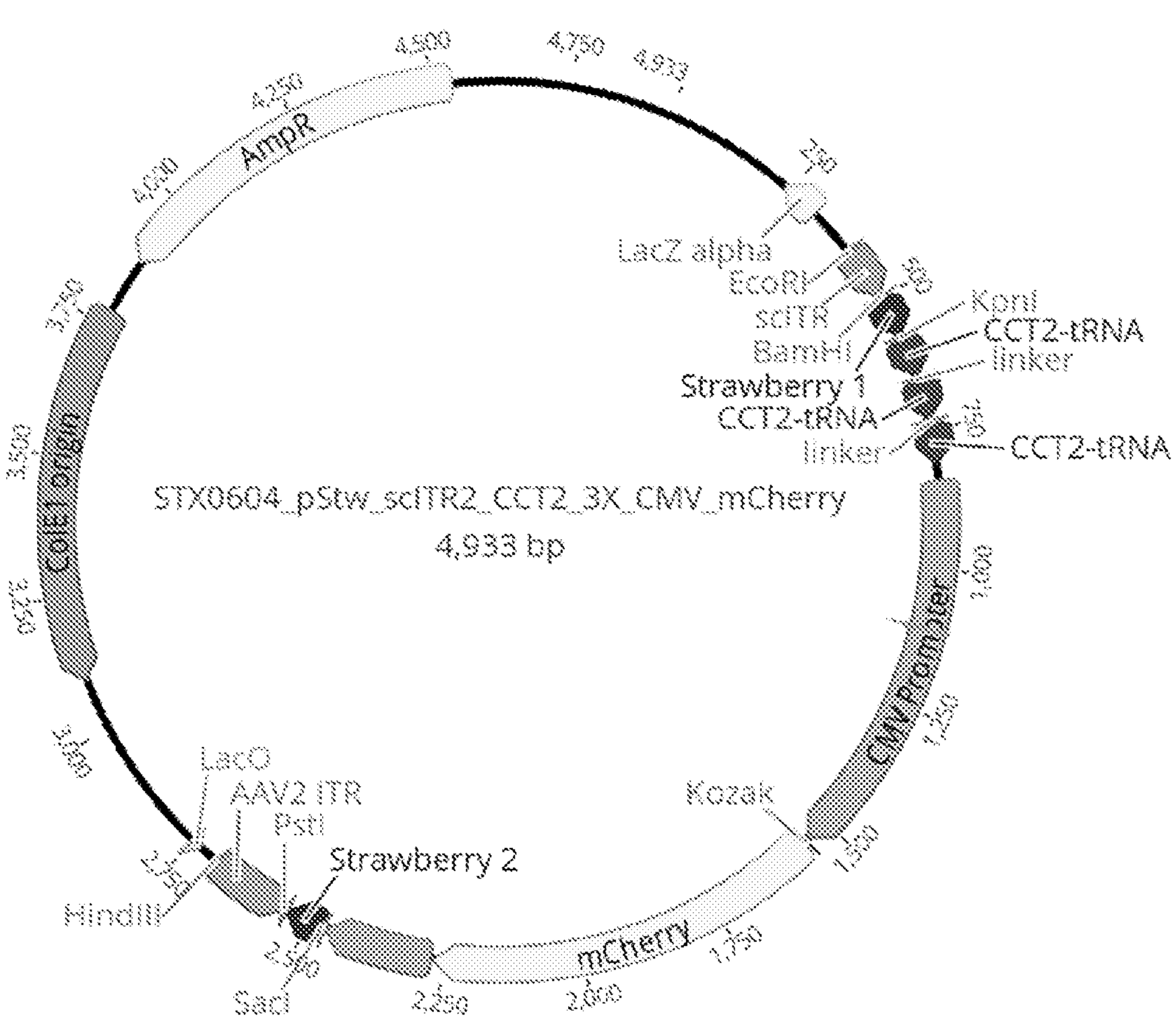

4,500
4,750
4,933
4,250
AmpR
4,000
5'
LacZ alpha
EcoRI
3'
scITR
KpnI
CCT2-tRNA
BamHI
linker
Strawberry 1
CCT2-tRNA
3'
linker
CCT2-tRNA
STX0604_pStw_scITR2_CCT2_3X_CMV_mCherry
4,933 bp
3,500
3,250
ColE1 origin
3,000
LacO
AAV2 ITR
PstI
Strawberry 2
Kozak
1,500
1,750
mCherry
HindIII
5'
2,500
2,750
SacI
2,250
2,000

