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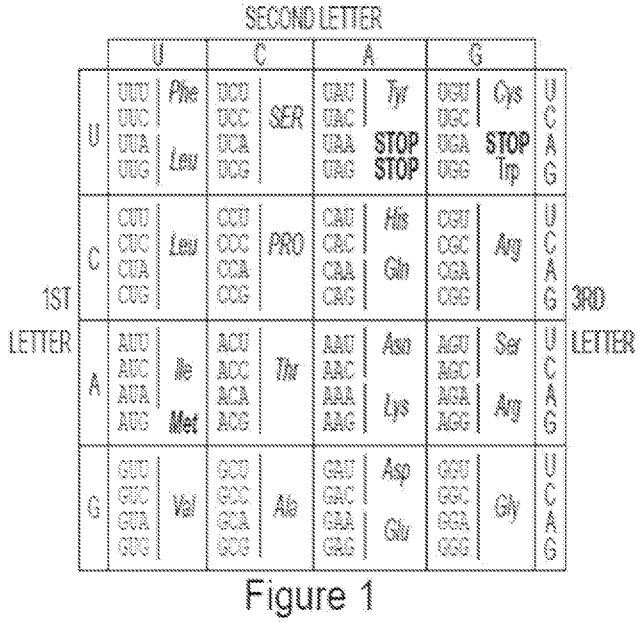

Figure 1

(57) Abstract: The present invention provides compositions and methods for treating diseases and disorders associated with premature termination codons (PTCs) comprising administration of nucleic acid molecules comprising or encoding anticodon edited (ACE) tRNAs (ACE-tRNAs). Spcifically, the methods comprsing administering to a subject at leat one ACE-tRNA specific for UGA, UAA, or UAG for treating associated disorders. Further disclosed are nucleic acid sequences of ACE-tRNAs.

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Methods of Rescuing Stop Codons via Genetic Reassignment with ACE-tRNA

CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority to U.S. Provisional Application Serial No. 62/580,887, filed November 2, 2017, and to U.S. Provisional Application Serial No. 62/687,015 filed June 20, 2018, the contents of which are incorporated by reference herein in their entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with government support under R01 GM106569 awarded by the National Institutes of Health. The Government has certain rights in the invention.

BACKGROUND

DNA molecules carry genetic information in the form of the sequence of the nucleotide bases that make up the DNA polymer. Only four nucleotide bases are utilized in DNA: adenine, guanine, cytosine, and thymine. This information, in the form of codons of three contiguous bases is transcribed into messenger RNA (mRNA), and then translated by transfer RNA (tRNA) and ribosomes to form proteins. Four nucleotide bases are utilized in RNA: adenine, guanine, cytosine, and uracil. The genetic code is the relation between a triplet codon and a particular amino acid. Sixty-four possible codon triplets form the genetic code, where three stop (also called terminating) codons, which provide a signal to the translation machinery (cellular ribosomes) to stop protein production at the particular codon. The other sixty-one triplets in the code correspond to one of the 20 standard amino acid. See **Figure 1**.

DNA is translated by ribosomes, causing each amino acid to be linked together one by one to form polypeptides, according to the genetic instructions specifically provided by the DNA. When the ribosome reaches a stop codon, the elongation of the protein terminates. The three stop codons are UAG (amber), UAA (ochre) and UGA (opal). Mutations that occur that change an amino acid-encoding codon to stop codon are called "nonsense mutations." These nonsense mutations can result in a significant truncation/shortening of the polypeptide sequence, and can cause a profound change in genetic phenotype. Thus, even though a gene directing

expression may be present, a crucial protein may not be produced because when the ribosome reaches the mutant stop signal, it terminates translation resulting in an unfinished protein.

Transfer RNAs translate mRNA into a protein on a ribosome. Each tRNA contains an "anti-codon" region that hybridizes with a complementary codon on the mRNA. A tRNA that carries its designated amino acid is called a "charged" tRNA. If the tRNA is one of the 61 amino-acid-associated (i.e., not a stop-signal-associated) tRNAs, it will normally attach its amino acid to the growing peptide. The structural gene of tRNA is about 72-90 nucleotides long and folds into a cloverleaf structure. tRNAs are transcribed by RNA polymerase III and contain their own intragenic split promoters that become a part of the mature tRNA coding sequence (Sharp S. J., Schaack J., Coolen L., Burke D. J. and Soll D., "Structure and transcription of eukaryotic tRNA genes", Crit. Rev. Biochem, 19:107-144 (1985); Geiduschek E. O., and Tocchini-Valentini, "Transcription by RNA polymerase III, Annu. Rev. Biochem. 57:873-914 (1988)).

"Nonsense suppressors" are alleles of tRNA genes that contain an altered anticodon, such that instead of triggering a "stop" signal, they insert an amino acid in response to a termination codon. For example, an ochre mutation results in the creation of a UAA codon in an mRNA. An ochre suppressor gene produces tRNA with an AUU anticodon that inserts an amino acid at the UAA site, which permits the continued translation of the mRNA despite the presence of a codon that would normally trigger a stop in translation.

A number of nonsense suppressor tRNA alleles have been identified in prokaryotes and eukaryotes such as yeast and *C. elegans*. The different suppressor tRNAs vary in their suppression efficiency. In *E. coli* and other systems, the amber suppressors are relatively more efficient, ochre suppressors are less efficient while opal are the least, this suggests that the amber codons are used infrequently to terminate protein synthesis, while ochre and opal codons are more frequently used as natural termination signals.

Unwanted errors in the DNA blueprint can cause disease. For example, the occurrence of an unexpected "stop" signal in the middle of the protein, rather than at the end of the blueprint, results in the production of a truncated or shortened protein that has an altered function, or no function at all. Many human diseases, including cystic fibrosis (Cheng et al., 1990, Cell, 63, 827-834), Duchenne muscular dystrophy, spinal muscular atrophy (Lefebvre et al., 1995, Cell, 80, 155-165), infantile neuronal ceroid lipofuscinosis (Das et al., 1998, J Clin Invest, 102, 361-370), β o-thalessemia (Chang and Kan, 1979, Proc Natl Acad Sci U S A, 76, 2886-2889), cystinosis (Kalatzis et al., 2002, Hum Mutat, 20, 439-446), X-linked nephrogenic diabetes

insipidus (Pan et al., 1992, Nat Genet, 2, 103-106), Hurler syndrome (Ballabio et al., 2009, Biochim Biophys Acta, 1793, 684-696), Usher syndrome 11, polycystic kidney disease, Liddle's syndrome, xeroderma pigmentosum, Fanconi's anemia, anemia, hypothyroidism, p53-associated cancers (e.g., p53 squamal cell carcinoma, p53 hepatocellular carcinoma, p53 ovarian carcinoma), esophageal carcinoma, osteocarcinoma, ovarian carcinoma, hepatocellular carcinoma, breast cancer, hepatocellular carcinoma, fibrous histiocytoma, ovarian carcinoma, SRY sex reversal, triosephosphate isomerase-anemia, diabetes and rickets result from unwanted stop signals in DNA reading frames for proteins. Additionally, nonsense mutations occur within the tumor suppressor genes p53 and ATM, further implicating their role in disease. As such, PTCs represent a unique constellation of diseases which afflict over 30 million people worldwide, accounting for 10-15% of all genetic diseases.

There is thus a need in the art for compositions and methods that are generalizable to multiple tRNA gene families for treatment of diseases and disorders associated with PTCs. The present invention addresses this unmet need in the art

SUMMARY

In certain embodiments, the present invention provides a modified transfer RNA (tRNA) comprising a T-arm, a D- arm, and anticodon arm and an acceptor arm, wherein the T-arm comprises a T-stem having nucleotides that interact with Elongation Factor 1-alpha 1 (EF1alpha). EF1alpha recruits aminoacyl-tRNA to the ribosome and protects the tRNA from being deacylated. Rational nucleotide replacement results in a tuned tRNA: EF1α interaction that enhances tRNA delivery to the ribosome and protection from deacylation.

In certain embodiments, the present invention provides a modified transfer RNA (tRNA) of SEQ ID NO: 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 or 55, wherein the thymidines are replaced with uracils.

In certain embodiments, the present invention provides a modified transfer RNA (tRNA) of any one of SEQ ID NO: 1-538, wherein the thymidines are replaced with uracils.

In certain embodiments, the modified tRNA is any one of SEQ ID NOs: 56-60, 62-66, 84-86, 90-111, 113, 128-143, 147-149, 153-156, 161-174, 176, 178, 181, 184-186, 192, 196-197, 199-201, 205, 213-240, 246, 255-256, 258-285, 299, 305-312, 314, 318-332, 335-344, 346, 350-354, 357-360, 362, 365-370, 372-383, 388-390, 392, 394-401, 403-407, 414-416, 418, 422, 425,

428-433, 437, 444-445, 452, 455, 459-463, 470, 472-474, 476, 487-492, 525, 530-539, 545-550, 553-555, 561-563, and 567-579, wherein the thymidines are replaced with uracils.

In certain embodiments, the present invention provides a modified transfer RNA (tRNA) comprising a T-stem, a D-stem, and anticodon-loop and an acceptor stem, wherein (a) wherein the anticodon-arm comprises a tri-nucleotide anticodon, wherein the anticodon is 5′-UCA-3′ and recognizes TGA stop codons, and wherein the acceptor arm is operably linked to a arginine, tryptophan or glycine; (b) wherein the anticodon-arm comprises a tri-nucleotide anticodon, wherein the anticodon is 5′-UUA-3′ and recognizes TAA stop codons, and wherein the acceptor arm is operably linked to a glutamine or, glutamate; or (c) wherein the anticodon-arm comprises a tri-nucleotide anticodon, wherein the anticodon is 5′-CUA-3′ and recognizes TAG stop codons, and wherein the acceptor arm is operably linked to a tryptophan, glutamate or glutamine. In certain embodiments, the T-arm comprises rationally altered nucleotide sequences that tune the interaction with the EF1α, enhancing its suppression activity and thereby increasing its therapeutic potential. tRNAs with tuned interaction with the EF1alpha have enhanced nonsense suppression and provide enhanced therapeutic properties.

In certain embodiments, the present invention provides an oligonucleotide sequence that encodes the modified tRNA as described above, wherein the oligonucleotide has a total length of less than 150 nucleotides. In certain embodiments, the oligonucleotide is DNA.

In certain embodiments, the present invention provides an oligonucleotide comprising a first oligonucleotide sequence and a second oligonucleotide sequence, wherein the first and second oligonucleotide sequences independently encode a modified tRNA as described above, wherein the first and second oligonucleotides independently have a total length of less than 150 nucleotides, and wherein the two sequences are in tandem.

In certain embodiments, the present invention provides an expression cassette comprising a promoter and a nucleic acid encoding the modified tRNA or oligonucleotides as described above.

In certain embodiments, the present invention provides a vector comprising the oligonucleotide or the expression cassette described above.

In certain embodiments, the vector is a viral or plasmid vector.

In certain embodiments, the present invention provides a composition comprising a modified tRNA, an oligonucleotide, or a vector described above, and a pharmaceutically acceptable carrier.

In certain embodiments, the carrier is a liposome.

In certain embodiments, the invention provides a cell comprising the vector described above.

The present invention provides a method of treating a stop-codon-associated genetic disease, comprising administering the modified tRNA composition described above to a patient in need thereof.

In certain embodiments, the genetic disease associated with a premature stop codon is cystic fibrosis, muscular dystrophy, β-thalassemia or Liddle's syndrome.

In certain embodiments, the present invention provides a method of restoring translation to a nucleotide sequence that includes a nonsense mutation in a cell, comprising introducing to the cell the composition described above.

In certain embodiments, the present invention provides a method of identifying anti-codon edited (ACE) tRNAs by high-throughput cloning and screening using suppression of a nonsense codon in luciferase enzymes including NanoLuc.

In certain embodiments, the present invention provides methods of treating a disease associated with a PTC in a subject in need thereof, comprising administering to the subject at least one composition comprising an ACE-tRNA or nucleic acid molecule encoding the same.

In certain embodiments, the disease is a disease or disorder associated with a UGA PTC, and the method comprises administering at least one ACE-tRNA specific for UGA.

In certain embodiments, the disease is a disease or disorder associated with a UAA PTC, and the method comprises administering at least one ACE-tRNA specific for UAA.

In certain embodiments, the disease is a disease or disorder associated with a UAG PTC, and the method comprises administering at least one ACE-tRNA specific for UAG.

In certain embodiments, the method comprises administering at least 2 ACE-tRNA, wherein each of the at least two ACE-tRNA are specific for incorporation of at least two different amino acid molecules onto a polypeptide chain.

In certain embodiments, the method comprises administering at least 2 ACE-tRNA, wherein each of the at least two ACE-tRNA are specific for incorporation of the same amino acid molecule onto a polypeptide chain.

In certain embodiments, at least 2 ACE-tRNA are encoded on the same nucleic acid molecule.

In certain embodiments, at least 2 ACE-tRNA are encoded on different nucleic acid molecules.

In certain embodiments, the method comprises administering at least one ACE-tRNA specific for UGA selected from the group consisting of ACE-tRNA^{Arg}, ACE-tRNA^{Gly} and ACE-tRNA^{Trp}.

In certain embodiments, the disease is selected from the group consisting of Duchenne and Becker muscular dystrophies, retinoblastoma, neurofibromatosis, ataxia-telangiectasia, Tay-Sachs disease, cystic fibrosis, Wilm's tumor, hemophilia A, hemophilia B, Menkes disease, Ullrich's disease, β-Thalassemia, type 2A and type 3 von Willebrand disease, Robinow syndrome, brachydactyly type B (shortening of digits and metacarpals), inherited susceptibility to mycobacterial infection, inherited retinal disease, inherited bleeding tendency, inherited blindness, congenital neurosensory deafness and colonic agangliosis and inherited neural develop-mental defect including neurosensory deafness, colonic agangliosis, peripheral neuropathy and central dysmyelinating leukodystrophy, Liddle's syndrome, xeroderma pigmentosum, Fanconi's anemia, anemia, hypothyroidism, p53-associated cancers (e.g., p53 squamal cell carcinoma, p53 hepatocellular carcinoma, p53 ovarian carcinoma), esophageal carcinoma, osteocarcinoma, ovarian carcinoma, hepatocellular carcinoma, breast cancer, hepatocellular carcinoma, fibrous histiocytoma, ovarian carcinoma, SRY sex reversal, triosephosphate isomerase-anemia, diabetes and rickets.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1. Table of the Genetic Code.

Figure 2. tRNAs have a general four-arm structure comprising a T-arm, a D-arm, and anticodon-arm, and an acceptor arm. These arms are also referred to as 'loops' throughout.

Figure 3. ACE-tRNA for nonsense suppression (*H. sapiens* tRNA^{Trp} $_{TGA}$).

Figure 4. Anti-codon edited (ACE)-tRNA encoded in a vector used to identify functional ACE tRNA sequences. This vector sequence includes a Nanoluciferase reporter system. The depicted vector was used to identify ACE tRNA with TGA suppression. TAA and TAG variants were used for the appropriate tRNA screens (*see* **Figures 14** through **17**).

Figure 5. Schematic of the rescue of proteins and ion channels with stop codons via suppressor tRNA.

Figures 6A and 6B. Nonsense codon rescue with human ACE-tRNA. **Fig. 6A**. Schematic of the Anti-Codon Edited (ACE) Trp tRNA and cherry-TGA-eGFP-HA construct. **Fig. 6B**. Rescue of the cherry TGA eGFP-HA construct by ACE tryptophan tRNA #4.

Figure 7. Nonsense codon rationale and prevalence observed in human disease. The twenty natural amino acids codons ranked as to their contribution to human disease, with dark cross-hatched codons being most prevalent (TGG, TAC, TAT, TCA, and TTA) and stippled codons being least prevalent. All cross-hatched codon sequences require a single nucleotide mutation to convert to a stop codon from the intended amino acid. Right panel, the most common disease causative nonsense codons within the cystic fibrosis transmembrane conductance regulator (CFTR). Herein, novel tRNA sequences have been discovered for the repair of the indicated mutation.

Figure 8. Identification of tRNA sequences for the repair of tryptophan-TGA and glycine-TGA. Left axis indicates fold above background for luciferase activity. A majority tRNA with mutant anti-codon loops lack rescue activity.

Figure 9. CFTR 1282x rescue with Trpchr17.trna39 and Glychr19.trna2 ACE-tRNAs. Biochemical western blot data of CFTR W1282X channels co-expressed in HEK cells with the indicated tRNA. Expression vectors containing four copies of the indicated tRNA display higher rescue of the CFTR protein. "C" band indicates rescue of the fully mature, glycosylated CFTR protein. Antibody used was M3A7 from Cystic Fibrosis Therapeutics at a 1:1000 dilution.

Figures 10A and 10B. Expression of ACE-tRNA$_{Trp}$ and ACE-tRNA$_{Gly}$ results in specific incorporation of cognate amino acids into nonsense codons. **Fig. 10A)** Co-expression of model protein histidinol dehydrogenase (HDH)-His-Strep N94-TGA and ACE-tRNA$_{Trp}$ (left) and ACE-tRNA$_{Gly}$ (right) results in full-length HDH protein (asterisks) that is detectable by silver stain following affinity purification. **Fig. 10B)** Spectra of WT HDH (top), HDH-N94 + ACE-tRNA$_{Gly}$ (middle), and HDH-N94 + ACE-tRNA$_{Trp}$ (bottom). Spectra highlight amino acid mass differences at position N94 that match specifically with Glycine (-57Da) and Tryptophan (+72Da), indicating insertion of ACE-tRNA cognate amino acids.

Figure 11. Cloning workflow for the construction of tRNA libraries.

Figures 12A-12B. Targeted mutations of nucleotides within the t-stem region further enhance ACE-tRNA rescue function. **Fig. 12A**. Trpchr17.tRNA 39 was systematically mutagenized within the t-stem region. These efforts identified ACE tRNA TS-10 52-62 G-C, (**Fig. 12B**) and cross-hatched bar in plot, which displays ~250% increased biological activity.

Figures 13A-13F. ACE-tRNAs are selective for nonsense codons and more efficient than aminoglycoside nonsense suppression. **Fig.13A)** ACE-tRNA$_{Trp}$#5 and **Fig.13B)** ACE-tRNA$_{Gly}$#16 were cloned into NanoLuc reporter plasmids containing TGA, TAA or TAG

nonsense codons. Nonsense suppression was only measured in NanoLuc-TGA contructs following transfection. **Fig.13C & Fig.13D)** Suppression of NanoLuc-TGA by addition of gentimicin (40uM) and G418 (150uM) and co-transfection with ACE-tRNA$_{Trp}$#5 and ACE-tRNA$_{Gly}$#16, was measured at **Fig.13C)** 24 and **Fig.13D)** 48hours in HEK293 cells. **Fig.13E & Fig.13F)** HEK293 cells stably expressing NanoLuc-TGA were treated with gentimicin (40uM) and G418 (150uM) and transfected with ACE-tRNA$_{Trp}$#5 and ACE-tRNA$_{Gly}$#16. Nonsense suppression was measured at **Fig.13E)** 24 and **Fig.13F)** 48hours post treatment.

Figure 14. ACE-tRNA-Arg-TGA. Identification of ACE-tRNA for repair of arginine-TGA nonsense codons.

Figure 15. ACE-tRNA-Gln TAG. Identification of ACE-tRNA for repair of glutamine TAG nonsense codons.

Figure 16. ACE-tRNA-Gln TAA Identification of ACE-tRNA for repair of glutamine TAA nonsense codons.

Figure 17. ACE-tRNA-Glu TAG Identification of ACE-tRNA for repair of glutamate-TAG nonsense codons.

Figure 18. ACE-tRNA-Gln TAA Identification of ACE-tRNA for repair of glutamate TAA nonsense codons.

Figure 19. ACE-tRNA-Trp TAG Identification of ACE tRNA for the repair of tryptophan TAG nonsense codons.

Figures 20A – 20D. Delivery of ACE-tRNA as small RNA supports robust suppression of G542X and W1282X nonsense mutations. Fig. 20A) CFTR cRNA with G542X or W1282X cystic fibrosis causing nonsense mutations was co-injected in Xenopus oocytes with serial dilutions of pre-folded ACE-tRNAGly and ACE-tRNATrp, respectively. Two-electrode voltage-clamp recordings of CFTR Cl- current were performed after 36 hours. Current-voltage relationships illustrate that increasing amounts of **Fig. 20B)** ACE-tRNATrp and **Fig. 20C)** ACE-tRNAGly pre-folded RNA results in increased CFTR function (measured CFTR Cl- currents) with WT CFTR achieved in ACE-tRNAGly experiments. **Fig. 20D)** Dose response of G542X ACE-tRNAGly (filled circles) and W1282X ACE-tRNATrp (open squares) rescue (CFTR Cl- currents elicited at +40mV were normalized to WT CFTR Cl- currents at +40mV). The dose dependence of ACE-tRNAGly (EC50= ~20ng; Hill coefficient ~1.4) shows clear saturation at WT CFTR levels, while ACE-tRNATrp is right shifted (EC50= ~94ng; Hill coefficient 1.24).

Figures 21A-21B. A nonsense mutation suppression screen to identify candidate anticodon edited tRNAs (ACE-tRNAs). **Fig. 21A,** Schematic illustrates requisite interactions of ACE-tRNAs with translational machinery. Following delivery, ACE-tRNAs are recognized by an endogenous aminoacyl-tRNA synthetase and charged (aminoacylated) with their cognate amino acid. The aminoacylated ACE-tRNA is recognized by the endogenous elongation factor 1-alpha , which protects the ACE-tRNA from being de-acylated and delivers the aminoacyl ACE-tRNA to the ribosome for suppression of a premature termination codon, in this instance UGA. **Fig. 21B**, Individual ACE-tRNAs were cloned into the High Throughput Cloning Nonsense Reporter plasmid using Golden Gate paired with CcdB negative selection. The all-in-one plasmid contains the NLuc luciferase reporter with either a UGA, UAG or UAA PTC at p.162 between the enzymatic large bit and requisite C-terminal small bit.

Figure 22 Screens of ACE-tRNA gene families with the high throughput cloning nonsense mutation reporter platform**.** The indicated anticodon edited PTC sequences were tested for each ACE-tRNA family that is one nucleotide away from the endogenous anticodon sequence, **Figure 25**. Multiple high performing suppressor tRNA were identified for each class. Data are shown in Log10 scale in terms of normalized NLuc luminescence. Each tRNA dataset were obtained in triplicates and are displayed at SEM, with the corresponding ANOVA statistical analysis in Table 2. Coded identities and corresponding tRNA sequences are shown in **Figure 26** and **Table 9**, respectively.

Figures 23A-23C Cognate Encoding and High-Fidelity Suppression by Engineered tRNA**. Fig. 23A**, Tryptic fragment of histidinol dehydrogenase (HDH), where "X" indicates suppressed PTC codon. MS/MS spectra of the tryptic fragment with masses of indicated *y* and *b* ions for WT (top), N94G (middle) and N94W (bottom) HDH. b9 ion mass is shifted by the predicted mass of -57 Da and +72 Da from the WT asparagine, indicating the encoding of cognate amino acids glycine and tryptophan by ACE-tRNA^{Gly} and ACE-tRNA^{Trp}, respectively. **Fig. 23B**, ACE-TGA - tRNA^{Gly} (Glychr19.t2) selectively suppresses the UGA stop codon in transiently transfected HEK293 cells. **Fig. 23C**) ACE-tRNA^{Gly} transfection outperforms both gentamicin (40uM) and G418 (140uM) following a 48 hour incubation in Hek293 cells stably expressing NLuc-UGA.

Figures 24A-24B. Ribosome profiling of ACE-tRNA on transcriptome-wide 3'UTRs. **Fig. 24A,** Ribosome footprint densities on 3'UTRs are plotted as log2-fold change for reads of treated cells versus control (puc57GG empty vector) as described in the materials and methods.

Transcripts were grouped by their endogenous TAA, TAG, and TGA stop codons. Each point represents the mean of two replicates for a transcript. Error bars show Mean ± SD of the log2-fold changes. **Fig. 24B**, The average log2-fold change of normalized ribosome footprint occupancy was plotted for each nucleotide from -50 to +50 nt surrounding stop codons of transcriptome (18,101 sequences). The cartoon illustrates the ~15 nt offset from the 5' end of ribosome footprint to the first base position of stop codon in the ribosome A-site.

Figure 25. Codon usage for common PTC. Cross-hatching indicates the most common codons and corresponding amino acid type that can be converted to stop codons via nucleotide substitution. Engineered tRNA have been developed for each type.

Figure 26. Number referenced ACE-tRNA activity plot.

Figure 27. Alignment of Glycine tRNA sequences. 21 tRNAGly human sequences demonstrate high sequence homology amongst tRNA clades. Pattern in in tRNA image corresponds to patterned boxes in sequences.

Figure 28. Side-chain identity at p.162 in Nanoluciferase does not effect activity. Total luminescence activity is indicated for each mutation at site.

Figures 29A-29C. Analysis of ACE-tRNA^{Trp} sequences from multiple species and suppressor tRNA mutations. **Figs. 29A-29B.** Sequence alignment. **Fig. 29CB.** NLuc-UGA + ACE-tRNA^{Trp}/NLuc-UGA.

Figures 30A-30C. Histidinol dehydrogenase (HDH) His(8)-streptactin expression construct allows for efficient one-step isolation of protein from HEK293 cells. **Fig. 30A**) Protein sequence of HDH expression construct. Underlined sequence indicates coverage by mass spectrometry. The bold, underlined asparagine (a.a. position 94) is the residue mutated to a TGA PTC for determining ACE-tRNA fidelity. The dual affinity tag is indicated in bold italics. Silver stain of HDH protein following PTC suppression with **Fig. 30B**) Trpchr17.trna39 and **Fig. 30C**) Glychr19.trna2 .

Figure 31. Stop codon specificity is maintained for ACE-tRNA^{Trp}. Suppression activity 36for tRNA Trp^{TGA} Trpchr17.trna39, the top performing Trp^{TGA} suppressor tRNA, Figure 22. This tRNA was co-expressed with the indicated pNano-STOP plasmid.

Figures 32A-32D. ACE-tRNAs are more efficient than aminoglycoside PTC suppression. **Fig. 32A**) Raw and **Fig. 32B**) normalized luminescence measured 24 hours following addition of gentamicin (40uM), G418 (150uM) and transfection with Trpchr17.trna39 and Glychr19.trna2 in HEK293 cells stably expressing PTC reporter Nluc-UGA. **Fig. 32C**) Raw

and **Fig. 32D**) normalized luminescence measured 24 hours following addition of gentamicin (40uM), G418 (150uM) and co-transfection with Trpchr17.trna39 and Glychr19.trna2 in HEK293 cells.

Figure 33. Comparison of time courses of ACE-tRNA activitiy following delivery as RNA or cDNA. ACE-tRNAs were delivered to HEK293 cells that stably express pNanoLuc-UGA, however only 5µl of the reaction mix was added to the cells to reduce the effect of transfection reagents on cell viability. ACE-tRNA delivered as RNA (open symbols), was more rapid in rescuing expression of the PTC reporter than cDNA constructs (close circles). However, ACE-tRNA activity continued to rise over the 48 hourrs when expressed from cDNA and decreased as an RNA deliverable.

Figures 34A-34F. In vivo delivery and suppression with ACE-tRNA as cDNA and RNA. **Fig. 34A**) Representative images of mice injected with NLuc-UGA with ACE-tRNAArg or pUC57 empty vector, NLuc-WT or water in the tibialis anterior muscle followed by electroporation at days 1, 2 and 7 after DNA administration. **Fig. 34B**) Quantification of luminescence emission by the tibialis anterior muscles of the abovementioned mouse groups at different timepoints after DNA injection and electroporation (n=3 mice per group). **Fig. 34C**) Rescued luminesce of stably expressed NLuc–UGA following transfection of Trpchr17.trna39cRNA and Glychr19.trna2 RNA transcripts (n=3). **Fig. 34D**) Representative western blot analysis of CFTR protein expressed in HEK293 cells 36 hours following transfection of WT, G542X, G542X + Glychr19.trna2, W1282X and W1282X + Trpchr17.trna39 CFTR cDNA. **Fig. 34E**) Exemplary families of CFTR Cl- current traces recorded using two-electrode voltage-clamp, 36 hours following injection with WT, G542X, G542X + ACE-tRNA-Glychr19.trna2, W1282X and W1282X + ACE-tRNA-Trpchr17.trna39 CFTR cRNA. Currents were elicited using 5mV voltage steps from -60 to +35 mV. The vertical and horizontal scale bars indicate 10 µA and 50 ms, respectively. **Fig. 34F**) Dose response of G542X ACE-tRNAGly (filled circles) and W1282X ACE-tRNATrp (open squares) rescue (CFTR Cl- currents elicited at +35 mV were normalized to WT CFTR Cl- currents at +35 mV). ACE-tRNAGly rescue achieves WT-level of expressed CFTR current.

Figure 35. ACE tRNA rescues PTC luciferase protein expression in muscle.

Figure 36. ACE tRNA rescues PTC luciferase protein expression in skin.

DETAILED DESCRIPTION OF THE INVENTION

Over the years, researchers have identified hundreds of unique point mutations that resulted in nonsense codons being established in human genes. These types of mutations result, for example, in Duchenne and Becker muscular dystrophies, retinoblastoma, neurofibromatosis, ataxia-telangiectasia, Tay-Sachs disease, cystic fibrosis, Wilm's tumor, hemophilia A, hemophilia B, , Menkes disease, Ullrich's disease, β-Thalassemia, type 2A and type 3 von Willebrand disease, Robinow syndrome, brachydactyly type B (shortening of digits and metacarpals), inherited susceptibility to mycobacterial infection, inherited retinal disease, inherited bleeding tendency, inherited blindness, congenital neurosensory deafness and colonic agangliosis and inherited neural develop-mental defect including neurosensory deafness, colonic agangliosis, peripheral neuropathy and central dysmyelinating leukodystrophy, Liddle's syndrome, xeroderma pigmentosum, Fanconi's anemia, anemia, hypothyroidism, p53-associated cancers (e.g., p53 squamal cell carcinoma, p53 hepatocellular carcinoma, p53 ovarian carcinoma), esophageal carcinoma, osteocarcinoma, ovarian carcinoma, hepatocellular carcinoma, breast cancer, hepatocellular carcinoma, fibrous histiocytoma, ovarian carcinoma, SRY sex reversal, triosephosphate isomerase-anemia, diabetes and rickets. The BRACA-1 and BRACA-2 genes associated with breast cancer also have similar mutations.

The nucleotide sequences encoding several hundred human tRNAs are known and generally available to those of skill in the art through sources such as Genbank. The structure of tRNAs is highly conserved and tRNAs are often functional across species. Thus, bacterial or other eukaryotic tRNA sequences are also potential sources for the oligonucleotides for the stabilized tRNAs of the invention. The determination of whether a particular tRNA sequence is functional in a desired mammalian cell can be ascertained through routine experimentation. Further additional potential tRNA sequences that are not yet known can be modified as described herein in order to be stabilized through routine experimentation.

tRNA genes have strong promoters that are active in all cell types. The promoters for eukaryotic tRNA genes are contained within the structural sequences encoding the tRNA molecule itself. Although there are elements that regulate transcriptional activity within the 5′ upstream region, the length of an active transcriptional unit may be considerably less than 500 base pairs and thus accommodation within a delivery vector is straightforward. Once they have been transcribed and processed, tRNAs have low rates of degradation. Finally, gene therapy with a nonsense suppressor maintains the endogenous physiological controls over the target gene that contains the nonsense codon.

Nonsense Mutations

Transfer RNA (tRNA) is a type of RNA molecule that functions in the decoding of a messenger RNA (mRNA) sequence into a protein. tRNAs function at specific sites in the ribosome during translation, which synthesizes a protein from an mRNA molecule. Nonsense mutations, also called Premature Termination Codons (PTCs), make up ~10-15% of the single base pair mutations that cause human disease, and cystic fibrosis follows suit. (Peltz et al., Annu Rev Med., 64:407-25, 2013). In general, nonsense mutations have more serious ramifications than missense mutations because of the almost complete loss of gene expression and activity and with the possibility of dominant negative effects of truncated products. PTCs result in premature translation termination and accelerated mRNA transcript decay through the Nonsense Mediated Decay (NMD) pathway.

The current studies show that the specific site within an RNA transcript to which a tRNA delivers its amino acid can be changed through molecular editing of the anti-codon sequence within the tRNA. This approach allowed for a premature termination codon (PTC) to be effectively and therapeutically reverted back into the originally lost amino acid. Anticodon-edited tRNA (ACE-tRNA) form a new class of biological therapeutics.

Engineered tRNAs allow for "re-editing" of a disease-causing nonsense codon to a specific amino acid. These engineered tRNAs target only one type of stop codon, such as TGA over TAC or TAA. The small size of these tRNA molecules makes them amenable to ready expression, as the tRNA + the promoter is only ~300 bp. Briefly, an oligonucleotide is synthesized that comprises the structural component of a tRNA gene functional in human cells. The sequence of this oligonucleotide is designed based upon the known sequence with substitutions made in the anticodon region of the tRNA causing the specific tRNA to recognize a nonsense or other specific mutation.

Several small molecule screens have been performed to suppress nonsense stop codons through interactions with the ribosome, the most outstanding molecules being G418, Gentamicin and PTC124. PTC124 or Ataluren has recently been relieved from Phase 3 clinical trials as use for a cystic fibrosis therapeutic. Ataluren and aminoglycosides promote read-through of each of the three nonsense codons by putting in a near cognate amino acid that turn a nonsense mutation into a missense mutation. (Roy et al., PNAS 2016 Nov 1;113(44):12508-12513)

Anticodon-edited tRNA (ACE-tRNA)

tRNAs have a general four-arm structure comprising a T-arm, a D-arm, and anticodon-arm, and an acceptor arm (**Figure 2**).

The T-arm is made up of a "T-stem" and a "TΨC loop." In certain embodiments, the T-stem is modified to increase the stability of the tRNA. In certain embodiments, the ACE-tRNA has a modified T-stem that increases the biological activity to suppress stop sites relative to the endogenous T-stem sequence.

The present invention in one embodiment includes compositions comprising stabilized tRNAs, which can be used with higher effectiveness in order to treat a wide variety of nonsense mutation-associated diseases. The following sequences in Tables 1-8 are written as DNA, but as RNA (transcribed DNA) the "T : thymidine" is "U : uracil." Therefore, tRNAs transcribed from the following sequences all contain uracils in place of the thymidines.

In certain embodiments, the tRNA has the following sequences (wherein the thymidines are replaced with uracils):

TS-36:
GGCCTCGTGGCGCAACGGTAGCGCGTCTGACTtCAGATCAGAAGGtTGCGgGTTCAAATCcCGT CGGGGTCA (SEQ ID NO: 1)

TS-37:
GGCCTCGTGGCGCAACGGTAGCGCGTCTGACTtCAGATCAGAAGGtTaCGgGTTCAAATCcCGT CGGGGTCA (SEQ ID NO: 2)

TS-38:
GGCCTCGTGGCGCAACGGTAGCGCGTCTGACTtCAGATCAGAAGGtTcCGgGTTCAAATCcCGg CGGGGTCA (SEQ ID NO: 3)

Table 1

Ranking	Identifier	Sequence	SEQ ID NO.
#1	ArgTGAchr9.trna6/nointron	CGTCGGCTCTGTGGCGCAATGGATAGCGCATTGGACTTCAAATTCAAAGGTTGTGGGTTCGAGTCCCACCAGAGTCG	4
#2	ArgTGAchr17.trna19	CGTCGCCCCAGTGGCCTAATGGATAAGGCACTGGCCTTCAAAGCCAGGGATTGTGGGTTCGAGTCCCACCTGGGGTG	5
#3	ArgTGAchr1.trna10/nointron	CGTCGGCTCCGTGGCGCAATGGATAGCGCATTGGACTTCAAATTCAAAGGTTCCGGGTTCGAGTCCCGGCGGAGTCG	6
#4	ArgTGAch	CGTCGCCCCAGTGGCCTAATGGATAAGGCATTGGCCTTCAAAGCCAGGGATTGTGGGTTCGAGTCCCATCTGGGGTG	7

Ranking	Identifier	Sequence	SEQ ID NO.
	r7.trna5		
#4	ArgTGAchr17.trna3/nointron	CGTCGGCTCTGTGGCGCAATGGATAGCGCATTGGACTTCAAATTCAAAGGTTGTGGGTTCGAATCCCACCAGAGTCG	8
#5	ArgTGAchr9.trna6/withintron	CGTCGGCTCTGTGGCGCAATGGATAGCGCATTGGACTTCAAGCTGAGCCTAGTGTGGTCATTCAAAGGTTGTGGGTTCGAGTCCCACCAGAGTCG	9
#5	ArgTGAchr16.trna3	CGTCGCCCCGGTGGCCTAATGGATAAGGCATTGGCCTTCAAAGCCAGGGATTGTGGGTTCGAGTCCCACCCGGGGTA	10
#6	ArgTGAchr1.trna10/withintron	CGTCGGCTCCGTGGCGCAATGGATAGCGCATTGGACTTCAAGAGGCTGAAGGCATTCAAAGGTTCCGGGTTCGAGTCCCGGCGGAGTCG	11
#7	ArgTGAchr17.trna3/withinron	CGTCGGCTCTGTGGCGCAATGGATAGCGCATTGGACTTCAAGTGACGAATAGAGCAATTCAAAGGTTGTGGGTTCGAATCCCACCAGAGTCG	12
	ArgTGAchr15.trna4	CGTCGGCCGCGTGGCCTAATGGATAAGGCGTCTGACTTCAGATCAGAAGATTGCAGGTTCGAGTCCTGCCGCGGTCG	13
	ArgTGAchr17.trna17	CGTCGACCGCGTGGCCTAATGGATAAGGCGTCTGACTTCAGATCAGAAGATTGAGGGTTCGAGTCCCTTCGTGGTCG	14
	ArgTGAchr11.trna3/withintron	CGTCGGCTCTGTGGCGCAATGGATAGCGCATTGGACTTCAAGATAGTTAGAGAAATTCAAAGGTTGTGGGTTCGAGTCCCACCAGAGTCG	15

Table 2

Ranking	Identifier	Sequence	SEQ ID NO.
#1	GlnTAGchr1.trna17	CGTCGGTTCCATGGTGTAATGGTgAGCACTCTGGACTctaAATCCAGCGaTCCGAGTTCGAGTCTCGGTGGAACCT	16
#2	GlnTAGchr6.trna175	CGTCGGCCCCATGGTGTAATGGTtAGCACTCTGGACTctaAATCCAGCGaTCCGAGTTCAAATCTCGGTGGGACCT	17
#3	GlnTAGchr6.trna63	CGTCGGTCCCATGGTGTAATGGTtAGCACTCTGGACTctaAATCCAGCAaTCCGAGTTCGAATCTCGGTGGGACCT	18
#4	GlnTAGchr17.trna14	CGTCGGTCCCATGGTGTAATGGTtAGCACTCTGGACTctaAATCCAGCGaTCCGAGTTCAAATCTCGGTGGGACCT	19
#5	GlnTAGchr6.trna132	CGTCGGCCCCATGGTGTAATGGTcAGCACTCTGGACTctaAATCCAGCGaTCCGAGTTCAAATCTCGGTGGGACCC	20
	GlnTAGchr1.trna101	CGTCGGTTCCATGGTGTAATGGTaAGCACTCTGGACTctaAATCCAGCGaTCCGAGTTCGAGTCTCGGTGGAACCT	21
	GlnTAGchr6.trna42	CGTCGGTTCCATGGTGTAATGGTtAGCACTCTGGACTctaAATCCGGTAaTCCGAGTTCAAATCTCGGTGGAACCT	22
	GlnTAGchr6.trna147	CGTCGGTTCCATGGTGTAATGGTtAGCACTCTGGACTctaAATCCAGCGaTCCGAGTTCAAGTCTCGGTGGAACCT	23

Table 3

Ranking	Identifier	Sequence	SEQ ID NO.
#1	GlnTAAchr1.trna101	CGTCGGTTCCATGGTGTAATGGTaAGCACTCTGGACTttaAATCCAGCGaTCCGAGTTCGAGTCTCGGTGGAACCT	24
#2	GlnTAAchr6.trna175	CGTCGGCCCCATGGTGTAATGGTtAGCACTCTGGACTttaAATCCAGCGaTCCGAGTTCAAATCTCGGTGGGACCT	25
#3	GlnTAAchr1.trna17	CGTCGGTTCCATGGTGTAATGGTgAGCACTCTGGACTttaAATCCAGCGaTCCGAGTTCGAGTCTCGGTGGAACCT	26
#4	GlnTAAchr6.trna1	CGTCGGTTCCATGGTGTAATGGTtAGCACTCTGGACTttaAATCCAGCGaTCCGAGTTCAAATCTCGGTGGAACCT	27
#5	GlnTAAchr17.trna14	CGTCGGTCCCATGGTGTAATGGTtAGCACTCTGGACTttaAATCCAGCGaTCCGAGTTCAAATCTCGGTGGGACCT	28
#5.2	GlnTAAchr6.trna63	CGTCGGTCCCATGGTGTAATGGTtAGCACTCTGGACTttaAATCCAGCAaTCCGAGTTCGAATCTCGGTGGGACCT	29
	GlnTAAchr6.trna42	CGTCGGTTCCATGGTGTAATGGTtAGCACTCTGGACTttaAATCCGGTAaTCCGAGTTCAAATCTCGGTGGAACCT	30
	GlnTAAchr6.trna132	CGTCGGCCCCATGGTGTAATGGTcAGCACTCTGGACTttaAATCCAGCGaTCCGAGTTCAAATCTCGGTGGGACCC	31
	GlnTAAchr6.trna147	CGTCGGTTCCATGGTGTAATGGTtAGCACTCTGGACTttaAATCCAGCGaTCCGAGTTCAAGTCTCGGTGGAACCT	32

Table 4

Ranking	Identifier	Sequence	SEQ ID NO.
#1	TrpTAGchr17.trna10	CGTCGACCTCGTGGCGCAATGGTAGCGCGTCTGACTctAGATCAGAAGGtTGCGTGTTCAAGTCACGTCGGGGTCA	33
#2	TrpTAGchr6.trna171	CGTCGACCTCGTGGCGCAACGGTAGCGCGTCTGACTctAGATCAGAAGGtTGCGTGTTCAAATCACGTCGGGGTCA	34
#3	TrpTAGchr17.trna39	CGTCGGCCTCGTGGCGCAACGGTAGCGCGTCTGACTctAGATCAGAAGGtTGCGTGTTCAAATCACGTCGGGGTCA	35
#4	TrpTAGchr12.trna6	CGTCGACCTCGTGGCGCAACGGTAGCGCGTCTGACTctAGATCAGAAGGcTGCGTGTTCGAATCACGTCGGGGTCA	36

Ranking	Identifier	Sequence	SEQ ID NO.
	TrpTAGchr7.trna3	CGTCGACCTCGTGGCGCAACGGCAGCGCGTCTGACTctAGATCAGAAGGtTGCGTGTTCAAATCACGTCGGGGTCA	37

Table 5

Ranking	Identifier	Sequence	SEQ ID NO.
#1	GluTAGchr13.trna2	CGTCTCCCACATGGTCTAGCGGTtAGGATTCCTGGTTctaACCCAGGCGGCCCGGGTTCGACTCCCGGTGTGGGAA	38
#2	GluTAGchr2.trna18	CGTCTCCCATATGGTCTAGCGGTtAGGATTCCTGGTTctaACCCAGGTGGCCCGGGTTCGACTCCCGGTATGGGAA	39
#3	GluTAGchr1.trna123	CGTCTCCCTGGTGGTCTAGTGGCtAGGATTCGGCGCTctaACCGCCGCGGCCCGGGTTCGATTCCCGGTCAGGGAA	40
#4	GluTAGchr1.trna106	CGTCTCCCTGGTGGTCTAGTGGTtAGGATTCGGCGCTctaACCGCCGCGGCCCGGGTTCGATTCCCGGTCAGGGAA	41
	GluTAGchr1.trna5	CGTCTCCCTGGTGGTCTAGTGGCtAGGATTCGGCGCTctaACCGCCGCGGCCCGGGTTCGATTCCCGGCCAGGGAA	42

Table 6

Ranking	Identifier	Sequence	SEQ ID NO.
	GluTAAchr13.trna2	CGTCTCCCACATGGTCTAGCGGTtAGGATTCCTGGTTctaACCCAGGCGGCCCGGGTTCGACTCCCGGTGTGGGAA	43
	GluTAAchr2.trna18	CGTCTCCCATATGGTCTAGCGGTtAGGATTCCTGGTTctaACCCAGGTGGCCCGGGTTCGACTCCCGGTATGGGAA	44
	GluTAAchr1.trna106	CGTCTCCCTGGTGGTCTAGTGGTtAGGATTCGGCGCTctaACCGCCGCGGCCCGGGTTCGATTCCCGGTCAGGGAA	45
	GluTAAchr1.trna55	CGTCTCCCTGGTGGTCTAGTGGTtAGGATTCGGCGCTctaACCGCCGCGGCCCGGGTTCGATTCCCGGTCAGGAAA	46
	GluTAAchr1.trna5	CGTCTCCCTGGTGGTCTAGTGGCtAGGATTCGGCGCTctaACCGCCGCGGCCCGGGTTCGATTCCCGGCCAGGGAA	47

5

Table 7

Ranking	Identifier	Sequence	SEQ ID NO.

Ranking	Identifier	Sequence	SEQ ID NO.
#1	TrpTGAchr17.trna39	GGCCTCGTGGCGCAACGGTAGCGCGTCTGACTtCAGATCA GAAGGtTGCGTGTTCAAATCACGTCGGGGTCA	48
#2	TrpTGAchr17.trna10	GACCTCGTGGCGCAATGGTAGCGCGTCTGACTtCAGATCA GAAGGtTGCGTGTTCAAGTCACGTCGGGGTCA	49
#3	TrpTGAchr6.trna171	GACCTCGTGGCGCAACGGTAGCGCGTCTGACTtCAGATCA GAAGGtTGCGTGTTCAAATCACGTCGGGGTCA	50
	TrpTGAchr12.trna6	GACCTCGTGGCGCAACGGTAGCGCGTCTGACTtCAGATCA GAAGGcTGCGTGTTCGAATCACGTCGGGGTCA	51
	TrpTGAchr7.trna3	GACCTCGTGGCGCAACGGCAGCGCGTCTGACTtCAGATCA GAAGGtTGCGTGTTCAAATCACGTCGGGGTCA	52

Table 8

Ranking	Identifier	Sequence	SEQ ID NO.
#1	GlyTGAchr19.trna2	GCGTTGGTGGTATAGTGGTtAGCATAGCTGCCTTCaAAG CAGTTGaCCCGGGTTCGATTCCCGGCCAACGCA	53
#2	GlyTGAchr1.trna107	GCGTTGGTGGTATAGTGGTgAGCATAGCTGCCTTCaAAG CAGTTGaCCCGGGTTCGATTCCCGGCCAACGCA	54
#3	GlyTGAchr17.trna9	GCGTTGGTGGTATAGTGGTaAGCATAGCTGCCTTCaAAG CAGTTGaCCCGGGTTCGATTCCCGGCCAACGCA	55

In one embodiment, the ACE-tRNA for nonsense suppression is as depicted in **Figure 3** (*H. sapiens* $tRNA^{Trp}_{TGA}$).

According to the invention, human UAA, UAG, and UGA suppressor tRNAs have been designed. The screen has identified codon edited tRNA for the repair of Trp-TGA, Trp-TAG, Arg-TGA, Gln-TAG, Gln-TA, Glu-TAG, Glu-TAA. The tRNAs are approximately 100 nucleotides in length and can be introduced to cells to suppress nonsense codons mutations where the wild-type amino acid should be present. The oligonucleotides can be introduced directly to recipient cells or can be ligated in tandem to increase efficacy of the oligonucleotide.

Expression Cassettes and Vectors

In certain embodiments, the ACT-tRNA is encoded by an expression cassette. In yet another embodiment, the suppressor tRNA of the invention may be introduced to the cells using standard conventional genetic engineering techniques through use of vectors. Because of the

internal promoter sequences of tRNA encoding sequences, the tRNA sequence need not be included in a separate transcription unit, although one may be provided.

In one embodiment of the present invention, the nucleotide expression system of the invention is included within an appropriate gene transfer vehicle which is then used to transduce cells to express the suppressor tRNA. The gene delivery vehicle can be any delivery vehicle known in the art, and can include naked DNA that is facilitated by a receptor and/or lipid mediated transfection, as well as any of a number of vectors. Such vectors include but are not limited to eukaryotic vectors, prokaryotic vectors (such as for example bacterial vectors) and viral vectors including, but not limited to, retroviral vectors, adenoviral vectors, adeno-associated viral vectors, lentivirus vectors (human and other including porcine), Herpes virus vectors, Epstein-Barr viral vectors, SV40 virus vectors, pox virus vectors, and pseudotyped viral vectors.

In certain embodiments, the ACT-tRNA (PTC) is encoded in a vector. **Figure 4**. In certain embodiments, the viral vector is a retroviral or adenoviral vector. Examples of retroviral vectors that may be employed include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, and vectors derived from retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, human immunodeficiency virus, myeloproliferative sarcoma virus, and mammary tumor virus.

Retroviruses; Retroviral Vectors

The term "retrovirus" is used in reference to RNA viruses that utilize reverse transcriptase during their replication cycle. The retroviral genomic RNA is converted into double-stranded DNA by reverse transcriptase. This double-stranded DNA form of the virus is capable of being integrated into the chromosome of the infected cell; once integrated, it is referred to as a "provirus." The provirus serves as a template for RNA polymerase II and directs the expression of RNA molecules that encode the structural proteins and enzymes needed to produce new viral particles. At each end of the provirus are structures called "long terminal repeats" or "LTRs." The LTR contains numerous regulatory signals including transcriptional control elements, polyadenylation signals and sequences needed for replication and integration of the viral genome. There are several genera included within the family Retroviridae, including Cisternavirus A, Oncovirus A, Oncovirus B, Oncovirus C, Oncovirus D, Lentivirus, and Spumavirus. Some of the retroviruses are oncogenic (*i.e.*, tumorigenic), while others are not. The oncoviruses induce sarcomas, leukemias, lymphomas, and mammary carcinomas in susceptible species. Retroviruses infect a wide variety of species, and may be transmitted both

horizontally and vertically. They are integrated into the host DNA, and are capable of transmitting sequences of host DNA from cell to cell. This has led to the development of retroviruses as vectors for various purposes including gene therapy.

Retroviruses, including human foamy virus (HFV) and human immunodeficiency virus (HIV) have gained much recent attention, as their target cells are not limited to dividing cells and their restricted host cell tropism can be readily expanded via pseudotyping with vesicular stomatitis virus G (VSV-G) envelope glycoproteins (See *e.g.*, J. C. Burns *et al.*, Proc. Natl. Acad. Sci. USA 90:8033-8037 [1993]; A. M. L. Lever, Gene Therapy. 3:470-471 [1996]; and D. Russell and A. D. Miller, J. Virol., 70:217-222 [1996]).

Vector systems generally have a DNA vector containing a small portion of the retroviral sequence (the viral long terminal repeat or "LTR" and the packaging or "psi" signal) and a packaging cell line. The gene to be transferred is inserted into the DNA vector. The viral sequences present on the DNA vector provide the signals necessary for the insertion or packaging of the vector RNA into the viral particle and for the expression of the inserted gene. The packaging cell line provides the viral proteins required for particle assembly (D. Markowitz *et al.*, J. Virol., 62:1120 [1988]). In one embodiment of the present invention, an FIV system employing a three-plasmid transfection production method in 293T cells was used (Johnston et al., J Virol. 1999 73:4991-5000). Replication incompetent virus was successfully produced.

The vector DNA is introduced into the packaging cell by any of a variety of techniques (*e.g.*, calcium phosphate coprecipitation, lipofection, electroporation). The viral proteins produced by the packaging cell mediate the insertion of the vector sequences in the form of RNA into viral particles, which are shed into the culture supernatant.

For cells that are naturally dividing, or are stimulated to divide by growth factors, simple retroviruses like murine leukemia virus (MLV) vectors are suitable delivery systems. A major limitation in the use of many commonly used retroviral vectors in gene transfer, however, is that many of the vectors are restricted to dividing cells. If a non-dividing cell is the target cell, then a lentivirus, which is capable of infecting non-dividing cells, may be used.

As used herein, the term "lentivirus" refers to a group (or genus) of retroviruses that give rise to slowly developing disease. Viruses included within this group include HIV (human immunodeficiency virus; including HIV type 1, and HIV type 2), the etiologic agent of the human acquired immunodeficiency syndrome (AIDS); visna-maedi, that causes encephalitis (visna) or pneumonia (maedi) in sheep, the caprine arthritis-encephalitis virus, which causes

immune deficiency, arthritis, and encephalopathy in goats; equine infectious anemia virus, which causes autoimmune hemolytic anemia, and encephalopathy in horses; feline immunodeficiency virus (FIV), which causes immune deficiency in cats; bovine immune deficiency virus (BIV), which causes lymphadenopathy, lymphocytosis, and possibly central nervous system infection in cattle; and simian immunodeficiency virus (SIV), which cause immune deficiency and encephalopathy in sub-human primates. Diseases caused by these viruses are characterized by a long incubation period and protracted course. Usually, the viruses latently infect monocytes and macrophages, from which they spread to other cells. HIV, FIV, and SIV also readily infect T lymphocytes (*i.e.*, T-cells).

Lentiviruses including HIV, SIV, FIV and equine infectious anemia virus (EIAV) depend on several viral regulatory genes in addition to the simple structural gag-pol-env genes for efficient intracellular replication. Thus, lentiviruses use more complex strategies than classical retroviruses for gene regulation and viral replication, with the packaging signals apparently spreading across the entire viral genome. These additional genes display a web of regulatory functions during the lentiviral life cycle. For example, upon HIV-1 infection, transcription is up-regulated by the expression of Tat through interaction with an RNA target (TAR) in the LTR. Expression of the full-length and spliced mRNAs is then regulated by the function of Rev, which interacts with RNA elements present in the gag region and in the env region (RRE) (S. Schwartz *et al.*, J. Virol., 66:150-159 [1992]). Nuclear export of gag-pol and env mRNAs is dependent on the Rev function. In addition to these two essential regulatory genes, a list of accessory genes, including vif, vpr, vpx, vpu, and nef, are also present in the viral genome and their effects on efficient virus production and infectivity have been demonstrated, although they are not absolutely required for virus replication (K. and F. Wong-Staal, Microbiol. Rev., 55:193-205 (1991]; R. A. Subbramanian and E. A. Cohen, J. Virol. 68:6831-6835 [1994]; and D. Trono, Cell 82:189-192 [1995]). A detailed description of the structure of an exemplary lentivirus, HIV-1, is given in US Patent no. 6,531,123.

A "source" or "original" retrovirus is a wild-type retrovirus from which a pseudotyped retrovirus is derived, or is used as a starting point, during construction of the packaging or transgene vector, for the preparation of one or more of the genetic elements of the vector. The genetic element may be employed unchanged, or it may be mutated (but not beyond the point where it lacks a statistically significant sequence similarity to the original element). A vector may have more than one source retrovirus, and the different source retroviruses may be, *e.g.*,

MLV, FIV, HIV-1 and HIV-2, or HIV and SIV. The term "genetic element" includes but is not limited to a gene.

A cognate retrovirus is the wild-type retrovirus with which the vector in question has the greatest percentage sequence identity at the nucleic acid level. Normally, this will be the same as the source retrovirus. However, if a source retrovirus is extensively mutated, it is conceivable that the vector will then more closely resemble some other retrovirus. It is not necessary that the cognate retrovirus be the physical starting point for the construction; one may choose to synthesize a genetic element, especially a mutant element, directly, rather than to first obtain the original element and then modify it. The term "cognate" may similarly be applied to a protein, gene, or genetic element (*e.g.*, splice donor site or packaging signal). When referring to a cognate protein, percentage sequence identities are determined at the amino acid level.