FIG. 47A

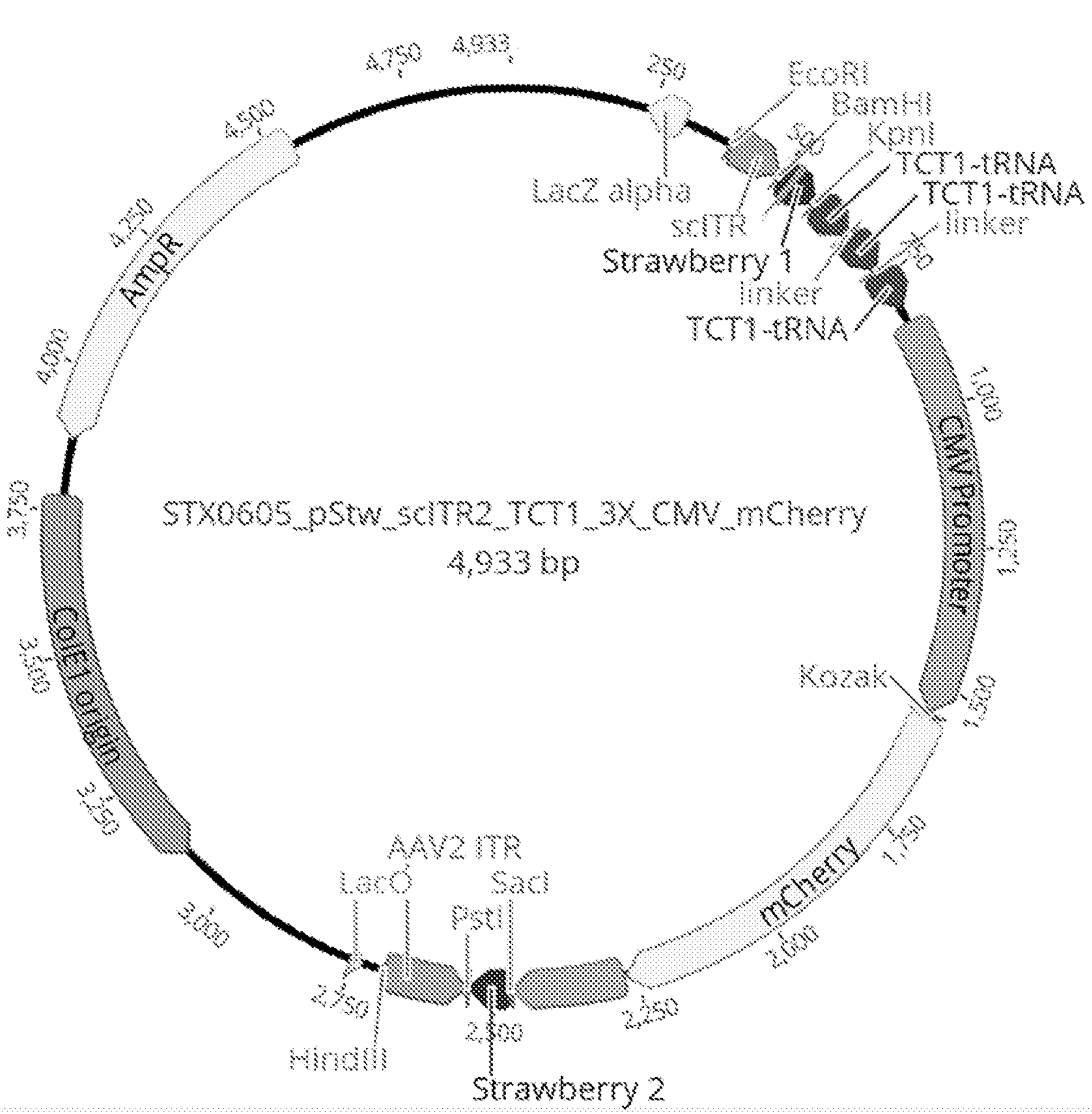

4,750
4,933
250
EcoRI
BamHI
Kpnl
TCT1-tRNA
TCT1-tRNA
linker
Lac2 alpha
scITR
Strawberry 1
linker
TCT1-tRNA
4,500
4,250
AmpR
4,000
1,000
CMV Promoter
750
3,750
ColE1 ori
3,500
3,250
Kozak
1,500
AAV2 ITR
LacO
SacI
PstI
3,000
mCherry
1,750
1,500
2,000
2,250
2,500
2,750
HindIII
Strawberry 2
STX0605_pStw_scITR2_TCT1_3X_CMV_mCherry
4,933 bp