The term "cognate" retrovirus may be difficult to interpret in the extreme case, *i.e.*, if all retroviral genetic elements have been replaced with surrogate non-lentiviral genetic elements. In this case, the source retrovirus strain mentioned previously is arbitrarily considered to be the cognate retrovirus.

The term "replication" as used herein in reference to a virus or vector, refers not to the normal replication of proviral DNA in a chromosome as a consequence of cell reproduction, or the autonomous replication of a plasmid DNA as a result of the presence of a functional origin of replication. Instead “replication” refers to the completion of a complete viral life cycle, wherein infectious viral particles containing viral RNA enter a cell, the RNA is reverse transcribed into DNA, the DNA integrates into the host chromosome as a provirus, the infected cell produces virion proteins and assembles them with full length viral genomic RNA into new, equally infectious particles.

The term "replication-competent" refers to a wild-type virus or mutant virus that is capable of replication, such that replication of the virus in an infected cell result in the production of infectious virions that, after infecting another, previously uninfected cell, causes the latter cell to likewise produce such infectious virions. The present invention contemplates the use of replication-defective virus.

As used herein, the term "attenuated virus" refers to any virus (*e.g.*, an attenuated lentivirus) that has been modified so that its pathogenicity in the intended subject is substantially reduced. The virus may be attenuated to the point it is nonpathogenic from a clinical standpoint,

i.e., that subjects exposed to the virus do not exhibit a statistically significant increased level of pathology relative to control subjects.

The present invention contemplates the preparation and use of a modified retrovirus. In some embodiments, the retrovirus is an mutant of murine leukemia virus, human immunodefciency virus type 1, human immunodeficiency virus type 2, feline immunodeficiency virus, simian immunodeficiency virus, visna-maedi, caprine arthritis-encephalitis virus, equine infectious anemia virus, and bovine immune deficiency virus, or a virus comprised of portions of more than one retroviral species (*e.g.*, a hybrid, comprised of portions of MLV, FIV, HIV-1 and HIV-2, or HIV-1 and/or SIV).

A reference virus is a virus whose genome is used in describing the components of a mutant virus. For example, a particular genetic element of the mutant virus may be said to differ from the cognate element of the reference virus by various substitutions, deletions or insertions. It is not necessary that the mutant virus actually be derived from the reference virus.

The preferred reference FIV sequence is found in Talbott et al., Proc Natl Acad Sci U S A. 1989 86:5743-7; Genbank access# NC_001482. In certain embodiments, a three-plasmid transient transfection method can be used to produce replication incompetent pseudotyped retrovirues (e.g., FIV). General methods are described in Wang et al., J Clin Invest. 1999 104:R55-62 and Johnston et al., J Virol. 1999 73:4991-5000.

Retroviral Vector System

The present invention contemplates a retroviral gene amplification and transfer system comprising a transgene vector, one or more compatible packaging vectors, an envelope vector, and a suitable host cell. The vectors used may be derived from a retrovirus (e.g., a lentivirus). Retrovirus vectors allow (1) transfection of the packaging vectors and envelope vectors into the host cell to form a packaging cell line that produces essentially packaging-vector-RNA-free viral particles, (2) transfection of the transgene vector into the packaging cell line, (3) the packaging of the transgene vector RNA by the packaging cell line into infectious viral particles, and (4) the administration of the particles to target cells so that such cells are transduced and subsequently express a transgene.

Either the particles are administered directly to the subject, *in vivo*, or the subject's cells are removed, infected *in vitro* with the particles, and returned to the body of the subject.

The packaging vectors and transgene vectors of the present invention will generate replication-incompetent viruses. The vectors chosen for incorporation into a given vector system

of the present invention are such that it is not possible, without further mutation of the packaging vector(s) or transgene vector, for the cotransfected cells to generate a replication-competent virus by homologous recombination of the packaging vector(s) and transgene vector alone. The envelope protein used in the present system can be a retroviral envelope, a synthetic or chimeric envelope, or the envelope from a non-retroviral enveloped virus (e.g., baculovirus).

Packaging Signal

As used herein, the term "packaging signal" or "packaging sequence" refers to sequences located within the retroviral genome or a vector that are required for, or at least facilitate, insertion of the viral or vector RNA into the viral capsid or particle. The packaging signals in an RNA identify that RNA as one that is to be packaged into a virion. The term "packaging signal" is also used for convenience to refer to a vector DNA sequence that is transcribed into a functional packaging signal. Certain packaging signals may be part of a gene, but are recognized in the form of RNA, rather than as a peptide moiety of the encoded protein.

The key distinction between a packaging vector and a transgene vector is that in the packaging vector, the major packaging signal is inactivated, and, in the transgene vector, the major packaging sign al is functional. Ideally, in the packaging vector, all packaging signals would be inactivated, and, in the transgene vector, all packaging signals would be functional. However, countervailing considerations, such as maximizing viral titer, or inhibiting homologous recombination, may lend such constructs less desirable.

Packaging System; Packaging Vectors; Packaging Cell Line

A packaging system is a vector, or a plurality of vectors, which collectively provide in expressible form all of the genetic information required to produce a virion that can encapsidate suitable RNA, transport it from the virion-producing cell, transmit it to a target cell, and, in the target cell, cause the RNA to be reverse transcribed and integrated into the host genome in a such a manner that a transgene incorporated into the aforementioned RNA can be expressed. However, the packaging system must be substantially incapable of packaging itself. Rather, it packages a separate transgene vector.

In the present invention, the packaging vector will provide functional equivalents of the gag and pol genes (a "GP" vector). The env gene(s) will be provided by the envelope vector. In theory, a three vector system ("G", "P", and "E" vectors) is possible if one is willing to construct distinct gag and pol genes on separate vectors, and operably link them to different regulatable

promoters (or one to a regulatable and the other to a constitutive promoter) such that their relative levels of expression can be adjusted appropriately.

A packaging cell line is a suitable host cell transfected by a packaging system that, under achievable conditions, produces viral particles. As used herein, the term "packaging cell lines" is typically used in reference to cell lines that express viral structural proteins (*e.g.*, gag, pol and env), but do not contain a packaging signal. For example, a cell line has been genetically engineered to carry at one chromosomal site within its genome, a 5'-LTR-gag-pol-3'-LTR fragment that lacks a functional psi^+ sequence (designated as Δ-psi), and a 5'-LTR-env-3'-LTR fragment that is also Δ-psi located at another chromosomal site. While both of these segments are transcribed constitutively, because the psi^+ region is missing and the viral RNA molecules produced are less than full-size, empty viral particles are formed.

If a host cell is transfected by the packaging vector(s) alone, it produces substantially only viral particles without the full-length packaging vector. In one example, less than 10% of the viral particles produced by the packaging cell contain full length packaging vector-derived RNA. However, since the packaging vector lacks a functional primer-binding site, even if these particles infect a new cell, the packaging vector RNA will not be reverse transcribed back into DNA and therefore the new cell will not produce virion. Thus, by itself, the packaging vector is a replication-incompetent virus.

In some embodiments, the packaging cell and/or cell line contains a transgene vector. The packaging cell line will package the transgene vector into infectious particles. Such a cell line is referred to herein as a "transgenic virion production cell line."

It is contemplated that packaging may be inducible, as well as non-inducible. In inducible packaging cells and packaging cell lines, retroviral particles are produced in response to at least one inducer. In non-inducible packaging cell lines and packaging cells, no inducer is required in order for retroviral particle production to occur.

The packaging vectors necessarily differ from wild-type, replication-competent retroviral genomes by virtue of the inactivation of at least one packaging signal of the cognate wild-type genome. More than one packaging signal may be inactivated. In one example, only the retroviral genes provided by the packaging vector are those encoding structural, or essential regulatory, proteins.

Transgene Vectors

A transgene vector is an expression vector that bears an expressible non-retroviral gene of interest and includes at least one functional retroviral packaging signal, so that, after the transgene vector is transfected into a packaging cell line, the transgene vector is transcribed into RNA, and this RNA is packaged into an infectious viral particle. These particles, in turn, infect target cells, their RNA is reverse transcribed into DNA, and the DNA is incorporated into the host cell genome as a proviral element, thereby transmitting the gene of interest to the target cells.

As used herein, the term "transduction" refers to the delivery of a gene(s) using a viral or retroviral vector by means of infection rather than by transfection. In certain embodiments, retroviral vectors are transduced. Thus, a "transduced gene" is a gene that has been introduced into the cell via retroviral or vector infection and provirus integration. In certain embodiments, viral vectors (*e.g.*, "transgene vectors") transduce genes into "target cells" or host cells. The, present invention encompasses transgene vectors that are suitable for use in the present invention that are linked to any gene of interest (or a "marker gene" or "reporter gene," used to indicate infection or expression of a gene).

As used herein, the term "long-term transduction" refers to vectors that are capable of remaining transduced in host or target cells for time periods that are longer than those observed with other vectors. For example, the present invention provides retroviral vectors that are capable of remaining transduced for at least 120 days, at least one year, or for the life of the subject or the necessary time course of treatment. The duration of expression is a function of the choice of promoter and the target cell type, more so than the choice of vector.

The term "stable transduction" or "stably transduced" refers to the introduction and integration of foreign DNA into the genome of the transducted cell. The term "stable transductant" refers to a cell that has stably integrated foreign DNA into the genomic DNA.

The term "transient transduction" or "transiently transduced" refers to the introduction of foreign DNA into a cell where the foreign DNA fails to integrate into the genome of the transducted cell. The foreign DNA persists in the nucleus of the transducted cell for several days. During this time the foreign DNA is subject to the regulatory controls that govern the expression of endogenous genes in the chromosomes. The term "transient transductant" refers to cells that have taken up foreign DNA but have failed to integrate this DNA.

In some embodiments, the target and/or host cells of the present invention are "non-dividing" cells. These cells include cells such as neuronal cells that do not normally divide.

However, it is not intended that the present invention be limited to non-dividing cells (including, but not limited to muscle cells, white blood cells, spleen cells, liver cells, eye cells, epithelial cells).

In some embodiments, the vector and the vector progeny are capable of transducing a plurality of target cells so as to achieve vector titers of at least 10^5 cfu/ml. The multiplicity of infection (MOI) may be at least one (*i.e.*, one hit on average per cell), or even at least two.

Expression Cassettes and Vectors

The present invention also provides an expression cassette comprising a sequence encoding ACE-tRNA.

In certain embodiments, the expression cassette further contains a promoter. In certain embodiments, the promoter is a regulatable promoter. In certain embodiments, the promoter is a constitutive promoter. In certain embodiments, the promoter is a PGK, CMV, RSV, H1 or U6 promoter (Pol II and Pol III promoters).

The present invention provides a vector containing the expression cassette described above. In certain embodiments, the vector is a viral vector. In certain embodiments, the viral vector is an adenoviral, lentiviral, adeno-associated viral (AAV), poliovirus, HSV, or murine Maloney-based viral vector.

"Expression cassette" as used herein means a nucleic acid sequence capable of directing expression of a particular nucleotide sequence in an appropriate host cell, which may include a promoter operably linked to the nucleotide sequence of interest that may be operably linked to termination signals. It also may include sequences required for proper translation of the nucleotide sequence. The coding region usually codes for a protein of interest. The expression cassette including the nucleotide sequence of interest may be chimeric. The expression cassette may also be one that is naturally occurring but has been obtained in a recombinant form useful for heterologous expression. The expression of the nucleotide sequence in the expression cassette may be under the control of a constitutive promoter or of a regulatable promoter that initiates transcription only when the host cell is exposed to some particular stimulus. In the case of a multicellular organism, the promoter can also be specific to a particular tissue or organ or stage of development.

"Operably-linked" refers to the association of nucleic acid sequences on single nucleic acid fragment so that the function of one of the sequences is affected by another. For example, a regulatory DNA sequence is said to be "operably linked to" or "associated with" a DNA

sequence that codes for an RNA or a polypeptide if the two sequences are situated such that the regulatory DNA sequence affects expression of the coding DNA sequence (i.e., that the coding sequence or functional RNA is under the transcriptional control of the promoter). Coding sequences can be operably-linked to regulatory sequences in sense or antisense orientation.

Adeno associated virus (AAV)

Adeno associated virus (AAV) is a small nonpathogenic virus of the parvoviridae family. AAV is distinct from the other members of this family by its dependence upon a helper virus for replication. In the absence of a helper virus, AAV may integrate in a locus specific manner into the q arm of chromosome 19. The approximately 5 kb genome of AAV consists of one segment of single stranded DNA of either plus or minus polarity. The ends of the genome are short inverted terminal repeats that can fold into hairpin structures and serve as the origin of viral DNA replication. Physically, the parvovirus virion is non-enveloped and its icosohedral capsid is approximately 20 nm in diameter.

To date, numerous serologically distinct AAVs have been identified, and more than a dozen have been isolated from humans or primates. The genome of AAV2 is 4680 nucleotides in length and contains two open reading frames (ORFs). The left ORF encodes the non-structural Rep proteins, Rep 40, Rep 52, Rep 68 and Rep 78, which are involved in regulation of replication and transcription in addition to the production of single-stranded progeny genomes. Furthermore, two of the Rep proteins have been associated with the preferential integration of AAV genomes into a region of the q arm of human chromosome 19. Rep68/78 has also been shown to possess NTP binding activity as well as DNA and RNA helicase activities. The Rep proteins possess a nuclear localization signal as well as several potential phosphorylation sites. Mutation of one of these kinase sites resulted in a loss of replication activity.

The ends of the genome are short inverted terminal repeats (ITR) which have the potential to fold into T-shaped hairpin structures that serve as the origin of viral DNA replication. Within the ITR region two elements have been described which are central to the function of the ITR, a GAGC repeat motif and the terminal resolution site (trs). The repeat motif has been shown to bind Rep when the ITR is in either a linear or hairpin conformation. This binding serves to position Rep68/78 for cleavage at the trs, which occurs in a site- and strand-specific manner. In addition to their role in replication, these two elements appear to be central to viral integration. Contained within the chromosome 19 integration locus is a Rep binding site

with an adjacent trs. These elements have been shown to be functional and necessary for locus specific integration.

The AAV virion is a non-enveloped, icosohedral particle approximately 25 nm in diameter, consisting of three related proteins referred to as VP1, VP2 and VP3. The right ORF encodes the capsid proteins VP1, VP2, and VP3. These proteins are found in a ratio of 1:1:10 respectively and are all derived from the right-hand ORF. The capsid proteins differ from each other by the use of alternative splicing and an unusual start codon. Deletion analysis has shown that removal or alteration of VP1 which is translated from an alternatively spliced message results in a reduced yield of infections particles. Mutations within the VP3 coding region result in the failure to produce any single-stranded progeny DNA or infectious particles. An AAV particle is a viral particle comprising an AAV capsid protein. An AAV capsid polypeptide can encode the entire VP1, VP2 and VP3 polypeptide. The particle can be a particle comprising AAV2 and other AAV capsid proteins (i.e., a chimeric protein, such as AAV1 and AAV2). Variations in the amino acid sequence of the AAV2 capsid protein are contemplated herein, as long as the resulting viral particle comprises the AAV2 capsid remains antigenically or immunologically distinct from AAV1, as can be routinely determined by standard methods. Specifically, for example, ELISA and Western blots can be used to determine whether a viral particle is antigenically or immunologically distinct from AAV1. Furthermore, the AAV2 viral particle preferably retains tissue tropism distinct from AAV1.

An AAV2 particle is a viral particle comprising an AAV2 capsid protein. An AAV2 capsid polypeptide encoding the entire VP1, VP2, and VP3 polypeptide can overall have at least about 63% homology (or identity) to the polypeptide having the amino acid sequence encoded by nucleotides set forth in NC_001401 (nucleotide sequence encoding AAV2 capsid protein). The capsid protein can have about 70% homology, about 75% homology, 80% homology, 85% homology, 90% homology, 95% homology, 98% homology, 99% homology, or even 100% homology to the protein encoded by the nucleotide sequence set forth in NC_001401. The capsid protein can have about 70% identity, about 75% identity, 80% identity, 85% identity, 90% identity, 95% identity, 98% identity, 99% identity, or even 100% identity to the protein encoded by the nucleotide sequence set forth in NC_001401. The particle can be a particle comprising another AAV and AAV2 capsid protein, i.e., a chimeric protein. Variations in the amino acid sequence of the AAV2 capsid protein are contemplated herein, as long as the resulting viral particle comprising the AAV2 capsid remains antigenically or immunologically distinct from

AAV4, as can be routinely determined by standard methods. Specifically, for example, ELISA and Western blots can be used to determine whether a viral particle is antigenically or immunologically distinct from AAV1. Furthermore, the AAV2 viral particle preferably retains tissue tropism distinction from AAV1, such as that exemplified in the examples herein, though an AAV2 chimeric particle comprising at least one AAV2 coat protein may have a different tissue tropism from that of an AAV2 particle consisting only of AAV2 coat proteins.

In certain embodiments, the invention further provides an AAV2 particle containing, i.e., encapsidating, a vector comprising a pair of AAV2 inverted terminal repeats. The nucleotide sequence of AAV2 ITRs is known in the art. Furthermore, the particle can be a particle comprising both AAV1 and AAV2 capsid protein, i.e., a chimeric protein. Moreover, the particle can be a particle encapsidating a vector comprising a pair of AAV inverted terminal repeats from other AAVs (e.g., AAV1-AAV9 and AAVrh10). The vector encapsidated in the particle can further comprise an exogenous nucleic acid inserted between the inverted terminal repeats.

The following features of AAV have made it an attractive vector for gene transfer. AAV vectors have been shown in vitro to stably integrate into the cellular genome; possess a broad host range; transduce both dividing and non-dividing cells in vitro and in vivo and maintain high levels of expression of the transduced genes. Viral particles are heat stable, resistant to solvents, detergents, changes in pH, temperature, and can be concentrated on CsCl gradients or by other means. The present invention provides methods of administering AAV particles, recombinant AAV vectors, and recombinant AAV virions. For example, an AAV2 particle is a viral particle comprising an AAV2 capsid protein, or an AAV1 particle is a viral particle comprising an AAV1 capsid protein. A recombinant AAV2 vector is a nucleic acid construct that comprises at least one unique nucleic acid of AAV2. A recombinant AAV2 virion is a particle containing a recombinant AAV2 vector. To be considered within the term "AAV2 ITRs" the nucleotide sequence must retain one or both features described herein that distinguish the AAV2 ITR from the AAV1 ITR: (1) three (rather than four as in AAV1) "GAGC" repeats and (2) in the AAV2 ITR Rep binding site the fourth nucleotide in the first two "GAGC" repeats is a C rather than a T.

The promoter to drive expression of the sequence encoding the tRNA to be delivered can be any desired promoter, selected by known considerations, such as the level of expression of a nucleic acid functionally linked to the promoter and the cell type in which the vector is to be used. Promoters can be an exogenous or an endogenous promoter. Promoters can include, for example, known strong promoters such as SV40 or the inducible metallothionein promoter, or an

AAV promoter, such as an AAV p5 promoter. Additional examples of promoters include promoters derived from actin genes, immunoglobulin genes, cytomegalovirus (CMV), adenovirus, bovine papilloma virus, adenoviral promoters, such as the adenoviral major late promoter, an inducible heat shock promoter, respiratory syncytial virus, Rous sarcomas virus (RSV), etc. Additional examples include regulated promoters.

The AAV vector can further comprise an exogenous (heterologous) nucleic acid functionally linked to the promoter. By "heterologous nucleic acid" is meant that any heterologous or exogenous nucleic acid can be inserted into the vector for transfer into a cell, tissue or organism. The nucleic acid can encode a tRNA, for example. By "functionally linked" is meant such that the promoter can promote expression of the heterologous nucleic acid, as is known in the art, such as appropriate orientation of the promoter relative to the heterologous nucleic acid. Furthermore, the heterologous nucleic acid preferably has all appropriate sequences for expression of the nucleic acid, as known in the art, to functionally encode, i.e., allow the nucleic acid to be expressed. The nucleic acid can include, for example, expression control sequences, such as an enhancer. The nucleic acid can encode more than one gene product, limited only by the size of nucleic acid that can be packaged.

An AAV1 particle is a viral particle comprising an AAV1 capsid protein. Variations in the amino acid sequence of the AAV1 capsid protein are contemplated herein, as long as the resulting viral particle comprising the AAV1 capsid remains antigenically or immunologically distinct from other AAV capsids, as can be routinely determined by standard methods. Specifically, for example, ELISA and Western blots can be used to determine whether a viral particle is antigenically or immunologically distinct from other AAV serotypes.

The term "polypeptide" as used herein refers to a polymer of amino acids and includes full-length proteins and fragments thereof. Thus, "protein" and “polypeptide" are often used interchangeably herein.

The present method provides a method of delivering a nucleic acid to a cell comprising administering to the cell an AAV particle containing a vector comprising the nucleic acid inserted between a pair of AAV inverted terminal repeats, thereby delivering the nucleic acid to the cell. Administration to the cell can be accomplished by any means, including simply contacting the particle, optionally contained in a desired liquid such as tissue culture medium, or a buffered saline solution, with the cells. The particle can be allowed to remain in contact with the cells for any desired length of time, and typically, the particle is administered and allowed to

remain indefinitely. For such in vitro methods, the virus can be administered to the cell by standard viral transduction methods, as known in the art and as exemplified herein. Titers of virus to administer can vary, particularly depending upon the cell type, but will be typical of that used for AAV transduction in general. Additionally the titers used to transduce the particular cells in the present examples can be utilized. The cells can include any desired cell in humans as well as other large (non-rodent) mammals, such as primates, horse, sheep, goat, pig, and dog.

The present invention further provides a method of delivering a nucleic acid to a cell in a subject comprising administering to the subject an AAV particle comprising the nucleic acid inserted between a pair of AAV inverted terminal repeats, thereby delivering the nucleic acid to a cell in the subject.

Certain embodiments of the present disclosure provide a cell comprising a viral vector as described herein.

AAV Vectors

In one embodiment, a viral vector of the disclosure is an AAV vector. An "AAV" vector refers to an adeno-associated virus, and may be used to refer to the naturally occurring wild-type virus itself or derivatives thereof. The term covers all subtypes, serotypes and pseudotypes, and both naturally occurring and recombinant forms, except where required otherwise. As used herein, the term "serotype" refers to an AAV, which is identified by, and distinguished from other AAVs based on capsid protein reactivity with defined antisera, e.g., there are eight known serotypes of primate AAVs, AAV-1 to AAV-9 and AAVrh10. For example, serotype AAV2 is used to refer to an AAV, which contains capsid proteins encoded from the cap gene of AAV2 and a genome containing 5' and 3' ITR sequences from the same AAV2 serotype. As used herein, for example, rAAV1 may be used to refer an AAV having both capsid proteins and 5'-3' ITRs from the same serotype or it may refer to an AAV having capsid proteins from one serotype and 5'-3' ITRs from a different AAV serotype, e.g., capsid from AAV serotype 2 and ITRs from AAV serotype 5. For each example illustrated herein, the description of the vector design and production describes the serotype of the capsid and 5'-3' ITR sequences. The abbreviation "rAAV" refers to recombinant adeno-associated virus, also referred to as a recombinant AAV vector (or "rAAV vector").

An "AAV virus" or "AAV viral particle" refers to a viral particle composed of at least one AAV capsid protein (preferably by all of the capsid proteins of a wild-type AAV) and an encapsidated polynucleotide. If the particle comprises heterologous polynucleotide (i.e., a

polynucleotide other than a wild-type AAV genome such as a transgene to be delivered to a mammalian cell), it is typically referred to as "rAAV".

In one embodiment, the AAV expression vectors are constructed using known techniques to at least provide as operatively linked components in the direction of transcription, control elements including a transcriptional initiation region, the DNA of interest and a transcriptional termination region. The control elements are selected to be functional in a mammalian cell. The resulting construct which contains the operatively linked components is flanked (5' and 3') with functional AAV ITR sequences.

By "adeno-associated virus inverted terminal repeats" or "AAV ITRs" is meant the art-recognized regions found at each end of the AAV genome which function together in cis as origins of DNA replication and as packaging signals for the virus. AAV ITRs, together with the AAV rep coding region, provide for the efficient excision and rescue from, and integration of a nucleotide sequence interposed between two flanking ITRs into a mammalian cell genome.

The nucleotide sequences of AAV ITR regions are known. As used herein, an "AAV ITR" need not have the wild-type nucleotide sequence depicted, but may be altered, e.g., by the insertion, deletion or substitution of nucleotides. Additionally, the AAV ITR may be derived from any of several AAV serotypes, including without limitation, AAV1, AAV2, AAV3, AAV4, AAV5, AAV7, etc. Furthermore, 5' and 3' ITRs which flank a selected nucleotide sequence in an AAV vector need not necessarily be identical or derived from the same AAV serotype or isolate, so long as they function as intended, i.e., to allow for excision and rescue of the sequence of interest from a host cell genome or vector, and to allow integration of the heterologous sequence into the recipient cell genome when AAV Rep gene products are present in the cell.

In one embodiment, AAV ITRs can be derived from any of several AAV serotypes, including without limitation, AAV1, AAV2, AAV3, AAV4, AAV5, AAV7, etc. Furthermore, 5' and 3' ITRs which flank a selected nucleotide sequence in an AAV expression vector need not necessarily be identical or derived from the same AAV serotype or isolate, so long as they function as intended, i.e., to allow for excision and rescue of the sequence of interest from a host cell genome or vector, and to allow integration of the DNA molecule into the recipient cell genome when AAV Rep gene products are present in the cell.

In one embodiment, AAV capsids can be derived from AAV2. Suitable DNA molecules for use in AAV vectors will be less than about 5 kilobases (kb), less than about 4.5 kb, less than

about 4kb, less than about 3.5 kb, less than about 3 kb, less than about 2.5 kb in size and are known in the art.

In one embodiment, the selected nucleotide sequence is operably linked to control elements that direct the transcription or expression thereof in the subject in vivo. Such control elements can comprise control sequences normally associated with the selected gene. Alternatively, heterologous control sequences can be employed. Useful heterologous control sequences generally include those derived from sequences encoding mammalian or viral genes. Examples include, but are not limited to, the SV40 early promoter, mouse mammary tumor virus LTR promoter; adenovirus major late promoter (Ad MLP); a herpes simplex virus (HSV) promoter, a cytomegalovirus (CMV) promoter such as the CMV immediate early promoter region (CMVIE), a rous sarcoma virus (RSV) promoter, pol II promoters, pol III promoters, synthetic promoters, hybrid promoters, and the like. In addition, sequences derived from non-viral genes, such as the murine metallothionein gene, will also find use herein. Such promoter sequences are commercially available from, e.g., Stratagene (San Diego, Calif.).

In one embodiment, both heterologous promoters and other control elements, such as tissue-specific and inducible promoters, enhancers and the like, will be of particular use. Examples of heterologous promoters include the CMV promoter. Examples of inducible promoters include DNA responsive elements for ecdysone, tetracycline, hypoxia and aufin.

In one embodiment, the AAV expression vector that harbors the DNA molecule of interest bounded by AAV ITRs, can be constructed by directly inserting the selected sequence(s) into an AAV genome, which has had the major AAV open reading frames ("ORFs"), excised therefrom. Other portions of the AAV genome can also be deleted, so long as sufficient portions of the ITRs remain to allow for replication and packaging functions. Such constructs can be designed using techniques well known in the art.

Alternatively, AAV ITRs can be excised from the viral genome or from an AAV vector containing the same and fused 5' and 3' of a selected nucleic acid construct that is present in another vector using standard ligation techniques. For example, ligations can be accomplished in 20 mM Tris-Cl pH 7.5, 10 mM MgCl2, 10 mM DTT, 33 μg/ml BSA, 10 mM-50 mM NaCl, and either 40 uM ATP, 0.01-0.02 (Weiss) units T4 DNA ligase at 0°C (for "sticky end" ligation) or 1 mM ATP, 0.3-0.6 (Weiss) units T4 DNA ligase at 14°C (for "blunt end" ligation). Intermolecular "sticky end" ligations are usually performed at 30-100 μg/ml total DNA concentrations (5-100 nM total end concentration). AAV vectors which contain ITRs.

Additionally, chimeric genes can be produced synthetically to include AAV ITR sequences arranged 5' and 3' of one or more selected nucleic acid sequences. The complete chimeric sequence is assembled from overlapping oligonucleotides prepared by standard methods.

In order to produce rAAV virions, an AAV expression vector is introduced into a suitable host cell using known techniques, such as by transfection. A number of transfection techniques are generally known in the art. See, e.g., Sambrook et al. (1989) Molecular Cloning, a laboratory manual, Cold Spring Harbor Laboratories, New York. Particularly suitable transfection methods include calcium phosphate co-precipitation, direct micro-injection into cultured cells, electroporation, liposome mediated gene transfer, lipid-mediated transduction, and nucleic acid delivery using high-velocity microprojectiles.

In one embodiment, suitable host cells for producing rAAV virions include microorganisms, yeast cells, insect cells, and mammalian cells, that can be, or have been, used as recipients of a heterologous DNA molecule. The term includes the progeny of the original cell that has been transfected. Thus, a "host cell" as used herein generally refers to a cell that has been transfected with an exogenous DNA sequence. Cells from the stable human cell line, 293 (readily available through, e.g., the American Type Culture Collection under Accession Number ATCC CRL1573) can be used in the practice of the present disclosure. Particularly, the human cell line 293 is a human embryonic kidney cell line that has been transformed with adenovirus type-5 DNA fragments, and expresses the adenoviral E1a and E1b genes. The 293 cell line is readily transfected, and provides a particularly convenient platform in which to produce rAAV virions.

By "AAV rep coding region" is meant the art-recognized region of the AAV genome which encodes the replication proteins Rep 78, Rep 68, Rep 52 and Rep 40. These Rep expression products have been shown to possess many functions, including recognition, binding and nicking of the AAV origin of DNA replication, DNA helicase activity and modulation of transcription from AAV (or other heterologous) promoters. The Rep expression products are collectively required for replicating the AAV genome. Suitable homologues of the AAV rep coding region include the human herpesvirus 6 (HHV-6) rep gene which is also known to mediate AAV-2 DNA replication.

By "AAV cap coding region" is meant the art-recognized region of the AAV genome that encodes the capsid proteins VP1, VP2, and VP3, or functional homologues thereof. These Cap

expression products supply the packaging functions, which are collectively required for packaging the viral genome.

In one embodiment, AAV helper functions are introduced into the host cell by transfecting the host cell with an AAV helper construct either prior to, or concurrently with, the transfection of the AAV expression vector. AAV helper constructs are thus used to provide at least transient expression of AAV rep and/or cap genes to complement missing AAV functions that are necessary for productive AAV infection. AAV helper constructs lack AAV ITRs and can neither replicate nor package themselves. These constructs can be in the form of a plasmid, phage, transposon, cosmid, virus, or virion. A number of AAV helper constructs have been described, such as the commonly used plasmids pAAV/Ad and pIM29+45 that encode both Rep and Cap expression products. A number of other vectors have been described that encode Rep and/or Cap expression products.

Methods of delivery of viral vectors include injecting the AAV into the subject. Generally, rAAV virions may be introduced into cells using either in vivo or in vitro transduction techniques. If transduced in vitro, the desired recipient cell will be removed from the subject, transduced with rAAV virions and reintroduced into the subject. Alternatively, syngeneic or xenogeneic cells can be used where those cells will not generate an inappropriate immune response in the subject.

Suitable methods for the delivery and introduction of transduced cells into a subject have been described. For example, cells can be transduced in vitro by combining recombinant AAV virions with cells e.g., in appropriate media, and screening for those cells harboring the DNA of interest can be screened using conventional techniques such as Southern blots and/or PCR, or by using selectable markers. Transduced cells can then be formulated into pharmaceutical compositions, described more fully below, and the composition introduced into the subject by various techniques, such as by grafting, intramuscular, intravenous, subcutaneous and intraperitoneal injection.

In one embodiment, pharmaceutical compositions will comprise sufficient genetic material to produce a therapeutically effective amount of the nucleic acid of interest, i.e., an amount sufficient to reduce or ameliorate symptoms of the disease state in question or an amount sufficient to confer the desired benefit. The pharmaceutical compositions will also contain a pharmaceutically acceptable excipient. Such excipients include any pharmaceutical agent that does not itself induce the production of antibodies harmful to the individual receiving the

composition, and which may be administered without undue toxicity. Pharmaceutically acceptable excipients include, but are not limited to, sorbitol, Tween80, and liquids such as water, saline, glycerol and ethanol. Pharmaceutically acceptable salts can be included therein, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles. A thorough discussion of pharmaceutically acceptable excipients is available in Remington's Pharmaceutical Sciences (Mack Pub. Co., N.J. 1991).

It should be understood that more than one transgene could be expressed by the delivered viral vector. Alternatively, separate vectors, each expressing one or more different transgenes, can also be delivered to the subject as described herein. Furthermore, it is also intended that the viral vectors delivered by the methods of the present disclosure be combined with other suitable compositions and therapies.

As is apparent to those skilled in the art in view of the teachings of this specification, an effective amount of viral vector that must be added can be empirically determined. Administration can be effected in one dose, continuously or intermittently throughout the course of treatment. Methods of determining the most effective means and dosages of administration are well known to those of skill in the art and will vary with the viral vector, the composition of the therapy, the target cells, and the subject being treated. Single and multiple administrations can be carried out with the dose level and pattern being selected by the treating physician.

In certain embodiments, the rAAV is administered at a dose of about 0.3-2 ml of 1x105 - 1x1016vg/ml. In certain embodiments, the rAAV is administered at a dose of about 1-3 ml of 1x107 -1x1014vg/ml. In certain embodiments, the rAAV is administered at a dose of about 1-2 ml of 1x108 -1x1013vg/ml.

Formulations containing the rAAV particles will contain an effective amount of the rAAV particles in a vehicle, the effective amount being readily determined by one skilled in the art. The rAAV particles may typically range from about 1% to about 95% (w/w) of the composition, or even higher or lower if appropriate. The quantity to be administered depends upon factors such as the age, weight and physical condition of the animal or the human subject considered for treatment. Effective dosages can be established by one of ordinary skill in the art through routine trials establishing dose response curves. The subject is treated by administration

of the rAAV particles in one or more doses. Multiple doses may be administered as is required to maintain adequate enzyme activity.

Vehicles including water, aqueous saline, artificial CSF, or other known substances can be employed with the subject invention. To prepare a formulation, the purified composition can be isolated, lyophilized and stabilized. The composition may then be adjusted to an appropriate concentration, optionally combined with an anti-inflammatory agent, and packaged for use.

The present invention provides a method of increasing the level of a target protein in a cell by introducing a protein, or nucleic acid molecule encoding a protein described above into a cell in an amount sufficient to increase the level of the target protein in the cell. In certain embodiments, the accumulation of target protein is increased by at least 10%. The accumulation of target protein is increased by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% 95%, or 99%. 39

Nucleic Acids Encoding Therapeutic Agents

The term "nucleic acid" refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form, composed of monomers (nucleotides) containing a sugar, phosphate and a base that is either a purine or pyrimidine. Unless specifically limited, the term encompasses nucleic acids containing known analogs of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides.

A "nucleic acid fragment" is a portion of a given nucleic acid molecule. The term "substantial identity" of polynucleotide sequences means that a polynucleotide comprises a sequence that has at least 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, or 79%, or at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, or 89%, or at least 90%, 91%, 92%, 93%, or 94%, or even at least 95%, 96%, 97%, 98%, or 99% sequence identity, compared to a reference sequence using one of the alignment programs described using standard parameters.

In one embodiment, the nucleic acid molecule is an RNA molecule comprising an ACE-tRNA.

In one embodiment, the nucleic acid molecule is a DNA or cDNA molecule which encodes one or more ACE-tRNA. In one embodiment, the nucleic acid molecule is a nucleic acid molecule encoding 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 11 or more, 12 or more, 13 or more, 14 or more, 15 or more, 16 or more,

17 or more, 18 or more, 19 or more or 20 or more ACE-tRNA molecules under the control of a single promoter.

In one embodiment, the invention provides a nucleic acid molecule encoding two or more ACE-tRNA separated by cleavage sites. In one embodiment, a cleavage site is a P2A site. In one embodiment, a cleavage site is a protease cleavage site. In various embodiments, a protease cleavage site may include, but is not limited to, a site targeted by a Caspase (e.g. Caspase 1 through Caspase 10), enterokinase, factor Xa, granzyme B, HRV3C protease, hydroxylamine, pancreatic elastase, pepsin A, prolyl endopeptidase, proteinase K, TEV protease, thermolysin, or thrombin. In one embodiment, a protease for cleavage of the protease cleavage sites is encoded downstream of two or more genes encoding reporter molecules as part of the same polypeptide. In one embodiment, the nucleotide sequence encoding the protease is linked to the nucleotide sequence of two or more genes encoding ACE-tRNA by a protease cleavage site.

In one embodiment, the nucleic acid molecule comprises one or more reporter molecule. A reporter is a molecule whose expression a cell confers a detectable trait to the cell. In various embodiments, reporters include, but are not limited to, chloramphenicol-acetyl transferase(CAT), β-galactosyltransferase, horseradish peroxidase, luciferase, NanoLuc®, alkaline phosphatase, and fluorescent proteins including, but not limited to, green fluorescent proteins (e.g. GFP, TagGFP, T-Sapphire, Azami Green, Emerald, mWasabi, mClover3), red fluorescent proteins (e.g. tdTomato, mRFP1, JRed, HcRed1, AsRed2, AQ143, mCherry, mRuby3, mPlum), yellow fluorescent proteins (e.g. EYFP, mBanana, mCitrine, PhiYFP, TagYFP, Topaz, Venus), orange fluorescent proteins (e.g. DsRed, Tomato, Kusabria Orange, mOrange, mTangerine, TagRFP), cyan fluorescent proteins (e.g. CFP, mTFP1, Cerulean, CyPet, AmCyan1), blue fluorescent proteins (e.g. Azurite, mtagBFP2, EBFP, EBFP2, Y66H), near-infrared fluorescent proteins (e.g. iRFP670, iRFP682, iRFP702, iRFP713 and iRFP720), infrared fluorescent proteins (e.g. IFP1.4) and photoactivatable fluorescent proteins (e.g. Kaede, Eos, IrisFP, PS-CFP).

Methods for Introducing Genetic Material into Cells

The exogenous genetic material (*e.g.*, a DNA encoding one or more therapeutic ACE-tRNAs) is introduced into the cell *in vivo* by genetic transfer methods, such as transfection or transduction, to provide a genetically modified cell. Various expression vectors (*i.e.*, vehicles for facilitating delivery of exogenous genetic material into a target cell) are known to one of ordinary skill in the art.

As used herein, "transfection of cells" refers to the acquisition by a cell of new genetic material by incorporation of added DNA. Thus, transfection refers to the insertion of nucleic acid into a cell using physical or chemical methods. Several transfection techniques are known to those of ordinary skill in the art including: calcium phosphate DNA co-precipitation; DEAE-dextran; electroporation; cationic liposome-mediated transfection; and tungsten particle-facilitated microparticle bombardment. Strontium phosphate DNA co-precipitation is another possible transfection method.

In contrast, "transduction of cells" refers to the process of transferring nucleic acid into a cell using a DNA or RNA virus. A RNA virus (*i.e.*, a retrovirus) for transferring a nucleic acid into a cell is referred to herein as a transducing chimeric retrovirus. Exogenous genetic material contained within the retrovirus is incorporated into the genome of the transduced cell. A cell that has been transduced with a chimeric DNA virus (*e.g.*, an adenovirus carrying a cDNA encoding a therapeutic agent), will not have the exogenous genetic material incorporated into its genome but will be capable of expressing the exogenous genetic material that is retained extrachromosomally within the cell.

Typically, the exogenous genetic material includes the heterologous gene (usually in the form of a cDNA comprising the exons coding for the therapeutic protein) together with a promoter to control transcription of the new gene. The promoter characteristically has a specific nucleotide sequence necessary to initiate transcription. Optionally, the exogenous genetic material further includes additional sequences (*i.e.*, enhancers) required to obtain the desired gene transcription activity. For the purpose of this discussion, an "enhancer" is simply any non-translated DNA sequence that works contiguous with the coding sequence (in cis) to change the basal transcription level dictated by the promoter. The exogenous genetic material may introduced into the cell genome immediately downstream from the promoter so that the promoter and coding sequence are operatively linked so as to permit transcription of the coding sequence. A retroviral expression vector may include an exogenous promoter element to control transcription of the inserted exogenous gene. Such exogenous promoters include both constitutive and inducible promoters.

Naturally-occurring constitutive promoters control the expression of essential cell functions. As a result, a gene under the control of a constitutive promoter is expressed under all conditions of cell growth. Exemplary constitutive promoters include the promoters for the following genes that encode certain constitutive or "housekeeping" functions: hypoxanthine

phosphoribosyl transferase (HPRT), dihydrofolate reductase (DHFR), adenosine deaminase, phosphoglycerol kinase (PGK), pyruvate kinase, phosphoglycerol mutase, the actin promoter, and other constitutive promoters known to those of skill in the art. In addition, many viral promoters function constitutively in eucaryotic cells. These include the early and late promoters of SV40; the long terminal repeats (LTRs) of Moloney Leukemia Virus and other retroviruses; and the thymidine kinase promoter of Herpes Simplex Virus, among many others. Accordingly, any of the above-referenced constitutive promoters can be used to control transcription of a heterologous gene insert.

Genes that are under the control of inducible promoters are expressed only or to a greater degree, in the presence of an inducing agent, (*e.g.*, transcription under control of the metallothionein promoter is greatly increased in presence of certain metal ions). Inducible promoters include responsive elements (REs) which stimulate transcription when their inducing factors are bound. For example, there are REs for serum factors, steroid hormones, retinoic acid and cyclic AMP. Promoters containing a particular RE can be chosen in order to obtain an inducible response and in some cases, the RE itself may be attached to a different promoter, thereby conferring inducibility to the recombinant gene. Thus, by selecting the appropriate promoter (constitutive versus inducible; strong versus weak), it is possible to control both the existence and level of expression of a therapeutic agent in the genetically modified cell. If the gene encoding the therapeutic agent is under the control of an inducible promoter, delivery of the therapeutic agent *in situ* is triggered by exposing the genetically modified cell *in situ* to conditions for permitting transcription of the therapeutic agent, *e.g.*, by intraperitoneal injection of specific inducers of the inducible promoters which control transcription of the agent. For example, *in situ* expression by genetically modified cells of a therapeutic agent encoded by a gene under the control of the metallothionein promoter, is enhanced by contacting the genetically modified cells with a solution containing the appropriate (*i.e.*, inducing) metal ions *in situ*.

Accordingly, the amount of therapeutic agent that is delivered *in situ* is regulated by controlling such factors as: (1) the nature of the promoter used to direct transcription of the inserted gene, (*i.e.*, whether the promoter is constitutive or inducible, strong or weak); (2) the number of copies of the exogenous gene that are inserted into the cell; (3) the number of transduced/transfected cells that are administered (*e.g.*, implanted) to the patient; (4) the size of the implant (*e.g.*, graft or encapsulated expression system); (5) the number of implants; (6) the length of time the transduced/transfected cells or implants are left in place; and (7) the

production rate of the therapeutic agent by the genetically modified cell. Selection and optimization of these factors for delivery of a therapeutically effective dose of a particular therapeutic agent is deemed to be within the scope of one of ordinary skill in the art without undue experimentation, taking into account the above-disclosed factors and the clinical profile of the patient.

In addition to at least one promoter and at least one heterologous nucleic acid encoding the therapeutic agent, the expression vector may include a selection gene, for example, a neomycin resistance gene, for facilitating selection of cells that have been transfected or transduced with the expression vector. Alternatively, the cells are transfected with two or more expression vectors, at least one vector containing the gene(s) encoding the therapeutic agent(s), the other vector containing a selection gene. The selection of a suitable promoter, enhancer, selection gene and/or signal sequence is deemed to be within the scope of one of ordinary skill in the art without undue experimentation.

An ACE-tRNA construct of the present invention can be inserted into any type of target or host cell. In the context of an expression vector, the vector can be readily introduced into a host cell, e.g., mammalian, bacterial, yeast, or insect cell by any method in the art. For example, the expression vector can be transferred into a host cell by physical, chemical, or biological means.

Physical methods for introducing a polynucleotide into a host cell include calcium phosphate precipitation, lipofection, particle bombardment, microinjection, electroporation, and the like. Methods for producing cells comprising vectors and/or exogenous nucleic acids are well-known in the art. See, for example, Sambrook et al. (2012, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York). A preferred method for the introduction of a polynucleotide into a host cell is calcium phosphate transfection.

Biological methods for introducing a polynucleotide of interest into a host cell include the use of DNA and RNA vectors. Viral vectors, and especially retroviral vectors, have become the most widely used method for inserting genes into mammalian, e.g., human cells. Other viral vectors can be derived from lentivirus, poxviruses, herpes simplex virus I, adenoviruses and adeno-associated viruses, and the like. See, for example, U.S. Pat. Nos. 5,350,674 and 5,585,362.

Chemical means for introducing a polynucleotide into a host cell include colloidal dispersion systems, such as macromolecule complexes, nanocapsules, microspheres, beads, and

lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. An exemplary colloidal system for use as a delivery vehicle in vitro and in vivo is a liposome (e.g., an artificial membrane vesicle).

In the case where a non-viral delivery system is utilized, an exemplary delivery vehicle is a liposome. The use of lipid formulations is contemplated for the introduction of the nucleic acids into a host cell (in vitro, ex vivo or in vivo). In another aspect, the nucleic acid may be associated with a lipid. The nucleic acid associated with a lipid may be encapsulated in the aqueous interior of a liposome, interspersed within the lipid bilayer of a liposome, attached to a liposome via a linking molecule that is associated with both the liposome and the oligonucleotide, entrapped in a liposome, complexed with a liposome, dispersed in a solution containing a lipid, mixed with a lipid, combined with a lipid, contained as a suspension in a lipid, contained or complexed with a micelle, or otherwise associated with a lipid. Lipid, lipid/DNA or lipid/expression vector associated compositions are not limited to any particular structure in solution. For example, they may be present in a bilayer structure, as micelles, or with a "collapsed" structure. They may also simply be interspersed in a solution, possibly forming aggregates that are not uniform in size or shape. Lipids are fatty substances which may be naturally occurring or synthetic lipids. For example, lipids include the fatty droplets that naturally occur in the cytoplasm as well as the class of compounds which contain long-chain aliphatic hydrocarbons and their derivatives, such as fatty acids, alcohols, amines, amino alcohols, and aldehydes.

Lipids suitable for use can be obtained from commercial sources. For example, dimyristyl phosphatidylcholine ("DMPC") can be obtained from Sigma, St. Louis, MO; dicetyl phosphate ("DCP") can be obtained from K & K Laboratories (Plainview, NY); cholesterol ("Choi") can be obtained from Calbiochem-Behring; dimyristyl phosphatidylglycerol ("DMPG") and other lipids may be obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). Stock solutions of lipids in chloroform or chloroform/methanol can be stored at about -20°C. Chloroform is used as the only solvent since it is more readily evaporated than methanol. "Liposome" is a generic term encompassing a variety of single and multilamellar lipid vehicles formed by the generation of enclosed lipid bilayers or aggregates. Liposomes can be characterized as having vesicular structures with a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous

solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh et al., 1991 Glycobiology 5: 505-10). However, compositions that have different structures in solution than the normal vesicular structure are also encompassed. For example, the lipids may assume a micellar structure or merely exist as nonuniform aggregates of lipid molecules. Also contemplated are lipofectamine-nucleic acid complexes.

The nucleic acid molecule of the invention can be administered via electroporation, such as by a method described in U.S. Patent No. 7,664,545, the contents of which are incorporated herein by reference. The electroporation can be by a method and/or apparatus described in U.S. Patent Nos. 6,302,874; 5,676,646; 6,241,701; 6,233,482; 6,216,034; 6,208,893; 6,192,270; 6,181,964; 6,150,148; 6,120,493; 6,096,020; 6,068,650; and 5,702,359, the contents of which are incorporated herein by reference in their entirety. The electroporation may be carried out via a minimally invasive device.

The minimally invasive electroporation device ("MID") may be an apparatus for injecting the composition described above and associated fluid into body tissue. The device may comprise a hollow needle, DNA cassette, and fluid delivery means, wherein the device is adapted to actuate the fluid delivery means in use so as to concurrently (for example, automatically) inject DNA into body tissue during insertion of the needle into the said body tissue. This has the advantage that the ability to inject the DNA and associated fluid gradually while the needle is being inserted leads to a more even distribution of the fluid through the body tissue. The pain experienced during injection may be reduced due to the distribution of the DNA being injected over a larger area.

The MID may inject the composition into tissue without the use of a needle. The MID may inject the composition as a small stream or jet with such force that the composition pierces the surface of the tissue and enters the underlying tissue and/or muscle. The force behind the small stream or jet may be provided by expansion of a compressed gas, such as carbon dioxide through a micro-orifice within a fraction of a second. Examples of minimally invasive electroporation devices, and methods of using them, are described in published U.S. Patent Application No. 20080234655; U.S. Patent No. 6,520,950; U.S. Patent No. 7,171,264; U.S. Patent No. 6,208,893; U.S. Patent NO. 6,009,347; U.S. Patent No. 6,120,493; U.S. Patent No. 7,245,963; U.S. Patent No. 7,328,064; and U.S. Patent No. 6,763,264, the contents of each of which are herein incorporated by reference.

The MID may comprise an injector that creates a high-speed jet of liquid that painlessly pierces the tissue. Such needle-free injectors are commercially available. Examples of needle-free injectors that can be utilized herein include those described in U.S. Patent Nos. 3,805,783; 4,447,223; 5,505,697; and 4,342,310, the contents of each of which are herein incorporated by reference.

A desired composition in a form suitable for direct or indirect electrotransport may be introduced (e.g., injected) using a needle-free injector into the tissue to be treated, usually by contacting the tissue surface with the injector so as to actuate delivery of a jet of the agent, with sufficient force to cause penetration of the composition into the tissue. For example, if the tissue to be treated is mucosa, skin or muscle, the agent is projected towards the mucosal or skin surface with sufficient force to cause the agent to penetrate through the stratum corneum and into dermal layers, or into underlying tissue and muscle, respectively.

Needle-free injectors are well suited to deliver compositions to all types of tissues, particularly to skin and mucosa. In some embodiments, a needle-free injector may be used to propel a liquid that contains the composition to the surface and into the subject's skin or mucosa. Representative examples of the various types of tissues that can be treated using the invention methods include pancreas, larynx, nasopharynx, hypopharynx, oropharynx, lip, throat, lung, heart, kidney, muscle, breast, colon, prostate, thymus, testis, skin, mucosal tissue, ovary, blood vessels, or any combination thereof.

The MID may have needle electrodes that electroporate the tissue. By pulsing between multiple pairs of electrodes in a multiple electrode array, for example set up in rectangular or square patterns, provides improved results over that of pulsing between a pair of electrodes. Disclosed, for example, in U.S. Patent No. 5,702,359 entitled “Needle Electrodes for Mediated Delivery of Drugs and Genes” is an array of needles wherein a plurality of pairs of needles may be pulsed during the therapeutic treatment. In that application, which is incorporated herein by reference as though fully set forth, needles were disposed in a circular array, but have connectors and switching apparatus enabling a pulsing between opposing pairs of needle electrodes. A pair of needle electrodes for delivering recombinant expression vectors to cells may be used. Such a device and system is described in U.S. Patent No. 6,763,264, the contents of which are herein incorporated by reference. Alternatively, a single needle device may be used that allows injection of the DNA and electroporation with a single needle resembling a

normal injection needle and applies pulses of lower voltage than those delivered by presently used devices, thus reducing the electrical sensation experienced by the patient.

The MID may comprise one or more electrode arrays. The arrays may comprise two or more needles of the same diameter or different diameters. The needles may be evenly or unevenly spaced apart. The needles may be between 0.005 inches and 0.03 inches, between 0.01 inches and 0.025 inches; or between 0.015 inches and 0.020 inches. The needle may be 0.0175 inches in diameter. The needles may be 0.5 mm, 1.0 mm, 1.5 mm, 2.0 mm, 2.5 mm, 3.0 mm, 3.5 mm, 4.0 mm, or more spaced apart.

The MID may consist of a pulse generator and a two or more-needle composition injectors that deliver the composition and electroporation pulses in a single step. The pulse generator may allow for flexible programming of pulse and injection parameters via a flash card operated personal computer, as well as comprehensive recording and storage of electroporation and patient data. The pulse generator may deliver a variety of volt pulses during short periods of time. For example, the pulse generator may deliver three 15 volt pulses of 100 ms in duration. An example of such a MID is the Elgen 1000 system by Inovio Biomedical Corporation, which is described in U.S. Patent No. 7,328,064, the contents of which are herein incorporated by reference.

The MID may be a CELLECTRA (Inovio Pharmaceuticals, Blue Bell PA) device and system, which is a modular electrode system, that facilitates the introduction of a macromolecule, such as a DNA, into cells of a selected tissue in a body or plant. The modular electrode system may comprise a plurality of needle electrodes; a hypodermic needle; an electrical connector that provides a conductive link from a programmable constant-current pulse controller to the plurality of needle electrodes; and a power source. An operator can grasp the plurality of needle electrodes that are mounted on a support structure and firmly insert them into the selected tissue in a body or plant. The macromolecules are then delivered via the hypodermic needle into the selected tissue. The programmable constant-current pulse controller is activated and constant-current electrical pulse is applied to the plurality of needle electrodes. The applied constant-current electrical pulse facilitates the introduction of the macromolecule into the cell between the plurality of electrodes. Cell death due to overheating of cells is minimized by limiting the power dissipation in the tissue by virtue of constant-current pulses. The Cellectra device and system is described in U.S. Patent No. 7,245,963, the contents of which are herein incorporated by reference.

The MID may be an Elgen 1000 system (Inovio Pharmaceuticals). The Elgen 1000 system may comprise device that provides a hollow needle; and fluid delivery means, wherein the apparatus is adapted to actuate the fluid delivery means in use so as to concurrently (for example automatically) inject fluid, the described composition herein, into body tissue during insertion of the needle into the said body tissue. The advantage is the ability to inject the fluid gradually while the needle is being inserted leads to a more even distribution of the fluid through the body tissue. It is also believed that the pain experienced during injection is reduced due to the distribution of the volume of fluid being injected over a larger area.

In addition, the automatic injection of fluid facilitates automatic monitoring and registration of an actual dose of fluid injected. This data can be stored by a control unit for documentation purposes if desired.

It will be appreciated that the rate of injection could be either linear or non-linear and that the injection may be carried out after the needles have been inserted through the skin of the subject to be treated and while they are inserted further into the body tissue.

Suitable tissues into which fluid may be injected by the apparatus of the present invention include tumor tissue, skin or liver tissue but may be muscle tissue.

The apparatus further comprises needle insertion means for guiding insertion of the needle into the body tissue. The rate of fluid injection is controlled by the rate of needle insertion. This has the advantage that both the needle insertion and injection of fluid can be controlled such that the rate of insertion can be matched to the rate of injection as desired. It also makes the apparatus easier for a user to operate. If desired means for automatically inserting the needle into body tissue could be provided.

A user could choose when to commence injection of fluid. Ideally however, injection is commenced when the tip of the needle has reached muscle tissue and the apparatus may include means for sensing when the needle has been inserted to a sufficient depth for injection of the fluid to commence. This means that injection of fluid can be prompted to commence automatically when the needle has reached a desired depth (which will normally be the depth at which muscle tissue begins). The depth at which muscle tissue begins could for example be taken to be a preset needle insertion depth such as a value of 4 mm which would be deemed sufficient for the needle to get through the skin layer.

The sensing means may comprise an ultrasound probe. The sensing means may comprise a means for sensing a change in impedance or resistance. In this case, the means may

not as such record the depth of the needle in the body tissue but will rather be adapted to sense a change in impedance or resistance as the needle moves from a different type of body tissue into muscle. Either of these alternatives provides a relatively accurate and simple to operate means of sensing that injection may commence. The depth of insertion of the needle can further be recorded if desired and could be used to control injection of fluid such that the volume of fluid to be injected is determined as the depth of needle insertion is being recorded.

The apparatus may further comprise: a base for supporting the needle; and a housing for receiving the base therein, wherein the base is moveable relative to the housing such that the needle is retracted within the housing when the base is in a first rearward position relative to the housing and the needle extends out of the housing when the base is in a second forward position within the housing. This is advantageous for a user as the housing can be lined up on the skin of a patient, and the needles can then be inserted into the patient's skin by moving the housing relative to the base.

As stated above, it is desirable to achieve a controlled rate of fluid injection such that the fluid is evenly distributed over the length of the needle as it is inserted into the skin. The fluid delivery means may comprise piston driving means adapted to inject fluid at a controlled rate. The piston driving means could for example be activated by a servo motor. However, the piston driving means may be actuated by the base being moved in the axial direction relative to the housing. It will be appreciated that alternative means for fluid delivery could be provided. Thus, for example, a closed container which can be squeezed for fluid delivery at a controlled or non-controlled rate could be provided in the place of a syringe and piston system.

The apparatus described above could be used for any type of injection. It is however envisaged to be particularly useful in the field of electroporation and so it may further comprises means for applying a voltage to the needle. This allows the needle to be used not only for injection but also as an electrode during, electroporation. This is particularly advantageous as it means that the electric field is applied to the same area as the injected fluid. There has traditionally been a problem with electroporation in that it is very difficult to accurately align an electrode with previously injected fluid and so users have tended to inject a larger volume of fluid than is required over a larger area and to apply an electric field over a higher area to attempt to guarantee an overlap between the injected substance and the electric field. Using the present invention, both the volume of fluid injected and the size of electric field applied may be reduced while achieving a good fit between the electric field and the fluid.

Regardless of the method used to introduce exogenous nucleic acids into a host cell, in order to confirm the presence of the recombinant nucleic acic sequence in the host cell, a variety of assays may be performed. Such assays include, for example, "molecular biological" assays well known to those of skill in the art, such as Southern and Northern blotting, RT-PCR and PCR; "biochemical" assays, such as detecting the presence or absence of a particular peptide, e.g., by immunological means (ELISAs and Western blots) or other assays known to those of skill in the art.

Disease Conditions and Methods of Treatment

The present invention in one embodiment includes compositions and methods for treating cystic fibrosis by reversing the effects of mutations present that are associated with nonsense mutations through introduction of the synthetic oligonucleotide suppressor tRNAs of the invention.

Certain embodiments of the present disclosure provide a method of treating a disease in a mammal comprising administering a protein or vector encoding a therapeutic agent (e.g., a modified and/or stabilized ACE-tRNA) as described herein to the mammal. In certain embodiments, the mammal is human.

Certain embodiments of the present disclosure provide a use of a therapeutic agent or vector encoding a therapeutic agent as described herein to prepare a medicament useful for treating disease in a mammal. Diseases or disorders associated with PTCs include, but are not limited to, variants of Duchenne and Becker muscular dystrophies due to a PTC in dystrophin, retinoblastoma due to a PTC in RB1, neurofibromatosis due to a PTC in NF1 or NF2, ataxia-telangiectasia due to a PTC in ATM, Tay-Sachs disease due to a PTC in HEXA, cystic fibrosis due to a PTC in CFTR, Wilm's tumor due to a PTC in WT1, hemophilia A due to a PTC in factor VIII, hemophilia B due to a PTC in factor IX, p53-associated cancers due to a PTC in p53, Menkes disease, Ullrich's disease, β-Thalassemia due to a PTC in betaglobin, type 2A and type 3 von Willebrand disease due to a PTC in Willebrand factor, Robinow syndrome, brachydactyly type B (shortening of digits and metacarpals), inherited susceptibility to mycobacterial infection due to a PTC in IFNGR1, inherited retinal disease due to a PTC in CRX, inherited bleeding tendency due to a PTC in Coagulation factor X, inherited blindness due to a PTC in Rhodopsin, congenital neurosensory deafness and colonic agangliosis due to a PTC in SOX10 and inherited neural develop-mental defect including neurosensory deafness, colonic agangliosis, peripheral

neuropathy and central dysmyelinating leukodystrophy due to a PTC in SOX10, Liddle's syndrome, xeroderma pigmentosum, Fanconi's anemia, anemia, hypothyroidism, p53-associated cancers (e.g., p53 squamal cell carcinoma, p53 hepatocellular carcinoma, p53 ovarian carcinoma), esophageal carcinoma, osteocarcinoma, ovarian carcinoma, hepatocellular carcinoma, breast cancer, hepatocellular carcinoma, fibrous histiocytoma, ovarian carcinoma, SRY sex reversal, triosephosphate isomerase-anemia, diabetes and ricketsand many others.

The present disclosure also provides a mammalian cell containing a vector described herein. The cell may be human.

Certain aspects of the disclosure relate to polynucleotides, polypeptides, vectors, and genetically engineered cells (modified *in vivo*), and the use of them. In particular, the disclosure relates to a method for gene therapy that is capable of both systemic delivery of a therapeutically effective dose of the therapeutic agent.

According to one aspect, a cell expression system for expressing a therapeutic agent in a mammalian recipient is provided. The expression system (also referred to herein as a "genetically modified cell") comprises a cell and an expression vector for expressing the therapeutic agent. Expression vectors include, but are not limited to, viruses, plasmids, and other vehicles for delivering heterologous genetic material to cells. Accordingly, the term "expression vector" as used herein refers to a vehicle for delivering heterologous genetic material to a cell. In particular, the expression vector is a recombinant adenoviral, adeno-associated virus, or lentivirus or retrovirus vector.

The expression vector further includes a promoter for controlling transcription of the heterologous gene. The promoter may be an inducible promoter (described herein). The expression system is suitable for administration to the mammalian recipient. The expression system may comprise a plurality of non-immortalized genetically modified cells, each cell containing at least one recombinant gene encoding at least one therapeutic agent.

The cell expression system is formed *in vivo*. According to yet another aspect, a method for treating a mammalian recipient *in vivo* is provided. The method includes introducing an expression vector for expressing a heterologous gene product into a cell of the patient *in situ*, such as via intravenous administration. To form the expression system *in vivo*, an expression vector for expressing the therapeutic agent is introduced *in vivo* into the mammalian recipient i.v.

According to yet another aspect, a method for treating a mammalian recipient *in vivo* is provided. The method includes introducing the target therapeutic agent into the patient *in vivo*.

The expression vector for expressing the heterologous gene may include an inducible promoter for controlling transcription of the heterologous gene product. Accordingly, delivery of the therapeutic agent *in situ* is controlled by exposing the cell *in situ* to conditions, which induce transcription of the heterologous gene.

The present disclosure provides methods of treating a disease in a mammal by administering an expression vector to a cell or patient. For the gene therapy methods, a person having ordinary skill in the art of molecular biology and gene therapy would be able to determine, without undue experimentation, the appropriate dosages and routes of administration of the expression vector used in the novel methods of the present disclosure.

The present disclosure provides methods of treating a disease in a mammal by administering at least one ACE-tRNA to a cell or patient. For the gene therapy methods, a person having ordinary skill in the art of molecular biology and gene therapy would be able to determine, without undue experimentation, the appropriate dosages and routes of administration of the ACE-tRNA used in the novel methods of the present disclosure.

In one embodiment, the disclosure provides methods of treating a disease in a mammal by administering at least two, at least 3, at least 4, or more than 4 ACE-tRNA or nucleic acid molecules encoding ACE-tRNA to a cell or patient. In one embodiment, the methods include administration of multiple ACE-tRNA or nucleic acid molecules encoding multiple ACE-tRNA, wherein each of the multiple ACE-tRNA are specific for incorporation of a distict amino acid from other ACE-tRNA in the composition. For example, in one embodiment, the method includes administration of a combination of a first ACE-tRNA for incorporation of Arg into a polypeptide and a second ACE-tRNA for incorporation of Gly into a polypeptide. In one embodiment, multiple ACE-tRNA are specific for the PTC (e.g., UGA). In one embodiment, multiple ACE-tRNA are specific for different PTC.

According to one embodiment, the cells are transformed or otherwise genetically modified *in vivo*. The cells from the mammalian recipient are transformed (*i.e.*, transduced or transfected) *in vivo* with a vector containing exogenous genetic material for expressing a heterologous (*e.g.*, recombinant) gene encoding a therapeutic agent and the therapeutic agent is delivered *in situ*.

As used herein, "exogenous genetic material" refers to a nucleic acid or an oligonucleotide, either natural or synthetic, that is not naturally found in the cells; or if it is naturally found in the cells, it is not transcribed or expressed at biologically significant levels by

the cells. Thus, "exogenous genetic material" includes, for example, a non-naturally occurring nucleic acid that can be transcribed into a tRNA.

The above-disclosed therapeutic agents and conditions amenable to gene therapy are merely illustrative and are not intended to limit the scope of the instant disclosure. The selection of a suitable therapeutic agent for treating a known condition is deemed to be within the scope of one of ordinary skill of the art without undue experimentation.

In certain embodiments, the therapy has potential use for the treatment/management of diseases that are caused by Premature Termination Codons (PTCs), including, but not limited to, Duchenne and Becker muscular dystrophies, retinoblastoma, neurofibromatosis, ataxia-telangiectasia, Tay-Sachs disease, cystic fibrosis, Wilm's tumor, hemophilia A, hemophilia B, Menkes disease, Ullrich's disease, β-Thalassemia, type 2A and type 3 von Willebrand disease, Robinow syndrome, brachydactyly type B (shortening of digits and metacarpals), inherited susceptibility to mycobacterial infection, inherited retinal disease, inherited bleeding tendency, inherited blindness, congenital neurosensory deafness and colonic agangliosis and inherited neural develop-mental defect including neurosensory deafness, colonic agangliosis, peripheral neuropathy and central dysmyelinating leukodystrophy, Liddle's syndrome, xeroderma pigmentosum, Fanconi's anemia, anemia, hypothyroidism, p53-associated cancers (e.g., p53 squamal cell carcinoma, p53 hepatocellular carcinoma, p53 ovarian carcinoma), esophageal carcinoma, osteocarcinoma, ovarian carcinoma, hepatocellular carcinoma, breast cancer, hepatocellular carcinoma, fibrous histiocytoma, ovarian carcinoma, SRY sex reversal, triosephosphate isomerase-anemia, diabetes and rickets. This therapy is advantageous in that it provides improved stop codon suppression specificity. The therapeutic ACE-tRNAs of the present invention target a specific stop-codon, TGA for instance, thus reducing off-target effects at stop-codons unrelated to disease. The present therapy is also advantageous in that it provides amino-acid specificity. The expressed tRNA is engineered to specifically replace the amino acid that was lost via insertion of the disease stop codon, thus negating any spurious effects on protein stability, folding and trafficking.

In certain embodiments, the present system is modular, and thus can be "personalized" to every possible disease PTC. For instnce, there are nine individual tryptophan tRNAs in the human genome that are recognized by the Trp synthetase, all of which suppress the mRNA UGG codon. Thus, each of these nine Trp tRNA provides an opportunity for codon re-editing tolerance (UGG → UGA). Additionally, given their proximity to stop codons in the genetic

code, the mutation of arginine codons to PTC nonsense codons are common in disease. There are over thirty Arg tRNA that could be tested for codon editing tolerance and suppression efficacy.

A further advantage of the present invention is that it provides facile expression and cell specific delivery, because the entire system (tRNA + promoter sequence) is compact.

Dosages, Formulations and Routes of Administration of the Agents of the Invention

The agents of the invention are administered so as to result in a reduction in at least one symptom associated with a genetic disease (e.g., cystic fibrosis). The amount administered will vary depending on various factors including, but not limited to, the composition chosen, the particular disease, the weight, the physical condition, and the age of the mammal, and whether prevention or treatment is to be achieved. Such factors can be readily determined by the clinician employing animal models or other test systems that are well known to the art.

The present invention envisions treating a disease or disorder associated with a PTC by the administration of an agent, e.g., ACE-tRNA, an expression vector, or a viral particle of the invention. Administration of the therapeutic agents in accordance with the present invention may be continuous or intermittent, depending, for example, upon the recipient's physiological condition, whether the purpose of the administration is therapeutic or prophylactic, and other factors known to skilled practitioners. The administration of the agents of the invention may be essentially continuous over a preselected period of time or may be in a series of spaced doses. Both local and systemic administration is contemplated.

One or more suitable unit dosage forms having the therapeutic agent(s) of the invention, which, as discussed below, may optionally be formulated for sustained release (for example using microencapsulation), can be administered by a variety of routes including parenteral, including by intravenous and intramuscular routes, as well as by direct injection into the diseased tissue. The formulations may, where appropriate, be conveniently presented in discrete unit dosage forms and may be prepared by any of the methods well known to pharmacy. Such methods may include the step of bringing into association the therapeutic agent with liquid carriers, solid matrices, semi-solid carriers, finely divided solid carriers or combinations thereof, and then, if necessary, introducing or shaping the product into the desired delivery system.

When the therapeutic agents of the invention are prepared for administration, they may be combined with a pharmaceutically acceptable carrier, diluent or excipient to form a pharmaceutical formulation, or unit dosage form. The total active ingredients in such formulations include from 0.1 to 99.9% by weight of the formulation. A "pharmaceutically

acceptable" is a carrier, diluent, excipient, and/or salt that is compatible with the other ingredients of the formulation, and not deleterious to the recipient thereof. The active ingredient for administration may be present as a powder or as granules; as a solution, a suspension or an emulsion.

Pharmaceutical formulations containing the therapeutic agents of the invention can be prepared by procedures known in the art using well-known and readily available ingredients. The therapeutic agents of the invention can also be formulated as solutions appropriate for parenteral administration, for instance by intramuscular, subcutaneous or intravenous routes.

The pharmaceutical formulations of the therapeutic agents of the invention can also take the form of an aqueous or anhydrous solution or dispersion, or alternatively the form of an emulsion or suspension.

Thus, the therapeutic agent may be formulated for parenteral administration (e.g., by injection, for example, bolus injection or continuous infusion) and may be presented in unit dose form in ampules, pre-filled syringes, small volume infusion containers or in multi-dose containers with an added preservative. The active ingredients may take such forms as suspensions, solutions, or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredients may be in powder form, obtained by aseptic isolation of sterile solid or by lyophilization from solution, for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water, before use.

It will be appreciated that the unit content of active ingredient or ingredients contained in an individual aerosol dose of each dosage form need not in itself constitute an effective amount for treating the particular indication or disease since the necessary effective amount can be reached by administration of a plurality of dosage units. Moreover, the effective amount may be achieved using less than the dose in the dosage form, either individually, or in a series of administrations.

The pharmaceutical formulations of the present invention may include, as optional ingredients, pharmaceutically acceptable carriers, diluents, solubilizing or emulsifying agents, and salts of the type that are well-known in the art. Specific non-limiting examples of the carriers and/or diluents that are useful in the pharmaceutical formulations of the present invention include water and physiologically acceptable buffered saline solutions such as phosphate buffered saline solutions pH 7.0-8.0 and water.

Methods of administration

Provided herein are methods of treating, protecting against, and/or preventing a PTC associated disease in a subject in need thereof by administering one or more composition described herein to the subject.

The composition dose can be between 1 μg to 10 mg active component/kg body weight/time, and can be 20 μg to 10 mg component/kg body weight/time. The composition can be administered every 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, or 31 days. The number of composition doses for effective treatment can be 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

The composition can be formulated in accordance with standard techniques well known to those skilled in the pharmaceutical art. Such compositions can be administered in dosages and by techniques well known to those skilled in the medical arts taking into consideration such factors as the age, sex, weight, and condition of the particular subject, and the route of administration.

The composition can be administered prophylactically or therapeutically. In therapeutic applications, the compositions are administered to a subject in need thereof in an amount sufficient to elicit a therapeutic effect. An amount adequate to accomplish this is defined as "therapeutically effective dose." Amounts effective for this use will depend on, e.g., the particular composition of the composition regimen administered, the manner of administration, the stage and severity of the disease, the general state of health of the subject, and the judgment of the prescribing physician.

The composition can be administered by methods well known in the art as described in Donnelly et al. (Ann. Rev. Immunol. 15:617-648 (1997)); Felgner et al. (U.S. Pat. No. 5,580,859, issued Dec. 3, 1996); Felgner (U.S. Pat. No. 5,703,055, issued Dec. 30, 1997); and Carson et al. (U.S. Pat. No. 5,679,647, issued Oct. 21, 1997), the contents of all of which are incorporated herein by reference in their entirety. The DNA of the composition can be complexed to particles or beads that can be administered to an individual, for example, using a vaccine gun. One skilled in the art would know that the choice of a pharmaceutically acceptable carrier, including a physiologically acceptable compound, depends, for example, on the route of administration of the expression vector.

The composition can be delivered via a variety of routes. Typical delivery routes include parenteral administration, e.g., intradermal, intramuscular or subcutaneous delivery. Other routes include oral administration, intranasal, and intravaginal routes. For the DNA of the composition in particular, the composition can be delivered to the interstitial spaces of tissues of an individual (Felgner et al., U.S. Pat. Nos. 5,580,859 and 5,703,055, the contents of all of which are incorporated herein by reference in their entirety). The composition can also be administered to muscle, or can be administered via intradermal or subcutaneous injections, or transdermally, such as by iontophoresis. Epidermal administration of the composition can also be employed. Epidermal administration can involve mechanically or chemically irritating the outermost layer of epidermis to stimulate an immune response to the irritant (Carson et al., U.S. Pat. No. 5,679,647, the contents of which are incorporated herein by reference in its entirety).

The composition can also be formulated for administration via the nasal passages. Formulations suitable for nasal administration, wherein the carrier is a solid, can include a coarse powder having a particle size, for example, in the range of about 10 to about 500 microns which is administered in the manner in which snuff is taken, i.e., by rapid inhalation through the nasal passage from a container of the powder held close up to the nose. The formulation can be a nasal spray, nasal drops, or by aerosol administration by nebulizer. The formulation can include aqueous or oily solutions of the composition.

The composition can be a liquid preparation such as a suspension, syrup or elixir. The composition can also be a preparation for parenteral, subcutaneous, intradermal, intramuscular or intravenous administration (e.g., injectable administration), such as a sterile suspension or emulsion.

The composition can be incorporated into liposomes, microspheres or other polymer matrices (Felgner et al., U.S. Pat. No. 5,703,055; Gregoriadis, Liposome Technology, Vols. Ito III (2nd ed. 1993), the contents of which are incorporated herein by reference in their entirety). Liposomes can consist of phospholipids or other lipids, and can be nontoxic, physiologically acceptable and metabolizable carriers that are relatively simple to make and administer.

The ACE-tRNA or nucleic acid molecule encoding the ACE-tRNA may be administered by different routes including orally, parenterally, sublingually, transdermally, rectally, transmucosally, topically, via inhalation, via buccal administration, intrapleurally, intravenous, intraarterial, intraperitoneal, subcutaneous, intramuscular, intranasal, intrathecal, and intraarticular or combinations thereof. For veterinary use, the composition may be administered

as a suitably acceptable formulation in accordance with normal veterinary practice. The veterinarian can readily determine the dosing regimen and route of administration that is most appropriate for a particular animal. The composition may be administered by traditional syringes, needleless injection devices, "microprojectile bombardment gone guns", or other physical methods such as electroporation ("EP"), "hydrodynamic method", or ultrasound.

The ACE-tRNA or nucleic acid molecule encoding the ACE-tRNA may be delivered to the mammal by several well-known technologies including DNA injection (also referred to as DNA vaccination) with and without in vivo electroporation, liposome mediated, nanoparticle facilitated, recombinant vectors such as recombinant adenovirus, recombinant adenovirus associated virus and recombinant vaccinia. The ACE-tRNA or nucleic acid molecule encoding the ACE-tRNA may be delivered via DNA injection and along with in vivo electroporation.

Electroporation

Administration of the composition via electroporation may be accomplished using electroporation devices that can be configured to deliver to a desired tissue of a mammal a pulse of energy effective to cause reversible pores to form in cell membranes, and preferable the pulse of energy is a constant current similar to a preset current input by a user. The electroporation device may comprise an electroporation component and an electrode assembly or handle assembly. The electroporation component may include and incorporate one or more of the various elements of the electroporation devices, including: controller, current waveform generator, impedance tester, waveform logger, input element, status reporting element, communication port, memory component, power source, and power switch. The electroporation may be accomplished using an in vivo electroporation device, for example CELLECTRA EP system (Inovio Pharmaceuticals, Plymouth Meeting, PA) or Elgen electroporator (Inovio Pharmaceuticals, Plymouth Meeting, PA) to facilitate transfection of cells by the plasmid.

The electroporation component may function as one element of the electroporation devices, and the other elements are separate elements (or components) in communication with the electroporation component. The electroporation component may function as more than one element of the electroporation devices, which may be in communication with still other elements of the electroporation devices separate from the electroporation component. The elements of the electroporation devices existing as parts of one electromechanical or mechanical device may not limited as the elements can function as one device or as separate elements in communication

with one another. The electroporation component may be capable of delivering the pulse of energy that produces the constant current in the desired tissue, and includes a feedback mechanism. The electrode assembly may include an electrode array having a plurality of electrodes in a spatial arrangement, wherein the electrode assembly receives the pulse of energy from the electroporation component and delivers same to the desired tissue through the electrodes. At least one of the plurality of electrodes is neutral during delivery of the pulse of energy and measures impedance in the desired tissue and communicates the impedance to the electroporation component. The feedback mechanism may receive the measured impedance and can adjust the pulse of energy delivered by the electroporation component to maintain the constant current.

A plurality of electrodes may deliver the pulse of energy in a decentralized pattern. The plurality of electrodes may deliver the pulse of energy in the decentralized pattern through the control of the electrodes under a programmed sequence, and the programmed sequence is input by a user to the electroporation component. The programmed sequence may comprise a plurality of pulses delivered in sequence, wherein each pulse of the plurality of pulses is delivered by at least two active electrodes with one neutral electrode that measures impedance, and wherein a subsequent pulse of the plurality of pulses is delivered by a different one of at least two active electrodes with one neutral electrode that measures impedance.

The feedback mechanism may be performed by either hardware or software. The feedback mechanism may be performed by an analog closed-loop circuit. The feedback occurs every 50 μs, 20 μs, 10 μs or 1 μs, but is preferably a real-time feedback or instantaneous (i.e., substantially instantaneous as determined by available techniques for determining response time). The neutral electrode may measure the impedance in the desired tissue and communicates the impedance to the feedback mechanism, and the feedback mechanism responds to the impedance and adjusts the pulse of energy to maintain the constant current at a value similar to the preset current. The feedback mechanism may maintain the constant current continuously and instantaneously during the delivery of the pulse of energy.

Examples of electroporation devices and electroporation methods that may facilitate delivery of the compositions of the present invention, include those described in U.S. Patent No. 7,245,963 by Draghia-Akli, et al., U.S. Patent Pub. 2005/0052630 submitted by Smith, et al., the contents of which are hereby incorporated by reference in their entirety. Other electroporation devices and electroporation methods that may be used for facilitating delivery of the

compositions include those provided in U.S. Patent Application, Serial No. 11/874072, filed October 17, 2007, which claims the benefit under 35 USC 119(e) to U.S. Provisional Applications Ser. Nos. 60/852,149, filed October 17, 2006, and 60/978,982, filed October 10, 2007, all of which are hereby incorporated in their entirety.

U.S. Patent No. 7,245,963 by Draghia-Akli, et al. describes modular electrode systems and their use for facilitating the introduction of a biomolecule into cells of a selected tissue in a body or plant. The modular electrode systems may comprise a plurality of needle electrodes; a hypodermic needle; an electrical connector that provides a conductive link from a programmable constant-current pulse controller to the plurality of needle electrodes; and a power source. An operator can grasp the plurality of needle electrodes that are mounted on a support structure and firmly insert them into the selected tissue in a body or plant. The biomolecules are then delivered via the hypodermic needle into the selected tissue. The programmable constant-current pulse controller is activated and constant-current electrical pulse is applied to the plurality of needle electrodes. The applied constant-current electrical pulse facilitates the introduction of the biomolecule into the cell between the plurality of electrodes. The entire content of U.S. Patent No. 7,245,963 is hereby incorporated by reference.

U.S. Patent Pub. 2005/0052630 submitted by Smith, et al. describes an electroporation device which may be used to effectively facilitate the introduction of a biomolecule into cells of a selected tissue in a body or plant. The electroporation device comprises an electro-kinetic device ("EKD device") whose operation is specified by software or firmware. The EKD device produces a series of programmable constant-current pulse patterns between electrodes in an array based on user control and input of the pulse parameters, and allows the storage and acquisition of current waveform data. The electroporation device also comprises a replaceable electrode disk having an array of needle electrodes, a central injection channel for an injection needle, and a removable guide disk. The entire content of U.S. Patent Pub. 2005/0052630 is hereby incorporated by reference.

The electrode arrays and methods described in U.S. Patent No. 7,245,963 and U.S. Patent Pub. 2005/0052630 may be adapted for deep penetration into not only tissues such as muscle, but also other tissues or organs. Because of the configuration of the electrode array, the injection needle (to deliver the biomolecule of choice) is also inserted completely into the target organ, and the injection is administered perpendicular to the target issue, in the area that is pre-

delineated by the electrodes The electrodes described in U.S. Patent No. 7,245,963 and U.S. Patent Pub. 2005/005263 are preferably 20 mm long and 21 gauge.

Additionally, contemplated in some embodiments that incorporate electroporation devices and uses thereof, there are electroporation devices that are those described in the following patents: US Patent 5,273,525 issued December 28, 1993, US Patents 6,110,161 issued August 29, 2000, 6,261,281 issued July 17, 2001, and 6,958,060 issued October 25, 2005, and US patent 6,939,862 issued September 6, 2005. Furthermore, patents covering subject matter provided in US patent 6,697,669 issued February 24, 2004, which concerns delivery of DNA using any of a variety of devices, and US patent 7,328,064 issued February 5, 2008, drawn to method of injecting DNA are contemplated herein. The above-patents are incorporated by reference in their entirety.

Method of Preparing the ACE-tRNA

Provided herein are methods for preparing the DNA plasmids that comprise the ACE-tRNA discussed herein. The DNA plasmids, after the final subcloning step into the mammalian expression plasmid, can be used to inoculate a cell culture in a large-scale fermentation tank, using known methods in the art.

The DNA plasmids for use with the EP devices of the present invention can be formulated or manufactured using a combination of known devices and techniques, but preferably they are manufactured using an optimized plasmid manufacturing technique that is described in a US published application no. 20090004716, which was filed on May 23, 2007. In some examples, the DNA plasmids used in these studies can be formulated at concentrations greater than or equal to 10 mg/mL. The manufacturing techniques also include or incorporate various devices and protocols that are commonly known to those of ordinary skill in the art, in addition to those described in U.S. Serial No. 60/939792, including those described in a licensed patent, US Patent No. 7,238,522, which issued on July 3, 2007. The above-referenced application and patent, US Serial No. 60/939,792 and US Patent No. 7,238,522, respectively, are hereby incorporated in their entirety.

DEFINITIONS

Disease state: For the purposes of the present invention, a “disease state” or “disease phenotype” is a characteristic of a mammalian cell that results from a stop codon within the

coding region of a gene inside the cell (e.g., that results from a nonsense mutation). For example, an increasing number of human genetic diseases are thought to be caused by nonsense mutations (see, for example, Atkinson et al., Nuc. Acids Res. 22:1327, 1994). To give but a few examples, β-thalessemia, Duchenne muscular dystrophy, xeroderma pigmentosum, Fanconi's anemia, and cystic fibrosis can all be caused by nonsense mutations in identified genes.

Endogenous tRNA synthetase: A tRNA synthetase is considered to be "endogenous" to a cell if it is present in the cell into which a tRNA is introduced according to the present invention. As will be the apparent to those of ordinary skill in the art, a tRNA synthetase may be considered to be endogenous for these purposes whether it is naturally found in cells of the relevant type, or whether the particular cell at issue has been engineered or otherwise manipulated by the hand of man to contain or express it.

Suppressor tRNA: A "suppressor tRNA" is one whose anti-codon is complementary with a codon that would otherwise terminate translation, so that detectable read-through occurs under the conditions of the experiment. Standard termination codons are amber (UAG), ochre (UAA), and opal (UGA) codons. However, non-standard termination codons (e.g., 4-nucleotide codons) have also been employed in the literature (see, for example, Moore et al., J. Mol. Biol. 298:195, 2000; Hohsaka et al., J. Am. Chem. Soc. 121:12194, 1999).

The invention is now illustrated by the following non-limiting Examples.

EXAMPLE 1

The genetic code uses four nucleotides that in turn form triplet codons, which form the basis for DNA to protein translation. There are 64 codons in total, 61 of which are used to encode amino acids, and three (TAG, TGA and TAA) of which encode protein termination "stop" or "nonsense" codons.

Five to ten percent of cystic fibrosis cases are caused by "nonsense" mutations that lead to premature truncation of the cystic fibrosis transmembrane conductance regulator (CFTR) protein. An example of this "class 1" mutation is p.Trp1282X, a premature termination codon (PTC) which causes a loss of CFTR function and severe cystic fibrosis phenotypes. Some compounds, such as ataluren, promote stop read-through of disease producing nonsense mutations but have been only modestly successful as therapeutics due to a number of caveats, including poor stop-codon specificity and unexpectedly low efficiency of codon skipping in

vivo. However, the widespread use of these compounds and the discovery that endogenous stop-codon read-through is common in metazoans, suggests that assisted suppression could be viable if delivered to a subset of cell types, *i.e.*, airway epithelium. Yet, when therapeutically assisted stop-codon read-through is successful, the nonselective incorporation of an amino acid at the location of the nonsense codon has the potential to affect protein folding, trafficking and function (as is the case with CFTR 1282X); and thus, requires additional therapeutic intervention. Thus, there is an acute unmet need to understand the nature of disease PTCs and potentially therapeutic suppressors and generally, more effective treatments of PTC diseases.

This Example characterizes anticodon edited (ACE) Trp-tRNA for the rescue of CFTR p.Trp1282X channels. Such tRNAs are engineered to 'suppress' the disease-causing TGA stop codon and incorporate the original amino acid, Trp at p.Trp1282X CFTR, in effect, genetically reconstructing the wild-type CFTR protein. Data demonstrate that this general approach (nonsense suppression) produces robust rescue of transcripts that carry in-frame stop codons, through either transient transfection of a tRNA and its cognate synthetase in adherent cells, or their virus-based delivery to more native airway cell-types, such as A549 airway cells. This approach offers a number of significant benefits over existing strategies: 1) Improved codon specificity – the expressed tRNA may be directed towards a specific stop-codon, thus reducing off-target effects at stop-codons unrelated to disease. 2) Amino-acid specificity – the expressed tRNA and/or synthetase can be engineered to replace the amino acid that was lost via insertion of the disease stop codon, thus negating any spurious effects on CFTR stability, folding and trafficking. 3) Tunability – the system can be theoretically personalized for each type of tRNA and PTC mutation. 4) Facile expression – the entire system is compact (<1kb) and can be easily packaged and expressed transiently or via nanoparticle delivery of tRNA. 5) Proof of principle for a general strategy – in-frame stop codons are a major cause of human disease and few treatment options exist; the experiments performed here on p.Trp1282X are expected to lead to insights into the mechanisms of other CFTR nonsense codons.

Data shows that ACE-tRNA stop-codon suppressor tRNA are efficient at "rescuing" transcripts which contain introduced stop-sites (**Figures 6A** and **6B**) suggesting that such tRNA have the potential to interfere with nonsense mediated decay (NMD) as the major biological hurdle in the therapeutic rescue of disease stop sites. Thus opening the possibility for the use of suppressor tRNA to gain more molecular insights into NMD in disease.

RESULTS

Eukaryotic tRNA that had been anticodon edited to suppress stop sites, TGA for instance, and not its designated codon were developed. They were tested in five human tryptophan tRNA on a test construct consisting of a fluorescent protein (cherry) in frame with eGFP sequence that are separated by a linker containing a TGA site. To indicate the production of the full-length protein an HA epitope was added to the C-terminus of the eGFP reading frame. This test system is useful because visual appearance of the cherry signal indicates plasmid delivery and expression and in combination with the eGFP rescue shows TGA suppression. Data in **Figures 6A** and **6B** show western blot data using this test construct to assay the ability of five anticodon edited Trp tRNA human to suppress the TGA stop site in the short linker between cherry and eGFP reading frames. Of these constructs, the candidates 1, 2, 3 & 5 show modest activity in this regard. This may be due to structural intolerance to the mutation or the possibility that altering the anticodon, even just by a single base, disrupted the ability of the Trp synthetase to recognize and/or acylate the tRNA with tryptophan. However, number 4 of these test tRNA (tRNA #4) shows significant suppression activity of the TGA site, producing a full-length cherry-eGFP-HA protein (**Figure 6B**). Further, no read-through was seen in the absence of co-expressed tRNA, last lane, **Figure 6B**.

METHODS

The Trp tRNA were examined for codon editing tolerance (TGG → TGA) and their ability to suppress a targeted TGA test site in a transiently transfected tandem-fluorophore (mCherry-TGA-GFP) and CFTR Trp1282X. Initial screening of 5/9 Trp tRNA discovered an anticodon edited Trp-tRNA that was transiently transfected in HEK cells and has 'stand alone' functionality to rescue a cherry-TGA-eGFP-HA test construct, **Figure 6B**. The selective presence of the HA epitope indicates successful rescue, as well as confocal examination of both cherry and eGFP fluorescence at the single cell level (not shown). This result provides proof of principle data that a) some ACE - tRNA can tolerate anticodon editing b) that these tRNA retain the ability to be acylated with Trp by endogenous tryptophan synthetases and c) these tRNA can suppress TGA sites embedded within open protein reading frames.

The remaining four Trp-tRNA are functionally examined for tolerance of anticodon editing from TAA to TGA suppressors. These anticodon edited tRNA are tested for their ability to rescue the cherry-TGA-eGFPHA clone. Biochemical (western blot) data are obtained for cherry and eGFP signals as well as HA epitopes. Here, cherry expression serves as the positive

transfection control. Confocal imaging verifies cherry and eGFP fluorescence at the single cell level.

The fidelity of endogenous Trp synthetases to charge ACE - Trp tRNA with the tryptophan amino acid is determined by mass spectroscopic analysis of tryptic fragments of purified rescued cherry-Trp-eGFPHAprotein. Predicted mass for the tryptic fragment generated from the linker between the cherry and eGFP reading frames is: KPINQ**W**PANTHER with a predicted mass of 1590.8135; bold **W** indicates incorporation site, **Figure 10**. Thus, this represents the first example of a nonsense codon repair and replacement with the wild-type amino acid and therefore is a significant advance over existing approaches, such as the therapeutic Ataluran. The later example, the compound promotes read-through of the nonsense codon with the incorrect amino acid, thus the discovery and identification of new tRNA sequences that provide stringent repair is significant.

Rescue of transiently transfected CFTR 1282X channels by ACE – tRNA identified above are assessed by standard biochemical methods for full maturation of the B and C glycosylated CFTR bands 20. Thus, the channel has been repaired with the wildtype amino acid, is fully functional and successfully trafficked to the plasma membrane.

The next step is to functionally characterize CFTR Trp1282X channels rescued with ACE - tRNA systems identified above using electrophysiological (single cell patch clamp and Ussing chamber) and biochemical approaches. The efficacy of expressed tRNA to diminish nonsense-mediated decay (NMD) of 1282X mRNA would be assessed with quantitative rtPCR. Reprogramed human airway cells are used to test expressed codon edited Trp-tRNA rescue of native 1282X CFTR channels.

It is demonstrated that anticodon editing is tolerated in an identified human Trp tRNA and this 75-base pair transfer RNA is capable of suppressing an in-frame TGA codon within a test construct. These experiments extrapolate this discovery to characterize the ability of this ACE – tRNA to interact with CFTR 1282TGA mRNA and produce functional CFTR channels in model cells (FRT and A549) as well as p. 1282X human reprogrammed airway cells.

Biochemical determination of rescue levels in transiently expressed CFTR 1282X channels as well as those in reprogrammed airway cells. Antibody M3A7 is used to recognize the rescued (epitope is aa 1370-1380) and to detect all CFTR, rescued and non-rescued, antibody binding to the N-terminus likeMM13-4 (epitope aa 25-36), available through EMD Millipore.

Alternatively, L12B4 (epitope aa 386-412, EMD Millipore) or 660 (epitope aa 576-585,) are available through Cystic Fibrosis Foundation Therapeutics.

Surface functionality is examined through electrophysiological approaches, patch-clamp and Ussing chamber recording. 1282X mRNA stability and abundance is assayed by quantitative rtPCR of RNA extracts from transiently expressing cells and reprogrammed airway cells.

Bioinformatic analysis of RNA transcriptome data from human airway cells identifies abundance, context and identity of TGA codon containing transcripts. The top 10 expressing transcripts using TGA for their normal stop sites are followed up at the level of individual transcript with protein biochemistry before and after ACE – tRNA expression. Biochemical and immunohistological probes of cellular apoptosis are also used to examine the impact of ACE-tRNA in cell death.

In conclusion, the data show that ion channel genes with in-frame stop sites are amenable to this type of "rescue" (**Figures 9**) and components of the system can be expressed virally in airway cells. Further, a highly simplified form of this idea, an ACE-tRNA of human origin, demonstrates the "stand alone" ability to rescue in-frame CFTR TGA codons in mammalian cell lines (**Figures 9**). This approach has many advantages over existing stop-codon strategies and merits closer examination in terms of the ability of ACE-tRNA to 1) abrogate nonsense mediated decay 2) function in lung cell preparations and 3) to specifically rescue CFTR 1282X.

EXAMPLE 2

Several different nonsense mutations cause CF, thus underlying roughly 10% of all CF disease. **Figure** 7. These cases are concentrated into ten specific genetic lesions: E60X, R75X, G542X, R553X, Q890X, Y1092X, R1158X, R1162X and W1282X. A screen was developed to screen existing human tRNA sequences for modification and tolerance to anti-codon editing. To this end, roughly 144 ACE-tRNAs were candidates to test for those that could be used to promote the repair of the disease causative nonsense codon and the expression of the full-length protein. Specifically, using the scheme described in **Figure 11**, tRNA libraries were generated to identify novel tRNA sequences that encode for ACE tRNA with the ability to repair the top CF causative nonsense mutations. Specifically, 10ng of annealed oligos encoding the ACE-tRNAs were combined with 50ng of NanoLuc reporter plasmid, 1µl 10x CutSmart Buffer (NEB), 1µl T4 ligase (NEB), 10mM ATP and 1µl BbsI (NEB) and cycled in a thermocycler as described in **Figure 11**. 1µl of the reaction was transformed into competent E. coli and the transformants

were plated on ampicillin agar plates. One transformant was picked per plate was picked, grown in 1ml of LB under ampicillin selection, miniprepped and sequence verified.

Screening studies were first performed to identify the best ACE-tRNA Candidates from tryptophan and glycine. 125ng of sequence verified miniprep cDNA of NanoLuc reporter plasmid with ACE-tRNA was transfected into HEK cells using calcium phosphate. HEK cells were plated in 96 well plates at $4x10^4$ the day prior. 24 hours after transfection the media was replaced with 20µl of PBS and 15µl of NanoGlo reagent (Promega) was added. Plates were read on a SpectraMax i3 (Molecular Devices). Data are of replicates of 3 or greater. **Figure 8**. The data show that most tRNA demonstrate poor codon editing tolerance. However, clear high-performing tRNA emerge from the screen, with identification of ACE-Trp and ACE-Gly tRNA which demonstrate rescue of nonsense codon containing protein of 20-fold to 130-fold over background.

To assess is these novel tRNA could rescue CFTR channels harboring nonsense codons, they were co-expressed in mammalian HEK cells with a CFTR W1282X cDNA plasmid. The cellular preparations were analyzed by standard biochemical approaches via Western blot assessment of CFTR protein. This method is highly advantageous for this purpose because the CFTR protein displays a multi-banded pattern that is well-established. Specifically, the "B" and "C" bands represent the full-length and fully mature, post-translationally proceeded CFTR protein at the cell surface, respectively. In this case, both rescue with Trpchr17.trna39 and Glychr19.trna2 ACT-tRNAs produce robust populations of 'B' and 'C' CFTR immunopositive (antibody MA37) bands, indicating the promotion by said tRNA of the full-length, successfully trafficked ion channel protein. **Figure 9**.

EXAMPLE 3

T-stem modification significantly improves nonsense suppression. **Figure 10**. An additional modification of the tRNA was made to further enable their function for the purpose of suppression of nonsense codons and the promotion of protein expression. Without being bound by theory, it was hypothesized that rationally introduced mutations within the tRNA 't-stem' loop, shown in **Figure 10**, will yield a tRNA molecule that is more stable and functionally more potent for nonsense codon suppression. To this end, single and double mutations were directly engineered into the t-stem loop of tRNA Trpchr17.trna39 – an ACE-tRNA identified with activity for the rescue of tryptophan TGA nonsense codons. Thirty-eight tRNA t-stem variants

were thus generated and screened in HEK cells transiently transfected with the nonsense rescue reporter construct shown in **Figure 4**. 24 hours post-transfection, cells were assayed for luciferase activity, shown in **Figure 10**. The data show strong variation and identify novel tRNA sequences with varied t-stem loop sequences with enhanced suppression activity. Notably, one such mutant, TS-38 52-62 G-C enhances the suppression ability of Trpchr17.trna39 by roughly 250% (**Figure 12**). This is a generalizable modification, that is, new tRNA sequences identified, by example 1 and 2, can be made better (for their ability to rescue nonsense codons) through further rationale modifications. Such approaches aid in the therapeutic utility of ACE-tRNA directed to tissue types with low abundance target RNA or where tRNA delivery may be limiting.

EXAMPLE 4

In order to enable the identification of the nucleotide composition and functional ability to suppress nonsense codons by new types of tRNA, an All-In-One Plasmid With A One Pot Cloning Reaction was invented for High Throughput Cloning **Figure 11.** This approach enables the facile investigation of ACE-tRNA activity via luciferase activity in a standard 96 well format. Briefly, synthetic nucleotide sequences encoding for tRNA are ligated into the NanoLuc Reporter plasmid, with an example of the TGA nonsense reporter plasmid variant shown in **Figure 11**. TAA (Opal) and TAG (amber) stop codon rescue vectors have been successfully designed and implemented in **Figures 16-19**. The benefits are the approach is that DNA oligos encoding for tRNA libraries can be ligated in the NanoLuc reporter plasmid with the presence of the restriction enzyme and ligase with the reaction pushed to nearly 100% incorporation of tRNA insert (**Fig. 11**)-thus the 'one-pot' designation. The reaction is transformed into E. coli, with the resultant cDNA purified by standard methods. Another benefit of the invented method is that the tRNA and reporter gen are within the single expression cassette, therefore lowering biological variability and improving data quality obtaining in resulting screens of tRNA suppression activity. The purified cDNA plasmids are then screened in high-throughput 96 well format for their ability to repair nonsense codons by inferred luciferase activity. The approach is suitable for the high-throughput screening of hundreds to thousands of tRNA for novel therapeutic activity.

The 'one-pot' cloning and expression system described in **Figure 11** has been used successfully to identify unique tRNA sequences for the repair of Tryptophan and Glycine ACE-tRNA (**Figure 13**), ACE-tRNA-Arg **(Figure 14)**, ACE-tRNA-Gln TAG (**Figure 15**), ACE-

tRNA-Gln TAA **(Figure 16)**, ACE-tRNA-Glu TAG (**Figure 17**), ACE-tRNA-Gln TAA **(Figure 18) and** ACE-tRNA-Trp TAG (**Figure 19**). **Figures 20A-20D** show that delivery of ACE-tRNA as small RNA supports robust suppression of G542X and W1282X nonsense mutations.

EXAMPLE 5

Engineered transfer RNAs for suppression of premature termination codons

ABSTRACT

Premature termination codons (PTCs) are responsible for 10-15% of all inherited disease. PTC suppression during translation offers a promising approach to treat a variety of genetic disorders, yet small molecules that promote PTC read-through have yielded mixed performance in clinical trials. A high-throughput, cell-based assay is presented to identify anticodon engineered transfer RNAs (ACE-tRNA) that can effectively suppress in-frame PTCs and faithfully encode their cognate amino acid. In total, ACE-tRNA were identified with a high degree of suppression activity targeting the most common human disease-causing nonsense codons. Genome-wide transcriptome ribosome profiling of cells expressing ACE-tRNA at levels which repair PTC indicate that there are limited interactions with translation termination codons. These ACE-tRNAs display high suppression potency in mammalian cells, *Xenopus* oocytes and mice *in vivo*, producing PTC repair in multiple genes, including disease causing mutations within the cystic fibrosis transmembrane conductance regulator (*CFTR*).

INTRODUCTION

Premature termination codons (PTCs) arise from single nucleotide mutations that convert a canonical triplet nucleotide codon into one of three stop codons, e.g., TAG, TGA, or TAA. PTCs are often more deleterious than missense mutations because they result in the loss of protein expression. Additionally, mRNA abundance is reduced through nonsense-mediated decay (NMD) and in some cases, truncated proteins may have a dominant negative function [1-3]. Therefore, it is not surprising that PTCs are associated with many severe disease phenotypes, including cystic fibrosis [4], Duchenne muscular dystrophy, spinal muscular atrophy [5], infantile neuronal ceroid lipofuscinosis [6], β-thalessemia [7], cystinosis [8], X-linked nephrogenic diabetes insipidus [9], Hurler syndrome [10], Usher syndrome [11], and polycystic kidney disease. Additionally, nonsense mutations occur within the tumor suppressor genes *p53* and *ATM* [12], further implicating their role in disease. Amino acid codons most vulnerable to PTC conversion are those with a single nucleotide substitution from a stop codon: tryptophan, tyrosine, cysteine, glutamic acid,

lysine, glutamine, serine, leucine, arginine, and glycine (**Figure 25**). As such, PTCs represent a unique constellation of diseases which afflict over 30 million people worldwide, accounting for 10-15% of all genetic diseases [13].

Small molecules, such as aminoglycosides [14], dipeptides [15], and oxadiazoles [16], promote the "read-through" or "suppression" of nonsense mutations. These compounds are effective in model organisms [17, 18], mammalian cell lines [19] and some animal disease models [16, 20]. However, this approach results in the encoding of a *near*-cognate amino acid [21], effectively generating a missense mutation at the PTC, which itself may have deleterious effects on protein folding, trafficking, and function. Furthermore, aminoglycosides are oto- and nephrotoxic [22], and the first-in-class oxadiazole, Ataluren, displayed unexpectedly low efficacy in patient populations (ACT DMD Phase 3 clinical trial, NCT01826487; ACT CF, NCT02139306), thus limiting their utility as PTC therapeutics. Recent and ongoing advances in CRISPR/Cas9-mediated genome editing provides potentially a permanent solution for diseases resulting from nonsense mutations. However, aspects of this technology impart hurdles for its rapid use as a therapeutic [23, 24]. This is not limited to the requirement of "precision" or "personalized" diagnostics for each mutation based on the context of each patient's genetic variability.

A PTC repair approach was identified that displays the versatility of small molecules and the precision of gene editing. tRNAs were investigated to fulfill these criteria, whereby their anticodons have been engineered via mutagenesis to recognize and suppress UGA, UAA or UAG PTC codons. In order to be effective, the anticodon edited tRNAs, aka ACE-tRNAs, should still be recognized by the endogenous translation cellular machinery, including the aminoacyl-tRNA synthetase for charging the ACE-tRNA with their cognate amino acid and the eukaryotic elongation factor 1a (eEF-1α) for delivery of the charged tRNA to the ribosome, **Figure 21A**. Such suppressor tRNAs have been shown, in a limited manner, to rescue in frame stop codons associated with β-thalassemia [25], xeroderma pigmentosum [26] and a transgenic PTC reporter gene [27].

Here it is shown that an anti-codon editing approach is generalizable to multiple tRNA gene families, indicating that many annotated tRNA are biologically viable. Further, it is demonstrate that anti-codon edited suppressor tRNA encode their cognate amino acid, lack significant interactions with termination stop codons and are efficacious *in vivo* to suppress PTC. In total, the data support the possibility that such engineered tRNA satisfy the broad requirement

for coverage of disease-causing PTCs and thus represent a promising new class of RNA therapeutic agent.

RESULTS

The rationale of this study is rooted in the observation that there are multiple tRNA genes with unique sequences (isodecoders) for a given cognate amino acid (isoacceptors), leading to >400 tRNAs annotated in the human genome (http:lowelab.ucsc.edu/GtRNAdb/) [28, 29]. First, tRNA genes were examined to identify individual ACE-tRNAs that retain suppression efficacy of PTCs in mammalian cells. In order to maximize sequence coverage, an all-in-one cDNA plasmid was generated that supports both high-throughput cloning (HTC) of ACE-tRNAs and quantitative measurement of PTC suppression using luminescence following delivery to mammalian cells, **Figure 21B**. ACE-tRNA sequences were cloned as DNA oligos into the HTC plasmid using Golden Gate cloning [30] paired with ccdB negative selection [31]. This strategy produced ~100% cloning efficiency. ACE-tRNA suppression efficiency was read out from a split NanoLuc luciferase (NLuc) NanoBiT platform whereby the PTC of interest (UGA, UAA, or UAG) was introduced in-frame at the junction between the large bit and small bit domains, **Figure 21B** [32], using a 96-well format and normalized to background obtained in NLuc-PTC expressing cells. Twenty-one glycine ACE-tRNAs were first evaluated for suppression of the UGA PTC, **Figure 22**, top left, column 1 (violet). A majority of the ACE-tRNA^{Gly} sequences failed to suppress the UGA NLuc PTC, however, three Gly-tRNA^{UGA} were identified with high suppression yields (~100-fold over background). Given the high sequence conservation among the Gly-tRNAs screened for anti-codon tolerance (**Figure 27**), it would be difficult to predict *de novo* which tRNA would be most amenable to anticodon-editing.

Next, performed screens were performed on codon-edited tRNA for the each of the possible single nucleotide mutations which could produce a disease-causing PTCs: Arg-tRNA^{UGA}, Gln-tRNA^{UAA}, Gln-tRNA^{UAG} Trp-tRNA^{UGA}, Trp-tRNA^{UAG}, Glu-tRNA^{UAA}, Glu-tRNA^{UAG}, Cys-tRNA^{UGA}, Tyr-tRNA^{UAG}, Tyr-tRNA^{UAA}, Ser-tRNAUAG, Leu-tRNA^{UAG}, Leu-tRNA^{UAA}, Lys-tRNA^{UAG}, Lys-tRNA^{UGA} and Ser-tRNA^{UAG}. The enzymatic activity of NLuc was not significantly influenced by the introduced amino acid (**Figure 28**), therefore owing the difference in NLuc luminescence to ACE-tRNA suppression ability. The screen identified multiple ACE-tRNAs for each of the amino acids and stop codon type, with suppression coverage for all three stop codons, **Figure 22**. Many of these ACE-tRNAs exhibited strong activity with >100-fold PTC suppression over background, which is significantly higher than the

aminoglycosides used in this study. Interestingly, some ACE-tRNAs displayed a clear preference for a particular anticodon editing, possibly reflecting altered aminoacyl-tRNA synthetase binding to the tRNA anticodon isoacceptor sequences [33]. For instance, tryptophan conversion to UAG suppression yielded rescue that was ten times higher than that of UGA editing of the same ACE-tRNA^{Trp}. Yet the opposite was true for glutamine, where a clear preference was shown for UAA over UAG. Notably, in each case, multiple high performing suppressors were identified, and this was especially evident with Arg^{UGA}, a PTC which plays an outsized role in human disease; where twenty efficient ACE-Arg^{UGA} suppressors were identified. In other cases, such as ACE-tRNA^{Glu}, of those which exhibited function, the suppression efficiency was roughly equal for UAA and UAG. And a similar pattern was found in ACE-tRNA^{Lys} where encoding via UAG or UGA suppression were strongly mirrored. For Gln-tRNA^{UAA}, the suppression activity resulted in suppression signals >2,000-fold over background. Of the ACE-tRNAs identified in the screen, the tryptophan tRNA gene family displayed the weakest suppression activity for UGA PTCs. With only 6 unique human ACE-tRNA^{Trp} sequences available to screen, the UGA suppressing ACE-tRNA^{Trp} library was expanded using tRNA from a range of species. UGA anticodon-editing tolerance was tested for tryptophan tRNA genes with unique sequences from yeast, fly, mouse, rat, rabbit, and frog; in addition to a miscoding A9C tRNA^{Trp} and bacterial Hirsh Trp suppressor [34-36], **Figure 29A-29B**. This effort was unsuccessful in identifying ACE-tRNA^{Trp} UGA PTC suppression activity that exceeded that of the human ACE Trp tRNA, **Figure 29C**. Overall, the tRNA screens identified multiple engineered tRNAs (for each amino acid and stop codon type) that displayed potent suppression, thus bearing general tolerance to anticodon editing.

Next it was established whether ACE-tRNAs identified in the screen were functionalized at the expense of aminoacylation stringency by the cognate aminoacyl-tRNA synthetase. To this end, mass spectrometry was used to examine PTC suppression in a model soluble protein, histidinol dehydrogenase (HDH), **Figure 23A**. A TGA codon was introduced at asparagine 94 (N94) (**Figure 30A-C**) and co-expressed in HEK293 cells in tandem with plasmids encoding Glychr19.trna2 or Trpchr17.trna39 ACE-tRNAs, the top performing glycine and tryptophan ACE-tRNA^{UGA}, respectively. The resulting full-length, suppressed, HDH proteins were purified via a Strep-Tactin® C-terminal affinity tag and analyzed by mass spectrometry, **Figure 23A** (**Figure 28**). Subsequent searches of the data identified the modification of Asn to Trp (+72 Da) for Trp chr17.trna39 and (-57 Da) for Glychr19.trna2, thus confirming the faithful encoding of

the cognate amino acid for each ACE-tRNA type. Importantly, in each case >98% of the peptide identified at the HDH p.N94X site had the encoded cognate tryptophan and glycine. Further, both ACE-tRNAs retained selectivity for the UGA stop codon, over UAA and UAG, **Figure 23B** (ACE-tRNA^{Gly}) and **Figure 31** (ACE-tRNA^{Trp}). Lastly, when transiently expressed, the ACE-tRNA^{Gly} outperformed the conventional small molecule suppressors gentamicin (40 μM) and G418 (140 μM) in their ability to suppress NLuc-UGA stably expressed in HEK293 cells, **Figure 23C**. The same was true even for ACE-tRNA^{Trp}, which had a lower suppression efficiency yet exceeded PTC rescue compared to G418, **Figure 33A-D**.

The question was raised whether ACE-tRNAs that show efficacious suppression of premature stop codons may also induce global readthrough of native stop codons. To address this potential "off target" suppression, a transcriptome-wide quantitative profile of actively engaged ribosomes on all cellular transcripts was obtained by generating libraries of ribosome footprints from HEK293 cells expressing exogenous ACE-tRNAs or a control mock plasmid (puc57GG). Streptomycin was removed from the growth media to prevent readthrough artifacts. For comparison, the ribosome footprint library was also generated from cells in the presence or absence of G418 (150 μM, 48 hours). **Figure 24A** shows ribosome footprint densities of G418 and five ACE-tRNAs compared against controls (log2-fold change) on 3'UTR regions. Only transcripts with a minimum threshold of 5 RPKM in the coding sequence and 0.5 RPKM in the 3'UTR in two replicate libraries were included for the quantitation comparison (254 transcripts in G418 and 495-748 transcripts in ACE-tRNAs). In this system, G418 had no observable effect on transcriptome-wide 3'UTR ribosome density for any of the three endogenous stop codon groups. ACE-tRNAs examined here had no detectable change of 3'UTR ribosome density with the exception of ACE-tRNA Gln-UAA and Arg-UGA which induced approximately a 2-fold increase in 3'UTR ribosome density for the cognate stop codon complimentary to the ACE-tRNA anticodon. Understanding the biological significance of 2-fold readthrough of protein stops will require further study, but this effect is substantially lower compared to the 100- to 1000-fold suppression of PTC for the same ACE-tRNA.