FIG. 47B

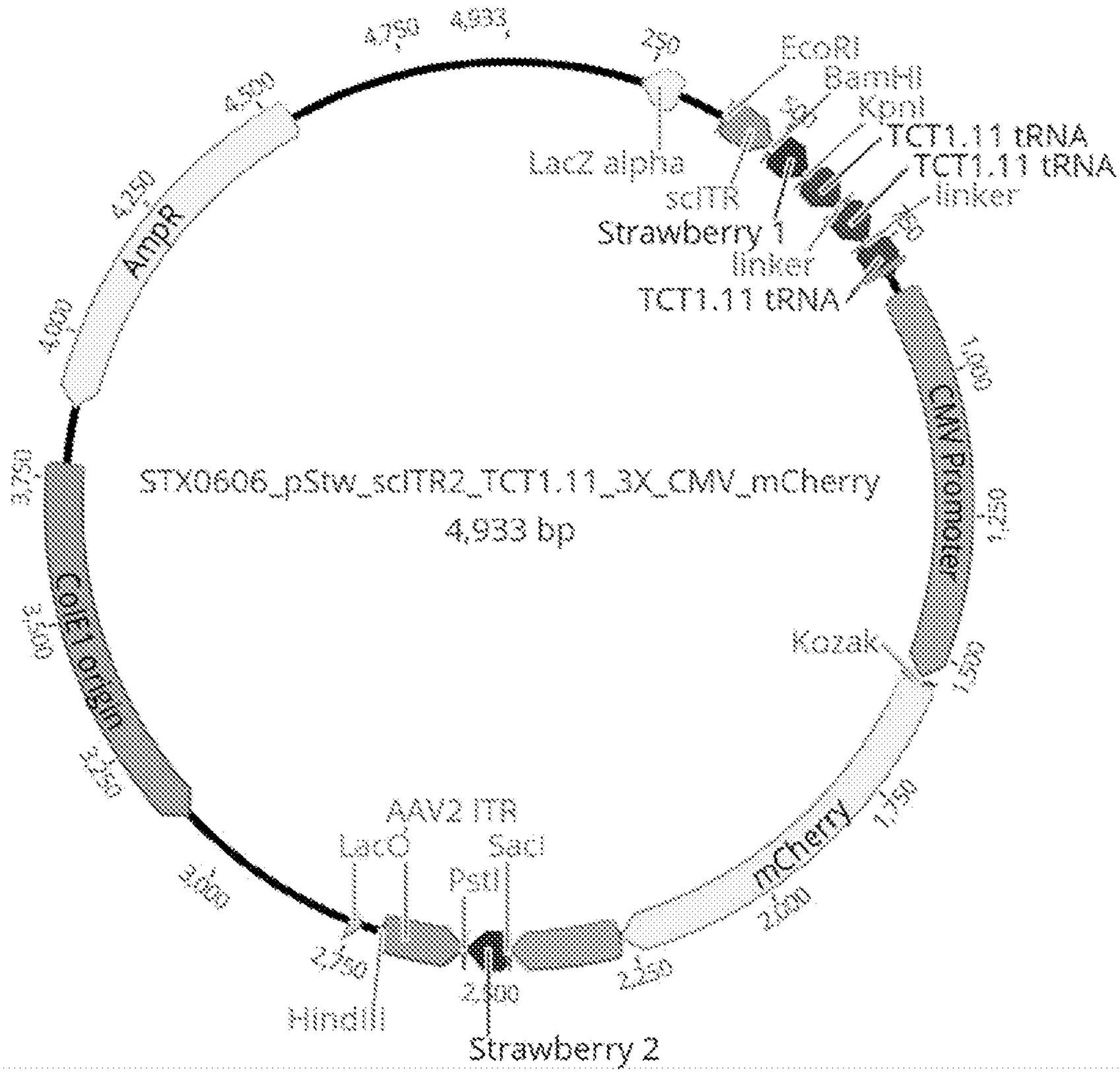

STX0606_pStw_scITR2_TCT1.11_3X_CMV_mCherry
4,933 bp
4,750
4,933
250
4,500
4,250
4,000
3,750
3,500
3,250
3,000
2,750
2,500
2,250
2,000
1,750
1,500
1,250
1,000
750
500
250
AmpR
ORI (pMB1)
CMV Promoter
mCherry
AAV2 ITR
scITR
LacZ alpha
Strawberry 1
Strawberry 2
EcoRI
BamHI
KpnI
TCT1.11 tRNA
TCT1.11 tRNA
linker
linker
TCT1.11 tRNA
Kozak
LacO
SacI
PstI
HindIII