Multiple in-frame stop codons are frequently found at the end of genes [37-39] and may cause a minor difference in overall 3'UTR ribosome density for ACE-tRNA and G418 treatment. Ribosome occupancy was examined at each nucleotide in the 3'UTR within a 60 nt region downstream of the stop codons. **Figure 24B** demonstrates the ribosome occupancy surrounding native stop codons for each nucleotide within the region from -35 to +65 nt relative to the first

nucleotide of stop codon. Reads were normalized per total million-mapped reads, compared against control cells, and reported as a log2-fold change as in panel A. More than 5,200 transcripts were mapped to at least 1 footprint in the region of interest. ACE-tRNA Gln-UAA and Arg-UGA showed not only notable increased ribosome occupancy in the early region but also characteristic 3-nt periodicity, indicating that the ribosomes were not randomly distributed but followed codon-by-codon movement. ACE-tRNAs for UGA-Trp, UGA-Gly and UAG-Glu, or G418, consistently showed no observable change of ribosome occupancy even in the early region of 3'UTR. Taken together, the ribosome profiling data argue that efficiency of native stop codon suppression by ACE-tRNAs is generally low, and markedly less than the level of PTC suppression.

DISCUSSION

PTCs cause a multitude of human diseases and there are no established therapeutic options for their therapeutic management. The high-throughput cloning and identification, characterization and functional analysis of anticodon-edited tRNA that display efficacious PTC reversion in eukaryotic cells and mouse skeletal muscle is reported herein. Notably, the screen identifies ACE-tRNA, in total, with the ability to repair a vast majority of known human disease-causing PTC. The engineered tRNA faithfully encode their cognate amino acid, thus abrogating spurious effects on downstream protein stability, folding, and trafficking, and consequently negating the need for tandem therapies involving protein folding or trafficking agents. When transfected as cDNA, ACE-tRNAs rescued multiple full-length proteins via PTC suppression; a NLuc luciferase reporter, a model protein HDH, and two disease nonsense mutations in *CFTR*. Potent and stable *in vivo* PTC suppression in mouse skeletal muscle was displayed by an ACE-$tRNA^{Arg}$ cDNA, suggesting a particularly high level of cellular tolerance for ACE-tRNA activity. The identification of an active ACE-tRNA for arginine in muscle is relevant for the treatment of dystrophinopathies caused by nonsense mutations. Following suit with most genetic diseases, greater than 10 percent of dystrophinopathies are caused by nonsense mutations [43], where CGA->TGA mutations are most prevalent [43]. Efficient suppression was also achieved with ACE-tRNAs delivered as synthetic RNA transcripts, thus enabling the development of nanoparticle formulations. Future studies will be needed to assess ideal tRNA delivery strategies for each tissue and disease type, where efforts will likely benefit from rapidly expanding technologies for nucleic acid delivery.

Agents that suppress PTCs have the potential to also produce readthrough of native stop codons. The RNA profiling data presented herein suggest this is, generally, not the case in the cells and for the codon-edited tRNA that were tested. While detectable readthrough was found with Arg-tRNA^{UGA} and Gln-tRNA^{UAA}, no significant effect on global translation termination was measured with Glu-tRNA^{UAG}, UGA-Gly-tRNA^{UGA} and Trp-tRNA^{UGA}. This behavior did not obviously segregate with stop codon type, or the intrinsic PTC suppression activity of the tRNA. One potential reason that ACE-tRNA ineffectually promote readthrough at real stop codons may be due to the contextual sequence landscapes near translation terminations [44]. This possibility is supported by the finding that the composition of termination complexes at PTCs differ from those at native stops [45, 46]. However, in cases where lower level readthrough occurs, there are multiple cellular mechanisms in place to limit both normal stop read-through and damaging effects thereof. Multiple in-frame stop codons are frequently found at the end of genes [37-39] and specialized ubiquitin ligases [47] and ribosome associated pathways [48] are known to identify and degrade proteins with erroneous translation termination. Nonetheless, despite the limited impact seen here in mammalian cells, similar ribosomal profiling experiments should be performed in the desired cell or tissue type for ACE-tRNA delivery and expression.

Previous studies have shown that the surrounding mRNA sequence influences inherent stop codon suppression efficacy of aminoglycosides and Ataluren PTC [49-52], and ACE-tRNA may be similarly affected. Further, while gene addition strategies to replace a PTC containing gene, via viral or non-viral delivery, have achieved short term benefit in some settings, it may be difficult to regulate transgene expression levels. In contrast, the abundance of protein rescue via ACE-tRNA suppression is coupled to native cellular RNA levels, and thus upper levels of expression will be intrinsically regulated. The biological purpose remains unknown for a majority of the variable isoacceptor tRNA sequences in the human genome, and almost half these genes have been speculated to be transcriptionally silent pseudogenes [53], however the data here suggest many annotated tRNA are viable. Consistent with this possibility, a suppression approach has been used to identify functional isodecoder tRNAs within Ser and Leu isoacceptor families [54]. The data presented here further demonstrate that the majority of tRNA gene sequences support viable activity when removed from the genomic context, further deepening the mystery for the biological need for a plurality of tRNA, and codon usage. Thus, the high-throughput suppression strategy described here will be useful to identify new types of tRNA sequences with unique suppression properties, and such studies have the potential to produce

new RNA reagents as well as advance the molecular understanding tRNA expression and suppression.

MATERIALS AND METHODS

Nonsense reporter HTC plasmid

The parent plasmid used was pcDNA3.1(+). The cDNA encoding pNLuc was Gibson Assembled (New England Biolabs, USA) into restriction sites HindIII and XhoI. A glycine (codon gga), tryptophan (tgc), amber (tag), opal (tga) and ochre (taa), were added to amino acid position 160 during cDNA pcr. The pcDNA3.1(+) polyA sequence was replaced for one with no BbsI restriction sites using pcr based Gibson Assembly. The high throughput ACE-tRNA Golden Gate cloning site was generated by first inserting the 5′ leader sequence of the human $tRNA^{Tyr}$ gene (bold) with a T7 promoter sequence upstream (italics)

(*TAATACGACTCACTATAG***AGCGCTCCGGTTTTTCTGTGCTGAACCTCAGGGGACG CCGACACACGTACACGTC**)

(Ye et al., 2008) followed by two BbsI restriction sites (***bold italics***) (TA***GTCTTC***GG (*ccdB cassette*) AA***GAAGAC***CG) and 3' termination sequence (bold) followed by a reverse T3 primer sequence (italics)

(**GTCCTTTTTTTG***CTTTAGTGAGGGTTAATT*).

HTC of ACE-tRNA library

tRNA gene sequences were obtained from the tRNA database tRNAscan-SE (http://gtrnadb.ucsc.edu/index.html; PMID: 26673694). Sequences of all tRNA genes used in this study are numbered in **Figure 26** and **Table 9**. tRNA sequences were synthesized as complementary Ultramers from Integrated DNA Technologies (IDT, USA) in 96 well format at 200pmol scale with their corresponding anticodons mutated appropriately (UAG, UGA or UAA). All tRNA sequences were synthesized with CGAC and GGAC overhangs (annotated 5'->3') on forward and reverse oligos, respectively. Ultramers were annealed by resuspending in annealing buffer (100 mM Potassium Acetate; 30 mM HEPES, pH 7.5) to 100ng/µl, heated to 96°C for 2 minutes and cooled at 1°C/minute in a thermocyler to 4°C. In 96 well PCR plates, each well contained 10ng of HTC plasmid with appropriate PTC codon, 2ng ACE-tRNA duplex, 1mM ATP, 10mM DTT, 400 Units T4 DNA Ligase, and 10 Units BbsI-HF, queued to 10µl with

ddH_2O. The 96 well plates were cycled as follows ([5 minute @37°C, 5 minute @20°C] x 30 cycles, 10 minutes @ 37°C, 10 minutes @ 80°C and cooled to 4°C in a thermocycler. In a deep welled 96 well plate 1µl of the Golden Gate reaction was added to 10µl of DH5α chemically competent cells (ThermoFisher, USA), heat-shocked @ 42°C for 30 sec and resuspended in 100µl of Super Optimal Broth (S.O.C.; Thermofisher, USA). Transformations were outgrown at 37°C for 1hour, 250 rpm and then added to 2ml of Luria-Bertani liquid media (LB) supplemented with 100ug/ml Carbenicillin and grown in covered deep 48 well plates @ 37°C for 20hours, 300 rpm. E. coli outgrowth was performed in deep well plates and clamps from Enzyscreen (http://www.enzyscreen.com). E. coli suspension cultures were spun down (10minutes, 4,000*g* at RT) and plasmid DNA was prepared and diluted to 125ng/µl (IBI scientific, USA). All clones were sequence verified. Using this method, 100% cloning efficiency was achieved.

HTS of ACE-tRNA library

The day before transfection, HEK293 cells (<40 passages) were plated at 1.4 x 10^4 cells/well in 96 well cell culture treated plates in Dulbecco's Modified Essential Medium (DMEM) supplemented with 10% FBS, 1% Pen/Step and 2mM L-Glutamine (Thermofisher, USA). The all-in-one nonsense reporter with ACE-tRNA genes were transfected in triplicate/plate using Calfectin (Signagen, USA). 16 hours post-transfection, the media was aspirated and 20µl of PBS was added to each well. 15µl of lytic Nano-Glo® Luciferase Assay Reagent was added to each well (1:50 reagent to buffer; Promega, USA). The plates were incubated for 2 minutes after rotational shaking and read using a SpectraMax i3 plate reader (Molecular Devices, USA; integration time, 200ms; All wavelengths collected in endpoint mode). Luminescence was averaged across three wells for each experiment and all ACE-tRNAs were repeated >3 times in this fashion. Each plate also contained in triplicate wells transfected with the all-in-one nonsense reporter with no ACE-tRNA to server as control for transfection efficiency and baseline PTC readthrough. All values are reported as ratios of ACE-tRNA luminescence over baseline PTC readthrough luminescence ± SEM. One-way ANOVAs were performed with Tukey's post-hoc analysis across all ACE-tRNAs in a given amino acid family.

CFTR, HDH-his-strep and 4xACE-tRNA expression plasmids

For expression in mammalian cells, the cDNA for the coding region and 200 base-pair of the 3' untranslated region (UTR) of human CFTR was ligated into pcDNA3.1(+) (Promega, USA) using the KpnI and XbaI restriction enzymes. The G542tga and W1282tga mutations were introduced using QuickChange XL II (Stratagene, USA). For expression in Xenopus laevis oocytes, the cDNA for the coding region and 140 base-pair of the 5' and 244 base-pair 3' UTR of human CFTR was ligated into pGEM-HE (Promega, USA). Bothe the G542tga and W1282tga mutations were introduced using QuickChange XL II. The cDNA encoding the E. coli histidinol dehydrogenase was codon optimized for *mus musculus* and synthesized (BioBasic Inc, Canada) with a c-terminal 8xHis-Strep- tag for protein purification from mammalian cells. The synthesized cDNA was ligated into pcDNA3.1(+) using EcoRI and XhoI restriction sites. The nonsense mutations tag, taa and tga were introduced using QuickChange XL II. To generate multiplexed ACE-tRNA expression plasmids, a novel parent Golden Gate pUC57(amp) plasmid was generated by inserting a BbsI "multiple cloning site" (5'-GAATTCTTCCCGAGACG**TTCC**AA*GTCTTC*AT*GAAGAC*TA**CAGG**CGTCTCCCAGGAAG CT-3'; directional BbsI recognition sequences are *italicized* and unique four base-pair overhangs for ligation are **bolded**) between the EcoRI and HindIII restriction sites. pUC57(amp) was chosen as a parent plasmid because it is relatively small in size and lacks backbone BbsI restriction sites and T7 and T3 promoter sequence. A feature included in the HTS plasmid is T7 and T3 promoter sequence flanking the ACE-tRNA cassette, giving universal primer binding sequences with comparable melting temperatures (T_m), ideal for pcr amplification. Using the NEB Golden Gate Assembly Tool (https://goldengate.neb.com/editor) pcr primers were generated that annealed to the T7 and T3 flanking sequence and created unique four base-pair overhangs following cleavage of distal BbsI recognition sequence. The end result was the generation of four ACE-tRNA pcr products using universal pcr primers that could be "daisy-chained" through complementary four base-pair overhangs and ligated into the puc57 Golden Gate plasmid using a one-pot Golden Gate reaction. All clones were sequence verified.

Cell culture, protein expression and Western blot

HEK293T cells (ATCC, USA) were grown in standard grown media containing (% in v/v) 10% FBS (HiClone, USA), 1% Pen Strep, 1 % L-Glut in high glucose DMEM (Gibco, USA) at 37°C, 5% CO2. cDNA was transfected at 75% confluency using Calfectin according to standard protocols (SignaGen Laboratories, USA). Following 36 hours the cells were scraped and pelleted at 7,000g for 8 minutes at 4°C in PBS supplemented with 0.5 μg/ml pepstatin, 2.5

μg/ml aprotinin, 2.5 μg/ml leupeptin, 0.1 mM PMSF, 0.75 mM benzamidine. For CFTR expressing cells, the cell pellet was vigorously dounced in 100mM sucrose, 150 mM NaCl, 1mM DTT, 0.5 μg/ml pepstatin, 2.5 μg/ml aprotinin, 2.5 μg/ml leupeptin, 0.1 mM PMSF, 0.75 mM benzamidine, 50 mM Tris-HCL ph 7.4 and centrifuged at 100,000g to separate total membranes from the soluble cytosolic proteins. Pellets were solubilized in a buffer containing 1% triton, 250mM NaCl, 50mM tris-HCl pH 7.4, and 0.5 μg/ml pepstatin, 2.5 μg/ml aprotinin, 2.5 μg/ml leupeptin, 0.1 mM PMSF, 0.75 mM benzamidine. Equal cell-lysate was loaded on a 3-15% separating gradient SDS-page with 4% stacking gel in the presence of 1% 2-mercaptoethanol, separated at 55 V O/N and transferred to 0.45 μM LF PVDF (Bio-Rad, USA). PVDF was immunoblotted using anti-CFTR antibody M3A7(1:1000; Millipore, USA) in 2% non-fat milk and imaged on LI-COR Odyssey Imaging System (LI-COR, USA). For HDH-His-Strep expressing cells, the cell pellet was vigorously dounce homogenized in 100mM sucrose, 1mM DTT, 1mM EDTA, 20mM tris-HCl pH 8.0, 0.5 μg/ml pepstatin, 2.5 μg/ml aprotinin, 2.5 μg/ml leupeptin, 0.1 mM PMSF and 0.75 mM benzamidine. The lysate was centrifuged at 100,000*g* for 30 minutes at 4 °C. The supernatant (soluble cellular protein) was separated on 4-12% Bis-Tris SDS-page acrylamide gels (ThermoFisher, USA) in the presence of 1% 2-mercaptoethanol, transferred to 0.22 μM LF PVDF (Bio-Rad, USA) and immunoblotted using anti-Strep antibody (1:5000; iba, Germany) in 2% non-fat milk and imaged on LI-COR Odyssey Imaging System (LI-COR, USA).

Mass spectrometry

Fragmentation data on purified HDH-His-Strep protein were obtained at the University of Iowa Proteomics Facility. Briefly, HDH-His-Strep protein from the soluble fraction of the high-speed spin was passed through StrepTrap HP columns (GE Healthcare, Sweden) and washed with 5 column volumes of 100mM sucrose, 1mM DTT, 1mM EDTA, 20mM tris-HCl pH 8.0, 0.5 μg/ml pepstatin, 2.5 μg/ml aprotinin, 2.5 μg/ml leupeptin, 0.1 mM PMSF and 0.75 mM benzamidine. The protein was eluted in wash buffer supplemented with 10mM d-desthbiotin and concentrated in 30kDA cutoff Amicon-Ultra filtration columns (Millipore, USA). The concentrated protein was loaded on NuPage 4-12% Bis-Tris precast gels (Invitrogen, USA) and separated at 150V for 1.5 hours. The gel was stained using a Pierce mass spec compatible silver stain kit (ThermoFisher Scientific, USA).

In-gel Trypsin Digestion. Briefly, the targeted protein bands from SDS-PAGE gel were manually excised, cut into 1 mm^3 pieces, and washed in 100 mM ammonium

bicarbonate:acetonitrile (1:1, v/v) and 25 mM ammonium bicarbonate /acetonitrile (1:1, v/v), respectively to achieve complete destaining. The gel pieces were further treated with ACN, and dried *via* speed vac. After drying, gel pieces were reduced in 50 µl of 10 mM DTT at 56 °C for 60 minutes and then alkylated by 55 mM IAM for 30 minutes at room temperature. The gel pieces were washed with 25 mM ammonium bicarbonate:acetonitrile (1:1, v/v) twice to removed excess DTT and IAM. After drying, the gel pieces were placed on ice in 50 µL of trypsin solution at 10 ng/µL in 25 mM ammonium bicarbonate and incubated on ice for 60 minutes. Then, digestion was performed at 37 °C for 16 h. Peptide extraction was performed twice for 0.5 h with 100 µl 50% acetonitrile/0.2% formic acid. The combined extracts were concentrated in a Speed Vac to ~15 µl.

LC-MS/MS. The mass spectrometry data were collected using an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific, San Jose, CA) coupled to an Eksigent Ekspert™ nanoLC 425 System (Sciex). A Trap-Elute Jumper Chip (P/N:800-00389) and a coupled to a 1/16" 10 port Valco directed loading performed by the gradient 1 pump and final elution (by the gradient 2 pump). The column assembly was was designed as two tandem 75 µmx15cm columns (ChromXP C18-CL, 3µm 120A, Eksigent part of AB SCIEX) mounted in the ekspert™ cHiPLC system. For each injection, an estimated 0.5 µg of total digest was loaded. Peptides were separated in-line with the mass spectrometer using a 120 min gradient composed of linear and static segments wherein Buffer A is is 0.1% formic acid and B is 95%ACN, 0.1% Formic acid. The gradient begins first holds at 4% for 3 minutes then makes the following transitions (%B, min): (26, 48), (35, 58), (35, 64), (50, 72), (50, 78), (94, 84), (94, 96), (4, 100), (4, 120).

Tandem mass spectrometry on the LUMOS Orbitrap. Scan sequences began with a full survey (m/z 350 -1500) acquired on an Orbitrap Fusion Lumos mass spectrometer (Thermo) at a resolution of 60,000 in the off axis Orbitrap segment (MS1). Every 3 seconds of the gradient MS1 scans were acquired during the 120 min gradient described above. The most abundant precursors were selected among 2-8 charge state ions at a 2.0E5 threshold. Ions were dynamically excluded for 30 seconds if they were targeted twice in the prior 30 sec. Selected ions were isolated by a multi-segment quadrupole with a mass window on m/z 2, then sequentially subjected to both CID and HCD activation conditions in the IT and the ioin routing multipole respectively. The AGC target for CID was 4.0E04, 35% collision energy, an activation Q of 0.25 and a 100 milliseconds maximum fill time. Targeted precursors were also fragmented by high energy collision-induced dissociation (HCD) at 40% collision energy, and an activation

Q of 0.25. HCD fragment ions were analyzed using the Orbitrap (AGC 1.2E05, maximum injection time 110 ms, and resolution set to 30,000 at 400 Th). Both MS2 channels were recorded as centroid and the MS1 survey scans were recorded in profile mode.

Proteomic Searches. Initial spectral searches were performed with Proteome Discoverer version 2.1.1.21 (ThermoFisher Scientific, USA) using Sequest HT. Spectra were also searched with Byonic search engine (Protein Metrics) ver. 2.8.2. Search databases were composed of the Uniprot KB for species 9606 (Human) downloaded 10/24/2016 containing 92645 sequences and Uniprot KB for taxonomy 562 (*E. coli*) downloaded on 11/08/2016 containing 10079 sequences. For Byonic searches, these two data bases were directly concatenated. In either search an equal number of decoy entries were created and searched simultaneously by reversing the original entries in the Target databases.

***In vitro* cRNA transcription.** G542X$_{UGA}$, W1282X$_{UGA}$, and WT CFTR pGEMHE (Mense et al., 2006; PMID:1703051) plasmids were linearized by 10 x excess of NheI-HF restriction enzyme (site positioned 3' of coding region)(New England BioLabs, USA) for 3 hours at 37°C and purified using standard cDNA precipition methods. All cRNAs were transcribed using the mMessage mMachine T7 Kit (ThermoFisher Scientific, USA). Purification of the cRNA from the transcription reaction was conducted on columns from the RNeasy Mini Kit (Qiagen, Germany). Concentration was determined by absorbance measurements at 260 nm and quality was confirmed on a 1% agarose gel (RNase-free). All cRNA was queued to 1µg/ml before use and all results were generated from ≥2 cRNA preparations.

***In vitro* tRNA transcription.** Trpchr17.trna39 and Glychr19.trna2, the top performing Trp and Gly ACE-tRNAs, were transcribed in vitro using CellScript T7-Scribe Standard RNA IVT Kit (CELLSCRIPT, USA). Equimolar concentration of T7 oligo (5'-taatacgactcactata-3') was annealed to ACE-tRNA PAGE-purified Ultramers (20ug; Integrated DNA Technologies, Coralville, IA) coding for the ACE-tRNA and preceded by a T7 promoter (italics). Importantly, the three terminal nucleotides containing CCA were included (bold).

Trpchr17.trna39 (3'->5'):

TGGTGACCCCGACGTGATTTGAACACGCAACCTTCTGATCTGAAGTCAGACGCGCT ACCGTTGCGCCACGAGGCC*TATAGTGAGTCGTATTA*

Glychr19.trna2 (3'->5'):

TGGTGCGTTGGCCGGGAATCGAACCCGGGTCAATGCTTTGAAGGAGCTATGCTAAC CATATACCACCAACGC*TATAGTGAGTCGTATTA*

The total reaction volume was adjusted to 100 µl and the kit reagents were added in the following amounts: 10 µl of 10X T7-Scribe transcription buffer, 7.5 µl of each nucleotide (100 mM stocks), 10 µl of 100 mM Dithiothreitol, 2.5 µl ScriptGuard RNase Inhibitor, 10 µl T7-Scribe enzyme solution. After the reaction was incubated for 4–5 hours at 37°C, the DNA template was digested with 5 µl DNase (1 U/µl) provided with the kit for 30–60 min. The ACE-tRNA was extracted from the reaction with acidic phenol chloroform (5:1, pH 4.5) and precipitated with ethanol. The precipitates ACE-tRNA was pelleted, washed, dried and resuspended in 100 µl DEPC-treated water and further purified with Chroma Spin-30 columns (Clontech, USA). The procedure yielded roughly 100 µl of ~5 µg/µl ACE-tRNA. ACE-tRNAs were re-pelleted in 20ug aliquots, washed, lyophilized and stored at -80°C until use. All results were generated from ≥2 ACE-tRNA preparations.

Ribosome Footprint Profiling Library preparation. HEK293 cells transiently transfected with ACE-tRNAs and control plasmid (puc57GG) were grown in standard grown media in the absence of Pen-Strep for 48 h. Libraries were prepared as described[55], with a few modifications. Briefly, cells were rapidly cooled by addition of ice-cold PBS, lysed in lysis buffer (20 mM Tris-HCl/pH7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 1% (v/v) Triton X-100, and 25 U ml^{-1} Turbo DNase I) for 10 min on ice, and triturated with ten times through a 26-G needle. After clearance by centrifugation at 16,000*g* for 10 min at 4°C, the lysates were digested with 100 U RNase I (Ambion, USA) per A_{260} lysate at room temperature for 45 min with gentle agitation prior to adding 200 U RiboLock RNase Inhibitor (Thermo Scientific). Ribosome protected mRNA fragments were then isolated by loading lysates onto a 1M sucrose cushion prepared in modified polysome buffer (20 mM Tris-HCl/pH7.4, 150 mM NaCl, 8.5 mM $MgCl_2$, 0.5 mM DTT, 20 U ml^{-1} RiboLock RNase Inhibitor) and centrifugated at 70,000 rpm at 4°C for 2 h using a Beckmen TLA-110 rotor. Ribosome pellets containing mRNA footprints were extracted using TRIzol and separated on a denaturing 12% polyacrylamide gel containing 8M urea. RNA fragments with sizes ranging from 26 to 34 nt were manually excised from the gel stained with SYBR Gold (Invitrogen) and isolated to generate the ribosome-protected fragment library. Contaminating rRNA fragments depleted using a Ribo-Zero kit (Illumina). 3'

Oligonucleotide adaptor ligation, reverse transcription, circularization, and secondary rRNA depletion using biotinylated rRNA depletion oligos (**Table 9**) were performed as described [55]. Libraries were barcoded using indexing primers for each sample during PCR amplification. Barcoded libraries were then pooled with 3% PhiX (Illumina) and sequenced in an Illumina NextSeq 500 as per manufacturer protocol to typically generate 18-27 million reads per sample.

Ribosome Footprint Data analysis. Data files for each barcoded sample (minus adaptor sequence at 3' end) were first mapped to four rRNA sequences (RNA5S1;NR_023363, RNA5-8SN5; NR_003285, RNA18SN5;NR_003286, and RNA28SN5;NR_003287) using HISAT 2.0.3 [56] to eliminate rRNA contaminant reads. The remaining reads were aligned to the sense stands of the longest transcript variant of each human gene (UCSC RefSeq GRCh38). Transcripts with 3'UTR length of at least 75 nt (18,101 sequences) were used for subsequence analysis. A maximum of two mismatches at the 5'end of reads was allowed. All multi-mapped reads were discarded. Fragment reads with lengths between 26 to 34 nt were defined as ribosome footprints and used for analysis. The 5' end nucleotide from each footprint was annotated and mapped on each transcript. Position of the ribosome A-site occupying the 16th-18th nucleotides of each footprint[57, 58] was used to infer the position of the ribosome on each transcript. RPKM (footprint Reads Per Kilobase of transcript per total Million-mapped reads) on each individual transcript (18,101 sequences) was calculated. Only transcripts with a minimum threshold of 5 RPKM in the coding sequence and 0.5 RPKM in 3'UTR region in two replicate libraries (254 transcripts in G418 and 495-748 transcripts in ACE-tRNAs) were included for analysis in **Figure 24A**. For transcriptome-wide metagene plots in **Figure 2B**, footprint counts for each nucleotide within the region from -35 to +65 nt relative to the first nucleotide of stop codon were normalized per total million-mapped reads. All transcripts (18,101 sequences) were used for mapping, and more than 5,200 transcripts were mapped to at least 1 footprint in the region of interest. Next, the in vivo bioactivity of ACE-tRNAs Glychr19.trna2 and Trpchr17.trna39 to rescue PTC was examined. The sequencing data was analyzed using Galaxy platform [59]. Graphs were generated using Prism 7 (GraphPad Software).

Generation of stable NLuc reporter cell lines. The cDNAs encoding pNLuc with tag, taa and tga stop codons at amino acid position 160 were inserted into AgeI and NotI restriction sites within the multiple cloning site of the retroviral vector pQCXIP (Clontech, USA) using Gibson Assembly (New England Biolabs, USA). PhoenixGP cells (PMID: 7690960) were co-transfected with pNLuc-STOP-pQCXIP and cmv-VSV-G (VSV-G envelope pseudotyping)

plasmids using Calfectin (SignaGen Laboratories, USA) and placed in a 33°C CO_2-controlled (5%) cell incubator for 48 hours. The culture media (20mls) containing retroviral particles was chilled to 4°C and spun at 10,000*g* to remove cell debris and filtered through a 0.45 μm MCE-membrane syringe filter (Millipore, USA) onto two 10cm dishes seeded with low-passage HEK293 cells at 30% confluency. Cell culture dishes were sealed with Parafilm and spun for 90 minutes at 3,500*g* at 24°C and placed in a 37°C CO_2 controlled (5%) cell culture incubator. Cells were selected 24 hours later with puromycin (1μg/ml) until the control dish (no infection) showed complete cell death. Cells were monodispersed into 96-well plates using FACS and clonal populations were subsequently. Puromycin was not used to maintain selected clones during experimentation and standard DMEM media (DMEM–Dulbecco's Modified Eagle Medium-high glucose with L-glutamine supplemented with 10% FBS, 1% Pen/Step and 2mM L-Glutamine; ThermoFisher, USA) was used in all studies.

RNA transfection. HEK293 cells stably expressing pNLuc-UGA were plated at 1.4 x 10^4 cells/well in 96 well cell culture treated plates in Dulbecco's Modified Essential Medium (DMEM) supplemented with 10% FBS, 1% Pen/Step and 2mM L-Glutamine (Thermofisher, USA). 16-24 hours later the cells were transfected with ACE-tRNAs using lipofectamine 2000 (ThermoFisher Scientific, USA). Briefly, 3μg of ACE-tRNA were suspended in 150μl of OptiMEM and 12μl of Lipofectamine 2000 was mixed with 150μl of OptiMEM. The volumes were combined, thoroughly mixed and incubated for 10 mins at RT. 75μl of the transfection complex was added to each well. PTC suppression by ACE-tRNA transcripts was quantified as described above.

Expression in *Xenopus laevis* oocytes. *Xenopus laevis* oocytes (stage V and VI) were purchased from Ecocyte (Austin, TX). Prior to injection, each ACE-tRNA pellet was resuspended in 2 μl of ddH_2O and debris was pelleted at 21,000 x g, 4°C for 25 min. To determine dose response of ACE-tRNAs on CFTR channel rescue, serial dilutions were generated of ACE-tRNA aliquots (200, 100, 50, 25, 12.5, 6.25, 3.125 and 1.562 ng/oocyte) balanced in volume with ddH_2O. In all experiments 25ng of CFTR cRNA was injected per oocyte and injection volumes were 50nl. ddH_2O was used in no ACE-tRNA background control experiments. After injection, oocytes were kept in OR-3 (50% Leibovitz's medium, 250 mg/l gentamycin, 1 mM L-glutamine, 10 mM HEPES (pH 7.6)) at 18°C for 36 hours.

Two-electrode voltage clamp (TEVC) recordings. CFTR Cl^- currents were recorded in ND96 bath solution that contained (in mM): 96 NaCl, 2 KCl, 1 $MgCl_2$, and 5 HEPES (pH 7.5) in

the presence of a maximal CFTR activation cocktail, forskolin (10μM; adenylate cyclase activator) and 3-isobutyl-1-methylxanthine (1mM; phosphodiesterase inhibitor). Glass microelectrodes backfilled with 3 M KCl had resistances of 0.5–2 MΩ. Data were filtered at 1 kHz and digitized at 10 kHz using a Digidata 1322A controlled by the pClamp 9.2 software (Molecular Devices, USA). *CFTR* currents were elicited using 5mV voltage steps from -60 to +35mV using an OC-725C voltage clamp amplifier (Warner Instruments, USA). Oocytes where the CFTR Cl^- current reversed positive of -20mV were discarded. Clampfit 9.2 software was used for current analysis. All values are presented as mean ± SEM.

Animals and *in vivo* imaging. Nu/J mice were purchased from Jackson labs. Animal experiments were approved by the Institutional Animal Care and Use Committee at the Wistar Institute (protocol number: 112762). Mice were treated by injecting 10-20 μg of DNA resuspended in 30μl of water into the tibialis anterior muscle followed by electroporation. 10 μg pNano-TGA + 10 μg Arg ACE-tRNA (right tibialis anterior) or 10 μg pNano-TGA + 10 μg empty pUC57 (left tibialis anterior) were injected into 3 mice. As controls 3 other mice were injected with 10 μg pNano-WT (right tibialis anterior; positive control) or water (left tibialis anterior; negative control). The DNA was formulated with 333IU/ml of hyaluronidase (Sigma). One minute after DNA injection, electroporation with CELLECTRA 3P device (Inovio Pharmaceuticals) was performed. Nanoluciferase activity was imaged in mice by injecting 100μl of furimazine (40x dilution of Nano-Glo substrate) intraperitoneally and imaged mice on an IVIS Spectrum (Perkin Elmer) 5 minutes after injection. Imaging was with open filter and images were acquired at 40 seconds. Tthe images were analyzed using Living Image Software (Perkin Elmer).

Table 9. Library of annotated sequences of tRNA screened for PTC suppression activity. Italicized text for each sequence shows the site of anti-codon editing. Bold text indicates tRNAs with suppression activity 5-fold above background. Note that in tRNA the thymidines are replaced with uracils.

	tRNAscan-SE ID	Sequence	SEQ ID NO
1	TrpTGAchr17.trna39	GGCCTCGTGGCGCAACGGTAGCGCGTCTGACT*tca*GA TCAGAAGGtTGCGTGTTCAAATCACGTCGGGGTCA	56

	tRNAscan-SE ID	Sequence	SEQ ID NO
2	**TrpTGAchr17.trna10**	GACCTCGTGGCGCAATGGTAGCGCGTCTGACT*tca*GA TCAGAAGGtTGCGTGTTCAAGTCACGTCGGGGTCA	57
3	**TrpTGAchr6.trna171**	GACCTCGTGGCGCAACGGTAGCGCGTCTGACT*tca*GA TCAGAAGGtTGCGTGTTCAAATCACGTCGGGGTCA	58
4	**TrpTGAchr12.trna6**	GACCTCGTGGCGCAACGGTAGCGCGTCTGACT*tca*GA TCAGAAGGcTGCGTGTTCGAATCACGTCGGGGTCA	59
5	**TrpTGAchr7.trna3**	GACCTCGTGGCGCAACGGCAGCGCGTCTGACT*tca*GA TCAGAAGGtTGCGTGTTCAAATCACGTCGGGGTCA	60
6	TrpTGAchr7.trna31	GGCCTCATGGTGCAACAGTAGTGTGTCTGACT*tca*GA TCAGAAGGtTGTATGTTCAAATCACGTAGGGGTCA	61
1	**TrpTAGchr17.trna39**	GGCCTCGTGGCGCAACGGTAGCGCGTCTGACT*cta*GA TCAGAAGGtTGCGTGTTCAAATCACGTCGGGGTCA	62
2	**TrpTAGchr17.trna10**	GACCTCGTGGCGCAATGGTAGCGCGTCTGACT*cta*GA TCAGAAGGtTGCGTGTTCAAGTCACGTCGGGGTCA	63
3	**TrpTAGchr6.trna171**	GACCTCGTGGCGCAACGGTAGCGCGTCTGACT*cta*GAT CAGAAGGtTGCGTGTTCAAATCACGTCGGGGTCA	64
4	**TrpTAGchr12.trna6**	GACCTCGTGGCGCAACGGTAGCGCGTCTGACT*cta*GA TCAGAAGGcTGCGTGTTCGAATCACGTCGGGGTCA	65
5	**TrpTAGchr7.trna3**	GACCTCGTGGCGCAACGGCAGCGCGTCTGACT*cta*GA TCAGAAGGtTGCGTGTTCAAATCACGTCGGGGTCA	66
6	TrpTAGchr7.trna31	GGCCTCATGGTGCAACAGTAGTGTGTCTGACT*cta*GA TCAGAAGGtTGTATGTTCAAATCACGTAGGGGTCA	67
1	GlyTGAchr1.trna122	GCATTGGTGGTTCAGTGGTAGAATTCTCGCCT*tca*AC GCGGGAGaCCCGGGTTCAATTCCCGGCCAATGCA	68
2	GlyTGAchr2.trna25	GCGCCGCTGGTGTAGTGGTATCATGCAAGATT*tcaa*A TTCTTGCGaCCCGGGTTCGATTCCCGGGCGGCGCA	69
3	GlyTGAchr17.trna11	GCATTGGTGGTTCAATGGTAGAATTCTCGCCT*tca*AC GCAGGAGaCCCAGGTTCGATTCCTGGCCAATGCA	70
4	GlyTGAchr1.trna120	GCGTTGGTGGTTTAGTGGTAGAATTCTCGCCT*tca*AT GCGGGAGaCCCGGGTTCAATTCCCGGCCACTGCA	71
5	GlyTGAchr1.trna2	GCCTTGGTGGTGCAGTGGTAGAATTCTCGCCT*tca*AC GTGGGAGaCCCGGGTTCAATTCCCGGCCAATGCA	72
6	GlyTGAchr1.trna83	GGTGGTTCAGTGGTAGAATTCTCGCCT*tca*ACGCGGG AGaCCCGGGTTTAATTCCCGGTCA	73
7	GlyTGAchr2.trna1	GTGGTCTAGTGGTTAGGATTCAGCGCT*tca*ACCGCCG CAGCCCGGGTTCGATTCCCGGtCA	74
8	GlyTGAchr1.random.trna2	GCGTCAGTGGTTTAGTGGTGGAATTCCTGCCT*tca*AT GCACGAGATCCGTGTTCAACTCCTGGTTGGTGCA	75
9	GlyTGAchr1.trna102	GCGTCAGTGgTTTTAGTGGTGGAATTCCTGCCT*tca*A TGCACGAGATCCGTGTTCAACTCCTGGTTGGTGCA	76
10	GlyTGAchr1.trna16	GCGTTGGCAGTTCAGTGGTAGAATTCTCGCCT*tca*AC CCGGGAGaCCTGGATTCCATTTCCGGCAAATGCA	77
11	GlyTGAchr1.trna34	GCATGGGTGGTTCAGTGGTAGAATTCTCGCCT*tca*AC GCGGGAGGCCCGGGTTCGATTCCCGGCCCATGCA	78

	tRNAscan-SE ID	Sequence	SEQ ID NO
12	GlyTGAchr1.trna61	GCATTGGTGGTTCAGTGGTAGAATTCTCGCCT*tca*AC GCGGGAGGCCCGGGTTCGATTCCCGGCCAATGCA	79
13	GlyTGAchr16.trna25	GCATTGGTGGTTCAGTGGTAGAATTCTCGCCT*tca*AC GCGGGAGGCCCGGGTTTGATTCCCGGCCAGTGCA	80
14	GlyTGAchr1.trna42	GCATAGGTGGTTCAGTGGTAGAATTCTTGCCT*tca*AC GCAGGAGGCCCAGGTTTGATTCCTGGCCCATGCA	81
15	GlyTGAchr16.trna19	GCATTGGTGGTTCAGTGGTAGAATTCTCGCCT*tca*AT GCGGGCGGCCGGGCTTCGATTCCTgGCCAATGCA	82
16	GlyTGAchr6.trna80	GCATGGGTGATTCAGTGGTAGAATTTTCACCT*tca*AT GCAGGAGGTCCAGGTTCATTTCCTGGCCTATGCA	83
17	**GlyTGAchr19.trna2**	GCGTTGGTGGTATAGTGGTtAGCATAGCTGCCT*tca*A AGCAGTTGaCCCGGGTTCGATTCCCGGCCAACGCA	84
18	**GlyTGAchr1.trna107**	GCGTTGGTGGTATAGTGGTgAGCATAGCTGCCT*tca*A AGCAGTTGaCCCGGGTTCGATTCCCGGCCAACGCA	85
19	**GlyTGAchr17.trna9**	GCGTTGGTGGTATAGTGGTaAGCATAGCTGCCT*tca*A AGCAGTTGaCCCGGGTTCGATTCCCGGCCAACGCA	86
20	GlyTGAchr1.trna75	GCGTTGGTGGTATAGTGGTgAGCATAGTTGCCT*tca*A AGCAGTTGaCCCGGGCTCGATTCCCGCCCAACGCA	87
21	GlyTGAchr1.trna75-mod	GCGTTGGTGGTATAGTGGTgAGCATAGTTGCCT*tca*A AGCAGTTGaCCCGGGCTCGATTCCCGgCCAACGCA	88
1	ArgTGAchr6.trna6	GGGCCAGTGGCGCAATGGAtAACGCGTCTGACT*tca*G ATCAGAAGAtTCCAGGTTCGACTCCTGGCTGGCTCG	89
2	**ArgTGAchr3.trna8**	GGGCCAGTGGCGCAATGGAtAACGCGTCTGACT*tca*G ATCAGAAGAtTCTAGGTTCGACTCCTGGCTGGCTCG	90
3	**ArgTGAchr6.trna115**	GGCCGCGTGGCCTAATGGAtAAGGCGTCTGATT*tca*G ATCAGAAGAtTGAGGGTTCGAGTCCCTTCGTGGTCG	91
4	**ArgTGAchr17.trna21**	GACCCAGTGGCCTAATGGAtAAGGCATCAGCCT*tca*G AGCTGGGGAtTGTGGGTTCGAGTCCCATCTGGGTCG	92
5	**ArgTGAchr17.trna16**	GCCCCAGTGGCCTAATGGAtAAGGCACTGGCCT*tca*A AGCCAGGGAtTGTGGGTTCGAGTCCCACCTGGGGTA	93
6	**ArgTGAchr17.trna19**	GCCCCAGTGGCCTAATGGAtAAGGCACTGGCCT*tca*A AGCCAGGGAtTGTGGGTTCGAGTCCCACCTGGGGTG	94
7	**ArgTGAchr16.trna3**	GCCCCGGTGGCCTAATGGAtAAGGCATTGGCCT*tca*A AGCCAGGGAtTGTGGGTTCGAGTCCCACCCGGGGTA	95
8	**ArgTGAchr7.trna5**	GCCCCAGTGGCCTAATGGAtAAGGCATTGGCCT*tca*A AGCCAGGGAtTGTGGGTTCGAGTCCCATCTGGGGTG	96
9	**ArgTGAchr16.trna13**	GCCCCAGTGGCCTGATGGAtAAGGTACTGGCCT*tca*A AGCCAGGGAtTGTGGGTTCGAGTTCCACCTGGGGTA	97
10	**ArgTGAchr15.trna4**	GGCCGCGTGGCCTAATGGAtAAGGCGTCTGACT*tca*G ATCAGAAGAtTGCAGGTTCGAGTCCTGCCGCGGTCG	98
11	**ArgTGAchr6.trna4**	GACCACGTGGCCTAATGGAtAAGGCGTCTGACT*tca*G ATCAGAAGAtTGAGGGTTCGAATCCCTCCGTGGTTA	99
12	**ArgTGAchr17.trna17**	GACCGCGTGGCCTAATGGAtAAGGCGTCTGACTtcaG ATCAGAAGAtTGAGGGTTCGAGTCCCTTCGTGGTCG	100

	tRNAscan-SE ID	Sequence	SEQ ID NO
13	**ArgTGAchr6.trna3**	GACCACGTGGCCTAATGGAtAAGGCGTCTGACT*tca*G ATCAGAAGAtTGAGGGTTCGAATCCCTTCGTGGTTA	101
14	**ArgTGAchr6.trna125**	GACCACGTGGCCTAATGGAtAAGGCGTCTGACT*tca*G ATCAGAAGAtTGAGGGTTCGAATCCCTTCGTGGTTG	102
15	**ArgTGAchr9.trna5**	GGCCGTGTGGCCTAATGGAtAAGGCGTCTGACT*tca*G ATCAAAAGAtTGCAGGTTTGAGTTCTGCCACGGTCG	103
16	**ArgTGAchr1.trna10**	GGCTCCGTGGCGCAATGGAtAGCGCATTGGACT*tca*A gaggctgaaggcATTCAAAGGtTCCGGGTTCGAGTCC CGGCGGAGTCG	104
17	**ArgTGAchr1.trna10/ nointron**	GGCTCCGTGGCGCAATGGAtAGCGCATTGGACT*tca*A ATTCAAAGGtTCCGGGTTCGAGTCCCGGCGGAGTCG	105
18	**ArgTGAchr17.trna3**	GGCTCTGTGGCGCAATGGAtAGCGCATTGGACT*tca*A gtgacgaatagagcaATTCAAAGGtTGTGGGTTCGAA TCCCACCAGAGTCG	106
19	**ArgTGAchr17.trna3/ nointron**	GGCTCTGTGGCGCAATGGAtAGCGCATTGGACT*tca*A ATTCAAAGGtTGTGGGTTCGAATCCCACCAGAGTCG	107
20	**ArgTGAchr9.trna6**	GGCTCTGTGGCGCAATGGAtAGCGCATTGGACT*tca*A gctgagcctagtgtggtcATTCAAAGGtTGTGGGTTC GAGTCCCACCAGAGTCG	108
21	**ArgTGAchr9.trna6/n ointron**	GGCTCTGTGGCGCAATGGAtAGCGCATTGGACT*tca*A ATTCAAAGGtTGTGGGTTCGAGTCCCACCAGAGTCG	109
22	**ArgTGAchr11.trna3**	GGCTCTGTGGCGCAATGGAtAGCGCATTGGACT*tca*A gatagttagagaaATTCAAAGGtTGTGGGTTCGAGTC CCACCAGAGTCG	110
23	**ArgTGAchr1.trna79**	GTCTCTGTGGCGCAATGGAcgAGCGCGCTGGACT*tca* AATCCAGAGGtTCCGGGTTCGAGTCCCGGCAGAGATG	111
24	ArgTGAchr6.trna52	GGCTCTGTGGCGCAATGGAtAGCGCATTGGACT*tca*A gcctaaatcaagagATTCAAAGGtTGCGGGTTCGAGT CCCTCCAGAGTCG	112
25	**ArgTGAchr6.trna52/ nointron**	GGCTCTGTGGCGCAATGGAtAGCGCATTGGACT*tca*A ATTCAAAGGtTGCGGGTTCGAGTCCCTCCAGAGTCG	113
26	ArgTGAchr5.trna4	GGCAGCATAGCAGAGTGGTtCAGGTTACAGGT*tca*AG ATGTAAACTGAGTTCAAATCCCAGTTCTGCCA	114
1	GlnTAGnmt-tRNA-Gln chr10.trna6	TGGTGTAATAGGTAGCACAGAGAATT*cta*GATTCTCA GGGGTAGGTTCAATTCCTAT	115
2	GlnTAGnmt-tRNA-Gln chrX.trna1	TAGGACATGGTGTGATAGGTAGCATGGAGAATT*cta*G ATTCTCAGGGGTAGGTTCAATTCCTACAGTTCTAG	116
3	GlnTAGnmt-tRNA-Gln chr7.trna32	TAGGACGTGGTGTGATAGGTAGCATGGGGAATT*cta*G ATTCTCAGGGGTGGGTTCAATTCCTATAGTTCTAG	117
4	GlnTAGnmt-tRNA-Gln chr7.trna7	TAGGACGTGGTGTAGTAGGTAGCATGGAGAATG*cta*A ATTCTCAGGGGTAGGTTCAATTCCTATAGTTCTAG	118
5	GlnTAGnmt-tRNA-Gln chr2.trna24	TAGGACATGGTGTAATAGGTAGAATGGAGAATT*cta*A ATTCTCAGGGGTAGGTTCAATTCCTATAGTTCTAG	119
6	GlnTAGnmt-tRNA-Gln	TAGGATGTGGTGTATTAGGTAGCACAGAGAATT*cta*G	120

	tRNAscan-SE ID	Sequence	SEQ ID NO
	chr3.trna7	ATTCTCAGGGGTAGGTTCGATTCCTATAATTCTAC	
7	GlnTAGnmt-tRNA-Gln chr16.trna15	TAGGACTTGGTGTAATGGGTAGCACAGAGAATT*cta*G ATTCTCAGGGGTGGGTTCAATTCCTTTCGTCCTAG	121
8	GlnTAGnmt-tRNA-Gln chr12.trna15	TCTAGGAtgTGGTGTGATAGGTAGCATGGAGAATT*ct a*GATTCTCAGGGGTAGGTTCAATTCCTATaTTCTAGA A	122
9	GlnTAGnmt-tRNA-Gln chr2.trna21	TAGGACGTGGTGTGATAGGTAGCATGGAGAATT*cta*G ATTCTCAGGGATGGGTTCAATTCCTATAGTCCTAG	123
10	GlnTAGnmt-tRNA-Glnchr2.trna9	TAGGACGTGGTGTGATAGGTAGCACGGAGAATT*cta*G ATTCTCAGGGATGGGTTCAATTCCTGTAGTTCTAG	124
11	GlnTAGchr6.trna1	GGTTCCATGGTGTAATGGTtAGCACTCTGGACT*cta*A ATCCAGCGaTCCGAGTTCAAATCTCGGTGGAACCT	125
12	GlnTAGchr1.trna104	GGTTCCATGGTGTAATGGTgACCACTTTGGACT*cta*A ATACAGTGATCAGAGTTCAAGTCTCACTGGAACCT	126
13	GlnTAGchr1.trna28	GGTTCCATGGTGTAATGGTgAGGGCTTTGGACT*cta*A CTACAGTGaTCAGAGTTCAAGTCTCAGTGGGACCT	127
14	**GlnTAGchr12.trna3**	GGTTCCATGGTGTAATGGTaAGCACCCTGGACT*cta*A ATCCAGCAaCCAGAGTTCCAGTCTCAGCGtGGACCT	128
15	**GlnTAGchr5.trna23**	GGTAGTGTAGTCTACTGGTTAAACGCTTGGgCT*cta*A CATTAAcGtCCTGGGTTCAAATCCCAGCTTTGTCA	129
16	**GlnTAGchr6.trna147**	GGTTCCATGGTGTAATGGTtAGCACTCTGGACT*cta*A ATCCAGCGaTCCGAGTTCAAGTCTCGGTGGAACCT	130
17	**GlnTAGchr1.trna17**	GGTTCCATGGTGTAATGGTgAGCACTCTGGACT*cta*A ATCCAGCGaTCCGAGTTCGAGTCTCGGTGGAACCT	131
18	**GlnTAGchr1.trna101**	GGTTCCATGGTGTAATGGTaAGCACTCTGGACT*cta*A ATCCAGCGaTCCGAGTTCGAGTCTCGGTGGAACCT	132
19	**GlnTAGchr6.trna42**	GGTTCCATGGTGTAATGGTtAGCACTCTGGACT*cta*A ATCCGGTAaTCCGAGTTCAAATCTCGGTGGAACCT	133
20	**GlnTAGchr6.trna132**	GGCCCCATGGTGTAATGGTcAGCACTCTGGACT*cta*A ATCCAGCGaTCCGAGTTCAAATCTCGGTGGGACCC	134
21	**GlnTAGchr1.trna23**	GGTTCCATGGTGTAATGGTaAGCACTCTGGACT*cta*A ATCCAGCCATCTGAGTTCGAGTCTCTGTGGAACCT	135
22	**GlnTAGchr1.trna111**	GGTTCCATGGTGTAATGGTgAGCACTTTGGACT*cta*A ATACAGTGATCAGAGTTCAAGTCTCACTGGGACCT	136
23	**GlnTAGchr1.trna24**	GGTTCCATGgGTTAATGGTgAGCACCCTGGACT*cta*A ATCAAGCGaTCCGAGTTCAAATCTCGGTGGTACCT	137
24	**GlnTAGchr19.trna4**	GTTTCCATGGTGTAATGGTgAGCACTCTGGACT*cta*A ATCCAGAAATACATTCAAAGAATTAAGAACA	138
25	**GlnTAGchr17.trna14**	GGTCCCATGGTGTAATGGTtAGCACTCTGGACT*cta*A ATCCAGCGaTCCGAGTTCAAATCTCGGTGGGACCT	139
26	**GlnTAGchr6.trna63**	GGTCCCATGGTGTAATGGTtAGCACTCTGGACT*cta*A ATCCAGCAaTCCGAGTTCGAATCTCGGTGGGACCT	140
27	**GlnTAGchr6.trna175**	GGCCCCATGGTGTAATGGTtAGCACTCTGGACT*cta*A ATCCAGCGaTCCGAGTTCAAATCTCGGTGGGACCT	141

	tRNAscan-SE ID	Sequence	SEQ ID NO
28	**GlnTAGchr6.trna82**	GGTCCCATGGTGTAATGGTtAGCACTCTGGGCT*cta*A ATCCAGCAaTCCGAGTTCGAATCTTGGTGGGACCT	142
29	**GlnTAGchr2.trna26**	GGCTGTGTACCTCAGTGGGcAAGGGTATGGACT*cta*A AGCCAGACTaTTTGGGTTCAAATCCCAGCTTGGCCT	143
30	GlnTAG chr4.trna4	GACCATGTGGCCTAAGGGAaAAGACATCTCACT*cta*G GTCAGAAGAtTGAGGGTTCAAGTCCTTTCATGGTCA	144
31	GlnTAGchr8.trna10	GGTACAGTGTTAAAGGGGagaAAAATTGCTGACT*cta* AATaCAGTAGaCCTAGGTTTGAATCCTGGCTTTACCA	145
1	GlnTAAnmt-tRNA-Gln chr10.trna6	TGGTGTAATAGGTAGCACAGAGAATT*tta*GATTCTCA GGGGTAGGTTCAATTCCTAT	146
2	**GlnTAAnmt-tRNA-Gln chrX.trna1**	TAGGACATGGTGTGATAGGTAGCATGGAGAATT*tta*G ATTCTCAGGGGTAGGTTCAATTCCTACAGTTCTAG	147
3	**GlnTAAnmt-tRNA-Glnc hr7.trna32**	TAGGACGTGGTGTGATAGGTAGCATGGGGAATT*tta*G ATTCTCAGGGGTGGGTTCAATTCCTATAGTTCTAG	148
4	**GlnTAAnmt-tRNA-Gln chr7.trna7**	TAGGACGTGGTGTAGTAGGTAGCATGGAGAATG*tta*A ATTCTCAGGGGTAGGTTCAATTCCTATAGTTCTAG	149
5	GlnTAAnmt-tRNA-Gln chr2.trna24	TAGGACATGGTGTAATAGGTAGAATGGAGAATT*tta*A ATTCTCAGGGGTAGGTTCAATTCCTATAGTTCTAG	150
6	GlnTAAnmt-tRNA-Gln chr3.trna7	TAGGATGTGGTGTATTAGGTAGCACAGAGAATT*tta*G ATTCTCAGGGGTAGGTTCGATTCCTATAATTCTAC	151
7	GlnTAAnmt-tRNA-Gln chr16.trna15	TAGGACTTGGTGTAATGGGTAGCACAGAGAATT*tta*G ATTCTCAGGGGTGGGTTCAATTCCTTTCGTCCTAG	152
8	**GlnTAAnmt-tRNA-Gln chr12.trna15**	TCTAGGAtgTGGTGTGATAGGTAGCATGGAGAATT*tt a*GATTCTCAGGGGTAGGTTCAATTCCTATaTTCTAGA A	153
9	**GlnTAAnmt-tRNA-Gln chr2.trna21**	TAGGACGTGGTGTGATAGGTAGCATGGAGAATT*tta*G ATTCTCAGGGATGGGTTCAATTCCTATAGTCCTAG	154
10	**GlnTAAnmt-tRNA-Glnchr2.trna9**	TAGGACGTGGTGTGATAGGTAGCACGGAGAATT*tta*G ATTCTCAGGGATGGGTTCAATTCCTGTAGTTCTAG	155
11	**GlnTAAchr6.trna1**	GGTTCCATGGTGTAATGGTtAGCACTCTGGACT*tta*A ATCCAGCGaTCCGAGTTCAAATCTCGGTGGAACCT	156
12	GlnTAAchr1.trna104	GGTTCCATGGTGTAATGGTgACCACTTTGGACT*tta*A ATACAGTGATCAGAGTTCAAGTCTCACTGGAACCT	157
13	GlnTAAchr1.trna28	GGTTCCATGGTGTAATGGTgAGGGCTTTGGACT*tta*A CTACAGTGaTCAGAGTTCAAGTCTCAGTGGGACCT	158
14	GlnTAAchr12.trna3	GGTTCCATGGTGTAATGGTaAGCACCCTGGACT*tta*A ATCCAGCAaCCAGAGTTCCAGTCTCAGCGtGGACCT	159
15	GlnTAAchr5.trna23	GGTAGTGTAGTCTACTGGTTAAACGCTTGGgCT*tta*A CATTAAcGtCCTGGGTTCAAATCCCAGCTTTGTCA	160
16	**GlnTAAchr6.trna147**	GGTTCCATGGTGTAATGGTtAGCACTCTGGACT*tta*A ATCCAGCGaTCCGAGTTCAAGTCTCGGTGGAACCT	161
17	**GlnTAAchr1.trna17**	GGTTCCATGGTGTAATGGTgAGCACTCTGGACT*tta*A ATCCAGCGaTCCGAGTTCGAGTCTCGGTGGAACCT	162
18	**GlnTAAchr1.trna101**	GGTTCCATGGTGTAATGGTaAGCACTCTGGACT*tta*A	163

	tRNAscan-SE ID	Sequence	SEQ ID NO
		ATCCAGCGaTCCGAGTTCGAGTCTCGGTGGAACCT	
19	**GlnTAAchr6.trna42**	GGTTCCATGGTGTAATGGTtAGCACTCTGGACT*tta*A ATCCGGTAaTCCGAGTTCAAATCTCGGTGGAACCT	164
20	**GlnTAAchr6.trna132**	GGCCCCATGGTGTAATGGTcAGCACTCTGGACT*tta*A ATCCAGCGaTCCGAGTTCAAATCTCGGTGGGACCC	165
21	**GlnTAAchr1.trna23**	GGTTCCATGGTGTAATGGTaAGCACTCTGGACT*tta*A ATCCAGCCATCTGAGTTCGAGTCTCTGTGGAACCT	166
22	**GlnTAAchr1.trna111**	GGTTCCATGGTGTAATGGTgAGCACTTTGGACT*tta*A ATACAGTGATCAGAGTTCAAGTCTCACTGGGACCT	167
23	**GlnTAAchr1.trna24**	GGTTCCATGgGTTAATGGTgAGCACCCTGGACT*tta*A ATCAAGCGaTCCGAGTTCAAATCTCGGTGGTACCT	168
24	**GlnTAAchr19.trna4**	GTTTCCATGGTGTAATGGTgAGCACTCTGGACT*tta*A ATCCAGAAATACATTCAAAGAATTAAGAACA	169
25	**GlnTAAchr17.trna14**	GGTCCCATGGTGTAATGGTtAGCACTCTGGACT*tta*A ATCCAGCGaTCCGAGTTCAAATCTCGGTGGGACCT	170
26	**GlnTAAchr6.trna63**	GGTCCCATGGTGTAATGGTtAGCACTCTGGACT*tta*A ATCCAGCAaTCCGAGTTCGAATCTCGGTGGGACCT	171
27	**GlnTAAchr6.trna175**	GGCCCCATGGTGTAATGGTtAGCACTCTGGACT*tta*A ATCCAGCGaTCCGAGTTCAAATCTCGGTGGGACCT	172
28	**GlnTAAchr6.trna82**	GGTCCCATGGTGTAATGGTtAGCACTCTGGGCT*tta*A ATCCAGCAaTCCGAGTTCGAATCTTGGTGGGACCT	173
29	**GlnTAAchr2.trna26**	GGCTGTGTACCTCAGTGGGcAAGGGTATGGACT*tta*A AGCCAGACTaTTTGGGTTCAAATCCCAGCTTGGCCT	174
30	GlnTAAchr4.trna4	GACCATGTGGCCTAAGGGAaAAGACATCTCACT*tta*G GTCAGAAGAtTGAGGGTTCAAGTCCTTTCATGGTCA	175
31	**GlnTAAchr8.trna10**	GGTACAGTGTTAAAGGGGagaAAAATTGCTGACT*tta* AATaCAGTAGaCCTAGGTTTGAATCCTGGCTTTACCA	176
1	**GluTAAchr1.trna106**	TCCCTGGTGGTCTAGTGGTtAGGATTCGGCGCT*tta*A CCGCCGCGGCCCGGGTTCGATTCCCGGTCAGGGAA	177
2	**GluTAAchr1.trna55**	TCCCTGGTGGTCTAGTGGTtAGGATTCGGCGCT*tta*A CCGCCGCGGCCCGGGTTCGATTCCCGGTCAGGAAA	178
3	GluTAAchr13.trna3	CCCCTGGTGGTCTAGTGCTtAGGATTCGGTGCT*tta*A CCGCTGCTGCCTGCGTTCGATTCCCGGTCAGGGAA	179
4	GluTAAchr8.trna1	TCCTTGATGTCTAGTGGTtAGGATTTGGTGCT*tta*AC TGCAGCAGCCTGGGTTCATTTCTCAGTCAGGGAA	180
5	**GluTAAchr2.trna18**	TCCCATATGGTCTAGCGGTtAGGATTCCTGGTT*tta*A CCCAGGTGGCCCGGGTTCGACTCCCGGTATGGGAA	181
6	GluTAAchr1.trna92	TCCGTGGTGGTCTAGTGGCtAGGATTCGGCGCT*tta*A CCGCCTGCAGCTCGAGTTCGATTCCTGGTCAGGGAA	182
7	GluTAAchr14.trna15	CCCTGTGGTCTAGTGGCtAAGACTTTGTGCT*tta*ATT GCTGCAtCCTAGGTTCAATTCCCAGTCAGGGA	183
8	**GluTAAchr13.trna2**	TCCCACATGGTCTAGCGGTtAGGATTCCTGGTT*tta*A CCCAGGCGGCCCGGGTTCGACTCCCGGTGTGGGAA	184
9	**GluTAAchr1.trna5**	TCCCTGGTGGTCTAGTGGCtAGGATTCGGCGCT*tta*A	185

	tRNAscan-SE ID	Sequence	SEQ ID NO
		CCGCCGCGGCCCGGGTTCGATTCCCGGCCAGGGAA	
10	**GluTAAchr1.trna123**	TCCCTGGTGGTCTAGTGGCtAGGATTCGGCGCT*tta*A CCGCCGCGGCCCGGGTTCGATTCCCGGTCAGGGAA	186
11	GluTAAchr1.trna45	GCGTTGGTGGTGTAGTGGTgAGCACAGCTGCCT*tta*A AGCAGTTAaCGCGGGTTCGATTCCCGGGTAACGAA	187
12	GluTAAchr1.trna99	TCCTTGGTGGTCTAGTGGCtAGGATTCGGTGCTttaA CCTGTGCGGCCCGGGTTCAATTCCCGATGAAGGAA	188
13	GluTAAchr1.trna95	TGTCTGGTGGTCAAGTGGCtAGGATTTGGCGCT*tta*A CTGCCGCGGCCCGCGTTCGATTCCCGGTCAGGGAA	189
14	GluTAAchr1.trna86	TCCCTGGTGGTCTAGTGGCtAGGATTCGGCGCT*tta*A CCGCCTGCAGCTCGAGTTCGATTCCTGGTCAGGGAA	190
15	GluTAAchr2.trna16	GCAATGGTGGTTCAGTGGTAGAATTCTCGCCT*tta*AC ACAGGAGaCCCGGGTTCAATTCCTGACCCATGTA	191
1	**GluTAGchr1.trna106**	TCCCTGGTGGTCTAGTGGTtAGGATTCGGCGCT*cta*A CCGCCGCGGCCCGGGTTCGATTCCCGGTCAGGGAA	192
2	GluTAGchr1.trna55	TCCCTGGTGGTCTAGTGGTtAGGATTCGGCGCT*cta*A CCGCCGCGGCCCGGGTTCGATTCCCGGTCAGGAAA	193
3	GluTAGchr13.trna3	CCCCTGGTGGTCTAGTGCTtAGGATTCGGTGCT*cta*A CCGCTGCTGCCTGCGTTCGATTCCCGGTCAGGGAA	194
4	GluTAGchr8.trna1	TCCTTGATGTCTAGTGGTtAGGATTTGGTGCT*cta*AC TGCAGCAGCCTGGGTTCATTTCTCAGTCAGGGAA	195
5	**GluTAGchr2.trna18**	TCCCATATGGTCTAGCGGTtAGGATTCCTGGTT*cta*A CCCAGGTGGCCCGGGTTCGACTCCCGGTATGGGAA	196
6	**GluTAGchr1.trna92**	TCCGTGGTGGTCTAGTGGCtAGGATTCGGCGCT*cta*A CCGCCTGCAGCTCGAGTTCGATTCCTGGTCAGGGAA	197
7	GluTAGchr14.trna15	CCCTGTGGTCTAGTGGCtAAGACTTTGTGCT*cta*ATT GCTGCAtCCTAGGTTCAATTCCCAGTCAGGGA	198
8	**GluTAGchr13.trna2**	TCCCACATGGTCTAGCGGTtAGGATTCCTGGTT*cta*A CCCAGGCGGCCCGGGTTCGACTCCCGGTGTGGGAA	199
9	**GluTAGchr1.trna5**	TCCCTGGTGGTCTAGTGGCtAGGATTCGGCGCT*cta*A CCGCCGCGGCCCGGGTTCGATTCCCGGCCAGGGAA	200
10	**GluTAGchr1.trna123**	TCCCTGGTGGTCTAGTGGCtAGGATTCGGCGCT*cta*A CCGCCGCGGCCCGGGTTCGATTCCCGGTCAGGGAA	201
11	GluTAGchr1.trna45	GCGTTGGTGGTGTAGTGGTgAGCACAGCTGCCT*cta*A AGCAGTTAaCGCGGGTTCGATTCCCGGGTAACGAA	202
12	GluTAGchr1.trna99	TCCTTGGTGGTCTAGTGGCtAGGATTCGGTGCT*cta*A CCTGTGCGGCCCGGGTTCAATTCCCGATGAAGGAA	203
13	GluTAGchr1.trna95	TGTCTGGTGGTCAAGTGGCtAGGATTTGGCGCT*cta*A CTGCCGCGGCCCGCGTTCGATTCCCGGTCAGGGAA	204
14	**GluTAGchr1.trna86**	TCCCTGGTGGTCTAGTGGCtAGGATTCGGCGCT*cta*A CCGCCTGCAGCTCGAGTTCGATTCCTGGTCAGGGAA	205
15	GluTAGchr2.trna16	GCAATGGTGGTTCAGTGGTAGAATTCTCGCCTcta*ct* *a*ACACAGGAGaCCCGGGTTCAATTCCTGACCCATGTA	206
1	TyrTAA chr2.trna13	CCTTCAATAGTTCAGCTGGTAGAGCAGAGGACT*tta*G	207

	tRNAscan-SE ID	Sequence	SEQ ID NO
		ctacttcctcagtaggagacGTCCTTAGGtTGCTGGT TCGATTCCAGCTTGAAGGA	
2	TyrTAA chr2.trna13/nointr on	CCTTCAATAGTTCAGCTGGTAGAGCAGAGGACT*tta*G GTCCTTAGGtTGCTGGTTCGATTCCAGCTTGAAGGA	208
3	TyrTAAchr1.trna11	GGTAAAATGGCTGAGTAAGCTTTAGACT*ttaa*AATCT AAAGAGAGATTGAGCTCTCTTTTTACCA	209
4	TyrTAAchr1.trna52	GGTAAAATGACTGAGTAAGCATTAGACT*tta*AATCTA AAGaCAGAGGTCAAGACCTCTTTTTACCA	210
5	TyrTAAchr11.trna9	GGTAAAATGGCTGAGTAAGCATTAGACT*tta*AATCTA AAGaCAGAGGTCAAGGCCTCTTTTTACCA	211
6	TyrTAAchr9.trna2	GGTAAAATGGCTGAGTAAGCATTAGACT*tta*AATCTA AAGaCAGAGGTCAAGGCCTTTTTACCA	212
7	**TyrTAAchr6.trna14**	CCTTCGATAGCTCAGTTGGTAGAGCGGAGGACT*tta*G ttggctgtgtccttagacATCCTTAGGtCGCTGGTTC GAATCCGGCTCGAAGGA	213
8	**TyrTAAchr6.trna14/ nointron**	CCTTCGATAGCTCAGTTGGTAGAGCGGAGGACT*tta*G ATCCTTAGGtCGCTGGTTCGAATCCGGCTCGAAGGA	214
9	**TyrTAA chr7.trna12**	GGGGGTATAGCTCAGGGCtAGAGCTtTTTGACT*tta*G AGCAAGAGGtCCCTGGTTCAAATCCAGGTTCTCCCT	215
10	**TyrTAAchr7.trna28**	TATAGCTCAGTGGTAGAGCATTTAACT*tta*GATCAAG AGGtCCCTGGATCAACTCTGGGTG	216
11	**TyrTAAchr15.trna6**	GTCAGTGTTGCACAACGGTtaAGTGAAGAGGCT*tta*A ACCCAGACTGGATGGGTTCAATTCCCATCTCTGCCG	217
12	**TyrTAA chr2.trna2**	CCTTCGATAGCTCAGTTGGTAGAGCGGAGGACT*tta*G tggatagggcgtggcaATCCTTAGGtCGCTGGTTCGA TTCCGGCTCGAAGGA	218
13	**TyrTAAchr2.trna2/n ointron**	CCTTCGATAGCTCAGTTGGTAGAGCGGAGGACT*tta*G ATCCTTAGGtCGCTGGTTCGATTCCGGCTCGAAGGA	219
14	**TyrTAAchr6.trna16**	CCTTCGATAGCTCAGTTGGTAGAGCGGAGGACT*tta*G gctcattaagcaaggtATCCTTAGGtCGCTGGTTCGA ATCCGGCTCGGAGGA	220
15	**TyrTAAchr6.trna16/ nointron**	CCTTCGATAGCTCAGTTGGTAGAGCGGAGGACT*tta*G ATCCTTAGGtCGCTGGTTCGAATCCGGCTCGGAGGA	221
16	**TyrTAAchr14.trna19**	CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACT*tta*G attgtatagacatttgcggacATCCTTAGGtCGCTGG TTCGATTCCAGCTCGAAGGA	222
17	**TyrTAAchr14.trna19 /nointron**	CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACT*tta*G ATCCTTAGGtCGCTGGTTCGATTCCAGCTCGAAGGA	223
18	**TyrTAAchr8.trna2**	CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACT*tta*G ctacttcctcagcaggagacATCCTTAGGtCGCTGGT TCGATTCCGGCTCGAAGGA	224
19	**TyrTAAchr8.trna2/n ointron**	CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACT*tta*G ATCCTTAGGtCGCTGGTTCGATTCCGGCTCGAAGGA	225

	tRNAscan-SE ID	Sequence	SEQ ID NO
20	TyrTAAchr8.trna3	CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACT*tta*G gcgcgcgcccgtggccATCCTTAGGtCGCTGGTTCGA TTCCGGCTCGAAGGA	226
21	TyrTAAchr8.trna3/nointron	CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACT*tta*G ATCCTTAGGtCGCTGGTTCGATTCCGGCTCGAAGGA	227
22	TyrTAAchr14.trna20	CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACT*ttaa* GcctgtagaaacatttgtggacATCCTTAGGtCGCTG GTTCGATTCCGGCTCGAAGGA	228
23	TyrTAAchr14.trna20/nointron	CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACT*tta*G ATCCTTAGGtCGCTGGTTCGATTCCGGCTCGAAGGA	229
24	TyrTAAchr14.trna17	CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACTttaG attgtacagacatttgcggacATCCTTAGGtCGCTGG TTCGATTCCGGCTCGAAGGA	230
25	TyrTAAchr14.trna17/nointron	CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACT*tta*G ATCCTTAGGtCGCTGGTTCGATTCCGGCTCGAAGGA	231
26	TyrTAAchr14.trna5	CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACT*tta*G tacttaatgtgtggtcATCCTTAGGtCGCTGGTTCGA TTCCGGCTCGAAGGA	232
27	TyrTAAchr14.trna5/nointron	CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACT*tta*G ATCCTTAGGtCGCTGGTTCGATTCCGGCTCGAAGGA	233
28	TyrTAAchr6.trna17	CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACT*tta*G gggtttgaatgtggtcATCCTTAGGtCGCTGGTTCGA ATCCGGCTCGGAGGA	234
29	TyrTAAchr6.trna17/nointron	CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACT*tta*G ATCCTTAGGtCGCTGGTTCGAATCCGGCTCGGAGGA	235
30	TyrTAAchr14.trna18	CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACT*tta*G actgcggaaacgtttgtggacATCCTTAGGtCGCTGG TTCAATTCCGGCTCGAAGGA	236
31	TyrTAAchr14.trna18/nointron	CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACT*tta*G ATCCTTAGGtCGCTGGTTCAATTCCGGCTCGAAGGA	237
32	TyrTAAchr6.trna15	CTTTCGATAGCTCAGTTGGTAGAGCGGAGGACT*tta*G gttcattaaactaaggcATCCTTAGGtCGCTGGTTCG AATCCGGCTCGAAGGA	238
33	TyrTAAchr6.trna15/nointron	CTTTCGATAGCTCAGTTGGTAGAGCGGAGGACT*tta*G ATCCTTAGGtCGCTGGTTCGAATCCGGCTCGAAGGA	239
34	TyrTAAchr8.trna11	TCTTCAATAGCTCAGCTGGTAGAGCGGAGGACT*ttaa* Ggtgcacgcccgtggcc ATTCTTAGGTGCTGGTTTGA TTCCGACTTGGAGAG	240
35	TyrTAAchr8.trna11/nointron	TCTTCAATAGCTCAGCTGGTAGAGCGGAGGACT*tta*G ATTCTTAGGTGCTGGTTTGATTCCGACTTGGAGAG	241
36	TyrTAAchr1.trna127	GGTAAAATGGCTGAGTGAAGCATTGGACT*tta*AATCT AAAGaCAGGGGTTAAGCCTCTTTTTACCA	242
37	TyrTAAchr10.trna3	GGTAAAATGGCTGAGCAAGCATTGGACT*tta*AATCTA AAGaCAGATGTTGAGCCATCTTTTTAGCA	243

	tRNAscan-SE ID	Sequence	SEQ ID NO
38	TyrTAAchr14.trna8	GGTAAAATGGCTGAGTGAAGCATTGGACT*tta*AATCT AAAGaCAGGGGCTAAGCCTCTTTTTACCA	244
39	TyrTAAchr2.trna12	GGTAAAATGGCTGAGCAAGCATTAGACT*tta*AATCTA AAGaCAGAGGTTAAGGCCTCTTTTTACCA	245
40	**TyrTAAchr7.trna1**	GGTAAAATGGCTGAGTAAGCATTAGACTttaAATCTA AAGaCAGAGGTCAAGGCCTCTTTTTTCCT	246
41	TyrTAAchr7.trna2	GGTAAAATGGCTGAGCAAGCATTAGACT*tta*AATCTG AAAaCAGAGGTCAAAGgTCTCTTTTTACCA	247
42	TyrTAAchr7.trna6	GGTAAAATGGCTGAGTAAGCATTAGACT*tta*AATCTA AAGaCAGAGGTCAAGGCCTCTTTTTACCA	248
43	TyrTAAchr8.trna7	GGTAAAATGACTGAATAAGCCTTAGACT*tta*AATCTG AAGaCAGAGGTCAAGGCCTCTTTTTACCA	249
44	TyrTAAchr9.trna10	GGTAAAATGGCTGAGTAAGCATTGGACT*tta*AATCTA AAGaCAGAGGTCAAGACCTCTTTTTACCA	250
45	TyrTAAchr9.trna4	GGTAAAATGGCTGAGTAAAGCATTAGACT*tta*AATCT AAGGaCAGAGGCTAAACCTCTTTTTACCA	251
1	TyrTAGchr2.trna13	CCTTCAATAGTTCAGCTGGTAGAGCAGAGGACT*cta*G ctacttcctcagtaggagacGTCCTTAGGtTGCTGGT TCGATTCCAGCTTGAAGGA	252
2	TyrTAGchr2.trna13/ nointron	CCTTCAATAGTTCAGCTGGTAGAGCAGAGGACT*cta*G GTCCTTAGGtTGCTGGTTCGATTCCAGCTTGAAGGA	253
3	TyrTAGchr1.trna11	GGTAAAATGGCTGAGTAAGCTTTAGACT*cta*aAATCT AAAGAGAGATTGAGCTCTCTTTTTACCA	254
4	**TyrTAGchr1.trna52**	GGTAAAATGACTGAGTAAGCATTAGACT*cta*AATCTA AAGaCAGAGGTCAAGACCTCTTTTTACCA	255
5	**TyrTAGchr11.trna9**	GGTAAAATGGCTGAGTAAGCATTAGACT*cta*AATCTA AAGaCAGAGGTCAAGGCCTCTTTTTACCA	256
6	TyrTAGchr9.trna2	GGTAAAATGGCTGAGTAAGCATTAGACT*cta*AATCTA AAGaCAGAGGTCAAGGCCTTTTTACCA	257
7	**TyrTAGchr6.trna14**	CCTTCGATAGCTCAGTTGGTAGAGCGGAGGACT*cta*G ttggctgtgtccttagacATCCTTAGGtCGCTGGTTC GAATCCGGCTCGAAGGA	258
8	**TyrTAGchr6.trna14/ nointron**	CCTTCGATAGCTCAGTTGGTAGAGCGGAGGACT*cta*G ATCCTTAGGtCGCTGGTTCGAATCCGGCTCGAAGGA	259
9	**TyrTAG chr7.trna12**	GGGGGTATAGCTCAGGGCtAGAGCTtTTTGACT*cta*a GAGCAAGAGGtCCCTGGTTCAAATCCAGGTTCTCCCT	260
10	**TyrTAGchr7.trna28**	TATAGCTCAGTGGTAGAGCATTTAACT*cta*GATCAAG AGGtCCCTGGATCAACTCTGGGTG	261
11	**TyrTAGchr15.trna6**	GTCAGTGTTGCACAACGGTtaAGTGAAGAGGCT*cta*A ACCCAGACTGGATGGGTTCAATTCCCATCTCTGCCG	262
12	**TyrTAG chr2.trna2**	CCTTCGATAGCTCAGTTGGTAGAGCGGAGGACT*cta*G tggatagggcgtggcaATCCTTAGGtCGCTGGTTCGA TTCCGGCTCGAAGGA	263
13	**TyrTAGchr2.trna2/n**	CCTTCGATAGCTCAGTTGGTAGAGCGGAGGACT*cta*G	264

	tRNAscan-SE ID	Sequence	SEQ ID NO
	ointron	ATCCTTAGGtCGCTGGTTCGATTCCGGCTCGAAGGA	
14	TyrTAGchr6.trna16	CCTTCGATAGCTCAGTTGGTAGAGCGGAGGACT*cta*G gctcattaagcaaggtATCCTTAGGtCGCTGGTTCGA ATCCGGCTCGGAGGA	265
15	TyrTAGchr6.trna16/ nointron	CCTTCGATAGCTCAGTTGGTAGAGCGGAGGACT*cta*G ATCCTTAGGtCGCTGGTTCGAATCCGGCTCGGAGGA	266
16	TyrTAGchr14.trna19	CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACT*cta*G attgtatagacatttgcggacATCCTTAGGtCGCTGG TTCGATTCCAGCTCGAAGGA	267
17	TyrTAG chr14.trna19/noint ron	CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACT*cta*G ATCCTTAGGtCGCTGGTTCGATTCCAGCTCGAAGGA	268
18	TyrTAGchr8.trna2	CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACT*cta*G ctacttcctcagcaggagacATCCTTAGGtCGCTGGT TCGATTCCGGCTCGAAGGA	269
19	TyrTAGchr8.trna2/n ointron	CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACT*cta*G ATCCTTAGGtCGCTGGTTCGATTCCGGCTCGAAGGA	270
20	TyrTAGchr8.trna3	CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACT*cta*G gcgcgcgcccgtggccATCCTTAGGtCGCTGGTTCGA TTCCGGCTCGAAGGA	271
21	TyrTAGchr8.trna3/n ointron	CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACT*cta*G ATCCTTAGGtCGCTGGTTCGATTCCGGCTCGAAGGA	272
22	TyrTAGchr14.trna20	CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACT*cta*G cctgtagaaacatttgtggacATCCTTAGGtCGCTGG TTCGATTCCGGCTCGAAGGA	273
23	TyrTAGchr14.trna20 /nointron	CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACT*cta*G ATCCTTAGGtCGCTGGTTCGATTCCGGCTCGAAGGA	274
24	TyrTAGchr14.trna17	CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACT*cta*G attgtacagacatttgcggacATCCTTAGGtCGCTGG TTCGATTCCGGCTCGAAGGA	275
25	TyrTAGchr14.trna17 /nointron	CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACT*cta*G ATCCTTAGGtCGCTGGTTCGATTCCGGCTCGAAGGA	276
26	TyrTAGchr14.trna5	CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACT*cta*G tacttaatgtgtggtcATCCTTAGGtCGCTGGTTCGA TTCCGGCTCGAAGGA	277
27	TyrTAGchr14.trna5/ nointron	CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACT*cta*G ATCCTTAGGtCGCTGGTTCGATTCCGGCTCGAAGGA	278
28	TyrTAGchr6.trna17	CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACT*cta*G gggtttgaatgtggtcATCCTTAGGtCGCTGGTTCGA ATCCGGCTCGGAGGA	279
29	TyrTAGchr6.trna17/ nointron	CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACT*cta*G ATCCTTAGGtCGCTGGTTCGAATCCGGCTCGGAGGA	280
30	TyrTAGchr14.trna18	CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACT*cta*G actgcggaaacgtttgtggacATCCTTAGGtCGCTGG	281

	tRNAscan-SE ID	Sequence	SEQ ID NO
		TTCAATTCCGGCTCGAAGGA	
31	**TyrTAGchr14.trna18 /nointron**	CCTTCGATAGCTCAGCTGGTAGAGCGGAGGACT*cta*G ATCCTTAGGtCGCTGGTTCAATTCCGGCTCGAAGGA	282
32	**TyrTAGchr6.trna15**	CTTTCGATAGCTCAGTTGGTAGAGCGGAGGACT*cta*G gttcattaaactaaggcATCCTTAGGtCGCTGGTTCG AATCCGGCTCGAAGGA	283
33	**TyrTAGchr6.trna15/ nointron**	CTTTCGATAGCTCAGTTGGTAGAGCGGAGGACT*cta*G ATCCTTAGGtCGCTGGTTCGAATCCGGCTCGAAGGA	284
34	**TyrTAGchr8.trna11**	TCTTCAATAGCTCAGCTGGTAGAGCGGAGGACT*cta*G gtgcacgcccgtggccATTCTTAGGTGCTGGTTTGAT TCCGACTTGGAGAG	285
35	TyrTAGchr8.trna11/ nointron	TCTTCAATAGCTCAGCTGGTAGAGCGGAGGACT*cta*G ATTCTTAGGTGCTGGTTTGATTCCGACTTGGAGAG	286
36	TyrTAGchr1.trna127	GGTAAAATGGCTGAGTGAAGCATTGGACT*cta*AATCT AAAGaCAGGGGTTAAGCCTCTTTTTACCA	287
37	TyrTAGchr10.trna3	GGTAAAATGGCTGAGCAAGCATTGGACT*cta*AATCTA AAGaCAGATGTTGAGCCATCTTTTTAGCA	288
38	TyrTAGchr14.trna8	GGTAAAATGGCTGAGTGAAGCATTGGACT*cta*AATCT AAAGaCAGGGGCTAAGCCTCTTTTTACCA	289
39	TyrTAGchr2.trna12	GGTAAAATGGCTGAGCAAGCATTAGACT*cta*AATCTA AAGaCAGAGGTTAAGGCCTCTTTTTACCA	290
40	TyrTAGchr7.trna1	GGTAAAATGGCTGAGTAAGCATTAGACT*cta*AATCTA AAGaCAGAGGTCAAGGCCTCTTTTTTCCT	291
41	TyrTAGchr7.trna2	GGTAAAATGGCTGAGCAAGCATTAGACT*cta*AATCTG AAAaCAGAGGTCAAAGgTCTCTTTTTACCA	292
42	TyrTAGchr7.trna6	GGTAAAATGGCTGAGTAAGCATTAGACT*cta*AATCTA AAGaCAGAGGTCAAGGCCTCTTTTTACCA	293
43	TyrTAGchr8.trna7	GGTAAAATGACTGAATAAGCCTTAGACT*cta*AATCTG AAGaCAGAGGTCAAGGCCTCTTTTTACCA	294
44	TyrTAGchr9.trna10	GGTAAAATGGCTGAGTAAGCATTGGACT*cta*AATCTA AAGaCAGAGGTCAAGACCTCTTTTTACCA	295
45	TyrTAGchr9.trna4	GGTAAAATGGCTGAGTAAAGCATTAGACT*cta*AATCT AAGGaCAGAGGCTAAACCTCTTTTTACCA	296
1	LeuTAAchr4.trna2	GTTAAGATGGCAGAGCCtGGTaATTGCA*tta*AACTTA AAATTTTATAAtCAGAGGTTCAACTCCTCTTCTTAAC A	297
2	LeuTAAnmtchrX.trna 2	GTTAAGATGGCAGAGCCcGGCaATTGCA*tta*GACTTA AAACTTTATAAtCAGAGGTTCAACTCCTCTCATTAAC A	298
3	**LeuTAAchr6.trna77**	GGTAGCGTGGCCGAGCGGTctAAGGCGCTGGATT*tta* GCTCCAGTCTCTTCGGGGGCGTGGGTTCAAATCCCAC CGCTGCCA	299
4	LeuTAAchr6.trna127	GGTAGCGTGGCCGAGTGGTctAAGACGCTGGATT*tta* GCTCCAGTCTCTTCGGGGGCGTGGGTTTGAATCCCAC	300

	tRNAscan-SE ID	Sequence	SEQ ID NO
		CGCTGCCA	
5	LeuTAAchr2.trna4	GGGCCAGTGGCTCAATGGAtAATGCGTCTGACT*tta*A ATCAGAAGAtTCCAGCCTTGACTCCTGGCTGGCTCA	301
6	LeuTAAchr20.trna1	GGTAGGGTGGCCGAGCGGTctAAGGCACTGTATT*tta* ACTCCAGTCTCTTCAGAGGCATGGGTTTGAATCCCAC TGCTGCCA	302
7	LeuTAAchr5.trna20	GCCGAGCGGTctAAGGCTCCGGATT*tta*GCGCCGGTG TCTTCGGAGgCATGGGTTCGAATTCCAC	303
8	LeuTAAchr6.trna100	GTCAGGATGGCCGAGTGGTctAAGGCGCCAGACT*tta* GctaagcttcctccgcggtggggaTTCTGGTCTCCAA TGGAGGCGTGGGTTCGAATCCCACTTCTGACA	304
9	**LeuTAAchr6.trna100 /nointron**	GTCAGGATGGCCGAGTGGTctAAGGCGCCAGACT*tta* GTTCTGGTCTCCAATGGAGGCGTGGGTTCGAATCCCA CTTCTGACA	305
10	**LeuTAAchr6.trna73**	GTCAGGATGGCCGAGTGGTctAAGGCGCCAGACT*tta* GcttggcttcctcgtgttgaggaTTCTGGTCTCCAAT GGAGGCGTGGGTTCGAATCCCACTTCTGACA	306
11	**LeuTAAchr6.trna73/ nointron**	GTCAGGATGGCCGAGTGGTctAAGGCGCCAGACT*tta* GTTCTGGTCTCCAATGGAGGCGTGGGTTCGAATCCCA CTTCTGACA	307
12	**LeuTAAchr6.trna141**	GTCAGGATGGCCGAGTGGTctAAGGCGCCAGACT*tta* GcttactgcttcctgtgttcgggtcTTCTGGTCTCCG TATGGAGGCGTGGGTTCGAATCCCACTTCTGACA	308
13	**LeuTAAchr6.trna141 /nointron**	GTCAGGATGGCCGAGTGGTctAAGGCGCCAGACT*tta* GTTCTGGTCTCCGTATGGAGGCGTGGGTTCGAATCCC ACTTCTGACA	309
14	**LeuTAAchr6.trna142**	GTCAGGATGGCCGAGTGGTctAAGGCGCCAGACT*tta* GttgctacttcccaggtttggggcTTCTGGTCTCCGC ATGGAGGCGTGGGTTCGAATCCCACTTCTGACA	310
15	**LeuTAAchr6.trna142 /nointron**	GTCAGGATGGCCGAGTGGTctAAGGCGCCAGACT*tta* GTTCTGGTCTCCGCATGGAGGCGTGGGTTCGAATCCC ACTTCTGACA	311
16	**LeuTAAchr1.trna54**	GTCAGGATGGCCGAGTGGTctAAGGCGCCAGACT*tta* GgtaagcaccttgcctgcgggctTTCTGGTCTCCGGA TGGAGGCGTGGGTTCGAATCCCACTTCTGACA	312
17	LeuTAAchr1.trna54/ nointron	GTCAGGATGGCCGAGTGGTctAAGGCGCCAGACT*tta* GtTTCTGGTCTCCGGATGGAGGCGTGGGTTCGAATCC CACTTCTGACA	313
18	**LeuTAAchr11.trna1**	GCCTCCTTAGTGCAGTAGGTAGCGCATCAGTCT*tta*A ATCTGAATGgtCCTGAGTTCAAGCCTCAGAGGGGGCA	314
19	LeuTAAchr1.trna59	GTCAGGATGGCCGAGCAGTcttAAGGCGCTGCGTT*tt a*ATCGCACCCTCCGCTGGAGGCGTGGGTTCGAATCCC ACTTTTGACA	315
20	LeuTAAchr9.trna3	GGTTCCATGGTGTAATGGTgAGCACTCTGGACT*tta*A	316

	tRNAscan-SE ID	Sequence	SEQ ID NO
		ATCCAGAAGtAGTgCTGGAACAA	
21	LeuTAAchr9.trna7	GTCAGGGTGGCTGAGCAGTctGAGGGGCTGCGTT*tta*GTCGCAGTCTGCCCTGGAGGCGTGGGTTCGAATCCCACTCCTGAAA	317
22	**LeuTAAchr6.trna81**	ACCAGGATGGCCGAGTGGTtAAGGCGTTGGACT*tta*GATCCAATGGACATATGTCCGCGTGGGTTCGAACCCCACTCCTGGTA	318
23	**LeuTAAchr6.trna135**	ACCGGGATGGCCGAGTGGTtAAGGCGTTGGACT*tta*GATCCAATGGGCTGGTGCCCGCGTGGGTTCGAACCCCACTCTCGGTA	319
24	**LeuTAAchr11.trna4**	ACCAGAATGGCCGAGTGGTtAAGGCGTTGGACT*tta*GATCCAATGGATTCATATCCGCGTGGGTTCGAACCCCACTTCTGGTA	320
25	**LeuTAAchr6.trna156**	ACCGGGATGGCTGAGTGGTtAAGGCGTTGGACT*tta*GATCCAATGGACAGGTGTCCGCGTGGGTTCGAGCCCCACTCCCGGTA	321
26	**LeuTAAchr6.trna79**	ACTCATTTGGCTGAGTGGTtAAGGCATTGGACT*tta*GATCCAATGGAGTAGTGGCTGTGTGGGTTTAAACCCCACTACTGGTA	322
27	**LeuTAAchr1.trna9**	GAGAAAGTcATCGTAGTTACGAAGTTGGCT*tta*ACCCAGTTTtGGGAGGTTCAATTCCTTCCTTTCTCT	323
28	**LeuTAAchr11.trna12**	ACCAGGATGGCCAAGTAGTTaAAGGCACTGGACT*tta*GAGCCAATGGACATATGTCTGTGTGGGTTTGAACCCCACTCCTGGTG	324
29	**LeuTAAchr17.trna42**	GGTAGCGTGGCCGAGCGGTctAAGGCGCTGGATT*tta*GCTCCAGTCTCTTCGGAGGCGTGGGTTCGAATCCCACCGCTGCCA	325
30	**LeuTAAchr14.trna2**	GGTAGTGTGGCCGAGCGGTctAAGGCGCTGGATT*tta*GCTCCAGTCTCTTCGGGGGCGTGGGTTCGAATCCCACCACTGCCA	326
31	**LeuTAAchr16.trna27**	GGTAGCGTGGCCGAGTGGTctAAGGCGCTGGATT*tta*GCTCCAGTCATTTCGATGgCGTGGGTTCGAATCCCACCGCTGCCA	327
32	**LeuTAAchr14.trna16**	GGTAGTGTGGTTGAATGGTctAAGGCACTGAATT*tta*GCTCCAGTCTCTTTGGGGaCGTGGGTTTAAATCCCACTGCTGCAA	328
1	**LeuTAGchr4.trna2**	GTTAAGATGGCAGAGCCtGGTaATTGCA*cta*AACTTAAAATTTTATAAtCAGAGGTTCAACTCCTCTTCTTAACA	329
2	**LeuTAGnmtchrX.trna2**	GTTAAGATGGCAGAGCCcGGCaATTGCA*cta*GACTTAAAACTTTATAAtCAGAGGTTCAACTCCTCTCATTAACA	330
3	**LeuTAGchr6.trna77**	GGTAGCGTGGCCGAGCGGTctAAGGCGCTGGATT*cta*GCTCCAGTCTCTTCGGGGGCGTGGGTTCAAATCCCAC	331

	tRNAscan-SE ID	Sequence	SEQ ID NO
		CGCTGCCA	
4	**LeuTAGchr6.trna127**	GGTAGCGTGGCCGAGTGGTctAAGACGCTGGATT*cta*GCTCCAGTCTCTTCGGGGGCGTGGGTTTGAATCCCACCGCTGCCA	332
5	LeuTAGchr2.trna4	GGGCCAGTGGCTCAATGGAtAATGCGTCTGACT*cta*AATCAGAAGAtTCCAGCCTTGACTCCTGGCTGGCTCA	333
6	LeuTAGchr20.trna1	GGTAGGGTGGCCGAGCGGTctAAGGCACTGTATT*cta*ACTCCAGTCTCTTCAGAGGCATGGGTTTGAATCCCACTGCTGCCA	334
7	**LeuTAGchr5.trna20**	GCCGAGCGGTctAAGGCTCCGGATT*cta*GCGCCGGTGTCTTCGGAGgCATGGGTTCGAATTCCAC	335
8	**LeuTAGchr6.trna100**	GTCAGGATGGCCGAGTGGTctAAGGCGCCAGACT*cta*GctaagcttcctccgcggtggggaTTCTGGTCTCCAATGGAGGCGTGGGTTCGAATCCCACTTCTGACA	336
9	**LeuTAGchr6.trna100 /nointron**	GTCAGGATGGCCGAGTGGTctAAGGCGCCAGACT*cta*GTTCTGGTCTCCAATGGAGGCGTGGGTTCGAATCCCACTTCTGACA	337
10	**LeuTAGchr6.trna73**	GTCAGGATGGCCGAGTGGTctAAGGCGCCAGACT*cta*GcttggcttcctcgtgttgaggaTTCTGGTCTCCAATGGAGGCGTGGGTTCGAATCCCACTTCTGACA	338
11	**LeuTAGchr6.trna73/ nointron**	GTCAGGATGGCCGAGTGGTctAAGGCGCCAGACT*cta*GTTCTGGTCTCCAATGGAGGCGTGGGTTCGAATCCCACTTCTGACA	339
12	**LeuTAGchr6.trna141**	GTCAGGATGGCCGAGTGGTctAAGGCGCCAGACT*cta*GcttactgcttcctgtgttcgggtcTTCTGGTCTCCGTATGGAGGCGTGGGTTCGAATCCCACTTCTGACA	340
13	**LeuTAGchr6.trna141 /nointron**	GTCAGGATGGCCGAGTGGTctAAGGCGCCAGACT*cta*GTTCTGGTCTCCGTATGGAGGCGTGGGTTCGAATCCCACTTCTGACA	341
14	**LeuTAGchr6.trna142**	GTCAGGATGGCCGAGTGGTctAAGGCGCCAGACT*cta*GttgctacttcccaggtttggggcTTCTGGTCTCCGCATGGAGGCGTGGGTTCGAATCCCACTTCTGACA	342
15	**LeuTAGchr6.trna142 /nointron**	GTCAGGATGGCCGAGTGGTctAAGGCGCCAGACT*cta*GTTCTGGTCTCCGCATGGAGGCGTGGGTTCGAATCCCACTTCTGACA	343
16	**LeuTAGchr1.trna54**	GTCAGGATGGCCGAGTGGTctAAGGCGCCAGACT*cta*Ggtaagcaccttgcctgcgggct TTCTGGTCTCCGGATGGAGGCGTGGGTTCGAATCCCACTTCTGACA	344
17	LeuTAGchr1.trna54/ nointron	GTCAGGATGGCCGAGTGGTctAAGGCGCCAGACT*cta*GtTTCTGGTCTCCGGATGGAGGCGTGGGTTCGAATCCCACTTCTGACA	345
18	**LeuTAGchr11.trna1**	GCCTCCTTAGTGCAGTAGGTAGCGCATCAGTCT*cta*AATCTGAATGgtCCTGAGTTCAAGCCTCAGAGGGGGCA	346
19	LeuTAGchr1.trna59	GTCAGGATGGCCGAGCAGTcttAAGGCGCTGCGTT*ct*	347

	tRNAscan-SE ID	Sequence	SEQ ID NO
		*a*ATCGCACCCTCCGCTGGAGGCGTGGGTTCGAATCCC ACTTTTGACA	
20	LeuTAGchr9.trna3	GGTTCCATGGTGTAATGGTgAGCACTCTGGACT*cta*A ATCCAGAAGtAGTgCTGGAACAA	348
21	LeuTAGchr9.trna7	GTCAGGGTGGCTGAGCAGTctGAGGGGCTGCGTT*cta* GTCGCAGTCTGCCCTGGAGGCGTGGGTTCGAATCCCA CTCCTGAAA	349
22	**LeuTAGchr6.trna81**	ACCAGGATGGCCGAGTGGTtAAGGCGTTGGACT*cta*G ATCCAATGGACATATGTCCGCGTGGGTTCGAACCCCA CTCCTGGTA	350
23	**LeuTAGchr6.trna135**	ACCGGGATGGCCGAGTGGTtAAGGCGTTGGACT*cta*G ATCCAATGGGCTGGTGCCCGCGTGGGTTCGAACCCCA CTCTCGGTA	351
24	**LeuTAGchr11.trna4**	ACCAGAATGGCCGAGTGGTtAAGGCGTTGGACT*cta*G ATCCAATGGATTCATATCCGCGTGGGTTCGAACCCCA CTTCTGGTA	352
25	**LeuTAGchr6.trna156**	ACCGGGATGGCTGAGTGGTtAAGGCGTTGGACT*cta*G ATCCAATGGACAGGTGTCCGCGTGGGTTCGAGCCCCA CTCCCGGTA	353
26	**LeuTAGchr6.trna79**	ACTCATTTGGCTGAGTGGTtAAGGCATTGGACT*ctaa* GATCCAATGGAGTAGTGGCTGTGTGGGTTTAAACCCC ACTACTGGTA	354
27	LeuTAGchr1.trna9	GAGAAAGTcATCGTAGTTACGAAGTTGGCT*cta*ACCC AGTTTtGGGAGGTTCAATTCCTTCCTTTCTCT	355
28	LeuTAGchr11.trna12	ACCAGGATGGCCAAGTAGTTaAAGGCACTGGACT*cta* GAGCCAATGGACATATGTCTGTGTGGGTTTGAACCCC ACTCCTGGTG	356
29	**LeuTAGchr17.trna42**	GGTAGCGTGGCCGAGCGGTctAAGGCGCTGGATT*cta* GCTCCAGTCTCTTCGGAGGCGTGGGTTCGAATCCCAC CGCTGCCA	357
30	**LeuTAGchr14.trna2**	GGTAGTGTGGCCGAGCGGTctAAGGCGCTGGATT*cta* GCTCCAGTCTCTTCGGGGGCGTGGGTTCGAATCCCAC CACTGCCA	358
31	**LeuTAGchr16.trna27**	GGTAGCGTGGCCGAGTGGTctAAGGCGCTGGATT*cta* GCTCCAGTCATTTCGATGgCGTGGGTTCGAATCCCAC CGCTGCCA	359
32	**LeuTAGchr14.trna16**	GGTAGTGTGGTTGAATGGTctAAGGCACTGAATT*cta* GCTCCAGTCTCTTTGGGGaCGTGGGTTTAAATCCCAC TGCTGCAA	360
1	LeuTGAchr4.trna2	GTTAAGATGGCAGAGCCtGGTaATTGCA*tca*AACTTA AAATTTTATAAtCAGAGGTTCAACTCCTCTTCTTAAC A	523
2	LeuTGAnmtchrX.trna 2	GTTAAGATGGCAGAGCCcGGCaATTGCA*tca*GACTTA AAACTTTATAAtCAGAGGTTCAACTCCTCTCATTAAC	524

	tRNAscan-SE ID	Sequence	SEQ ID NO
		A	
3	**LeuTGAchr6.trna77**	GGTAGCGTGGCCGAGCGGTctAAGGCGCTGGATT*tca*GCTCCAGTCTCTTCGGGGGCGTGGGTTCAAATCCCACCGCTGCCA	525
4	LeuTGAchr6.trna127	GGTAGCGTGGCCGAGTGGTctAAGACGCTGGATT*tca*GCTCCAGTCTCTTCGGGGGCGTGGGTTTGAATCCCACCGCTGCCA	526
5	LeuTGAchr2.trna4	GGGCCAGTGGCTCAATGGAtAATGCGTCTGACT*tca*AATCAGAAGAtTCCAGCCTTGACTCCTGGCTGGCTCA	527
6	LeuTGAchr20.trna1	GGTAGGGTGGCCGAGCGGTctAAGGCACTGTATT*tca*ACTCCAGTCTCTTCAGAGGCATGGGTTTGAATCCCACTGCTGCCA	528
7	LeuTGAchr5.trna20	GCCGAGCGGTctAAGGCTCCGGATT*tca*GCGCCGGTGTCTTCGGAGgCATGGGTTCGAATTCCAC	529
8	**LeuTGAchr6.trna100**	GTCAGGATGGCCGAGTGGTctAAGGCGCCAGACT*tca*GctaagcttcctccgcggtggggaTTCTGGTCTCCAATGGAGGCGTGGGTTCGAATCCCACTTCTGACA	530 531
9	**LeuTGAchr6.trna100/nointron**	GTCAGGATGGCCGAGTGGTctAAGGCGCCAGACT*tca*GTTCTGGTCTCCAATGGAGGCGTGGGTTCGAATCCCACTTCTGACA	532
10	**LeuTGAchr6.trna73**	GTCAGGATGGCCGAGTGGTctAAGGCGCCAGACT*tca*GcttggcttcctcgtgttgaggaTTCTGGTCTCCAATGGAGGCGTGGGTTCGAATCCCACTTCTGACA	533
11	**LeuTGAchr6.trna73/nointron**	GTCAGGATGGCCGAGTGGTctAAGGCGCCAGACT*tca*GTTCTGGTCTCCAATGGAGGCGTGGGTTCGAATCCCACTTCTGACA	534
12	**LeuTGAchr6.trna141**	GTCAGGATGGCCGAGTGGTctAAGGCGCCAGACT*tca*GcttactgcttcctgtgttcgggtcTTCTGGTCTCCGTATGGAGGCGTGGGTTCGAATCCCACTTCTGACA	535
13	**LeuTGAchr6.trna141/nointron**	GTCAGGATGGCCGAGTGGTctAAGGCGCCAGACT*tca*GTTCTGGTCTCCGTATGGAGGCGTGGGTTCGAATCCCACTTCTGACA	536
14	**LeuTGAchr6.trna142**	GTCAGGATGGCCGAGTGGTctAAGGCGCCAGACT*tca*GttgctacttcccaggtttggggcTTCTGGTCTCCGCATGGAGGCGTGGGTTCGAATCCCACTTCTGACA	537
15	**LeuTGAchr6.trna142/nointron**	GTCAGGATGGCCGAGTGGTctAAGGCGCCAGACT*tca*GTTCTGGTCTCCGCATGGAGGCGTGGGTTCGAATCCCACTTCTGACA	538
16	**LeuTGAchr1.trna54**	GTCAGGATGGCCGAGTGGTctAAGGCGCCAGACT*tca*Ggtaagcaccttgcctgcgggct TTCTGGTCTCCGGATGGAGGCGTGGGTTCGAATCCCACTTCTGACA	539
17	LeuTGAchr1.trna54/nointron	GTCAGGATGGCCGAGTGGTctAAGGCGCCAGACT*tca*GtTTCTGGTCTCCGGATGGAGGCGTGGGTTCGAATCC	540

	tRNAscan-SE ID	Sequence	SEQ ID NO
		CACTTCTGACA	
18	LeuTGAchr11.trna1	GCCTCCTTAGTGCAGTAGGTAGCGCATCAGTCT*tca*A ATCTGAATGgtCCTGAGTTCAAGCCTCAGAGGGGGCA	541
19	LeuTGAchr1.trna59	GTCAGGATGGCCGAGCAGTcttAAGGCGCTGCGTT*tc a*ATCGCACCCTCCGCTGGAGGCGTGGGTTCGAATCCC ACTTTTGACA	542
20	LeuTGAchr9.trna3	GGTTCCATGGTGTAATGGTgAGCACTCTGGACT*tca*A ATCCAGAAGtAGTgCTGGAACAA	543
21	LeuTGAchr9.trna7	GTCAGGGTGGCTGAGCAGTctGAGGGGCTGCGTT*tca* GTCGCAGTCTGCCCTGGAGGCGTGGGTTCGAATCCCA CTCCTGAAA	544
22	**LeuTGAchr6.trna81**	ACCAGGATGGCCGAGTGGTtAAGGCGTTGGACT*tca*GATC CAATGGACATATGTCCGCGTGGGTTCGAACCCCACTCCTG GTA	545
23	**LeuTGAchr6.trna135**	ACCGGGATGGCCGAGTGGTtAAGGCGTTGGACT*tca*GATC CAATGGGCTGGTGCCCGCGTGGGTTCGAACCCCACTCTCG GTA	546 547
24	**LeuTGAchr11.trna4**	ACCAGAATGGCCGAGTGGTtAAGGCGTTGGACT*tca*GATC CAATGGATTCATATCCGCGTGGGTTCGAACCCCACTTCTG GTA	548
25	**LeuTGAchr6.trna156**	ACCGGGATGGCTGAGTGGTtAAGGCGTTGGACT*tca*GATC CAATGGACAGGTGTCCGCGTGGGTTCGAGCCCCACTCCCG GTA	549
26	**LeuTGAchr6.trna79**	ACTCATTTGGCTGAGTGGTtAAGGCATTGGACT*tca*GATC CAATGGAGTAGTGGCTGTGTGGGTTTAAACCCCACTACTG GTA	550
27	LeuTGAchr1.trna9	GAGAAAGTcATCGTAGTTACGAAGTTGGCT*tca*ACCCAGT TTtGGGAGGTTCAATTCCTTCCTTTCTCT	551
28	LeuTGAchr11.trna12	ACCAGGATGGCCAAGTAGTTaAAGGCACTGGACT*tca*GAG CCAATGGACATATGTCTGTGTGGGTTTGAACCCCACTCCT GGTG	552
29	**LeuTGAchr17.trna42**	GGTAGCGTGGCCGAGCGGTctAAGGCGCTGGATT*tca*GCT CCAGTCTCTTCGGAGGCGTGGGTTCGAATCCCACCGCTGC CA	553
30	**LeuTGAchr14.trna2**	GGTAGTGTGGCCGAGCGGTctAAGGCGCTGGATT*tca*GCT CCAGTCTCTTCGGGGGCGTGGGTTCGAATCCCACCACTGC CA	554
31	**LeuTGAchr16.trna27**	GGTAGCGTGGCCGAGTGGTctAAGGCGCTGGATT*tca*GCT CCAGTCATTTCGATGgCGTGGGTTCGAATCCCACCGCTGC CA	555
32	LeuTGAchr14.trna16	GGTAGTGTGGTTGAATGGTctAAGGCACTGAATT*tca*GCT CCAGTCTCTTTGGGGaCGTGGGTTTAAATCCCACTGCTGC AA	556
1	SerTAGnmtchr2.trna 19	GAGAAGGTcACAGAGGTtATGGGATTGGCT*cta*AACC AGTCTGtGGGGGGTTCGATTCCCTCCTTTTTCA	361
2	**SerTAGnmtchr2.trna 7**	GAGAAGGTcATAGAGGTtATGGGATTGGCT*cta*AACC AGTCTCTGGGGGGTTCGATTCCCTCCTTTTTCA	362

	tRNAscan-SE ID	Sequence	SEQ ID NO
3	SerTAGnmtchr17.trna31	GAAAAAGTCATAGGGGTTATGAGGCTGGCT*cta*AACCAGCCTtAGGAGGTTCAATTCCTTCCTTTTTTG	363
4	SerTAGchr6.trna41	GGCCGGTTAGCTCAGTTGGTtAGAGCGTGCTGCT*cta*AATGCCAGGGtCGAGGTTTCGATCCCCGTACGGGCCT	364
5	**SerTAGchr6.trna148**	GTAGTCGTGGCCGAGTGGTtAAGGCGATGGACT*cta*AATCCATTGGGGTTTCCCCGCGCAGGTTCGAATCCTGCCGACTACG	365
6	**SerTAGchr6.trna50**	GTAGTCGTGGCCGAGTGGTtAAGGCGATGGACT*cta*AATCCATTGGGGTTTCCCCACGCAGGTTCGAATCCTGCCGACTACG	366
7	**SerTAGchr6.trna146**	GTAGTCGTGGCCGAGTGGTtAAGGTGATGGACT*ctaa*AACCCATTGGGGTCTCCCCGCGCAGGTTCGAATCCTGCCGACTACG	367
8	**SerTAGchr7.trna15**	GGGTGTATGGCTCAGGGGTAGAGAATTTGACTctaGATCAAGAGGtCCCTGGTTCAAATCCAGGTGCCCCCT	368
9	**SerTAGchr11.trna10**	AGTTGTAGCTGAGTGGTtAAGGCAACGAGCT*cta*AATTCGTTGGTTTCTCTCTgTGCAGGTTTGAATCCTGCTAATTA	369
10	**SerTAGchr11.trna8**	CAAGAAATTCATAGAGGTTATGGGATTGGCT*cta*AACCAGTTTcAGGAGGTTCGATTCCTTCCTTTTTGG	370
11	SerTAGchr17.trna41	GCTGTGATGGCCGAGTGGTtAAGGCGTTGGACT*cta*AATCCAATGGGGTCTCCCCGCGCAGGTTCGAATCCTGCTCACAGCG	371
12	**SerTAGchr6.trna34**	GCTGTGATGGCCGAGTGGTtAAGGCGTTGGACT*cta*AATCCAATGGGGTCTCCCCGCGCAGGTTCAAATCCTGCTCACAGCG	372
13	**SerTAGchr6.trna138**	GCTGTGATGGCCGAGTGGTtAAGGTGTTGGACT*cta*AATCCAATGGGGGTTCCCCGCGCAGGTTCAAATCCTGCTCACAGCG	373
14	**SerTAGchr12.trna2**	GTCACGGTGGCCGAGTGGTtAAGGCGTTGGACT*cta*AATCCAATGGGGTTTCCCCGCACAGGTTCGAATCCTGTTCGTGACG	374
15	**SerTAGchr6.trna30**	GACGAGGTGGCCGAGTGGTtAAGGCGATGGACT*cta*AATCCATTGTGCTCTGCACGCGTGGGTTCGAATCCCACCCTCGTCG	375
16	**SerTAGchr6.trna43**	GACGAGGTGGCCGAGTGGTtAAGGCGATGGACT*cta*AATCCATTGTGCTCTGCACGCGTGGGTTCGAATCCCACCTTCGTCG	376
17	**SerTAGchr11.trna6**	GGCCGGTTAGCTCAGTTGGTtAGAGCGTGCT*cta*ACTAATGCCAGGGtCGAGGTTTCGATCCCCGTACGGGCCT	377
18	**SerTAGchr6.trna61**	GACGAGGTGGCCGAGTGGTtAAGGCGATGGACT*cta*AATCCATTGTGCTCTGCACACGTGGGTTCGAATCCCATCCTCGTCG	378
19	**SerTAGchr6.trna176**	GAGGCCTGGCCGAGTGGTtAAGGCGATGGACT*cta*AA	379