FIG. 47C

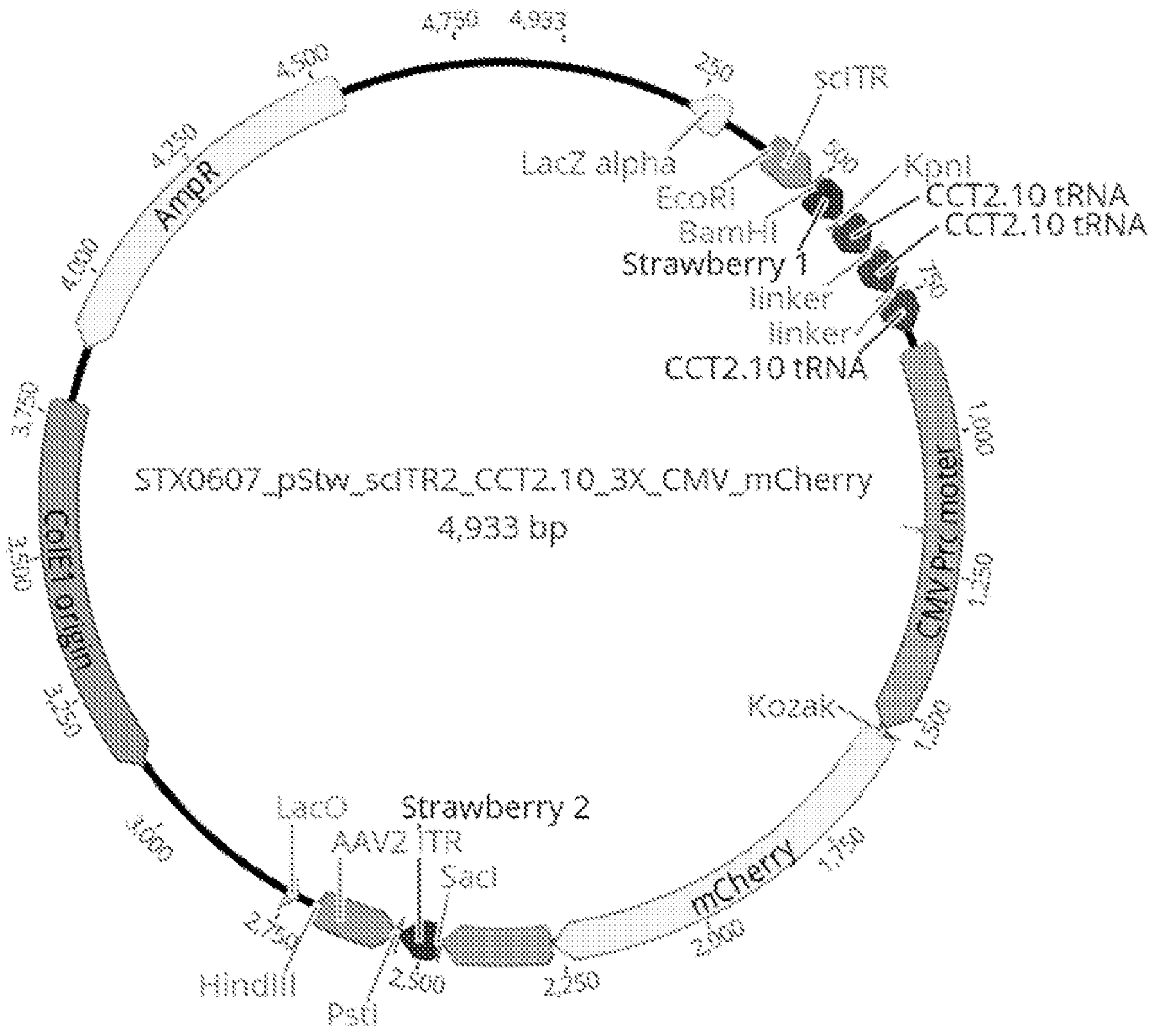

STX0607_pStw_scITR2_CCT2.10_3X_CMV_mCherry
4,933 bp
4,750
4,933
4,500
4,250
4,000
AmpR
3,750
3,500
3,250
3,000
ColE1 ori
scITR
LacZ alpha
EcoRI
BamHI
Strawberry 1
KpnI
CCT2.10 tRNA
CCT2.10 tRNA
linker
linker
CCT2.10 tRNA
1,000
1,250
1,500
1,750
2,000
2,250
2,500
CMV Promoter
Kozak
mCherry
LacO
AAV2 ITR
Strawberry 2
SacI
HindIII
PstI

FIG. 47D

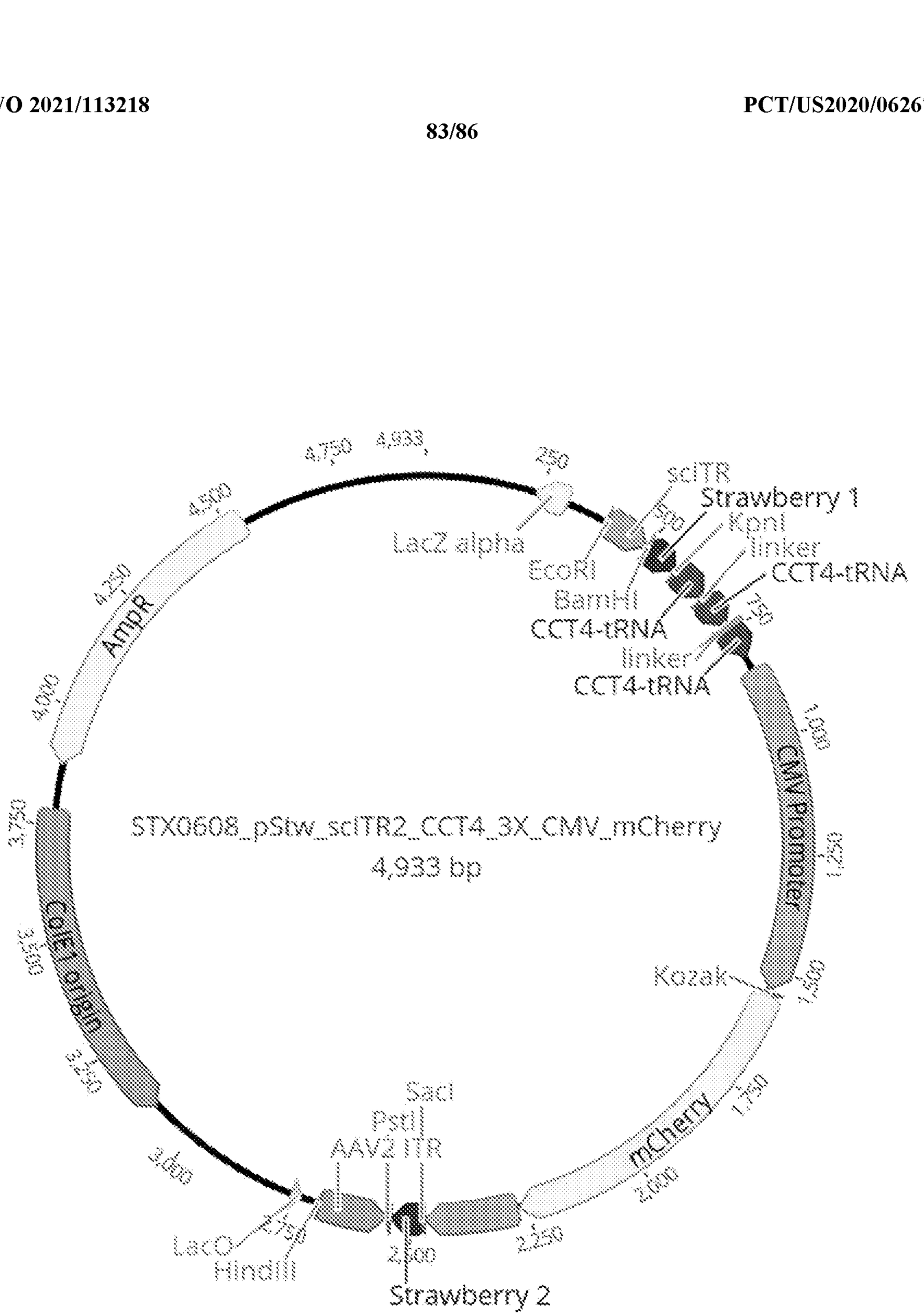

STX0608_pStw_scITR2_CCT4_3X_CMV_mCherry
4,933 bp
scITR
Strawberry 1
KpnI
linker
CCT4-tRNA
linker
CCT4-tRNA
linker
CCT4-tRNA
BamHI
EcoRI
LacZ alpha
AmpR
CMV promoter
Kozak
mCherry
ColE1 ori
LacO
HindIII
PstI
AAV2 ITR
SacI
Strawberry 2