	tRNAscan-SE ID	Sequence	SEQ ID NO
		TCCATTGTGCTCTGCACGCGTGGGTTCGAATCCCATCCTCG	
20	**SerTAGchr10.trna2**	GCAGCGATGGCCGAGTGGTtAAGGCGTTGGACT*cta*AATCCAATGGGGTCTCCCCGCGCAGGTTCGAACCCTGCTCGCTGCG	380
21	**SerTAGchr6.trna51**	GTAGTCGTGGCCGAGTGGTtAAGGCGATGGACT*cta*AATCCATTGGGGTTTCCCCGCGCAGGTTCGAATCCTGCCGACTACG	381
22	**SerTAGchr6.trna173**	GTAGTCGTGGCCGAGTGGTtAAGGCGATGGACT*cta*AATCCATTGGGGTCTCCCCGCGCAGGTTCGAATCCTGCCGACTACG	382
23	**SerTAGchr6.trna149**	GTAGTCGTGGCCGAGTGGTtAAGGCGATGGACT*cta*AATCCATTGGGGTTTCCCCGCGCAGGTTCGAATCCTGTCGGCTACG	383
1	SerTGAnmtchr2.trna19	GAGAAGGTcACAGAGGTtATGGGATTGGCT*tca*AACCAGTCTGtGGGGGGTTCGATTCCCTCCTTTTTCA	384
2	SerTGAnmt-chr2.trna7	GAGAAGGTcATAGAGGTtATGGGATTGGCT*tca*AACCAGTCTCTGGGGGGTTCGATTCCCTCCTTTTTCA	385
3	SerTGAnmtchr17.trna31	GAAAAAGTCATAGGGGTTATGAGGCTGGCT*tca*AACCAGCCTtAGGAGGTTCAATTCCTTCCTTTTTTG	386
4	SerTGAchr6.trna41	GGCCGGTTAGCTCAGTTGGTtAGAGCGTGCTGCT*tca*AATGCCAGGGtCGAGGTTTCGATCCCCGTACGGGCCT	387
5	**SerTGAchr6.trna148**	GTAGTCGTGGCCGAGTGGTtAAGGCGATGGACT*tca*AATCCATTGGGGTTTCCCCGCGCAGGTTCGAATCCTGCCGACTACG	388
6	**SerTGAchr6.trna50**	GTAGTCGTGGCCGAGTGGTtAAGGCGATGGACT*tca*AATCCATTGGGGTTTCCCCACGCAGGTTCGAATCCTGCCGACTACG	389
7	**SerTGAchr6.trna146**	GTAGTCGTGGCCGAGTGGTtAAGGTGATGGACT*tca*AACCCATTGGGGTCTCCCCGCGCAGGTTCGAATCCTGCCGACTACG	390
8	SerTGAchr7.trna15	GGGTGTATGGCTCAGGGGTAGAGAATTTGACT*tca*GATCAAGAGGtCCCTGGTTCAAATCCAGGTGCCCCCT	391
9	**SerTGAchr11.trna10**	AGTTGTAGCTGAGTGGTtAAGGCAACGAGCT*tca*AATTCGTTGGTTTCTCTCTgTGCAGGTTTGAATCCTGCTAATTA	392
10	SerTGAchr11.trna8	CAAGAAATTCATAGAGGTTATGGGATTGGCT*tca*AACCAGTTTcAGGAGGTTCGATTCCTTCCTTTTTGG	393
11	**SerTGAchr17.trna41**	GCTGTGATGGCCGAGTGGTtAAGGCGTTGGACT*tca*AATCCAATGGGGTCTCCCCGCGCAGGTTCGAATCCTGCTCACAGCG	394
12	**SerTGAchr6.trna34**	GCTGTGATGGCCGAGTGGTtAAGGCGTTGGACT*tca*AATCCAATGGGGTCTCCCCGCGCAGGTTCAAATCCTGCTCACAGCG	395

	tRNAscan-SE ID	Sequence	SEQ ID NO
13	**SerTGAchr6.trna138**	GCTGTGATGGCCGAGTGGTtAAGGTGTTGGACT*tca*A ATCCAATGGGGGTTCCCCGCGCAGGTTCAAATCCTGC TCACAGCG	396
14	**SerTGAchr12.trna2**	GTCACGGTGGCCGAGTGGTtAAGGCGTTGGACT*tca*A ATCCAATGGGGTTTCCCCGCACAGGTTCGAATCCTGT TCGTGACG	397
15	**SerTGAchr6.trna30**	GACGAGGTGGCCGAGTGGTtAAGGCGATGGACT*tca*A ATCCATTGTGCTCTGCACGCGTGGGTTCGAATCCCAC CCTCGTCG	398
16	**SerTGAchr6.trna43**	GACGAGGTGGCCGAGTGGTtAAGGCGATGGACT*tca*A ATCCATTGTGCTCTGCACGCGTGGGTTCGAATCCCAC CTTCGTCG	399
17	**SerTGAchr11.trna6**	GGCCGGTTAGCTCAGTTGGTtAGAGCGTGCT*tca*ACT AATGCCAGGGtCGAGGTTTCGATCCCCGTACGGGCCT	400
18	**SerTGAchr6.trna61**	GACGAGGTGGCCGAGTGGTtAAGGCGATGGACT*tca*A ATCCATTGTGCTCTGCACACGTGGGTTCGAATCCCAT CCTCGTCG	401
19	SerTGAchr6.trna176	GAGGCCTGGCCGAGTGGTtAAGGCGATGGACT*tca*AA TCCATTGTGCTCTGCACGCGTGGGTTCGAATCCCATC CTCG	402
20	**SerTGAchr10.trna2**	GCAGCGATGGCCGAGTGGTtAAGGCGTTGGACT*tca*A ATCCAATGGGGTCTCCCCGCGCAGGTTCGAACCCTGC TCGCTGCG	403
21	**SerTGAchr6.trna51**	GTAGTCGTGGCCGAGTGGTtAAGGCGATGGACT*tca*A ATCCATTGGGGTTTCCCCGCGCAGGTTCGAATCCTGC CGACTACG	404
22	**SerTGAchr6.trna173**	GTAGTCGTGGCCGAGTGGTtAAGGCGATGGACT*tca*A ATCCATTGGGGTCTCCCCGCGCAGGTTCGAATCCTGC CGACTACG	405
23	**SerTGAchr6.trna149**	GTAGTCGTGGCCGAGTGGTtAAGGCGATGGACT*tca*A ATCCATTGGGGTTTCCCCGCGCAGGTTCGAATCCTGT CGGCTACG	406
1	SerTAAnmtchr2.trna19	GAGAAGGTcACAGAGGTtATGGGATTGGCT*tta*AACC AGTCTGtGGGGGGTTCGATTCCCTCCTTTTTCA	557
2	SerTAAnmtchr2.trna7	GAGAAGGTcATAGAGGTtATGGGATTGGCT*tta*AACC AGTCTCTGGGGGGTTCGATTCCCTCCTTTTTCA	558
3	SerTAAnmtchr17.trna31	GAAAAAGTCATAGGGGTTATGAGGCTGGCT*tta*AACC AGCCTtAGGAGGTTCAATTCCTTCCTTTTTTG	559
4	SerTAAchr6.trna41	GGCCGGTTAGCTCAGTTGGTtAGAGCGTGCTGCT*tta* AATGCCAGGGtCGAGGTTTCGATCCCCGTACGGGCCT	560
5	**SerTAAchr6.trna148**	GTAGTCGTGGCCGAGTGGTtAAGGCGATGGACT*tta*A ATCCATTGGGGTTTCCCCGCGCAGGTTCGAATCCTGC CGACTACG	561
6	**SerTAAchr6.trna50**	GTAGTCGTGGCCGAGTGGTtAAGGCGATGGACT*tta*A	562

	tRNAscan-SE ID	Sequence	SEQ ID NO
		ATCCATTGGGGTTTCCCCACGCAGGTTCGAATCCTGC CGACTACG	
7	**SerTAAchr6.trna146**	GTAGTCGTGGCCGAGTGGTtAAGGTGATGGACT*tta*A ACCCATTGGGGTCTCCCCGCGCAGGTTCGAATCCTGC CGACTACG	563
8	SerTAAchr7.trna15	GGGTGTATGGCTCAGGGGTAGAGAATTTGACT*tta*GA TCAAGAGGtCCCTGGTTCAAATCCAGGTGCCCCCT	564
9	SerTAAchr11.trna10	AGTTGTAGCTGAGTGGTtAAGGCAACGAGCT*tta*AAT TCGTTGGTTTCTCTCTgTGCAGGTTTGAATCCTGCTA ATTA	565
10	SerTAAchr11.trna8	CAAGAAATTCATAGAGGTTATGGGATTGGCT*tta*AAC CAGTTTcAGGAGGTTCGATTCCTTCCTTTTTGG	566
11	**SerTAAchr17.trna41**	GCTGTGATGGCCGAGTGGTtAAGGCGTTGGACT*tta*A ATCCAATGGGGTCTCCCCGCGCAGGTTCGAATCCTGC TCACAGCG	567
12	**SerTAAchr6.trna34**	GCTGTGATGGCCGAGTGGTtAAGGCGTTGGACT*tta*A ATCCAATGGGGTCTCCCCGCGCAGGTTCAAATCCTGC TCACAGCG	568
13	**SerTAAchr6.trna138**	GCTGTGATGGCCGAGTGGTtAAGGTGTTGGACT*tta*A ATCCAATGGGGGTTCCCCGCGCAGGTTCAAATCCTGC TCACAGCG	569
14	**SerTAAchr12.trna2**	GTCACGGTGGCCGAGTGGTtAAGGCGTTGGACT*tta*A ATCCAATGGGGTTTCCCCGCACAGGTTCGAATCCTGT TCGTGACG	570
15	**SerTAAchr6.trna30**	GACGAGGTGGCCGAGTGGTtAAGGCGATGGACT*tta*A ATCCATTGTGCTCTGCACGCGTGGGTTCGAATCCCAC CCTCGTCG	571
16	**SerTAAchr6.trna43**	GACGAGGTGGCCGAGTGGTtAAGGCGATGGACT*tta*A ATCCATTGTGCTCTGCACGCGTGGGTTCGAATCCCAC CTTCGTCG	572
17	**SerTAAchr11.trna6**	GGCCGGTTAGCTCAGTTGGTtAGAGCGTGCT*tta*ACT AATGCCAGGGtCGAGGTTTCGATCCCCGTACGGGCCT	573
18	**SerTAAchr6.trna61**	GACGAGGTGGCCGAGTGGTtAAGGCGATGGACT*tta*A ATCCATTGTGCTCTGCACACGTGGGTTCGAATCCCAT CCTCGTCG	574
19	**SerTAAchr6.trna176**	GAGGCCTGGCCGAGTGGTtAAGGCGATGGACT*tta*AA TCCATTGTGCTCTGCACGCGTGGGTTCGAATCCCATC CTCG	575
20	**SerTAAchr10.trna2**	GCAGCGATGGCCGAGTGGTtAAGGCGTTGGACT*tta*A ATCCAATGGGGTCTCCCCGCGCAGGTTCGAACCCTGC TCGCTGCG	576
21	**SerTAAchr6.trna51**	GTAGTCGTGGCCGAGTGGTtAAGGCGATGGACT*tta*A ATCCATTGGGGTTTCCCCGCGCAGGTTCGAATCCTGC CGACTACG	577

	tRNAscan-SE ID	Sequence	SEQ ID NO
22	**SerTAAchr6.trna173**	GTAGTCGTGGCCGAGTGGTtAAGGCGATGGACT*tta*A ATCCATTGGGGTCTCCCCGCGCAGGTTCGAATCCTGC CGACTACG	578
23	**SerTAAchr6.trna149**	GTAGTCGTGGCCGAGTGGTtAAGGCGATGGACT*tta*A ATCCATTGGGGTTTCCCCGCGCAGGTTCGAATCCTGT CGGCTACG	579
1	**LysTAAchr19.trna6**	GCCCAGCTAGCTCAGTCGGTAGAGCATAAGACT*tta*A ATCTCAGGGtTGTGGATTCGTGCCCCATGCTGGGTG	407
2	LysTAAchr19.trna7	CTGCAGCTAGCTCAGTCGGTAGAGCATGAGACT*tta*A ATCTCAGGGtCATGGGTTCGTGCCCCATGTTGGG	408
3	LysTAAchr1.trna8	CCAGCATGTCTCAGTCGGTATAGTGTGAGACT*tta*AA TCTCAGGGtCGTGGGTTCAAGCCCCACATTGGG	409
4	LysTAAchr1.trna47	GTCTAGCTAGATCAGTTGGTAGAGCATAAGACT*tta*A ATCTCAGGGtCATGGGTTTGAGCCCTACGTTGGGCG	410
5	LysTAAchr16.trna14	GCCCAGCTAGCTCAGCCGGTAGAGCACAAGACT*tta*A ATCTCAGGGtCGTGGGTTTGAGCCCTGTGTTGAGCA	411
6	LysTAAchr11.trna2	CCGAATAGCTTAGTTGATgAAGCGTGAGACT*tta*AAT CTCAGGGtAGTGGGTTCAAGCCCCACATTGGA	412
7	LysTAAchr15.trna7	GCCTGGCTACCTCAGTTGGTAGAGCATGGGACT*tta*A ATCCCAGAGtcAGTGGGTTCAAGCCTCACATTGAGTG	413
8	**LysTAAchr16.trna31**	GCCCGGCTAGCTCAGTCGGTAGAGCATGAGACC*tta*A ATCTCAGGGtCGTGGGTTCGAGCCCCACGTTGGGCG	414
9	**LysTAAchr16.trna11**	GCCCGGCTAGCTCAGTCGGTAGAGCATGGGACT*tta*A ATCTCAGGGtCGTGGGTTCGAGCCCCACGTTGGGCG	415
10	**LysTAAchr16.trna30**	GCCCGGCTAGCTCAGTCGATAGAGCATGAGACT*tta*A ATCTCAGGGtCGTGGGTTCGAGCCGCACGTTGGGCG	416
11	LysTAAchr1.trna117	GCCCAGCTAGCTCAGTCGGTAGAGCATGAGACT*tta*A ATCTCAGGGtCATGGGTTTGAGCCCCACGTTTGGTG	417
12	**LysTAAchr16.trna6**	GCCTGGCTAGCTCAGTCGGCAAAGCATGAGACT*tta*A ATCTCAGGGtCGTGGGCTCGAGCTCCATGTTGGGCG	418
13	LysTAAchr5.trna25	GCCCGACTACCTCAGTCGGTgGAGCATGGGACT*tta*C ATCCCAGGGtTGTGGGTTCGAGCCCCACATTGGGCA	419
14	LysTAAchr16.trna1	CCCCGGCTGGCTCAGTCAGTAGATCATGAGACT*tta*A ATCTCAGGGtCGTGGGTTCACGCCCCACACTGGGCG	420
15	LysTAAchr7.trna30	GCGCTAGTCAGTAGAGCATGAGACT*tta*AATCTCAGG GtCGTGGGTTCGAGCCCCACATCGGGCG	421
16	**LysTAAchr16.trna23**	GCCTGGATAGCTCAGTTGGTAGAGCATCAGACT*tta*A ATCTGAGGGtCCAGGGTTCAAGTCCCTGTTCAGGCA	422
17	LysTAAchr19.trna10	GCCAGGATAGTTCAGGTGGTAGAGCATCAGACT*ttaa* AACCTGAGGGtTCAGGGTTCAAGTCTCTGTTTGGGCG	423
18	LysTAAchr12.trna1	ACCCAGATAGCTCAGTCAGTAGAGCATCAGACT*tta*A ATCTGAGGGtCCAAGGTTCATGTCCCTTTTTGGGTG	424
19	**LysTAAchr19.trna8**	ACCTGGGTAGCTTAGTTGGTAGAGCATTGGACT*tta*A ATTTGAGGGcCCAGGTTTCAAGTCCCTGTTTGGGTG	425

	tRNAscan-SE ID	Sequence	SEQ ID NO
20	LysTAAchr6.trna119	GCCTGGGTAGCTCAGTCGGTAGAGCTaTCAGACT*tta*AGCCTGAGGAtTCAGGGTTCAATCCCTTGCTGGGGCG	426
21	LysTAAchr14.trna13	GATAGCTCAGTTGATAGAGCATCAGACT*tta*AATCTGAGGGtCCAGGGTTCATGTCCCTGTT	427
22	**LysTAAchr2.trna15**	GTTGGGGTAACTCAGTTGGTAGAGTAGCAGACT*tta*CATCTGAGGGtCCAGGGTTTAAGTCCATGTCCAGGCA	428
23	**LysTAAchr11.trna11**	GCCTGGATAGCTCAGTTGGTAGAGCATCAGACT*tta*AATCTGAGGGtCCAGGGTTCAAGTCCCTGTTCAGGCG	429
24	**LysTAAchr6.trna144**	GCCTGGATAGCTCAGTCGGTAGAGCATCAGACTttaAATCTGAGGGtCCAGGGTTCAAGTCCCTGTTCAGGCG	430
25	**LysTAAchr11.trna5**	GCCCGGATAGCTCAGTCGGTAGAGCATCAGACT*tta*AATCTGAGGGtCCGGGGTTCAAGTCCCTGTTCGGGCG	431
26	**LysTAAchr6.trna150**	GCCTGGGTAGCTCAGTCGGTAGAGCATCAGACT*tta*AATCTGAGGGtCCAGGGTTCAAGTCCCTGTCCAGGCG	432
27	**LysTAAchr6.trna70**	GCCTGGATAGCTCAGTTGGTAGAACATCAGACT*tta*AATCTGACGGtGCAGGGTTCAAGTCCCTGTTCAGGCG	433
28	LysTAAchr1.trna50	GCCCGGAGAGCTCAGTGGGTAGAGCATCAGACT*tta*AATCTGAGGGtCCAGGGTTCAAGTCCTCGTTCGGGCA	434
29	**LysTAAchr6.trna53**	ACCTGGGTAGCTCAGTAGGTAGAACATCAGACTttaAATCTGAGGGtCTAGGGTTCAAGTCCCTGTCCAGGCG	435
30	LysTAAchr3.trna2	GCCTGGATAGCTCCTTCGGTAGAGCATCATcagACT*tta*AATGTGAGGGtCCAGGGTTCAAGTTCCTGTTTGGGCG	436
1	**LysTAGchr19.trna6**	GCCCAGCTAGCTCAGTCGGTAGAGCATAAGACT*cta*AATCTCAGGGtTGTGGATTCGTGCCCCATGCTGGGTG	437
2	LysTAGchr19.trna7	CTGCAGCTAGCTCAGTCGGTAGAGCATGAGACT*cta*AATCTCAGGGtCATGGGTTCGTGCCCCATGTTGGG	438
3	LysTAGchr1.trna8	CCAGCATGTCTCAGTCGGTATAGTGTGAGACT*cta*AATCTCAGGGtCGTGGGTTCAAGCCCCACATTGGG	439
4	LysTAGchr1.trna47	GTCTAGCTAGATCAGTTGGTAGAGCATAAGACT*cta*AATCTCAGGGtCATGGGTTTGAGCCCTACGTTGGGCG	440
5	LysTAGchr16.trna14	GCCCAGCTAGCTCAGCCGGTAGAGCACAAGACT*cta*AATCTCAGGGtCGTGGGTTTGAGCCCTGTGTTGAGCA	441
6	LysTAGchr11.trna2	CCGAATAGCTTAGTTGATgAAGCGTGAGACT*cta*AATCTCAGGGtAGTGGGTTCAAGCCCCACATTGGA	442
7	LysTAGchr15.trna7	GCCTGGCTACCTCAGTTGGTAGAGCATGGGACT*cta*AATCCCAGAGtcAGTGGGTTCAAGCCTCACATTGAGTG	443
8	**LysTAGchr16.trna31**	GCCCGGCTAGCTCAGTCGGTAGAGCATGAGACC*cta*AATCTCAGGGtCGTGGGTTCGAGCCCCACGTTGGGCG	444
9	**LysTAGchr16.trna11**	GCCCGGCTAGCTCAGTCGGTAGAGCATGGGACT*cta*AATCTCAGGGtCGTGGGTTCGAGCCCCACGTTGGGCG	445
10	LysTAGchr16.trna30	GCCCGGCTAGCTCAGTCGATAGAGCATGAGACT*cta*AATCTCAGGGtCGTGGGTTCGAGCCGCACGTTGGGCG	446
11	LysTAGchr1.trna117	GCCCAGCTAGCTCAGTCGGTAGAGCATGAGACT*cta*A	447

	tRNAscan-SE ID	Sequence	SEQ ID NO
		ATCTCAGGGtCATGGGTTTGAGCCCCACGTTTGGTG	
12	LysTAGchr16.trna6	GCCTGGCTAGCTCAGTCGGCAAAGCATGAGACT*cta*A ATCTCAGGGtCGTGGGCTCGAGCTCCATGTTGGGCG	448
13	LysTAGchr5.trna25	GCCCGACTACCTCAGTCGGTgGAGCATGGGACT*cta*C ATCCCAGGGtTGTGGGTTCGAGCCCCACATTGGGCA	449
14	LysTAGchr16.trna1	CCCCGGCTGGCTCAGTCAGTAGATCATGAGACT*cta*A ATCTCAGGGtCGTGGGTTCACGCCCCACACTGGGCG	450
15	LysTAGchr7.trna30	GCGCTAGTCAGTAGAGCATGAGACT*cta*AATCTCAGG GtCGTGGGTTCGAGCCCCACATCGGGCG	451
16	**LysTAGchr16.trna23**	GCCTGGATAGCTCAGTTGGTAGAGCATCAGACT*cta*A ATCTGAGGGtCCAGGGTTCAAGTCCCTGTTCAGGCA	452
17	LysTAGchr19.trna10	GCCAGGATAGTTCAGGTGGTAGAGCATCAGACT*cta*A ACCTGAGGGtTCAGGGTTCAAGTCTCTGTTTGGGCG	453
18	LysTAGchr12.trna1	ACCCAGATAGCTCAGTCAGTAGAGCATCAGACT*cta*A ATCTGAGGGtCCAAGGTTCATGTCCCTTTTTGGGTG	454
19	**LysTAGchr19.trna8**	ACCTGGGTAGCTTAGTTGGTAGAGCATTGGACT*cta*A ATTTGAGGGcCCAGGTTTCAAGTCCCTGTTTGGGTG	455
20	LysTAGchr6.trna119	GCCTGGGTAGCTCAGTCGGTAGAGCTaTCAGACT*cta* aAGCCTGAGGAtTCAGGGTTCAATCCCTTGCTGGGGC G	456
21	LysTAGchr14.trna13	GATAGCTCAGTTGATAGAGCATCAGACT*cta*AATCTG AGGGtCCAGGGTTCATGTCCCTGTT	457
22	LysTAGchr2.trna15	GTTGGGGTAACTCAGTTGGTAGAGTAGCAGACT*cta*C ATCTGAGGGtCCAGGGTTTAAGTCCATGTCCAGGCA	458
23	**LysTAGchr11.trna11**	GCCTGGATAGCTCAGTTGGTAGAGCATCAGACT*cta*A ATCTGAGGGtCCAGGGTTCAAGTCCCTGTTCAGGCG	459
24	**LysTAGchr6.trna144**	GCCTGGATAGCTCAGTCGGTAGAGCATCAGACT*cta*A ATCTGAGGGtCCAGGGTTCAAGTCCCTGTTCAGGCG	460
25	**LysTAGchr11.trna5**	GCCCGGATAGCTCAGTCGGTAGAGCATCAGACT*cta*A ATCTGAGGGtCCGGGGTTCAAGTCCCTGTTCGGGCG	461
26	**LysTAGchr6.trna150**	GCCTGGGTAGCTCAGTCGGTAGAGCATCAGACT*cta*A ATCTGAGGGtCCAGGGTTCAAGTCCCTGTCCAGGCG	462
27	**LysTAGchr6.trna70**	GCCTGGATAGCTCAGTTGGTAGAACATCAGACT*cta*A ATCTGACGGtGCAGGGTTCAAGTCCCTGTTCAGGCG	463
28	LysTAGchr1.trna50	GCCCGGAGAGCTCAGTGGGTAGAGCATCAGACT*cta*A ATCTGAGGGtCCAGGGTTCAAGTCCTCGTTCGGGCA	464
29	LysTAGchr6.trna53	ACCTGGGTAGCTCAGTAGGTAGAACATCAGACT*cta*A ATCTGAGGGtCTAGGGTTCAAGTCCCTGTCCAGGCG	465
30	LysTAGchr3.trna2	GCCTGGATAGCTCCTTCGGTAGAGCATCATcagACT*c ta*AATGTGAGGGtCCAGGGTTCAAGTTCCTGTTTGGG CG	466
1	CysTGAUndchr17.trn a20	GGCAGAATGGTGCAGCGGTtcAGCACCCAGgCTCT*tc a*GcCAGCTGTTGCCTGGGCTCAAATCCCAGCTCTGCC A	467

	tRNAscan-SE ID	Sequence	SEQ ID NO
2	CysTGAchr5.trna30	GGCTGTATAGCTCAGTGGTAGAGCATTTGACT*tca*Ga atcctatactcaggggaaggagaactgggggtttctc agtgggtcaaaggacttgtagtggtaaatcaaaagca actctataagctatgtaacaaaCTTTAAAGTCATAtG TAGcTGGGTTCAAATCCTGTTTCTGCCA	468
3	CysTGAchr5.trna3/n ointron	GGCTGTATAGCTCAGTGGTAGAGCATTTGACT*tca*GC TTTAAAGTCATAtGTAGcTGGGTTCAAATCCTGTTTC TGCCA	469
4	**CysTGAchr7.trna8**	GGGGGCATAGCTCAGTGGTAGAGCATTTGACT*tca*GA TCAAGAGGtCCCTGGTTCAAATCCAGGTGCCCCCT	470
5	CysTGAchr7.trna26	GGGGGTATAGCTCAGGGGTAGAGCATTTGACT*tca*GA TCAAGAGGtCCCTGGTTCAAATCCAGGTGCCCCCC	471
6	**CysTGAchr7.trna24**	GGGGGTATAGCTTAGCGGTAGAGCATTTGACT*tca*GA TCAAGAGGtCCCCGGTTCAAATCCGGGTGCCCCCT	472
7	**CysTGAchr7.trna20**	GGGGGTATAGCTTAGGGGTAGAGCATTTGACT*tca*GA TCAAAAGGtCCCTGGTTCAAATCCAGGTGCCCCTT	473
8	**CysTGAchr7.trna29**	GGGGGTATAGCTCAGGGGTAGAGCATTTGACT*tca*GA TCAAGAGGtCCCCAGTTCAAATCTGGGTGCCCCCT	474
9	CysTGAchr17.trna28	GGGGGTATAGCTCAGGGGTAGAGCATTTGACT*tca*GA TCAAGAAGtCCCCGGTTCAAATCCGGGTGCCCCCT	475
10	**CysTGAchr7.trna13**	GGGGGTATAGCTCAGGGGTAGAGCATTTGACT*tca*GA TCAAGAGGtCTCTGGTTCAAATCCAGGTGCCCCCT	476
11	CysTGAchr7.trna10	GGGGGTATAGCTCAGGGGTAGAGCACTTGACT*tca*GA TCAAGAAGtCCTTGGTTCAAATCCAGGTGCCCCCT	477
12	CysTGAchr7.trna19	GGGGATATAGCTCAGGGGTAGAGCATTTGACT*tca*GA TCAAGAGGtCCCCGGTTCAAATCCGGGTGCCCCCC	478
13	CysTGAchr7.trna27	GGGGGTATAGTTCAGGGGTAGAGCATTTGACT*tca*GA TCAAGAGGtCCCTGGTTCAAATCCAGGTGCCCCCT	479
14	CysTGAchr7.trna21	GGGGGTATAGCTCAGGGGTAGAGCATTTGACT*tca*AA TCAAGAGGtCCCTGATTCAAATCCAGGTGCCCCCT	480
15	CysTGAchr7.trna14	GGGCGTATAGCTCAGGGGTAGAGCATTTGACT*tca*GA TCAAGAGGtCCCCAGTTCAAATCTGGGTGCCCCCT	481
16	CysTGAchr7.trna17	GGGGGTATAGCTCACAGGTAGAGCATTTGACT*tca*GA TCAAGAGGtCCCCGGTTCAAATCTGGGTGCCCCCT	482
17	CysTGAchr7.trna11	GGGCGTATAGCTCAGGGGTAGAGCATTTGACT*tca*GA TCAAGAGGtCCCCAGTTCAAATCTGGGTGCCCA	483
18	CysTGAchr7.trna22	GGGGGTATAGCTCACAGGTAGAGCATTTGACT*tca*GA TCAAGAGGtCCCCGGTTCAAATCCGGTTACTCCCT	484
19	CysTGAchr17.trna29	GGGGGTAGGGCTCAGGGAtAGAGCATTTGACT*tca*GA TCAAGAGGtCCCCGGTTCGAATCTAGGTGCCCCCT	485
20	CysTGAchr3.trna9	GGTATATCTCAGGGGGcAGAGCATTTGACT*tca*GATC AAGAGGtCCCCGGTTGAAATCCGGGTGCT	486
21	**CysTGAchr7.trna23**	GGGGGTATAGCTCAGGGGTAGAGCACTTGACT*tca*GA TCAAGAGGtCCCTGGTTCAAATCCAGGTGCCCCCT	487

	tRNAscan-SE ID	Sequence	SEQ ID NO
22	**CysTGAchr17.trna27**	GGGGGTATAGCTCAGTGGTAGAGCATTTGACT*tca*GA TCAAGAGGtCCCTGGTTCAAATCCGGGTGCCCCCT	488
23	**CysTGAchr15.trna3**	GGGGGTATAGCTCAGTGGGTAGAGCATTTGACT*tca*G ATCAAGAGGtCCCCGGTTCAAATCCGGGTGCCCCCT	489
24	**CysTGAchr3.trna6**	GGGGGTGTAGCTCAGTGGTAGAGCATTTGACT*tca*GA TCAAGAGGtCCCTGGTTCAAATCCAGGTGCCCCCT	490
25	**CysTGAchr14.trna9**	GGGGGTATAGCTCAGGGGTAGAGCATTTGACT*tca*GA TCAAGAGGtCCCCGGTTCAAATCCGGGTGCCCCCT	491
26	**CysTGAchr3.trna5**	GGGGGTATAGCTCAGGGGTAGAGCATTTGACT*tca*GA TCAAGAGGtCCCTGGTTCAAATCCAGGTGCCCCCT	492
	Mus_musculuschr11.trna817-Trp	GACCTCGTGGCGCAATGGTAGCGCGTCTGACT*tca*GA TCAGAAGGtTGCGTGTTCAAATCACGTCGGGGTCA	493
	Mus_musculuschr10.trna567	GACCTCGTGGCACAATGGTAGCACGTCTGACT*tca*GA TCAGAAGGtTGCGTGTTCAAATCACGTCGGGGTCA	494
	Saccharomyces_cerevisiaechrVII.trna33	GAAGCGGTGGCTCAATGGTAGAGCTTTCGACT*tca*At taaatcttggaaattccacggaataagattgcaATCG AAGGGtTGCAGGTTCAATTCCTGTCCGTTTCA	495
	Saccharomyces_cerevisiaechrVII.trna33	GAAGCGGTGGCTCAATGGTAGAGCTTTCGACT*tca*AA TCGAAGGGtTGCAGGTTCAATTCCTGTCCGTTTCA	496
	Pan_troglodyteschr7.trna28	GGCCTCATGGTGCAACAGTAGTGTGTCTGACT*tca*GA TCAGAAGGtTGTATGTTCAAATCACATAGGGGTCA	497
	Oryctolagus_cuniculuschrUn0422.trna1	GACCTCGTGGTGAAATGGTAGCATGTTTGACT*tca*AA TCAGGAGGTTGTGTGTTCAAGTCACATCAGGGTCA	498
	Oryctolagus_cuniculus_chrUn0563.trna1	GACCTTGTGGCGCAATGGTAGCATGTTTGACT*tca*AA TCAGGAGGTTGTGTGTTCAAGTCACATCAGGGTCA	499
	Oryctolagus_cuniculus_chrUn0062.trna12	GACCTCGTGGCGCAACGGTAGCGCGTCTGACT*tca*GA TCAGAAGGCTGCGTGTTCGAATCACGCCGGGGTCA	500
	Rattus_norvegicus_chr13.trna4571	GACCTTGTGGCTCAATGGTAGCGCATCTGACT*tca*GA TCAGGAGGTTGCACGTTCAAATCATGCCGGGGTCA	501
	Rattus_norvegicus_chr17.trna3948	GACCTTGTGGCGCAACGGTAGCGCGTCTGACT*tca*GA TCAGAAGGTTGCGTGTTCAAATCACGTCGGGGTCA	502
	Xenopus_tropicalis_tRNA-Trp-CCA-10-1	GACCTCGTGGCGCAACGGTAGCGCGTCTGACT*tca*GA TCAGAAGGtTGCGTATTCAAATCACGTCGGGGTCA	503
	Xenopus_tropicalis_tRNA-Trp-CCA-11-1	GACCTCGTGGCGCAACGGCAGCGCGTCTGACT*tca*CA TTAGAAGGtTGCGTGTTCAAATCACGTCGGGGTCA	504
	Xenopus_tropicalis_tRNA-Trp-CCA-12-1	GACCTCATGGCGCAACGGTAGCGCGTCTGACT*tca*GA TCAGAAGGtTGCGTGTTCAAATCACATCGGGGTCA	505
	Xenopus_tropicalis_tRNA-Trp-CCA-13-1	GACCTCGTGGTGCAACGGTAGCGCGTATGATT*tca*GA TCAGAAGGtTGCGTGTTCAAATCACGTCGGGGTCA	506
	Xenopus_tropicalis_tRNA-Trp-CCA-3-1	GACCTCGTAGCGCAACGGTAGCGCGTCTGACT*tca*GA TCAGAAGGtTGCGTGTTCAAATCACGTCGGGGTCA	507

	tRNAscan-SE ID	Sequence	SEQ ID NO
	Xenopus_tropicalis_tRNA-Trp-CCA-5-1	AGGGGTATAGCTCAATTGGCAGAGCGTCGGTCT*tca*AAACCGAAGGtTGTAGGTTCGATTCCTACTGCCCCTGCCA	508
	Xenopus_tropicalis_tRNA-Trp-CCA-6-1	GACCTCATGGCGCAACGGTAGCGCGTCTGACT*tca*GATCAGAAGGtTGCGTGTTCAAATCACGTCGGGGTCA	509
	Xenopus_tropicalis_tRNA-Trp-CCA-7-1	GACCTCGTGGCGCAACGGTAGCGCGTCTAACT*tca*GATCAGAAGGtTGCGTGTTCAAATCACGTCGGGGTCA	510
	Xenopus_tropicalis_tRNA-Trp-CCA-8-1	ACGGGAGTAGCTCAGTTGGTAGAGCACCGGTCT*tca*AAACCGGGTGtCGGGAGTTCGAGCCTCTCCTCCCGTG	511
	Xenopus_tropicalis_tRNA-Trp-CCA-9-1	GACCTCGTGGCGCAACGGTAGCGCGTCTGACT*tca*GATCAGAAGGtTGCATGTTCAAATCACGTCGGGGTCA	512
	Drosophila_melanogaster_tRNA-Trp-CCA-2-1	GACTCCGTGGCGCAACGGTAGCGCGTCCGACT*tca*GATCGGAAGGtTGCGTGTTCAAATCACGTCGGGGTCA	513
	Drosophila_melanogaster_tRNA-Trp-CCA-1-1	GACTCCGTGGCGCAACGGTAGCGCGTCTGACT*tca*GATCAGAAGGtTGCGTGTTCAAATCACGTCGGGGTCA	514
	TrpWT-chr17.trna39	GGCCTCGTGGCGCAACGGTAGCGCGTCTGACT*cca*GATCAGAAGGtTGCGTGTTCAAATCACGTCGGGGTCA	515
	HirshWT	GGCCTCGTGGCGCAACGGTAGCaCGTCTGACT*cca*GATCAGAAGGtTGCGTGTTCAAATCACGTCGGGGTCA	516
	HirshACE-tRNA	CGGCCTCGTGGCGCAACGGTAGCaCGTCTGACT*tca*GATCAGAAGGtTGCGTGTTCAAATCACGTCGGGGTCA	517
	G9CWT	GGCCTCGTcGCGCAACGGTAGCGCGTCTGACT*cca*GATCAGAAGGtTGCGTGTTCAAATCACGTCGGGGTCA	518
	G9CACE-tRNA	GGCCTCGTcGCGCAACGGTAGCGCGTCTGACT*tca*GATCAGAAGGtTGCGTGTTCAAATCACGTCGGGGTCA	519
	G9C+HirshWT	GGCCTCGTcGCGCAACGGTAGCaCGTCTGACT*cca*GATCAGAAGGtTGCGTGTTCAAATCACGTCGGGGTCA	520
	G9C+HirshACE-tRNA	GGCCTCGTcGCGCAACGGTAGCaCGTCTGACT*tca*GATCAGAAGGtTGCGTGTTCAAATCACGTCGGGGTCA	521

Example 5 REFERENCES

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Example 6: ACE-tRNA are active in vivo

Here it is demonstrated that anticodon-edited tRNA display efficacious PTC reversion in eukaryotic cells and mouse skeletal muscle.

The *in vivo* activity and stability of ACE-tRNA was examined. The NLuc-UGA PTC reporter cDNA was delivered together with a plasmid encoding four copies of the ACE-$tRNA^{Arg}$ UGA or an 'empty vector control' into mouse skeletal muscle (tibialis anterior) using electroporation (Wheeler et al., 2009, Science, 325, 336-339; Wheeler et al., 2007, J Clin Invest, 117, 3952-3957; Muthumani et al., 2017, Cancer Immunol Immunother, 66, 1577-1588). These data were compared to the expression of the WT NLuc. The results showed that the ACE-$tRNA^{Arg}$ UGA is a potent *in vivo* PTC suppressor, yielding expression profiles equal to or at some time points, greater than, the full-length WT NLuc, Figure 34A and Figure 35. The signal from the NLuc-UGA plasmid and non-electroporated muscle was undetectable. Further, ACE-$tRNA^{Arg}$ suppression activity was stable, as evidenced by the similar duration of NLuc activity between rescued and WT protein, Figure 34B. Furthermore, this duration and intensity of luciferase expression argues in favor of a high *in vivo* tolerability and negligible repercussion of increased readthrough observed with ACE-$tRNA^{Arg}$. Next the ability of functional ACE-tRNAs to be delivered as RNA was demonstrated. To this end, ACE-$tRNA^{Trp}$ and ACE-$tRNA^{Gly}$ RNA

transcripts were transfected into HEK293 cells that stably express the NLuc-UGA reporter. Here the results indicated that both ACE-tRNAs functioned similarly as when expressed as cDNA plasmids, with comparable fold rescue when delivered as small RNA (Figure 34C). ACE-$tRNA^{Arg}$ suppression activity was also observed when the NLuc-UGA PTC reporter cDNA was delivered into mouse skin tissue using electroporation (Figure 36).

Next, experiments were designed to rescue two disease causing mutations in cystic fibrosis transmembrane conductance regulator (*CFTR*). This large membrane protein controls anion transport across epithelia in multiple organs and missense and nonsense mutations within its reading frame cause cystic fibrosis. To this end, *CFTR* p.G542X (c.16524G>T; UGA stop codon) and p.W1282X (c.3846G>A; UGA stop codon) cDNAs were transiently co-expressed with their respective ACE-tRNA expression plasmids in HEK293 cells and analyzed by western blot using a C-terminal antibody to identify production of the full-length protein, Figure 34D. Both rescue conditions, as well as WT CFTR expression, resulted in successfully trafficked CFTR protein as evidence by the presence of both the fully glycosylated band C form and the core glycosylated band B CFTR protein. No signal was seen for either p.G542X or p.W1282X transfected alone, indicating a low rate of spontaneous read-through of the indicated PTC under these conditions. To better quantify the PTC suppression properties of each ACE-tRNA in the absence of delivery or expression caveats, *Xenopus leavis* oocyte, a non-dividing model cell in which the ACE-tRNA concentration (as RNA) can be controlled and functional expression can be quantitated were used. Specifically, this expression system is amenable to microinjection and two-electrode voltage-clamp (TEVC) analysis, a facile electrophysiological method for assessing ion channel function at the plasma membrane. CFTR cRNA (complementary RNA produced *in vitro* from a cDNA template) was injected alone or together with the indicated ACE-tRNA RNA at increasing concentrations (Figure 34E and Figure 34F). Functional CFTR channels were not seen for either mutant lacking co-injected ACE-tRNA, even in the presence of a maximal CFTR activation cocktail, forskolin (10 μM; adenylate cyclase activator) and 3-isobutyl-1-methylxanthine (1 mM; phosphodiesterase inhibitor), Figure 34E, left). However, under the same conditions, when co-injected with 200 ng of ACE-tRNA Gly chr19.trna2 (Figure 34E, top right) or Trp chr17.trna39 (Figure 34E, bottom right) CFTR chloride conductance was measured in response to transient changes in membrane potential, indicating that both ACE-tRNAs were highly efficacious at suppressing two disease-causing UGA PTCs. To better quantify the relative expression of rescued channels, this rescue was

compared to WT *CFTR* cRNA alone (25 ng), and suppression of PTCs in *CFTR* was evaluated across a range of ACE-tRNA concentrations. The resulting ACE-tRNA dose response 'current-voltage' relationships are shown in Figure 34F. These data were generated by plotting the steady state ionic current at each voltage versus the voltage used to elicit the measured currents and are a direct measure of channel function and abundance. WT-like current levels of expression were achieved by Gly chr19.trna2, and ~50% for Trp chr17.trna39 ACE-tRNAs, consistent with the predetermined suppression activity and cognate amino acid encoding for these tRNA.

Although the foregoing specification and examples fully disclose and enable the present invention, they are not intended to limit the scope of the invention, which is defined by the claims appended hereto.

All publications, patents and patent applications are incorporated herein by reference. While in the foregoing specification this invention has been described in relation to certain embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details described herein may be varied considerably without departing from the basic principles of the invention.

The use of the terms "a" and "an" and "the" and similar referents in the context of describing the invention are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The terms "comprising," "having," "including," and "containing" are to be construed as open-ended terms (*i.e.*, meaning "including, but not limited to") unless otherwise noted. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (*e.g.*, "such as") provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

Embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

WHAT IS CLAIMED IS:

1. A composition for generating one or more anticodon edited tRNA (ACE-tRNA) or fragments thereof in a subject, comprising one or more nucleic acid molecules or fragments thereof.

2. The composition of claim 1, comprising a cDNA molecule encoding an ACE-tRNA.

3. The composition of claim 1, comprising a RNA molecule comprising an ACE-tRNA.

4. The composition of any one of claims 1-3, wherein the one or more nucleic acid molecules are engineered to be in an expression vector.

5. The composition of any one of claims 1-4, further comprising a pharmaceutically acceptable excipient.

6. A method of treating a disease associated with a PTC in a subject in need thereof, the method comprising administering to the subject at least one composition of any one of claims 1-5.

7. The method of claim 6, wherein the disease is a disease or disorder associated with a UGA PTC, and further wherein the method comprises administering at least one ACE-tRNA specific for UGA.

8. The method of claim 6, wherein the disease is a disease or disorder associated with a UAA PTC, and further wherein the method comprises administering at least one ACE-tRNA specific for UAA.

9. The method of claim 6, wherein the disease is a disease or disorder associated with a UAG PTC, and further wherein the method comprises administering at least one ACE-tRNA specific for UAG.

10. The method of any one of claim 6-9, wherein the method comprises administering at least 2 ACE-tRNA, wherein each of the at least two ACE-tRNA are specific for at least two different amino acid molecules onto a polypeptide chain.

11. The method of any one of claim 6-9, wherein the method comprises administering at least 2 ACE-tRNA, wherein each of the at least two ACE-tRNA are specific for incorporating the same amino acid molecule onto a polypeptide chain.

12. The method of any one of claims 10 or 11, wherein the at least 2 ACE-tRNA are encoded on the same nucleic acid molecule.

13. The method of any one of claims 10 or 11, wherein the at least 2 ACE-tRNA are encoded on different nucleic acid molecules.

14. The method of claim 7, wherein the method comprises administering at least one ACE-tRNA specific for UGA selected from the group consisting of ACE-$tRNA^{Arg}$, ACE-$tRNA^{Gly}$ and ACE-$tRNA^{Trp}$.

15. The method of claim 6, wherein the disease is selected from the group consisting of Duchenne and Becker muscular dystrophies, retinoblastoma, neurofibromatosis, ataxia-telangiectasia, Tay-Sachs disease, cystic fibrosis, Wilm's tumor, hemophilia A, hemophilia B, Menkes disease, Ullrich's disease, β-Thalassemia, type 2A and type 3 von Willebrand disease, Robinow syndrome, brachydactyly type B (shortening of digits and metacarpals), inherited susceptibility to mycobacterial infection, inherited retinal disease, inherited bleeding tendency, inherited blindness, congenital neurosensory deafness and colonic agangliosis and inherited neural develop-mental defect including neurosensory deafness, colonic agangliosis, peripheral neuropathy and central dysmyelinating leukodystrophy, Liddle's syndrome, xeroderma pigmentosum, Fanconi's anemia, anemia, hypothyroidism, p53-associated cancers (e.g., p53 squamal cell carcinoma, p53 hepatocellular carcinoma, p53 ovarian carcinoma), esophageal carcinoma, osteocarcinoma, ovarian carcinoma, hepatocellular carcinoma, breast cancer, hepatocellular carcinoma, fibrous histiocytoma, ovarian carcinoma, SRY sex reversal, triosephosphate isomerase-anemia, diabetes and rickets.

1ST LETTER	SECOND LETTER U		SECOND LETTER C		SECOND LETTER A		SECOND LETTER G		3RD LETTER
U	UUU	Phe	UCU	SER	UAU	Tyr	UGU	Cys	U
	UUC		UCC		UAC		UGC		C
	UUA	Leu	UCA		UAA	STOP	UGA	STOP	A
	UUG		UCG		UAG	STOP	UGG	Trp	G
C	CUU	Leu	CCU	PRO	CAU	His	CGU	Arg	U
	CUC		CCC		CAC		CGC		C
	CUA		CCA		CAA	Gln	CGA		A
	CUG		CCG		CAG		CGG		G
A	AUU	Ile	ACU	Thr	AAU	Asn	AGU	Ser	U
	AUC		ACC		AAC		AGC		C
	AUA		ACA		AAA	Lys	AGA	Arg	A
	AUG	Met	ACG		AAG		AGG		G
G	GUU	Val	GCU	Ala	GAU	Asp	GGU	Gly	U
	GUC		GCC		GAC		GGC		C
	GUA		GCA		GAA	Glu	GGA		A
	GUG		GCG		GAG		GGG		G

Figure 1

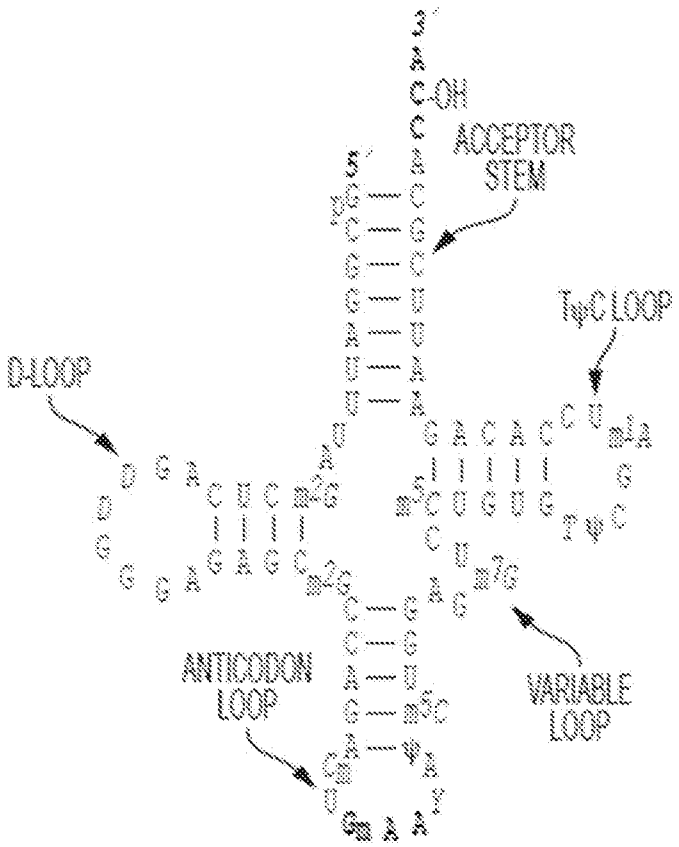


Figure 2

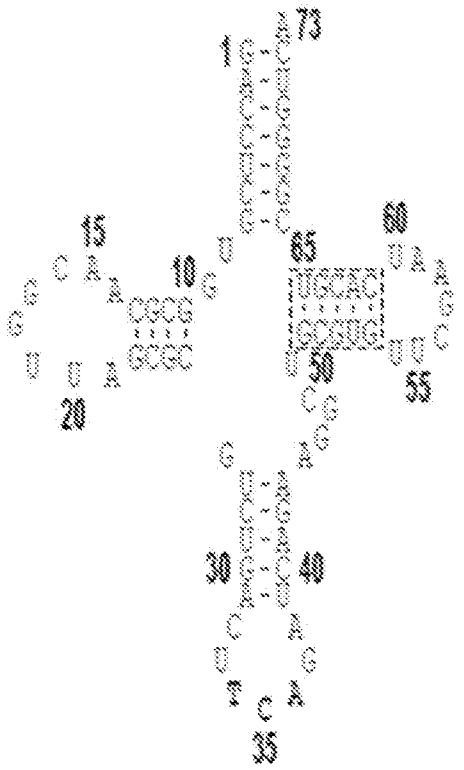


Figure 3

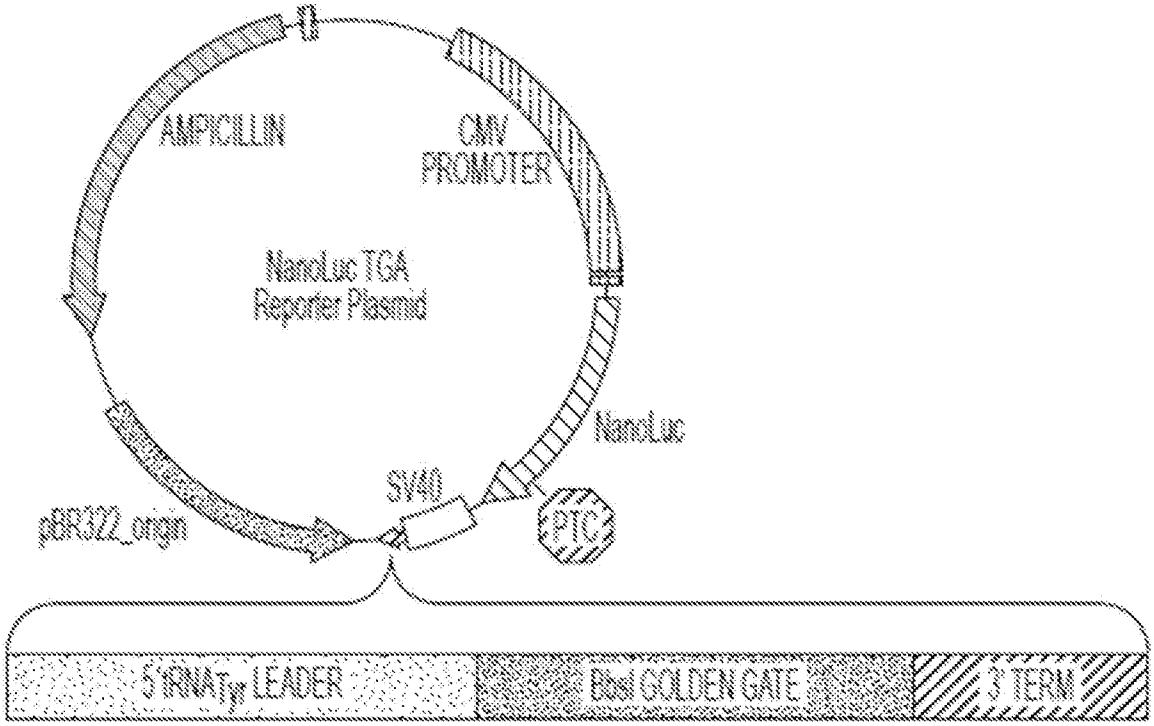


Figure 4

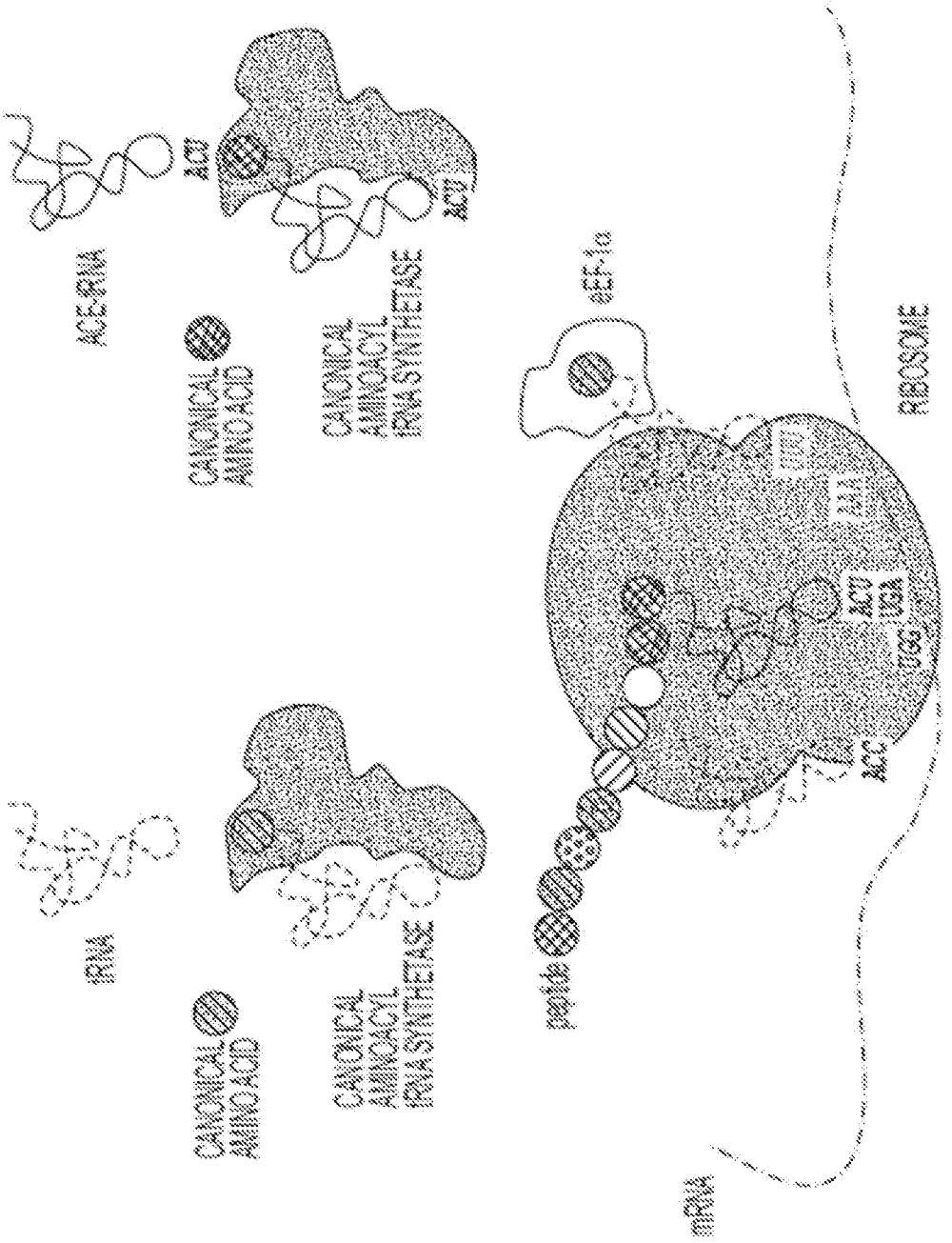

ACE-tRNA
ACT
CANONICAL AMINOACYL tRNA SYNTHETASE
CANONICAL AMINO ACID
tRNA
CANONICAL AMINO ACID
CANONICAL AMINOACYL tRNA SYNTHETASE
eEF-1α
peptide
AAA
ACU
UGA
UGG
RIBOSOME
mRNA