FIG. 47E

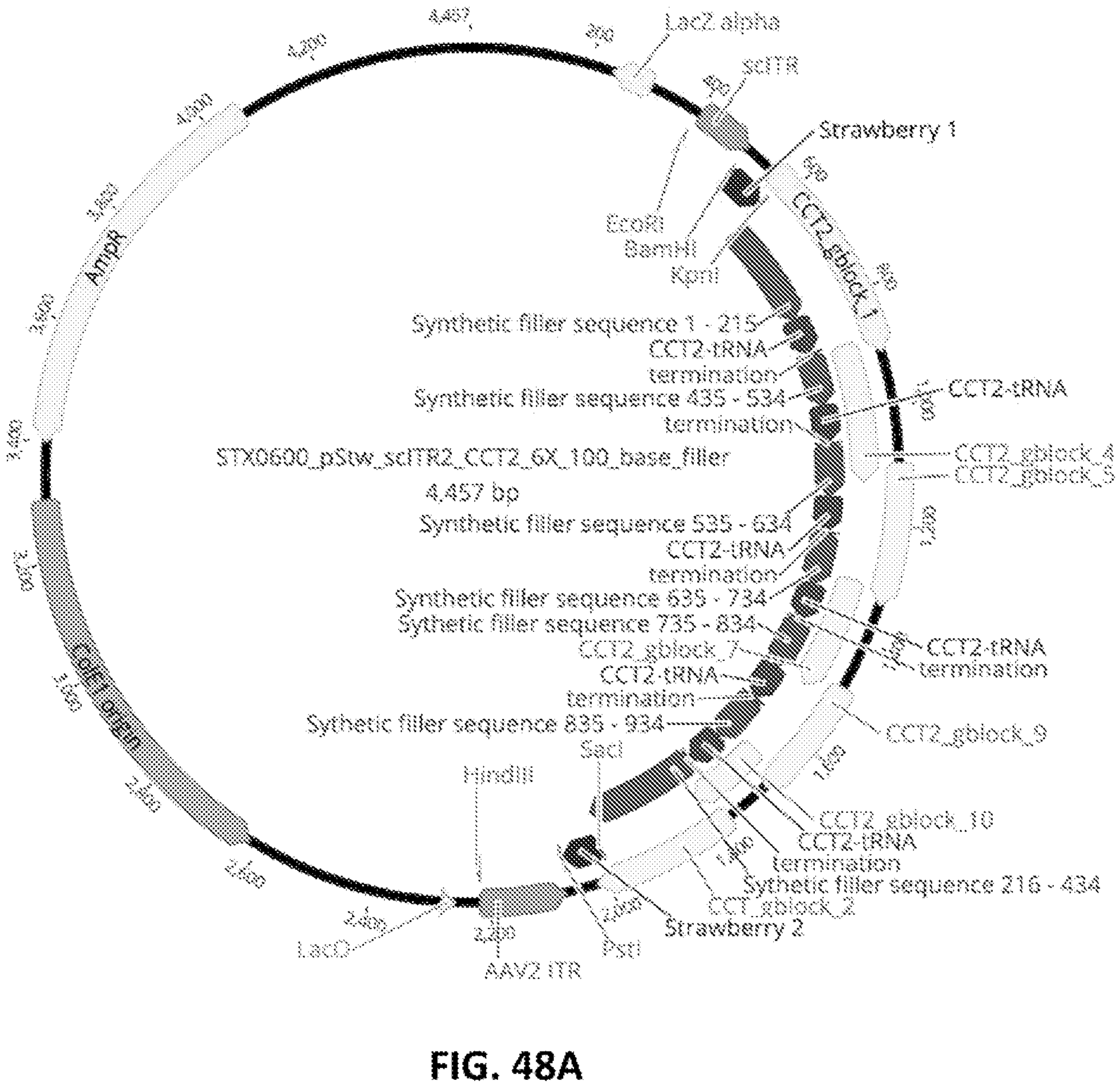

LacZ alpha
SciTR
Strawberry 1
EcoRI
BamHI
KpnI
CCT2_gblock_1
Synthetic filler sequence 1 - 215
CCT2-tRNA
termination
Synthetic filler sequence 435 - 534
termination
CCT2-tRNA
STX0600_pStw_scITR2_CCT2_6X_100_base_filler
4,457 bp
CCT2_gblock_4
CCT2_gblock_5
Synthetic filler sequence 535 - 634
CCT2-tRNA
termination
Synthetic filler sequence 635 - 734
Sythetic filler sequence 735 - 834
CCT2_gblock_7
CCT2-tRNA
termination
CCT2-tRNA
termination
Sythetic filler sequence 835 - 934
CCT2_gblock_9
SacI
HindIII
CCT2_gblock_10
CCT2-tRNA
termination
Sythetic filler sequence 216 - 434
CCT_gblock_2
Strawberry 2
PstI
LacO
AAV2 ITR
AmpR
ColE1 origin

FIG. 48A

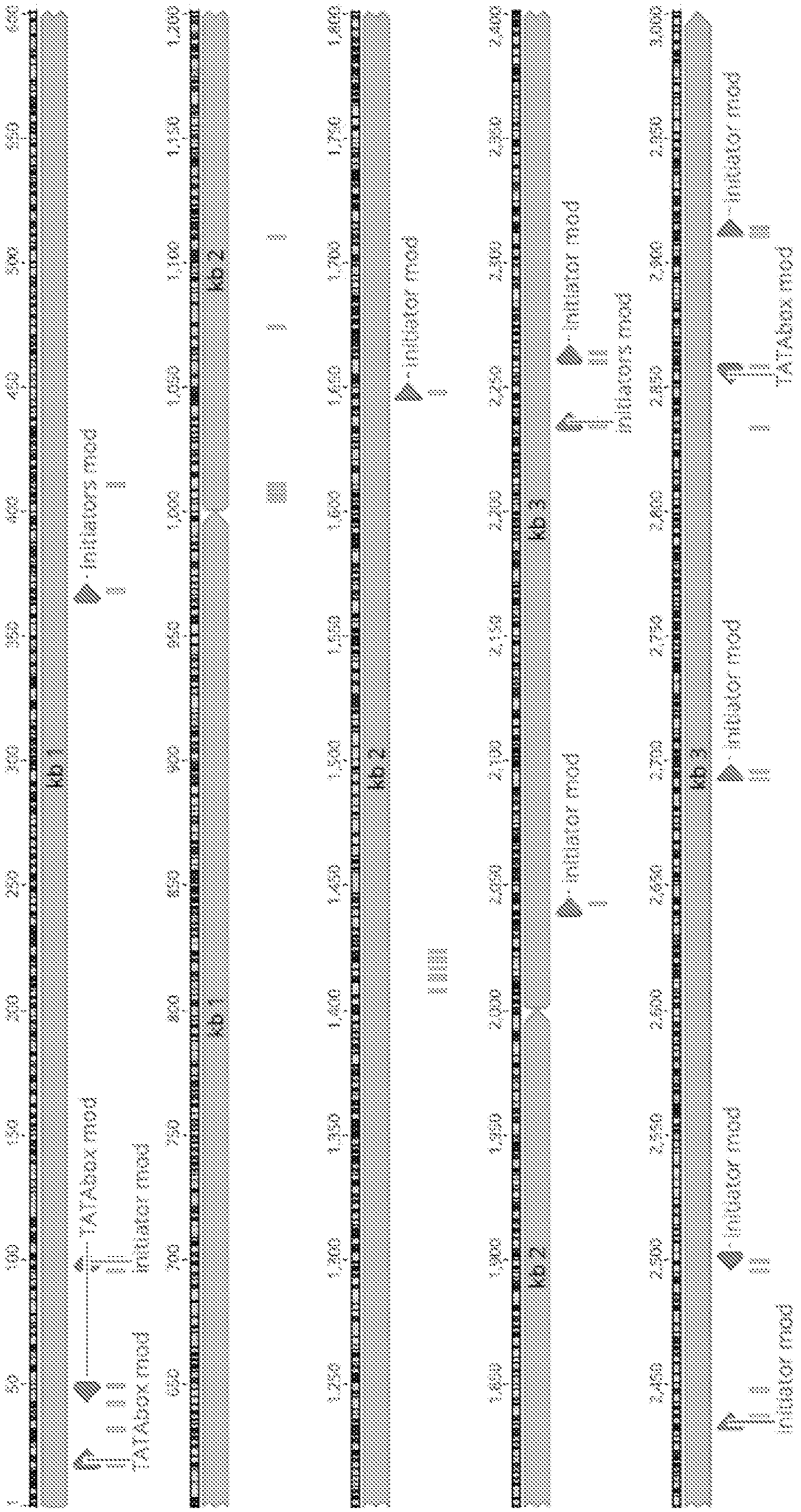


FIG. 48B

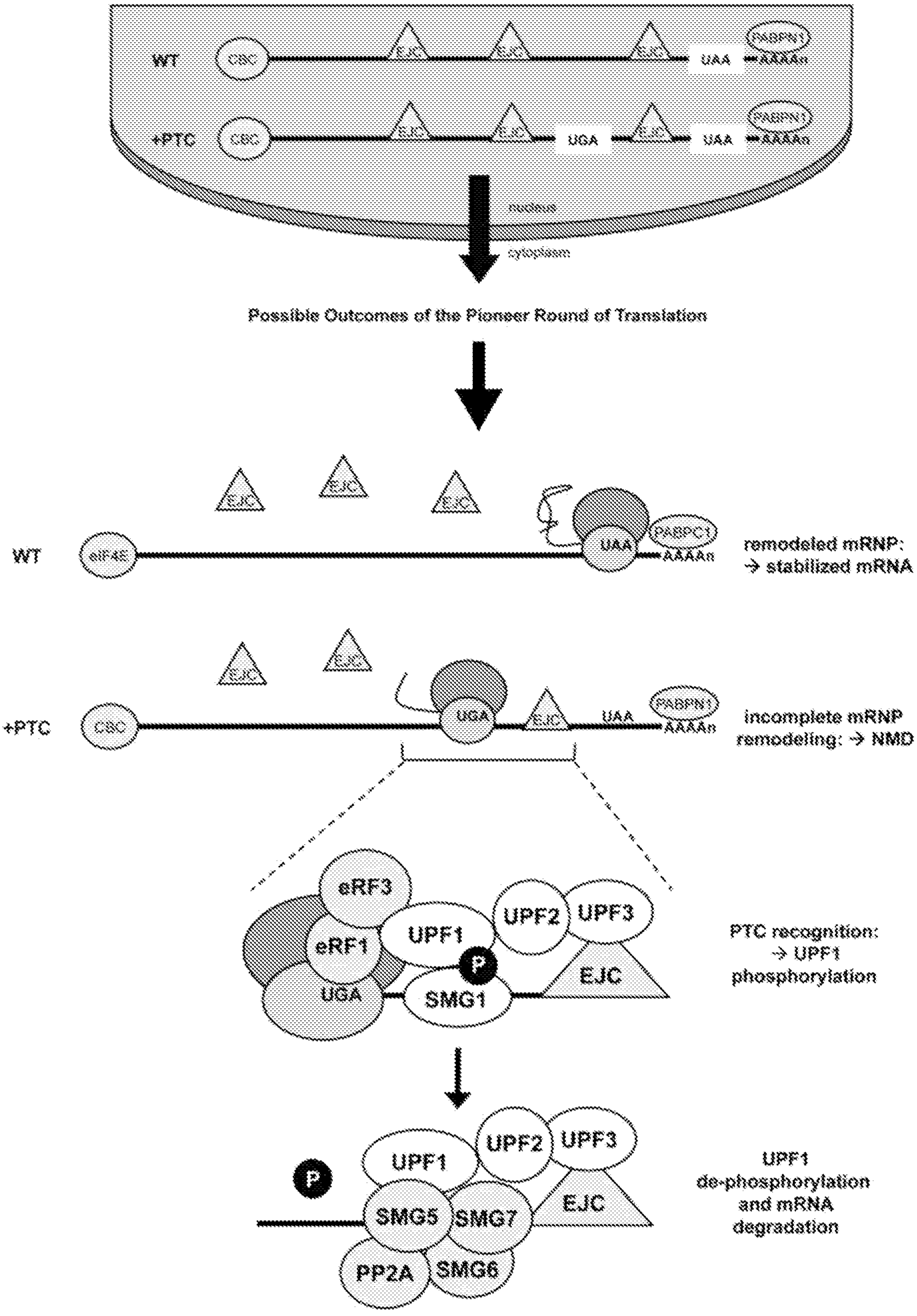

WT
CBC
EJC
EJC
EJC
UAA
PABPN1
AAAAn
+PTC
CBC
EJC
EJC
UGA
EJC
UAA
PABPN1
AAAAn
nucleus
cytoplasm
Possible Outcomes of the Pioneer Round of Translation
EJC
EJC
EJC
WT
eIF4E
UAA
PABPC1
AAAAn
remodeled mRNP:
→ stabilized mRNA
EJC
EJC
+PTC
CBC
UGA
EJC
UAA
PABPN1
AAAAn
incomplete mRNP
remodeling: → NMD
eRF3
eRF1
UPF1
P
UPF2
UPF3
EJC
UGA
SMG1
PTC recognition:
→ UPF1
phosphorylation
P
UPF1
UPF2
UPF3
EJC
SMG5
SMG7
PP2A
SMG6
UPF1
de-phosphorylation
and mRNA
degradation

FIG. 49

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2020/062671

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/11 A61K48/00 A61K31/7088
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, BIOSIS, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2019/090169 A1 (WISTAR INST [US]; UNIV IOWA RES FOUND [US]) 9 May 2019 (2019-05-09) sequences 89-113 claims; examples	1-69, 71-73, 76-79
Y		69,74, 75,80
Y	WO 2018/161032 A1 (UNIV CALIFORNIA [US]) 7 September 2018 (2018-09-07) the whole document	69,74, 75,80
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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 February 2021	09/03/2021

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

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US2020/062671

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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X,P	WO 2020/150608 A1 (FLAGSHIP PIONEERING INC [US]) 23 July 2020 (2020-07-23) claims; examples; sequences 32-60 -----	1-4,6,8, 9,11,13, 14, 16-26, 33-35, 38-47, 50,51, 58-60, 63-68, 71-73, 78,79

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