Figure 5

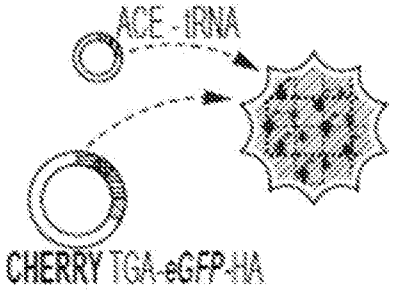


Figure 6A

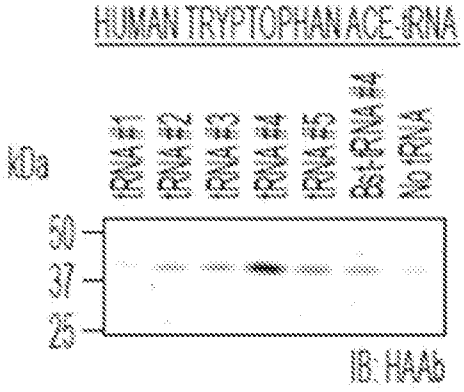


Figure 6B

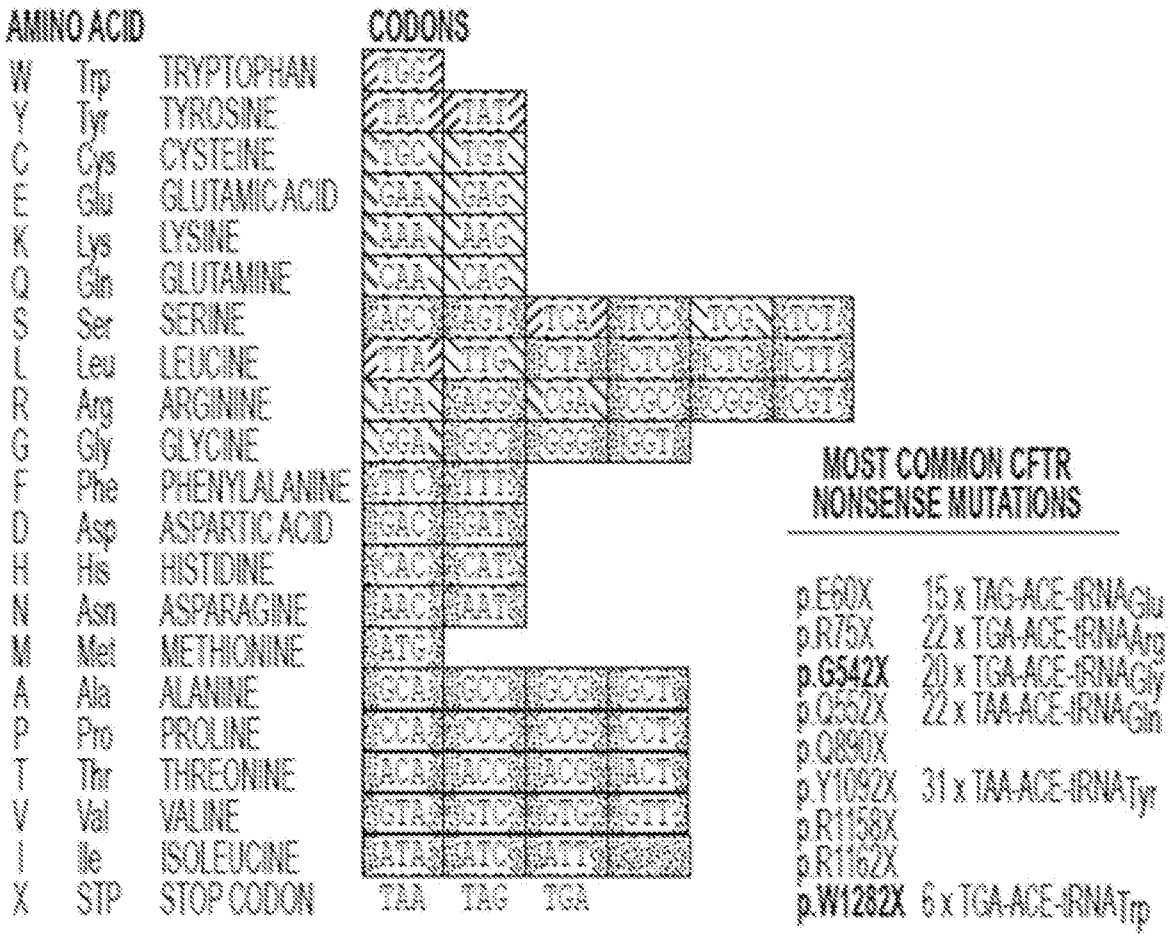


Figure 7

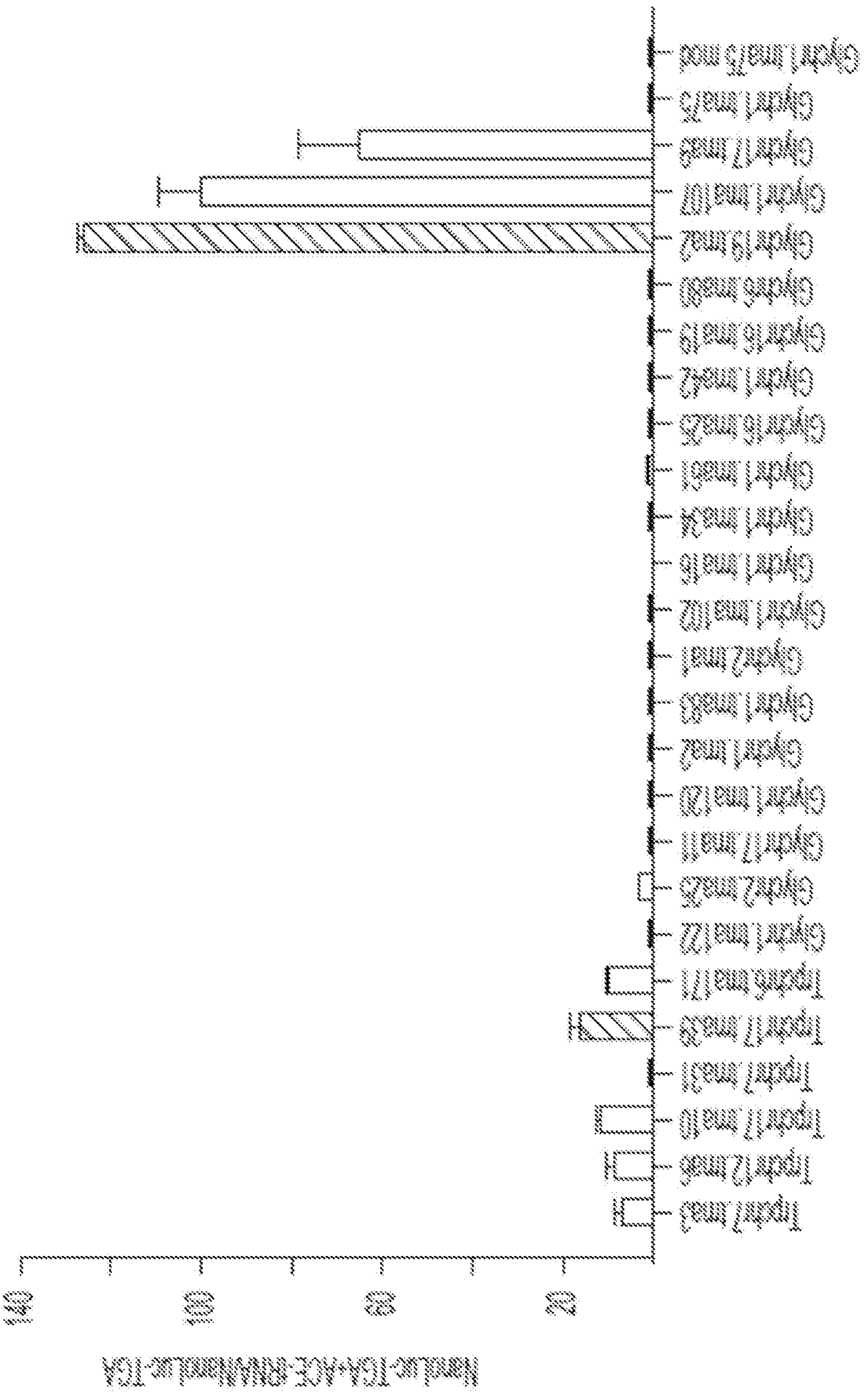

NanoLuc-TGA+ACE-tRNA/NanoLuc-TGA
20
60
100
140
Trpcthr7.ma3
Trpcthr2.ma6
Trpcthr17.ma10
Trpcthr7.ma31
Trpcthr17.ma38
Trpcthr6.ma171
Glycthr1.ma122
Glycthr2.ma25
Glycthr17.ma11
Glycthr1.ma120
Glycthr1.ma7
Glycthr1.ma83
Glycthr2.ma1
Glycthr1.ma102
Glycthr1.ma16
Glycthr1.ma34
Glycthr1.ma61
Glycthr16.ma25
Glycthr1.ma42
Glycthr16.ma19
Glycthr6.ma80
Glycthr19.ma2
Glycthr1.ma107
Glycthr17.ma6
Glycthr1.ma75
Glycthr1.ma75 mod

Figure 8

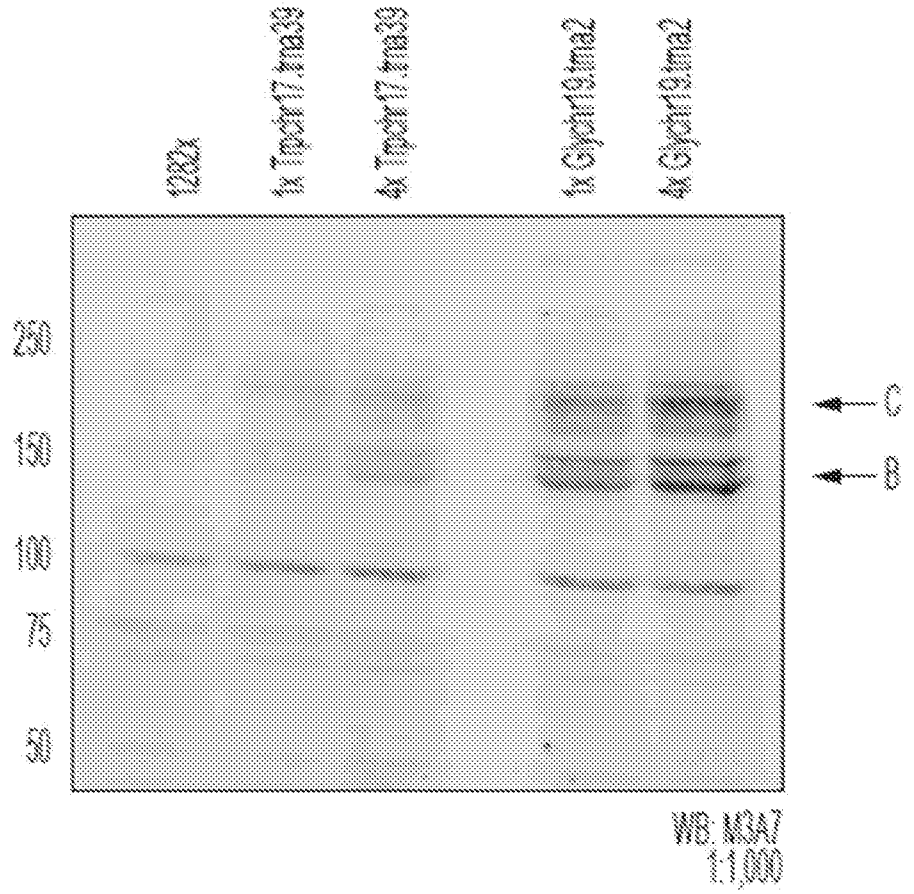


Figure 9

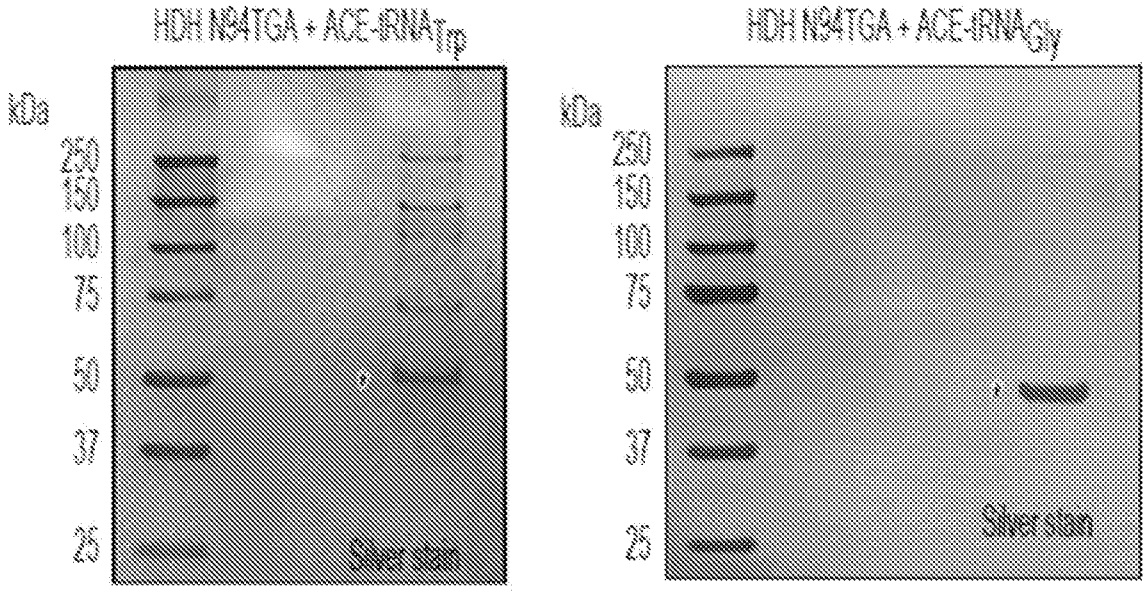


Figure 10A

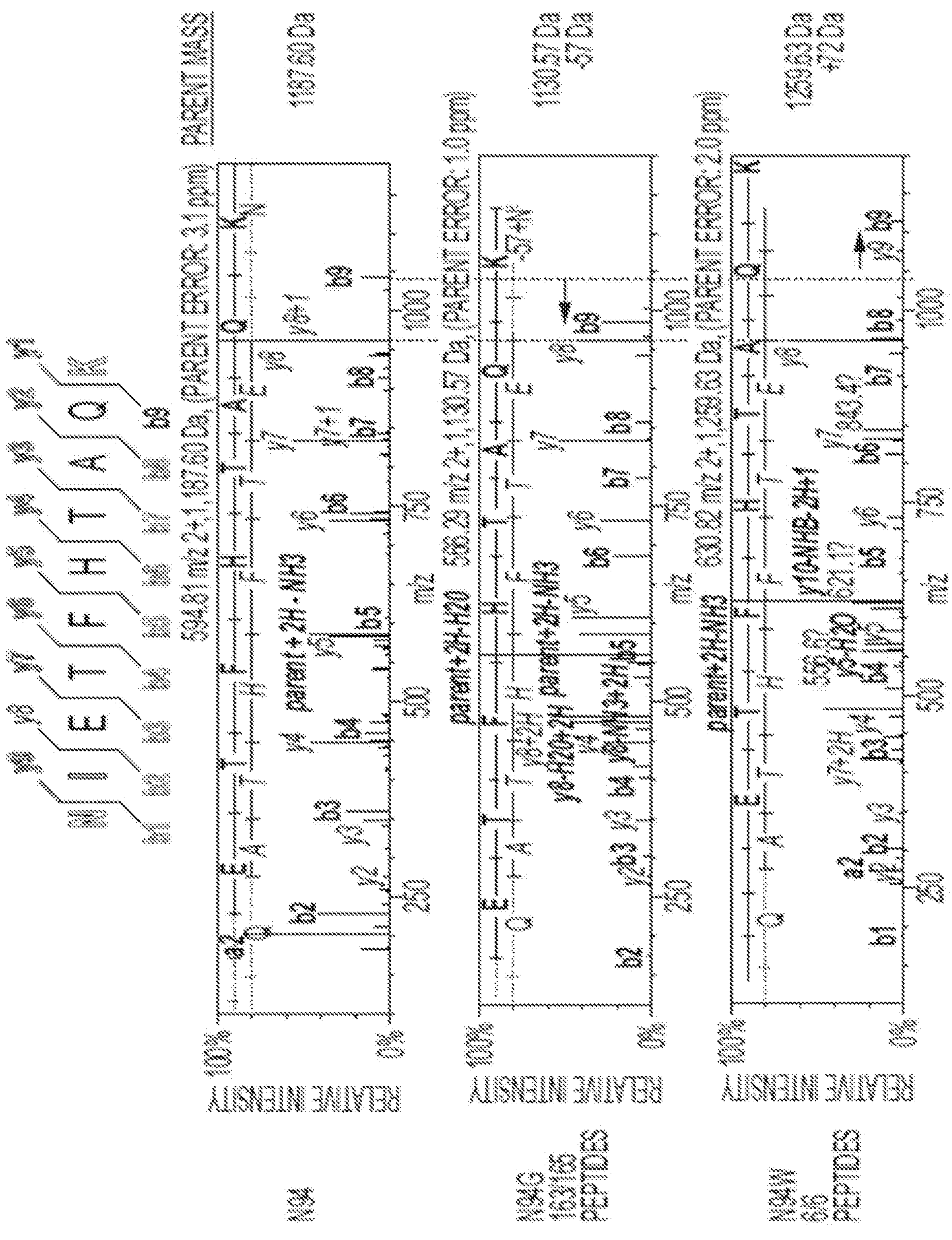

N I E T F H T A Q K
594.81 m/z 2+, 1,187.60 Da, (PARENT ERROR: 3.1 ppm)
PARENT MASS
1187.60 Da
565.29 m/z 2+, 1,130.57 Da, (PARENT ERROR: 1.0 ppm)
1130.57 Da
-57 Da
630.82 m/z 2+, 1,259.63 Da, (PARENT ERROR: 2.0 ppm)
1259.63 Da
+72 Da
N34
N34G
163165
PEPTIDES
N34W
686
PEPTIDES
RELATIVE INTENSITY
100%
0%
m/z
250
500
750
1000

Figure 10B

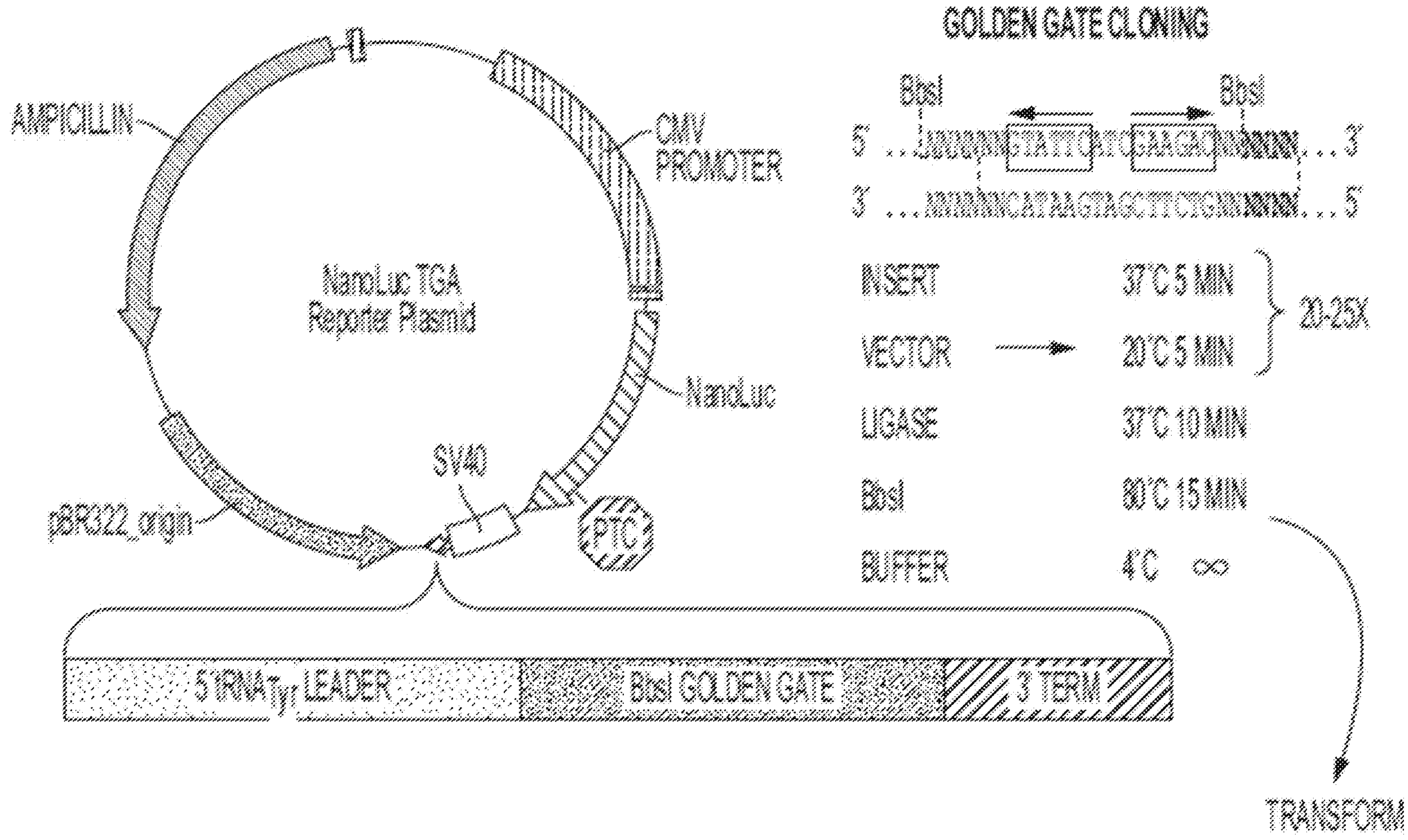

GOLDEN GATE CLONING
BbsI
BbsI
5'
3'
3'
5'
INSERT
VECTOR
LIGASE
BbsI
BUFFER
37°C 5 MIN
20°C 5 MIN
37°C 10 MIN
80°C 15 MIN
4°C ∞
20-25X
TRANSFORM
AMPICILLIN
CMV PROMOTER
NanoLuc TGA Reporter Plasmid
NanoLuc
SV40
PTC
pBR322_origin
5'tRNATyr LEADER
BbsI GOLDEN GATE
3' TERM

Figure 11

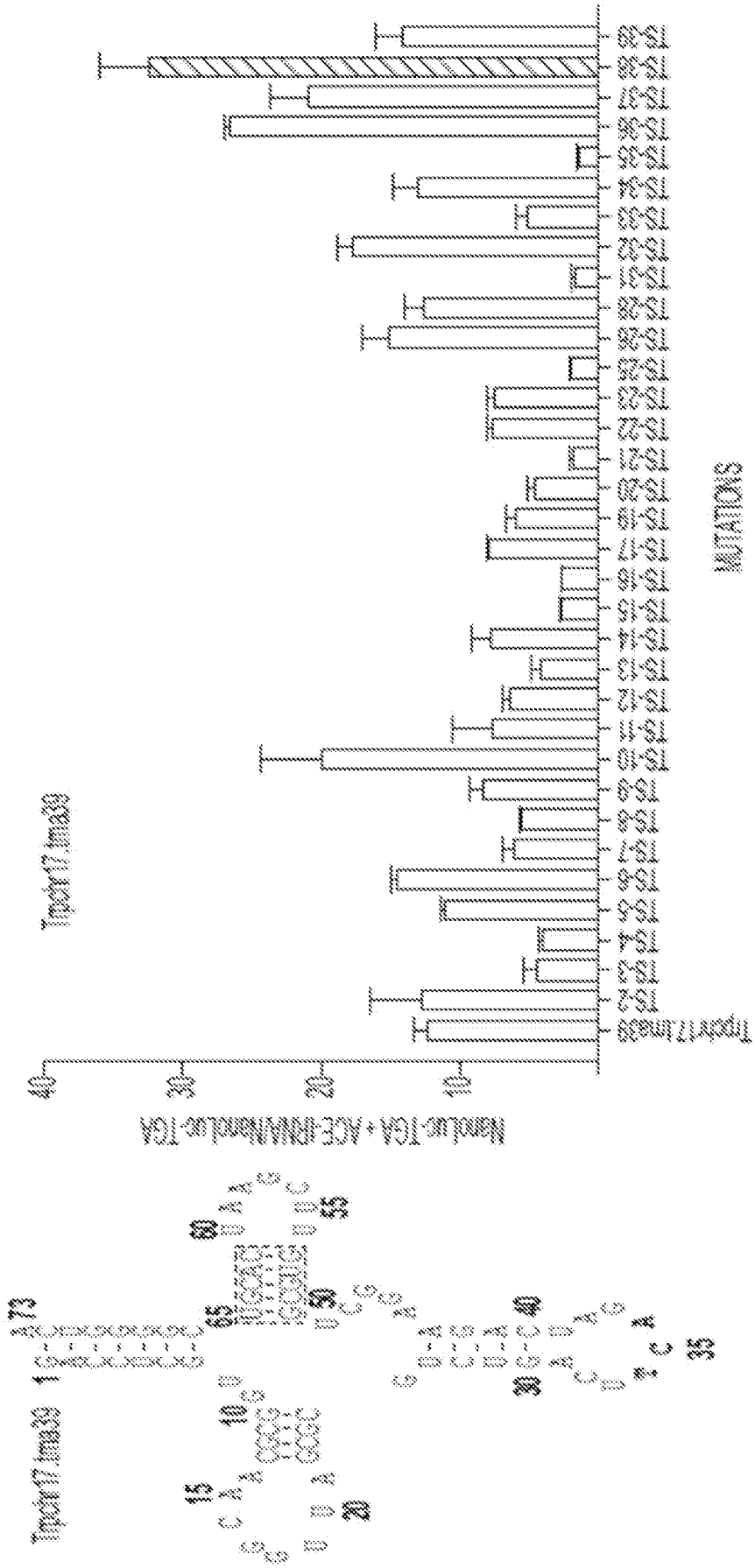

Tpcbn17.lma39
TS-39
TS-38
TS-37
TS-36
TS-35
TS-34
TS-33
TS-32
TS-31
TS-28
TS-29
TS-23
TS-22
TS-21
TS-20
TS-19
TS-17
TS-16
TS-15
TS-14
TS-13
TS-12
TS-11
TS-10
TS-9
TS-8
TS-7
TS-6
TS-5
TS-4
TS-3
TS-2
Tpcbn17.lma39
MUTATIONS
NanoLuc-TGA + ACE-tRNANanoLuc-TGA
40
30
20
10
Tpcbn17.lma39
173
1
65
55
50
40
35
30
25
20
15
10
5
1

Figure 12A

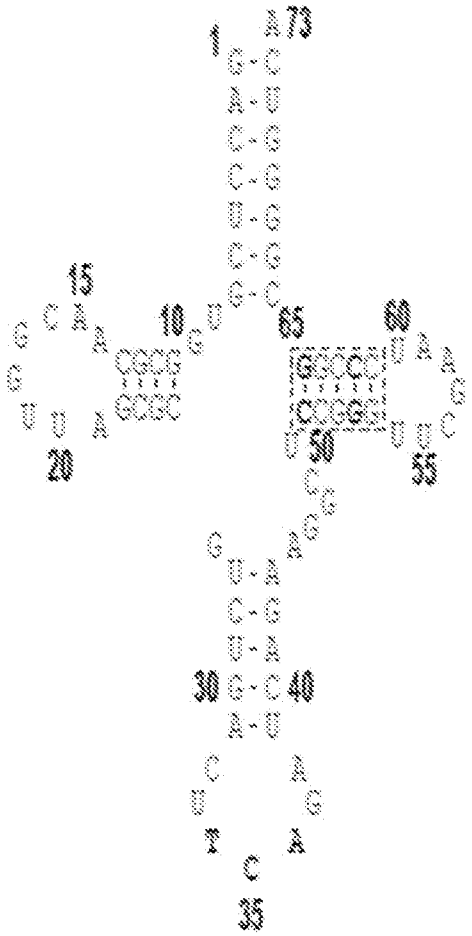


Figure 12B

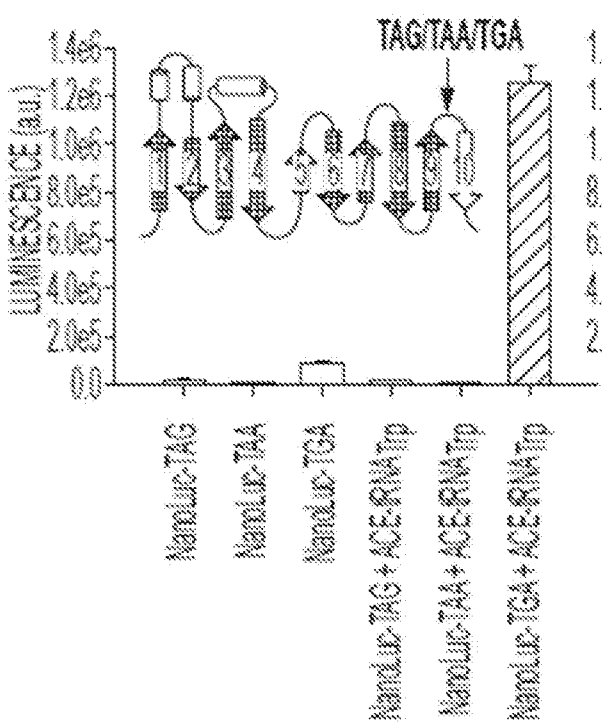


Figure 13A

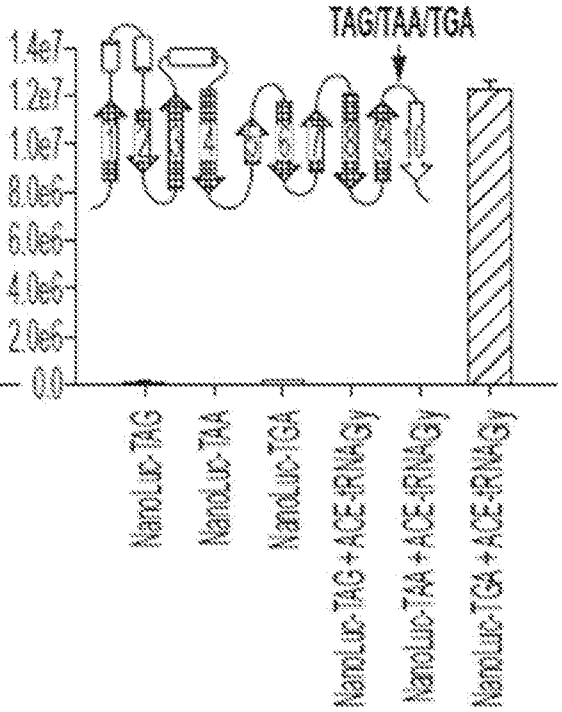


Figure 13B

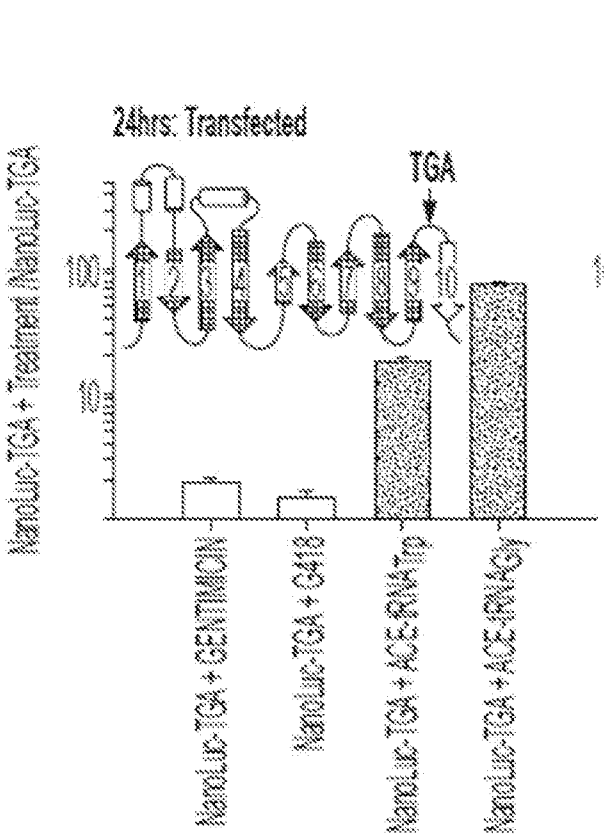


Figure 13C

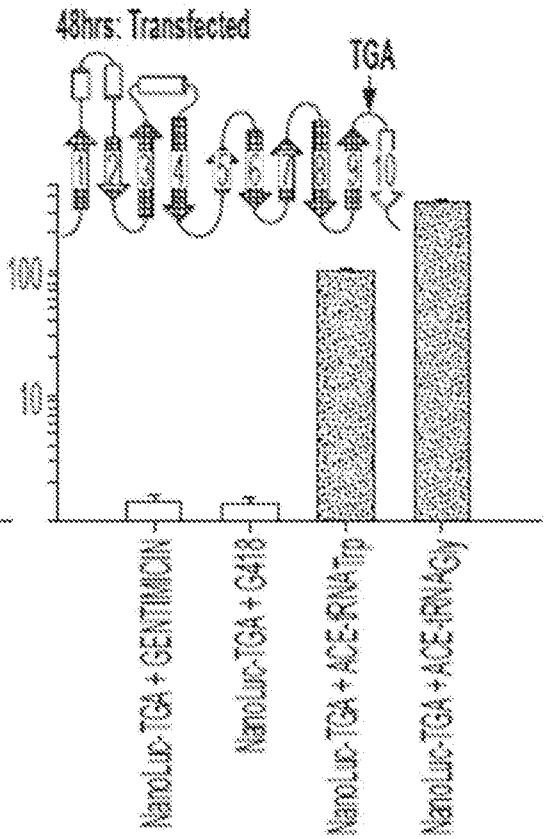


Figure 13D

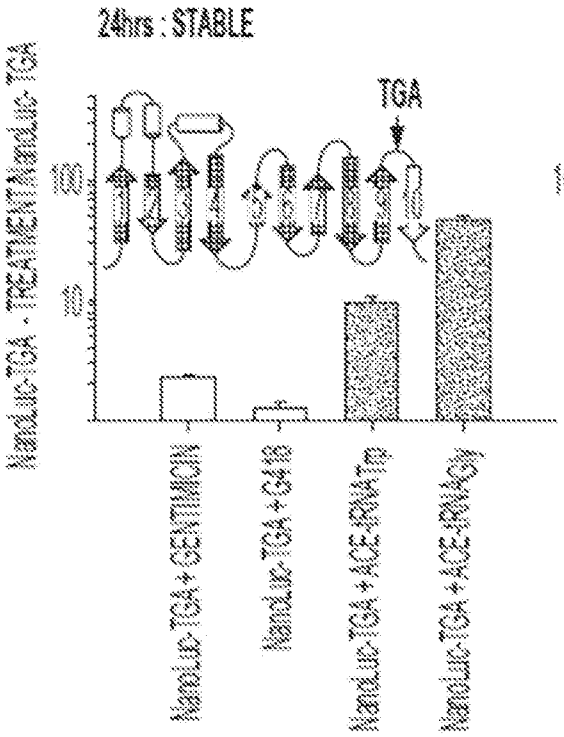


Figure 13E

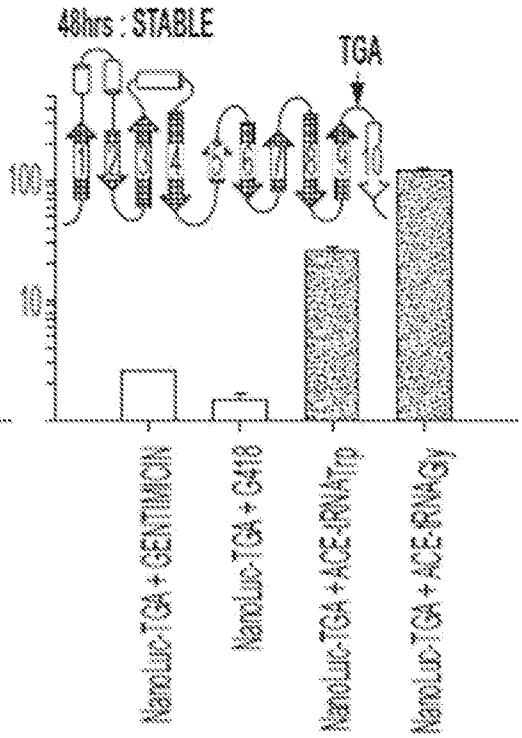


Figure 13F

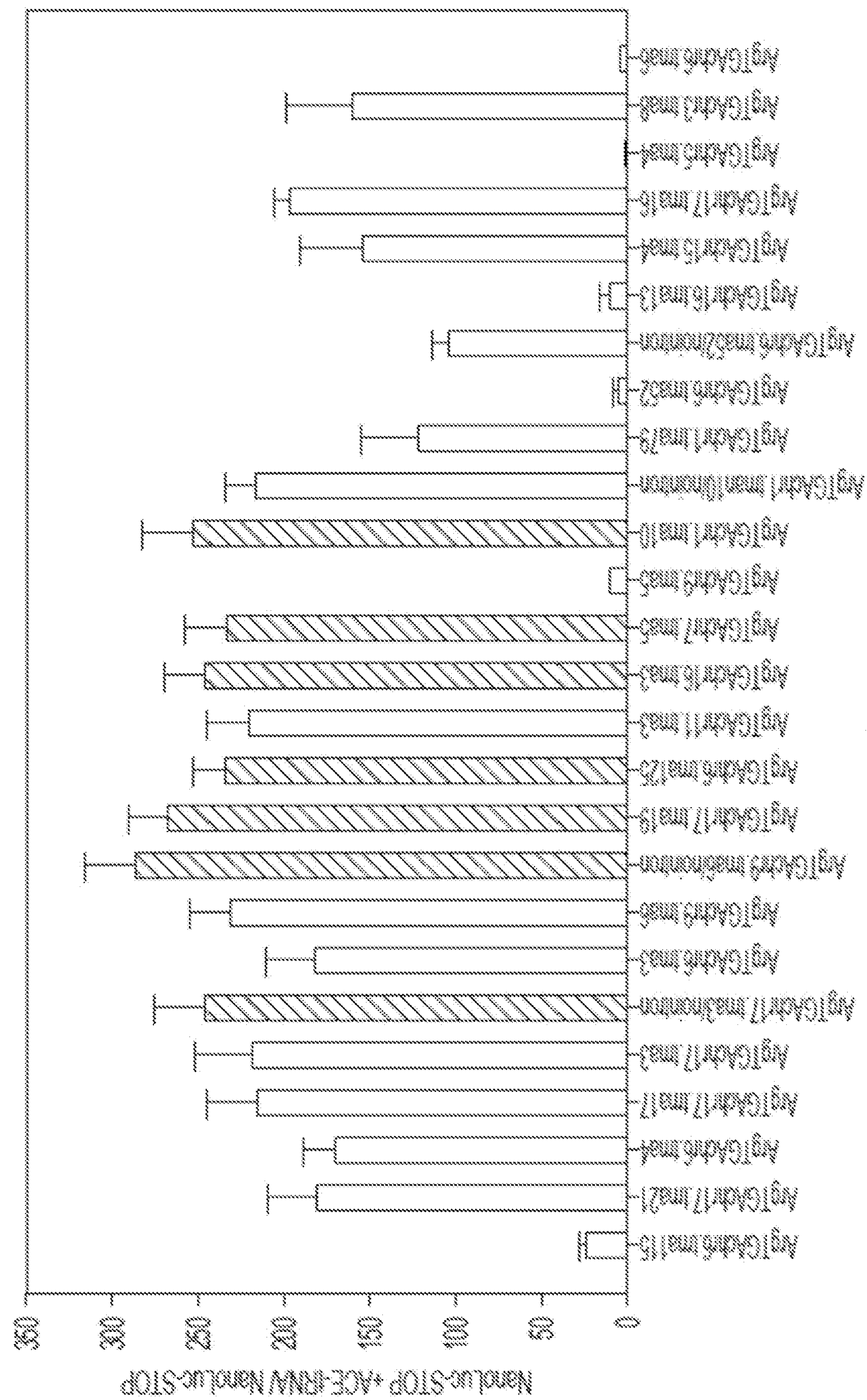


Figure 14

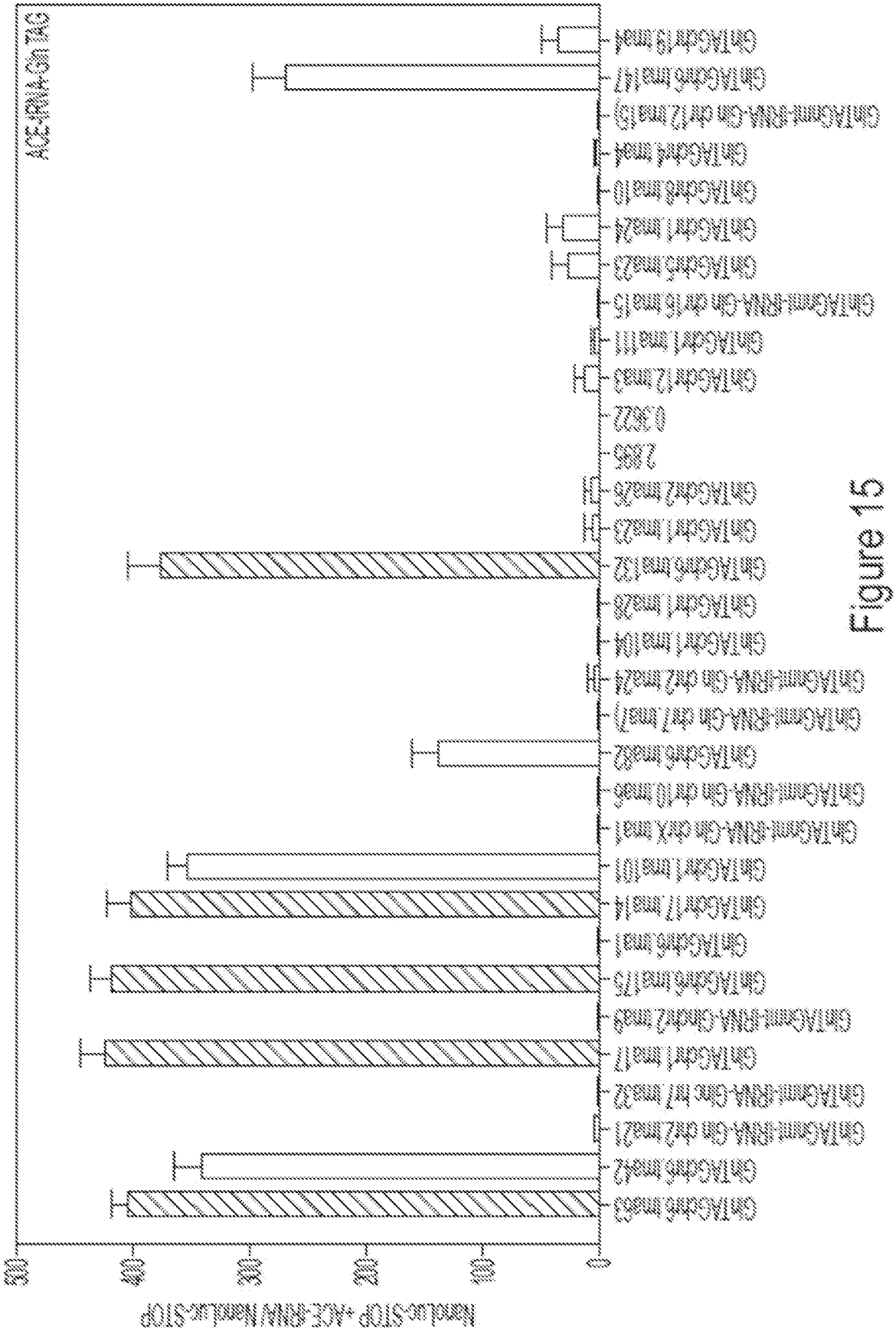

ACE-tRNA-Gln TAG
GmTAGchr9 tma4
GmTAGchr6 tma17
GmTAGcm-RNA-Gln chr12 tma15
GmTAGchr4 tma4
GmTAGchr6 tma10
GmTAGchr1 tma24
GmTAGchr6 tma23
GmTAGcm-RNA-Gln chr16 tma15
GmTAGchr1 tma11
GmTAGchr12 tma3
0.3Q2
2.8S
GmTAGchr2 tma26
GmTAGchr1 tma23
GmTAGchr6 tma12
GmTAGchr1 tma28
GmTAGchr1 tma10
GmTAGcm-RNA-Gln chr2 tma24
GmTAGcm-RNA-Gln chr7 tma7
GmTAGchr6 tma22
GmTAGcm-RNA-Gln chr10 tma36
GmTAGcm-RNA-Gln chrX tma1
GmTAGchr1 tma10
GmTAGchr17 tma14
GmTAGchr6 tma1
GmTAGchr6 tma175
GmTAGcm-RNA-Gln chr2 tma9
GmTAGchr1 tma17
GmTAGcm-RNA-Gln chr7 tma32
GmTAGcm-RNA-Gln chr2 tma21
GmTAGchr6 tma42
GmTAGchr6 tma63
Nanoluc-STOP +ACE-RNA/ Nanoluc-STOP
0.0
0.2
0.4
0.6
0.8
1.0

Figure 15

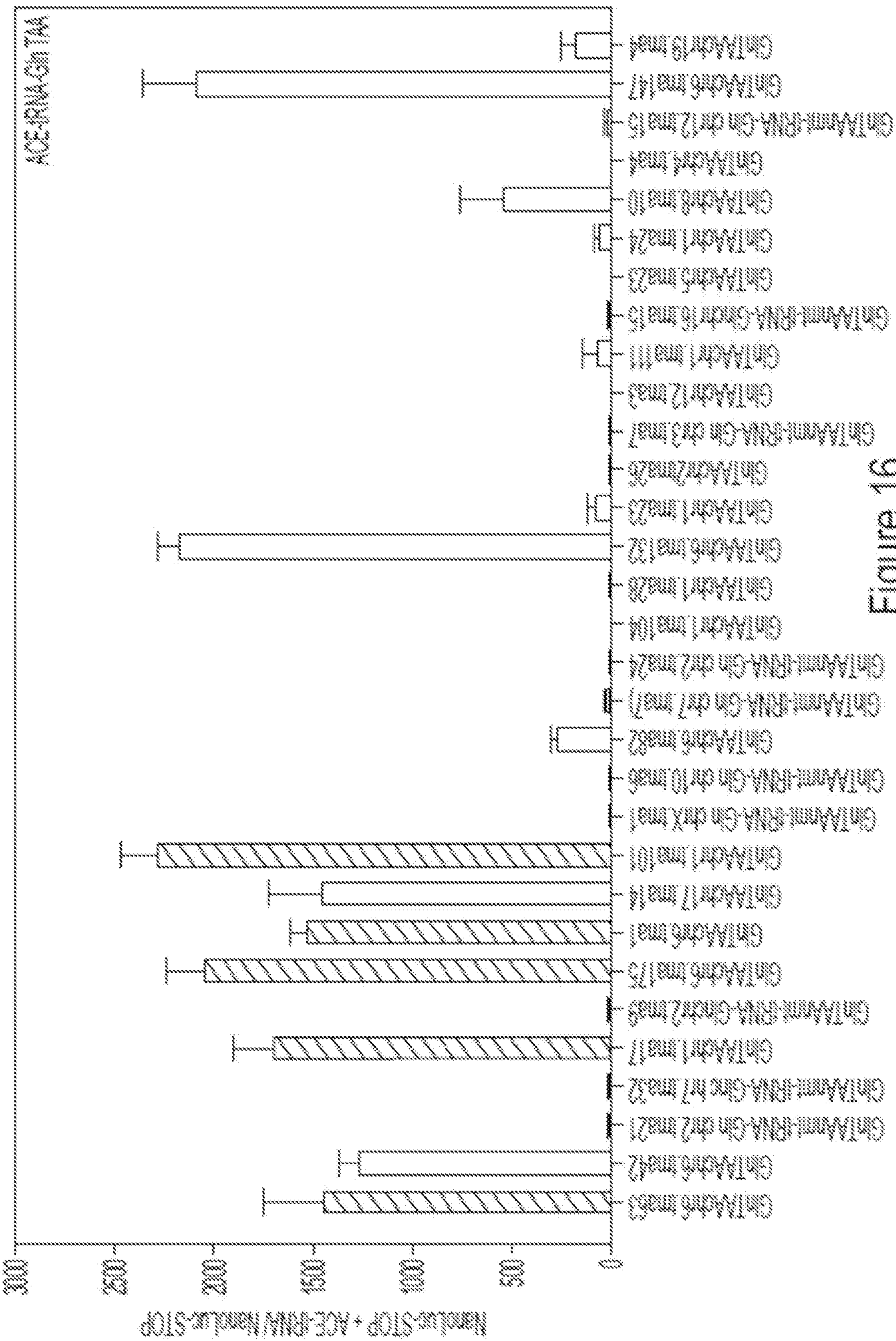

ACE-tRNA-Gln TAA
NanoLuc-STOP + ACE-tRNA/ NanoLuc-STOP
0
500
1000
1500
2000
2500
3000
GlnTAAchr6.trna53
GlnTAAchr6.trna42
GlnTAAnnnt-tRNA-Gln chr2.trna21
GlnTAAnnnt-tRNA-Glnc hr7.trna32
GlnTAAchr1.trna17
GlnTAAnnnt-tRNA-Glnchr2.trna9
GlnTAAchr6.trna175
GlnTAAchr6.trna1
GlnTAAchr17.trna14
GlnTAAchr1.trna101
GlnTAAnnnt-tRNA-Gln chrX.trna1
GlnTAAnnnt-tRNA-Gln chr10.trna6
GlnTAAchr6.trna82
GlnTAAnnnt-tRNA-Gln chr7.trna7)
GlnTAAnnnt-tRNA-Gln chr2.trna24
GlnTAAchr1.trna104
GlnTAAchr1.trna28
GlnTAAchr6.trna132
GlnTAAchr1.trna23
GlnTAAchr2trna35
GlnTAAnnnt-tRNA-Gln chr3.trna7
GlnTAAchr12.trna3
GlnTAAchr1.trna111
GlnTAAnnnt-tRNA-Glnchr16.trna15
GlnTAAchr6.trna23
GlnTAAchr1.trna24
GlnTAAchr6.trna10
GlnTAAchr4.trna1
GlnTAAnnnt-tRNA-Gln chr12.trna15
GlnTAAchr6.trna147
GlnTAAchr19.trna1

Figure 16

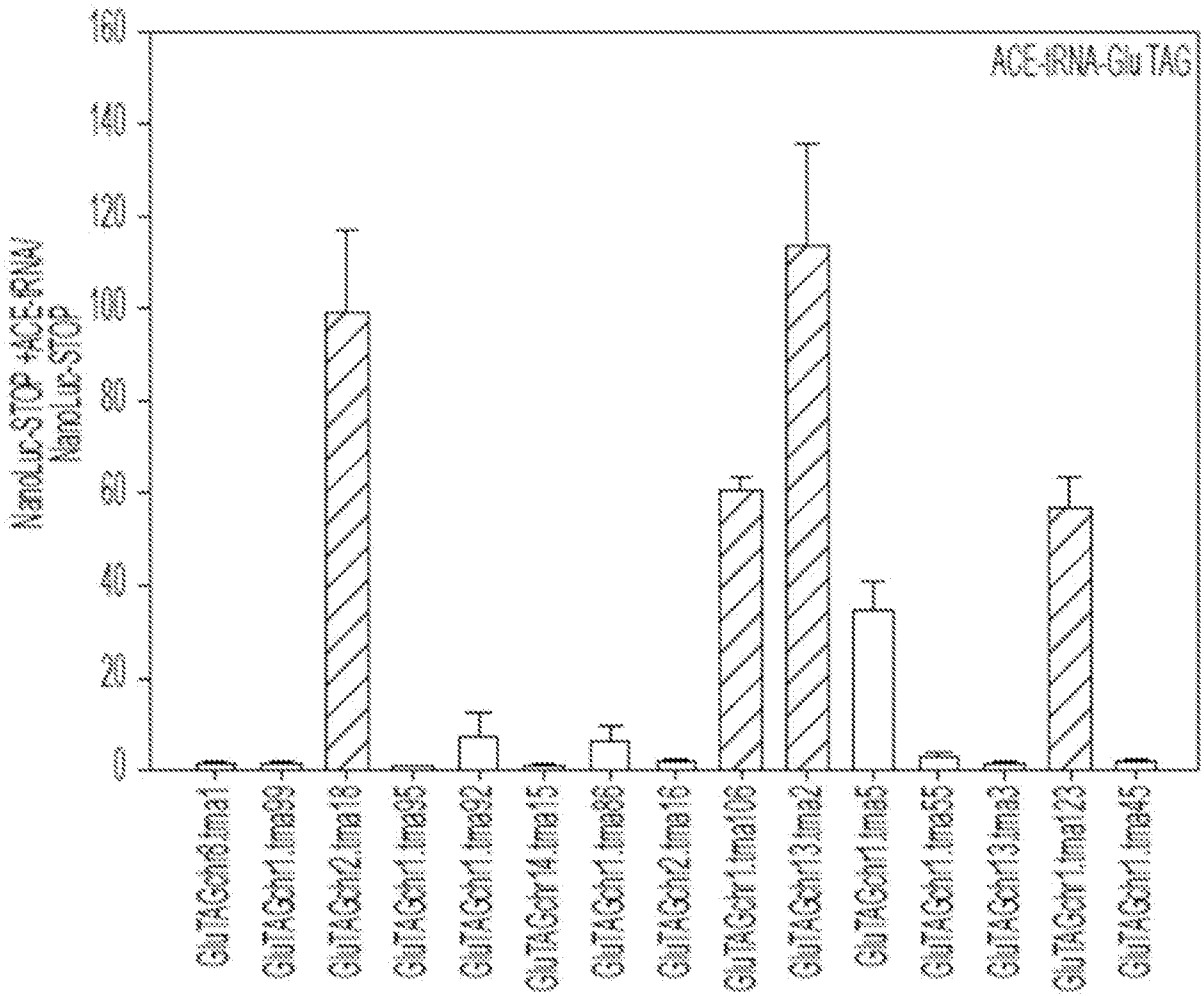

NanoLuc-STOP +ACE-tRNA/
NanoLuc-STOP
0
20
40
60
80
100
120
140
160
ACE-tRNA-GluTAG
GluTAGchr6.lma1
GluTAGchr1.lma99
GluTAGchr2.lma18
GluTAGchr1.lma65
GluTAGchr1.lma92
GluTAGchr14.lma15
GluTAGchr1.lma86
GluTAGchr2.lma16
GluTAGchr1.lma106
GluTAGchr13.lma2
GluTAGchr1.lma5
GluTAGchr1.lma55
GluTAGchr13.lma3
GluTAGchr1.lma123
GluTAGchr1.lma45

Figure 17

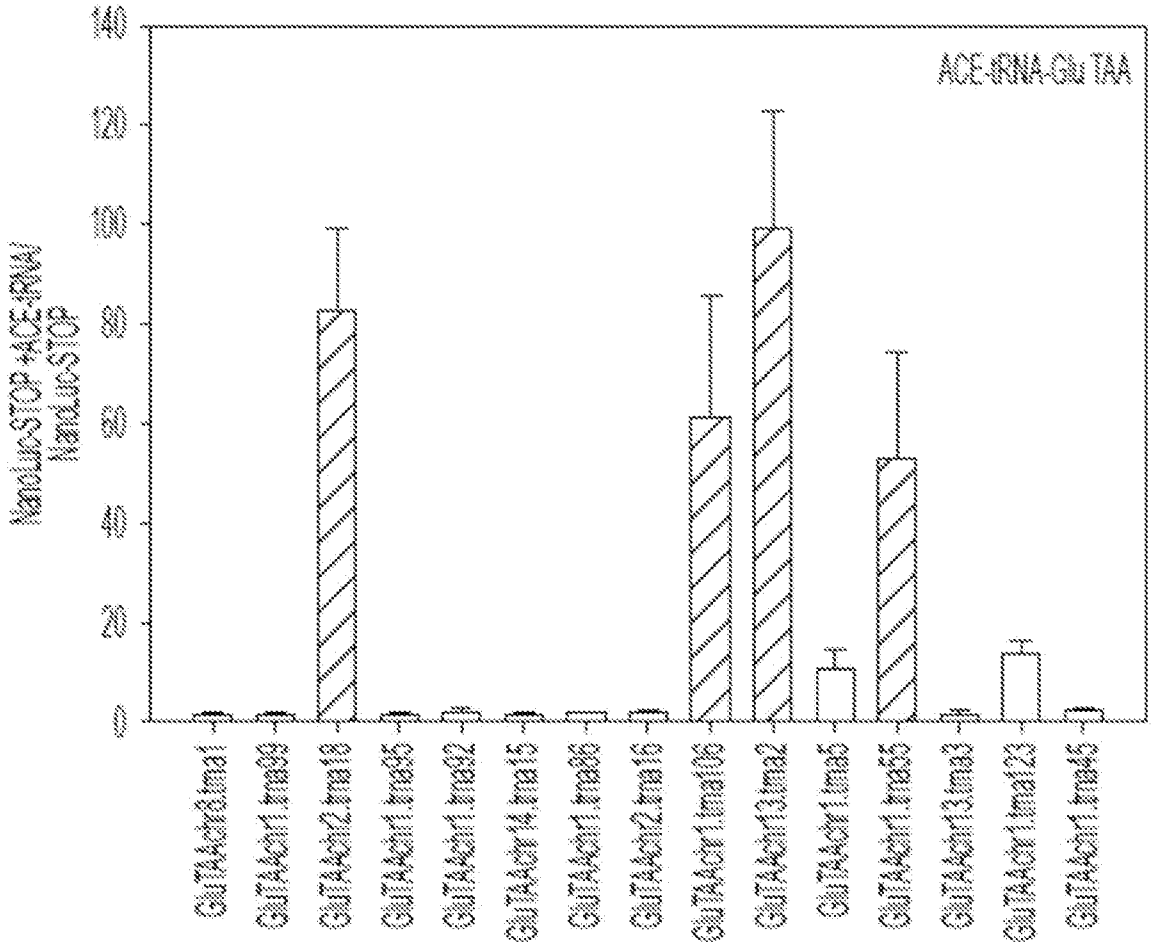

ACE-tRNA-GluTAA
NanoLuc-STOP +ACE-tRNA/ NanoLuc-STOP
0
20
40
60
80
100
120
140
GluTAAchr3.tma1
GluTAAchr1.tma39
GluTAAchr2.tma18
GluTAAchr1.tma45
GluTAAchr1.tma92
GluTAAchr14.tma15
GluTAAchr1.tma86
GluTAAchr2.tma16
GluTAAchr1.tma106
GluTAAchr13.tma2
GluTAAchr1.tma5
GluTAAchr1.tma55
GluTAAchr13.tma3
GluTAAchr1.tma123
GluTAAchr1.tma45

Figure 18

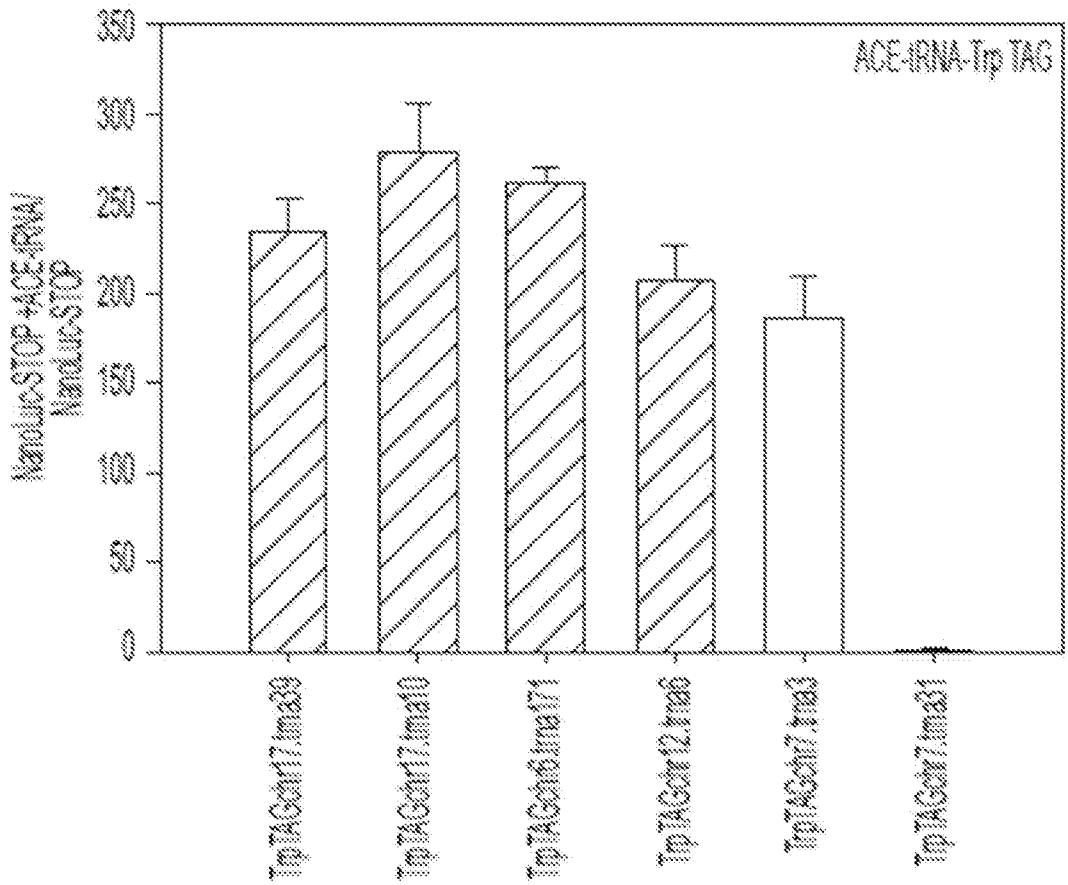

ACE-tRNA-Trp TAG
NanoLuc STOP +ACE-tRNA/
NanoLuc STOP
0
50
100
150
200
250
300
350
Trp TAG chr17.trna39
Trp TAG chr17.trna10
Trp TAG chr6.trna171
Trp TAG chr12.trna6
Trp TAG chr7.trna3
Trp TAG chr7.trna31

Figure 19

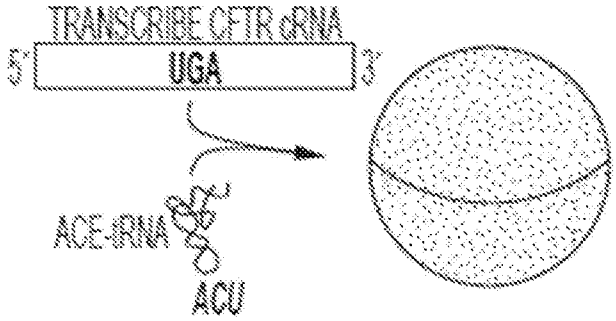


Figure 20A

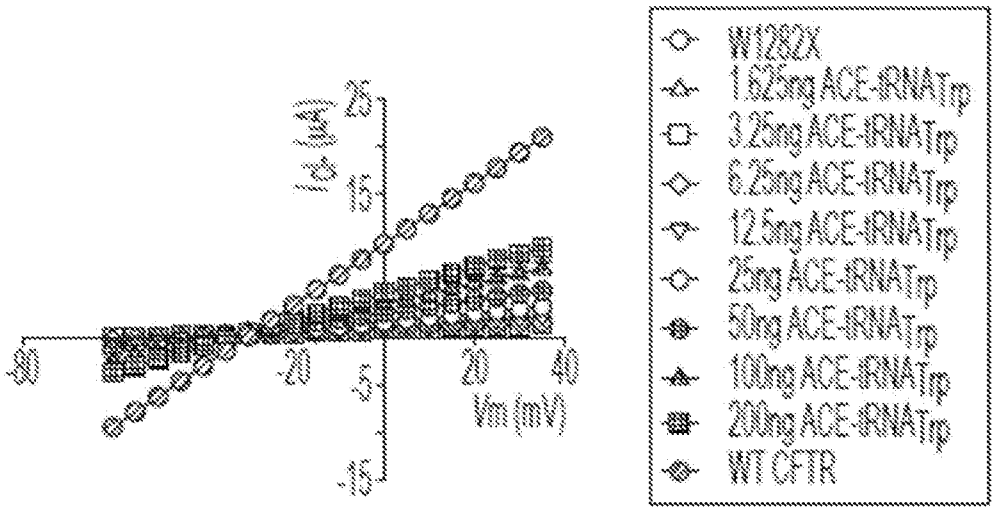


Figure 20B

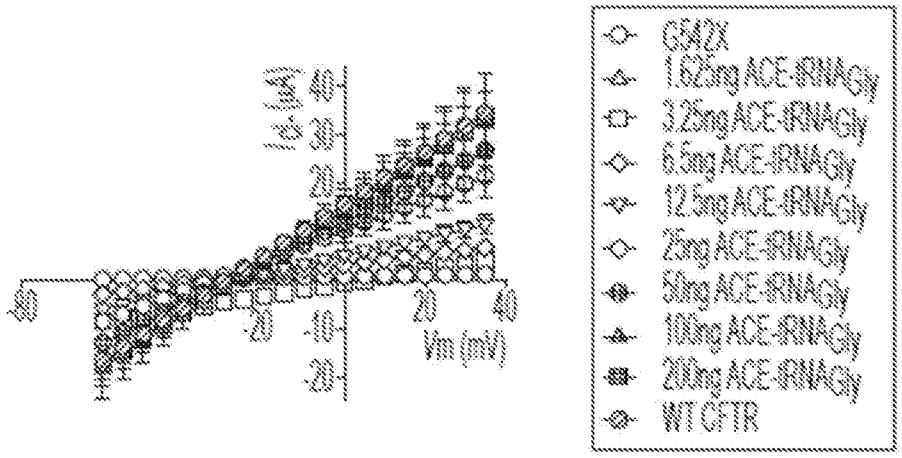


Figure 20C

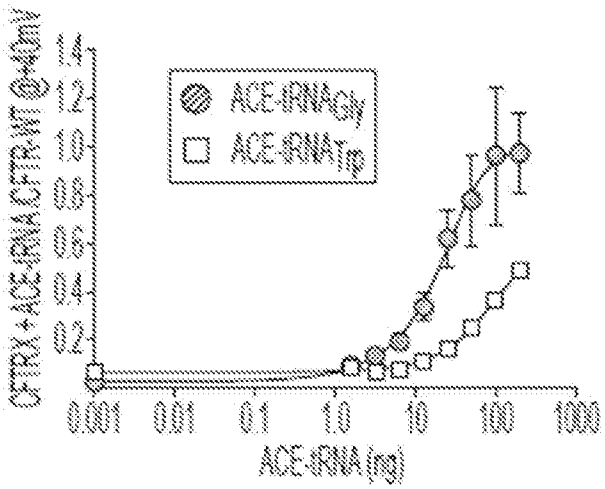


Figure 20D

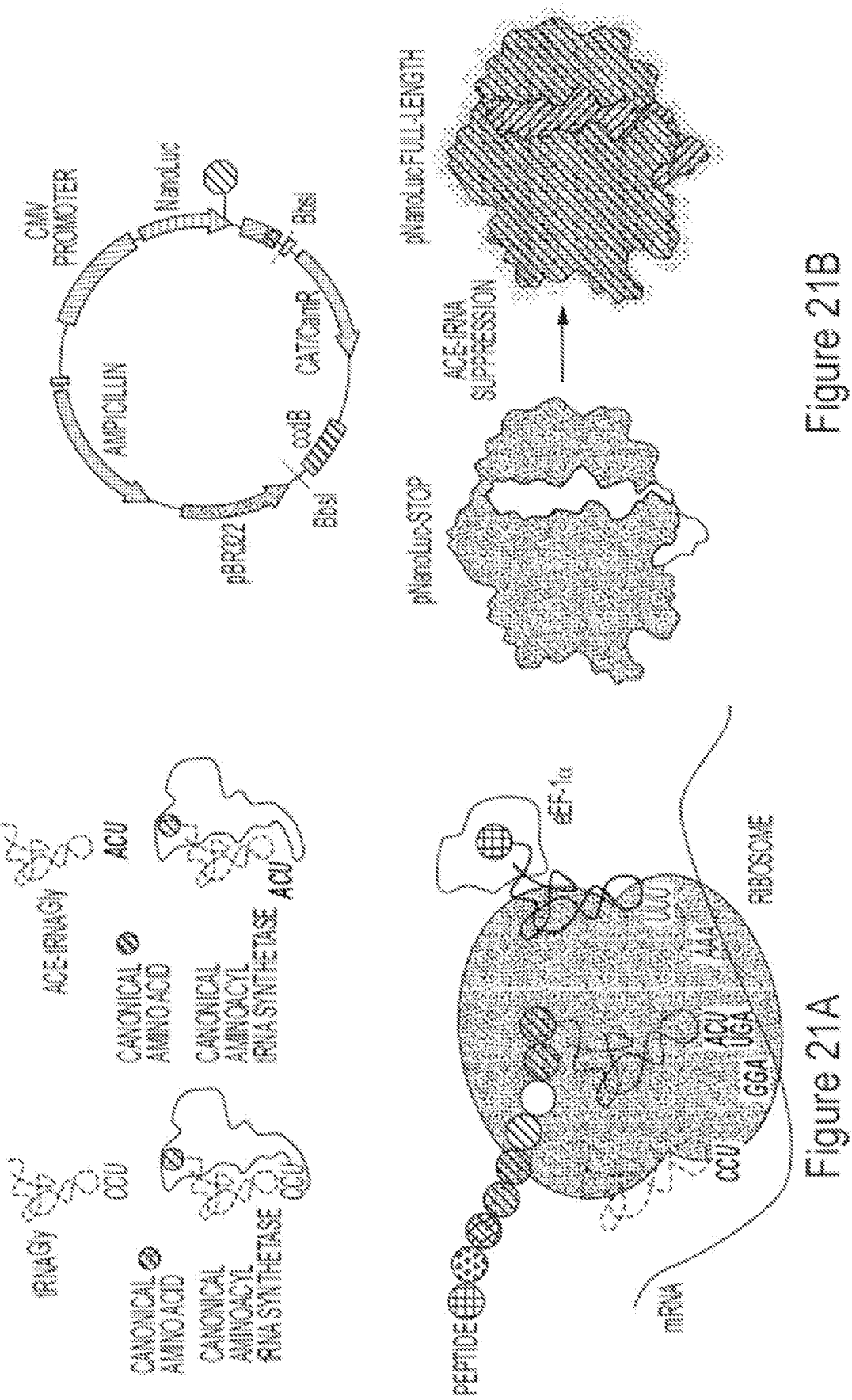


Figure 21A

Figure 21B

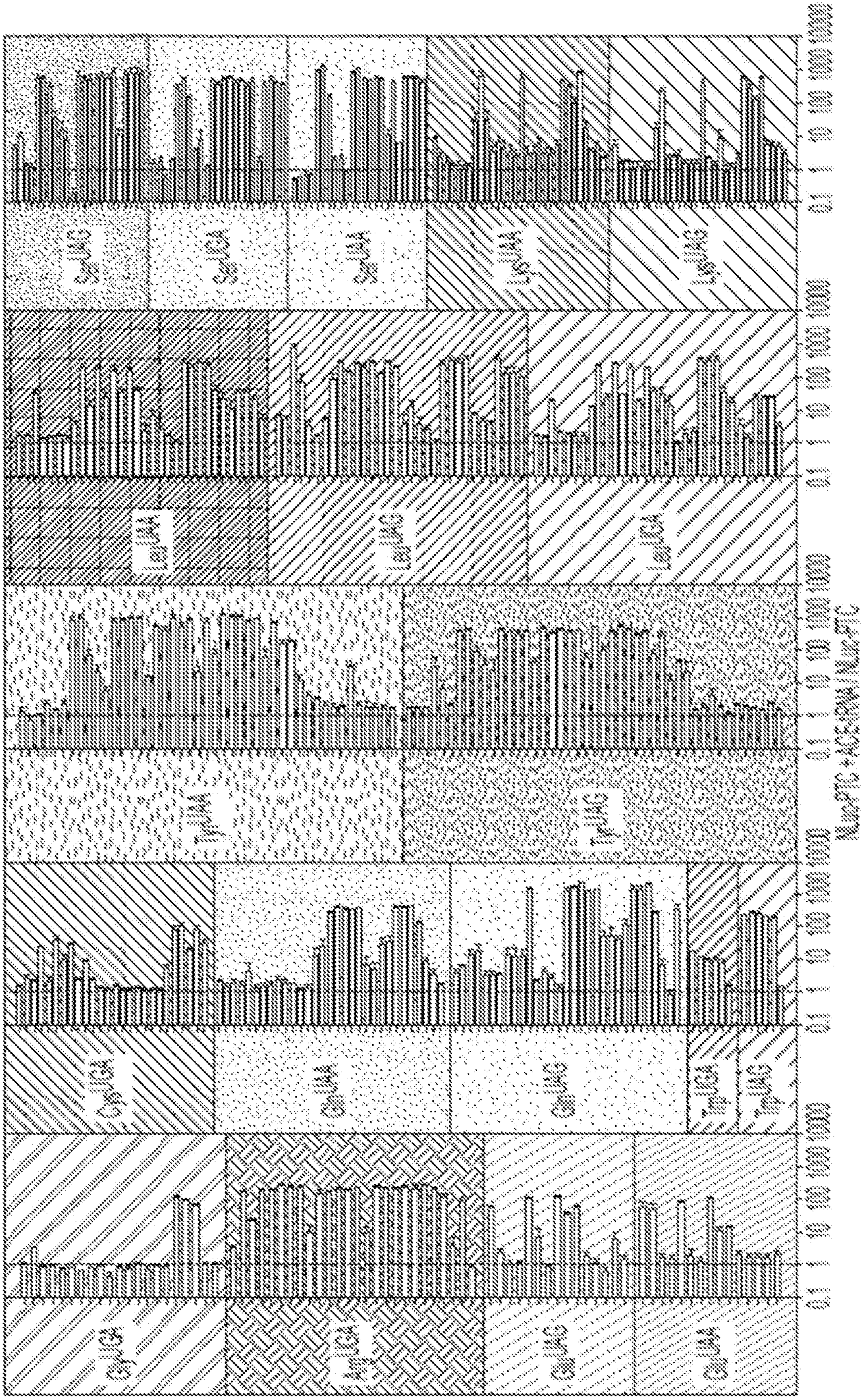


Figure 22

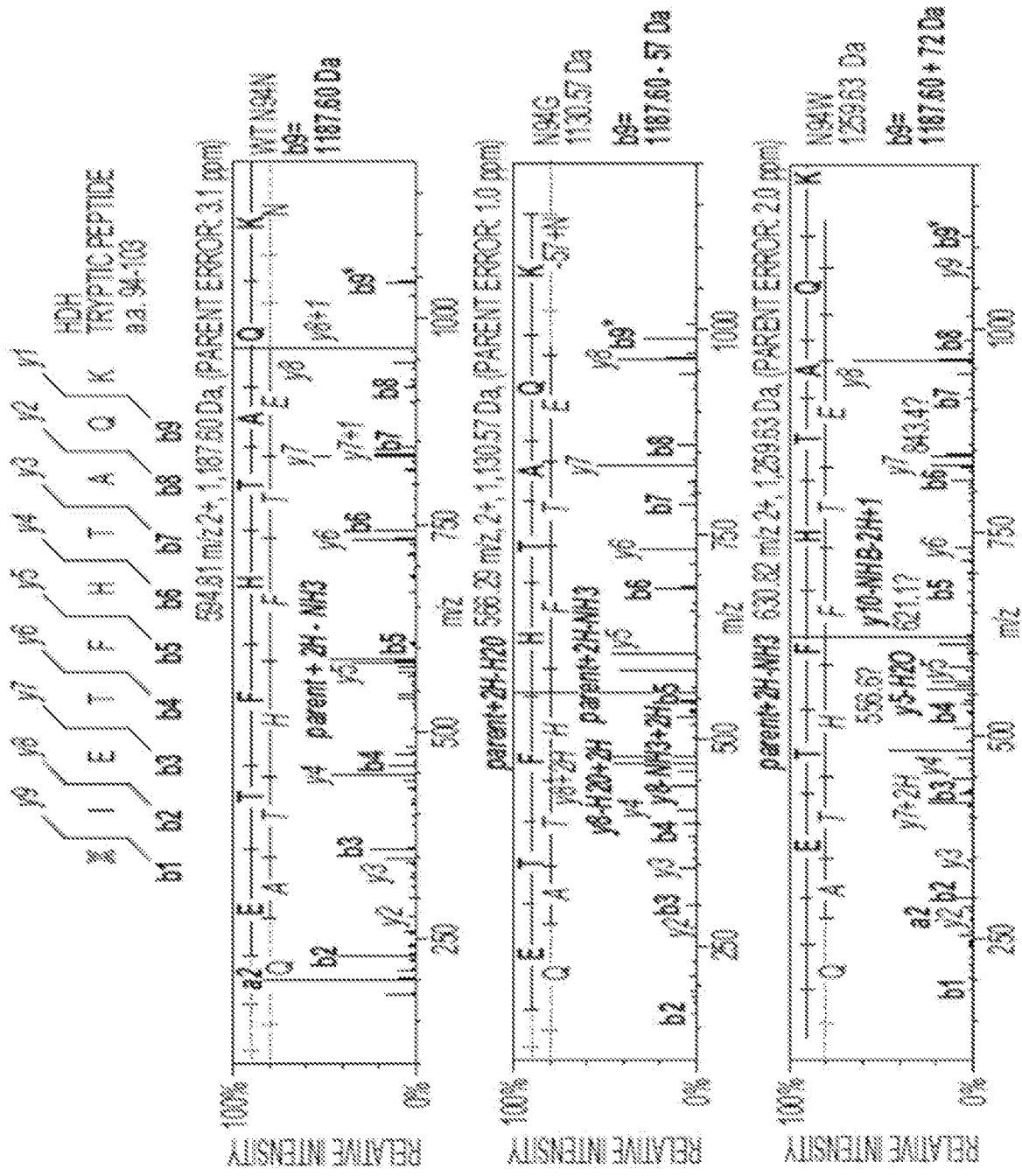

HDH
TRYPTIC PEPTIDE
a.a. 94-103
WT N94N
1187.60 Da
N94G
1130.57 Da
N94W
1259.63 Da
RELATIVE INTENSITY
m/z

Figure 23A

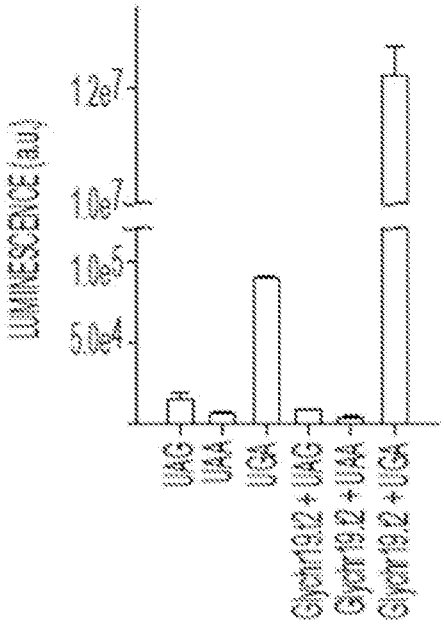


Figure 23B

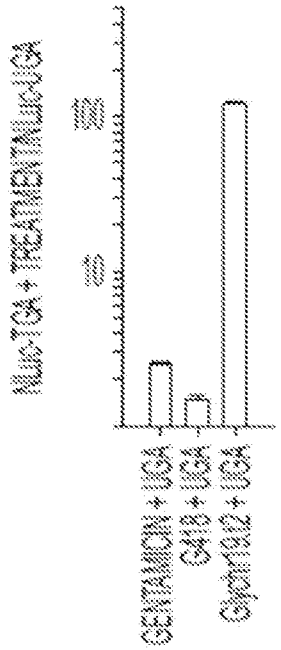


Figure 23C

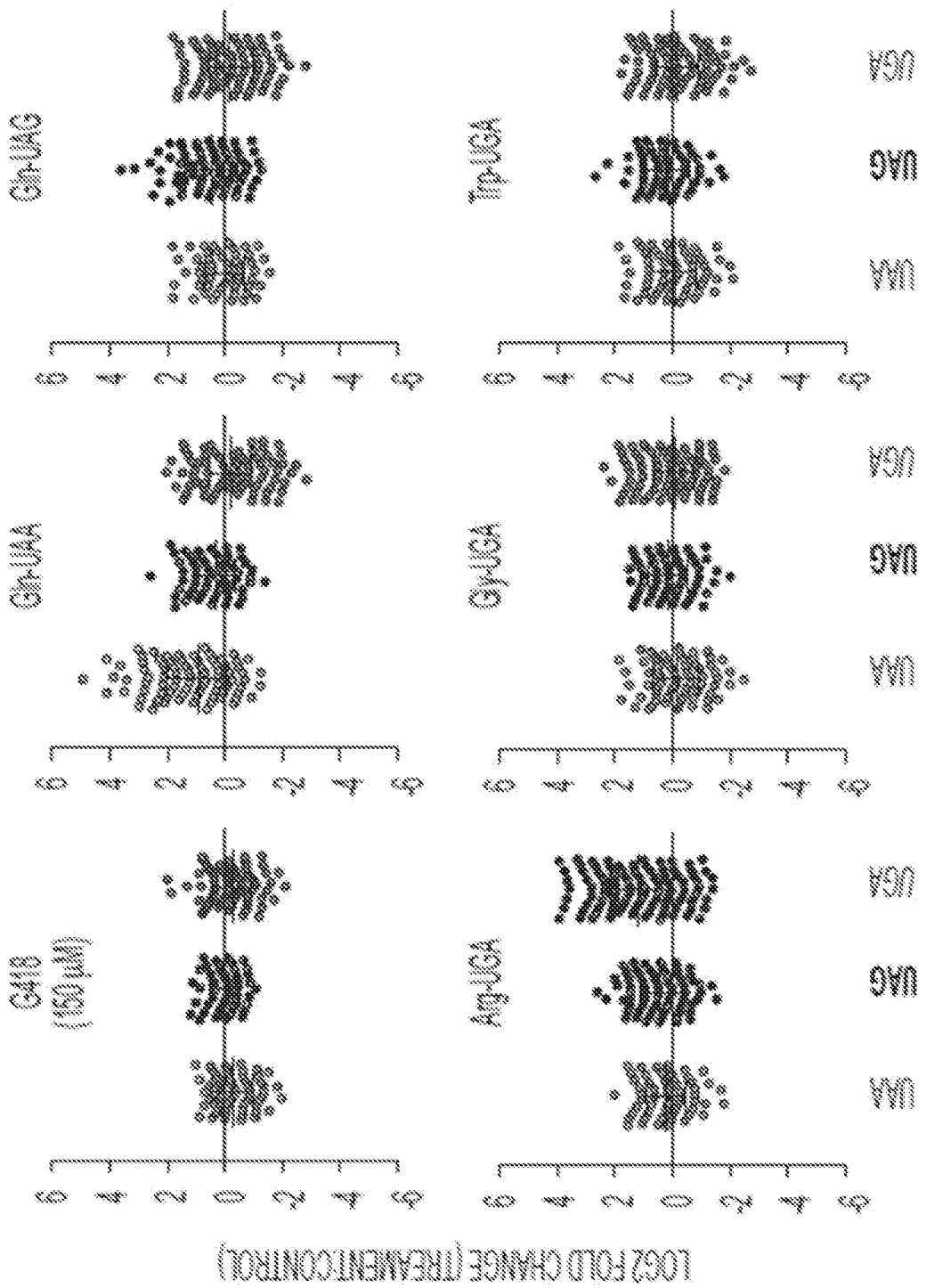

Gln-UAG
Trp-UGA
Gln-UAA
Gly-UGA
G418 (150 μM)
Arg-UGA
UAA
UAG
UGA
6 4 2 0 2 4 6
LOG2 FOLD CHANGE (TREATMENT:CONTROL)
STOP CODON OF TRANSCRIPT

Figure 24A

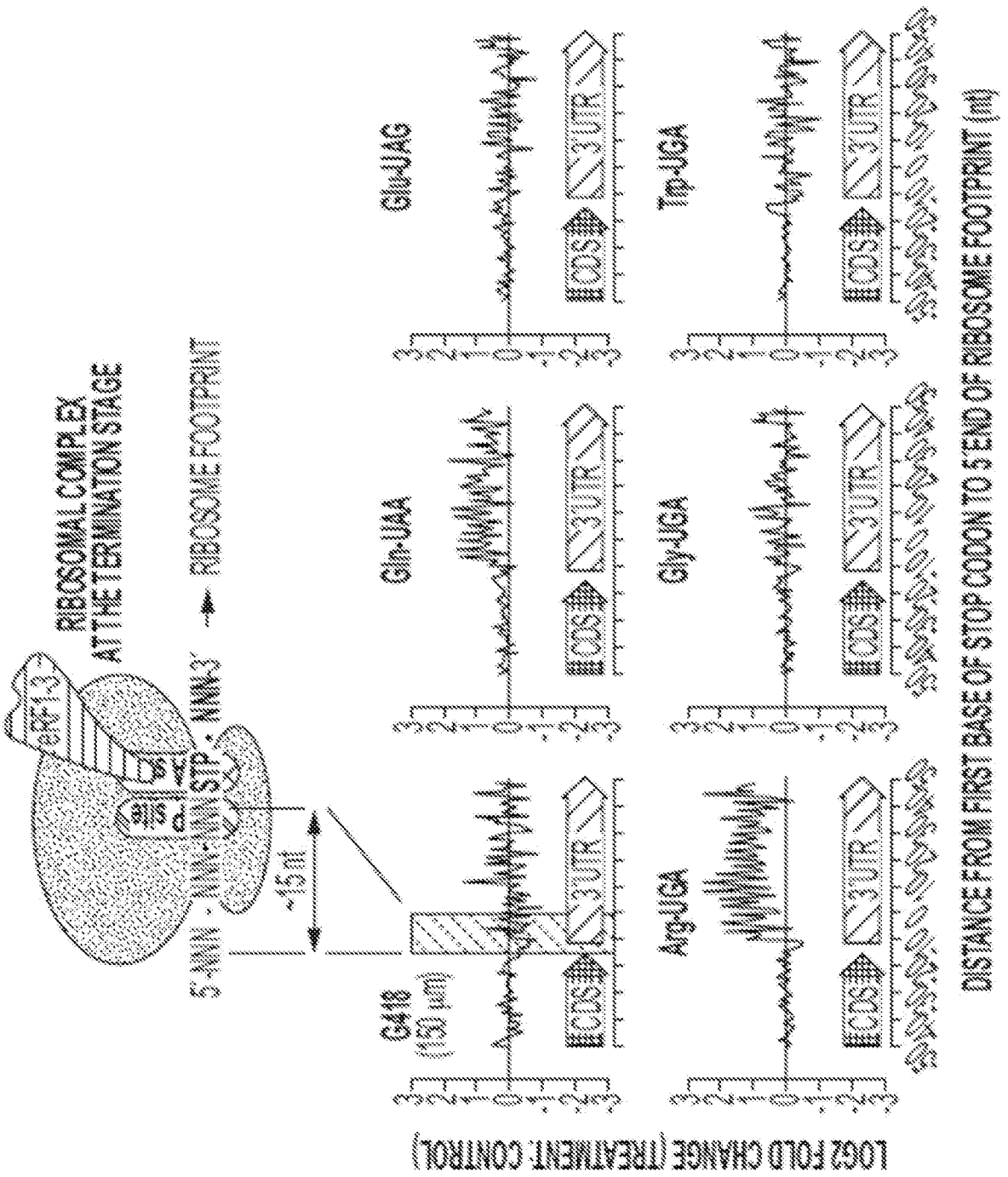

RIBOSOMAL COMPLEX AT THE TERMINATION STAGE
eRF1/3
P site
A site
5'-NNN - NNN-NNN STP - NNN-3'
~15 nt
RIBOSOME FOOTPRINT
G418 (150 μM)
Gln-UAA
Gln-UAG
Arg-UGA
Gly-UGA
Trp-UGA
5'UTR
CDS
3'UTR
LOG2 FOLD CHANGE (TREATMENT: CONTROL)
DISTANCE FROM FIRST BASE OF STOP CODON TO 5'END OF RIBOSOME FOOTPRINT (nt)

Figure 24B

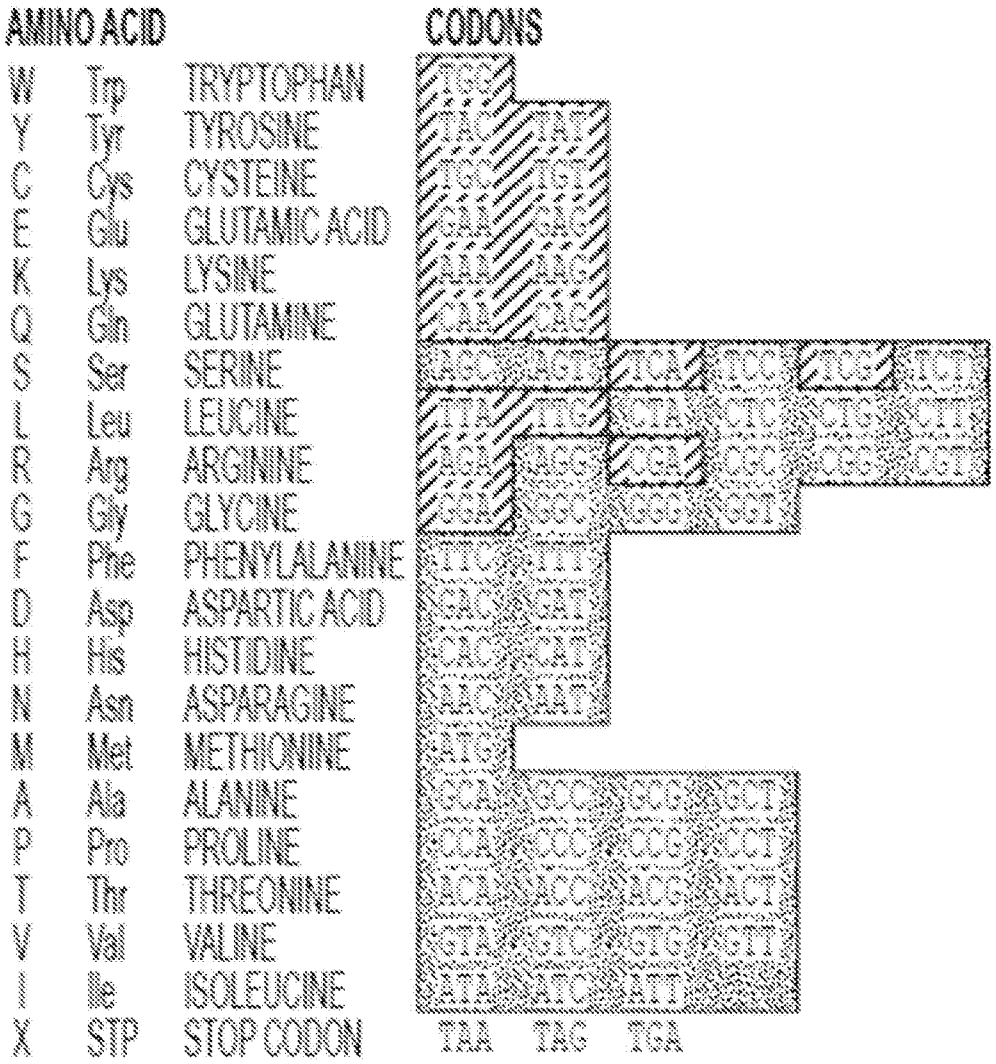

AMINO ACID
CODONS
W Trp TRYPTOPHAN TGG
Y Tyr TYROSINE TAC TAT
C Cys CYSTEINE TGC TGT
E Glu GLUTAMIC ACID GAA GAG
K Lys LYSINE AAA AAG
Q Gln GLUTAMINE CAA CAG
S Ser SERINE AGC AGT TCA TCC TCG TCT
L Leu LEUCINE TTA TTG CTA CTC CTG CTT
R Arg ARGININE AGA AGG CGA CGC CGG CGT
G Gly GLYCINE GGA GGC GGG GGT
F Phe PHENYLALANINE TTC TTT
D Asp ASPARTIC ACID GAC GAT
H His HISTIDINE CAC CAT
N Asn ASPARAGINE AAC AAT
M Met METHIONINE ATG
A Ala ALANINE GCA GCC GCG GCT
P Pro PROLINE CCA CCC CCG CCT
T Thr THREONINE ACA ACC ACG ACT
V Val VALINE GTA GTC GTG GTT
I Ile ISOLEUCINE ATA ATC ATT
X STP STOP CODON TAA TAG TGA

Figure 25

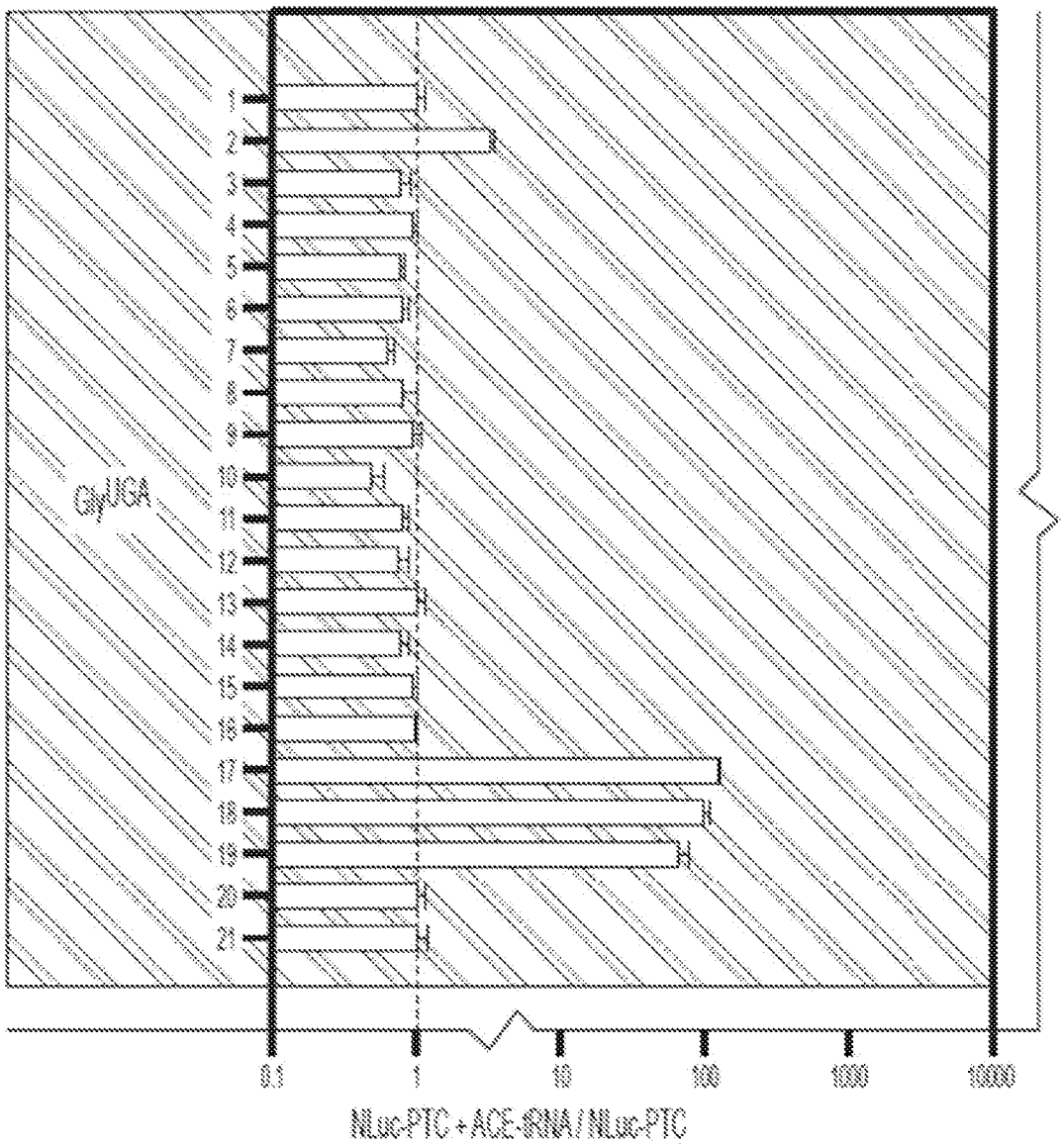

Gly/UGA
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
0.1
1
10
100
1000
10000
NLuc-PTC + ACE-tRNA / NLuc-PTC

Figure 26

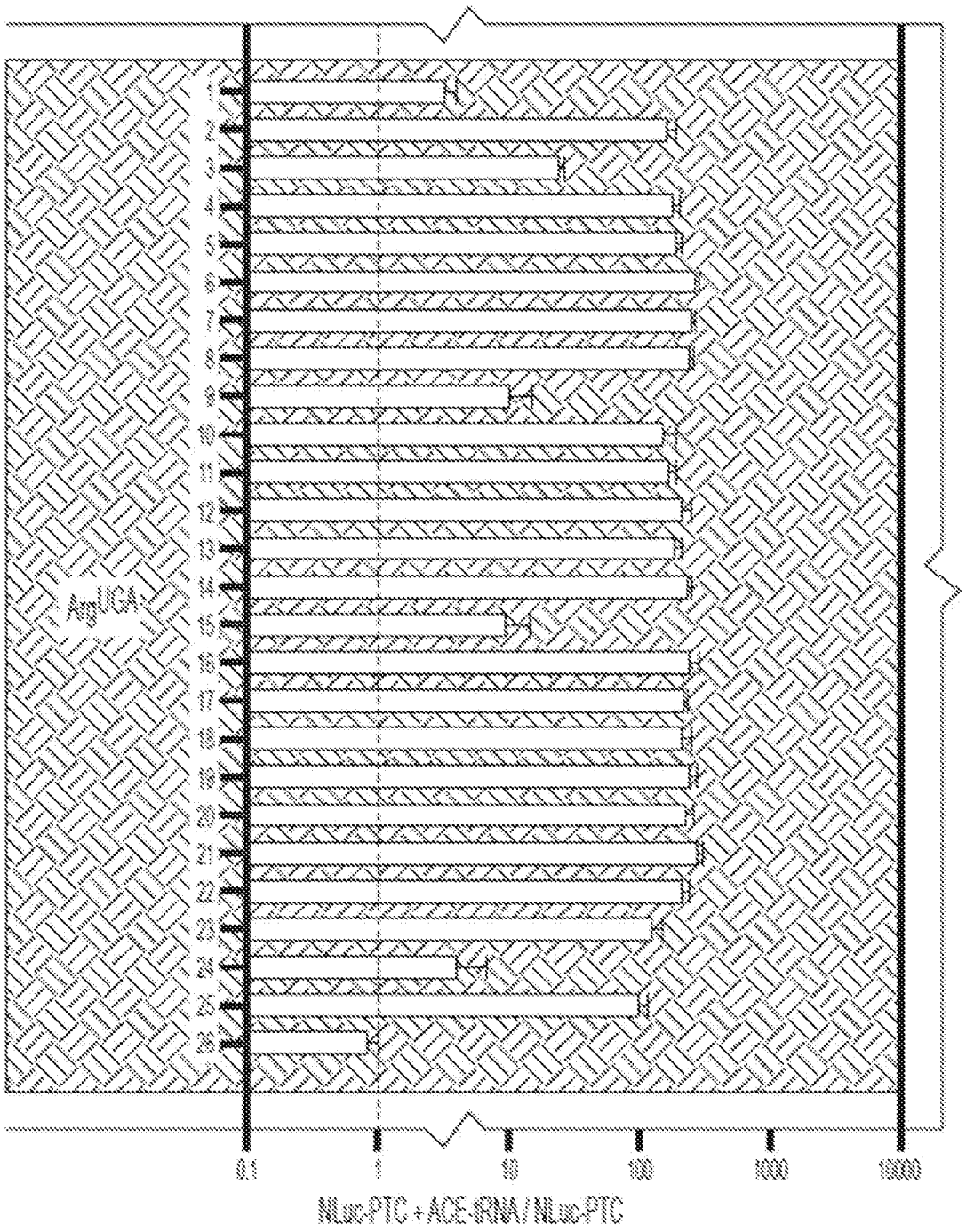


Figure 26
CONTINUED

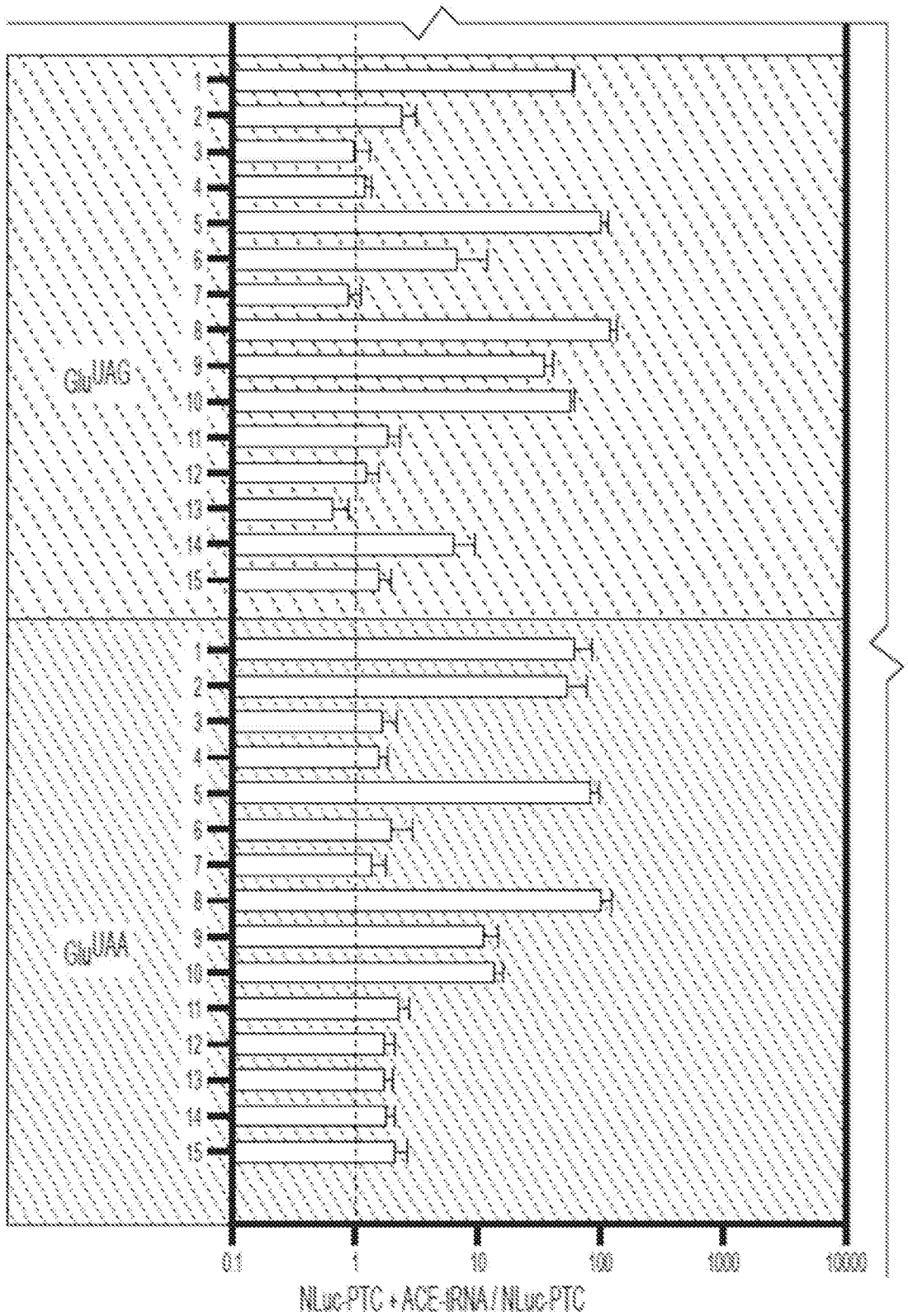


Figure 26
CONTINUED

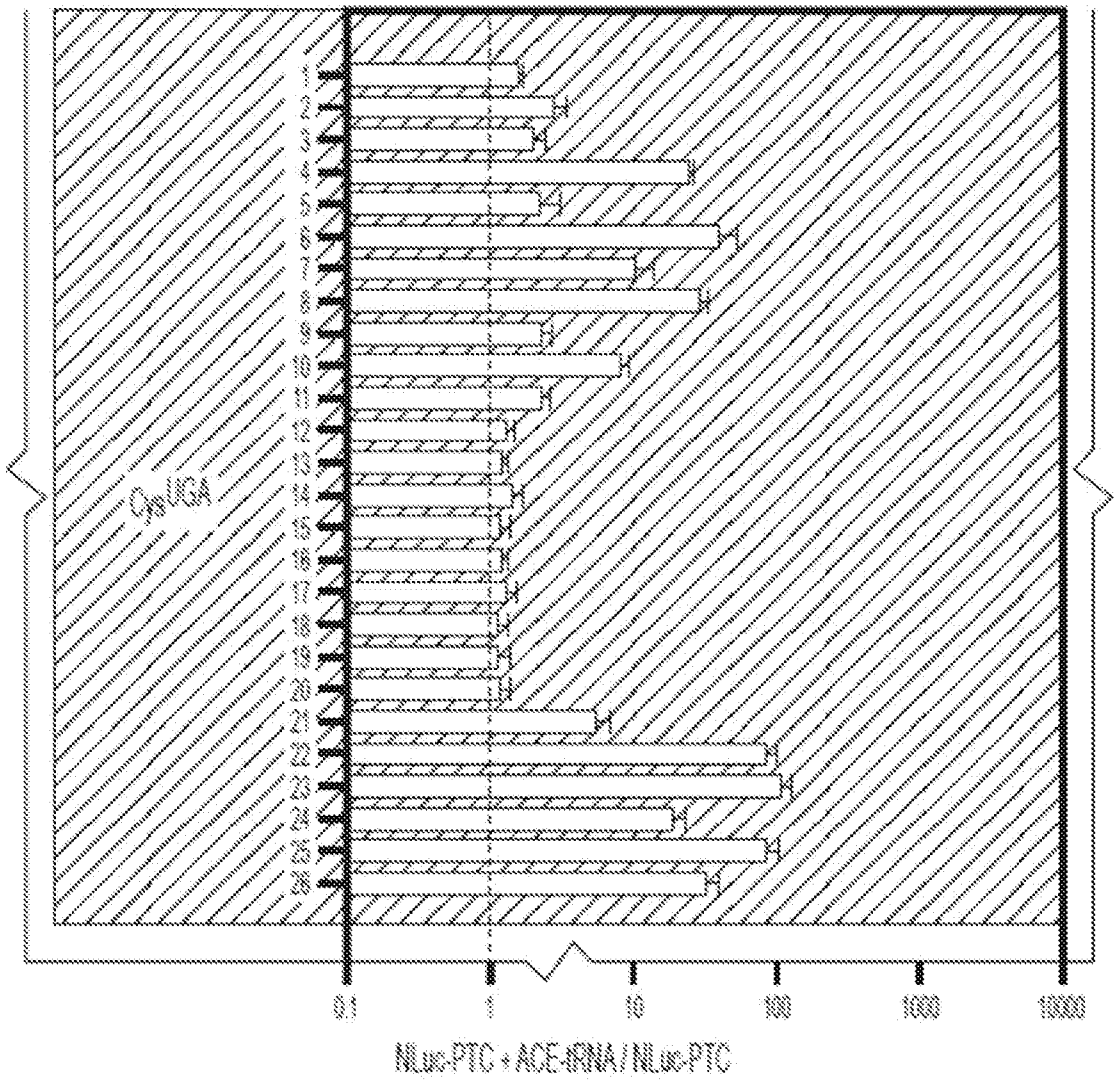


Figure 26
CONTINUED

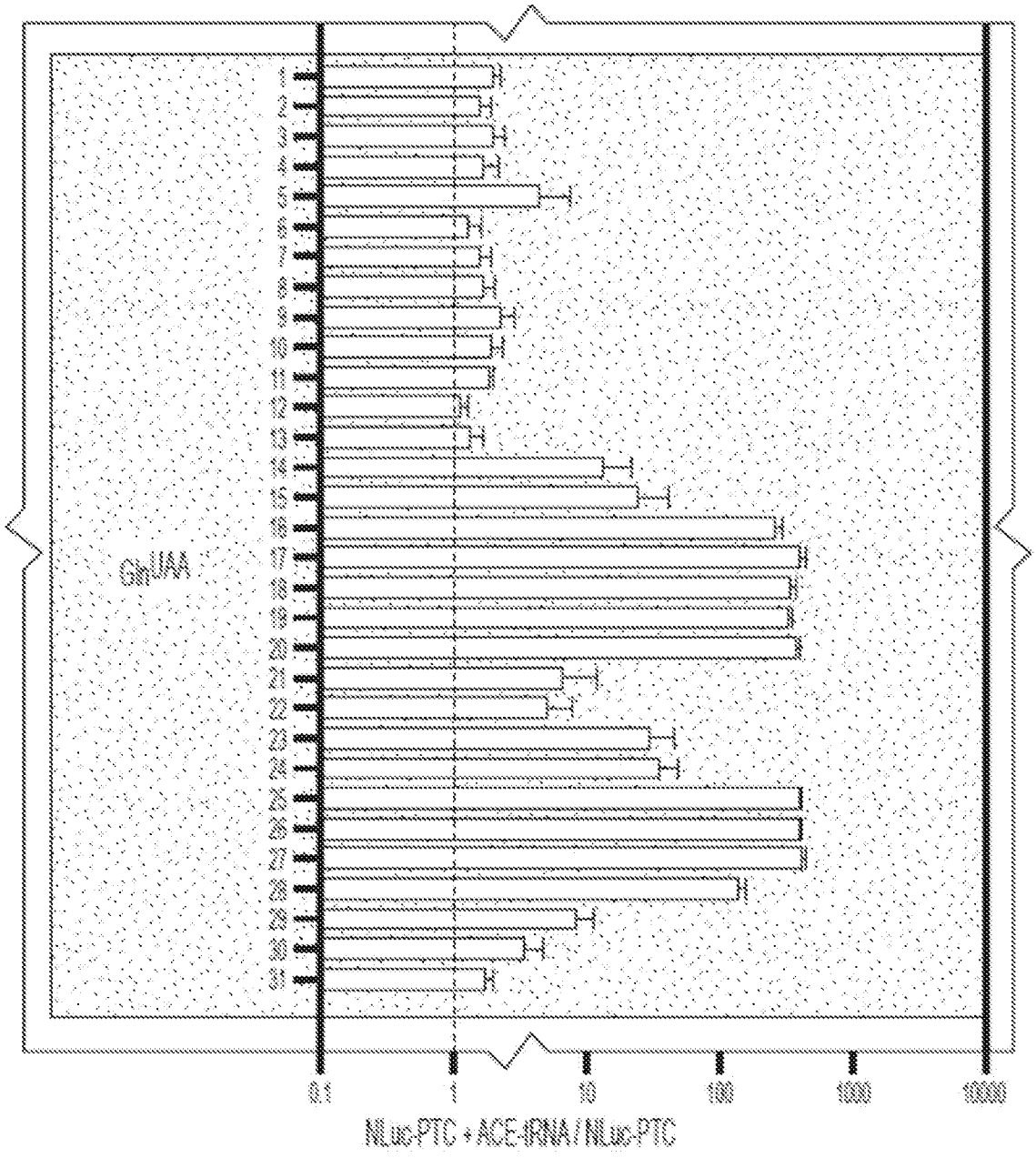


Figure 26
CONTINUED

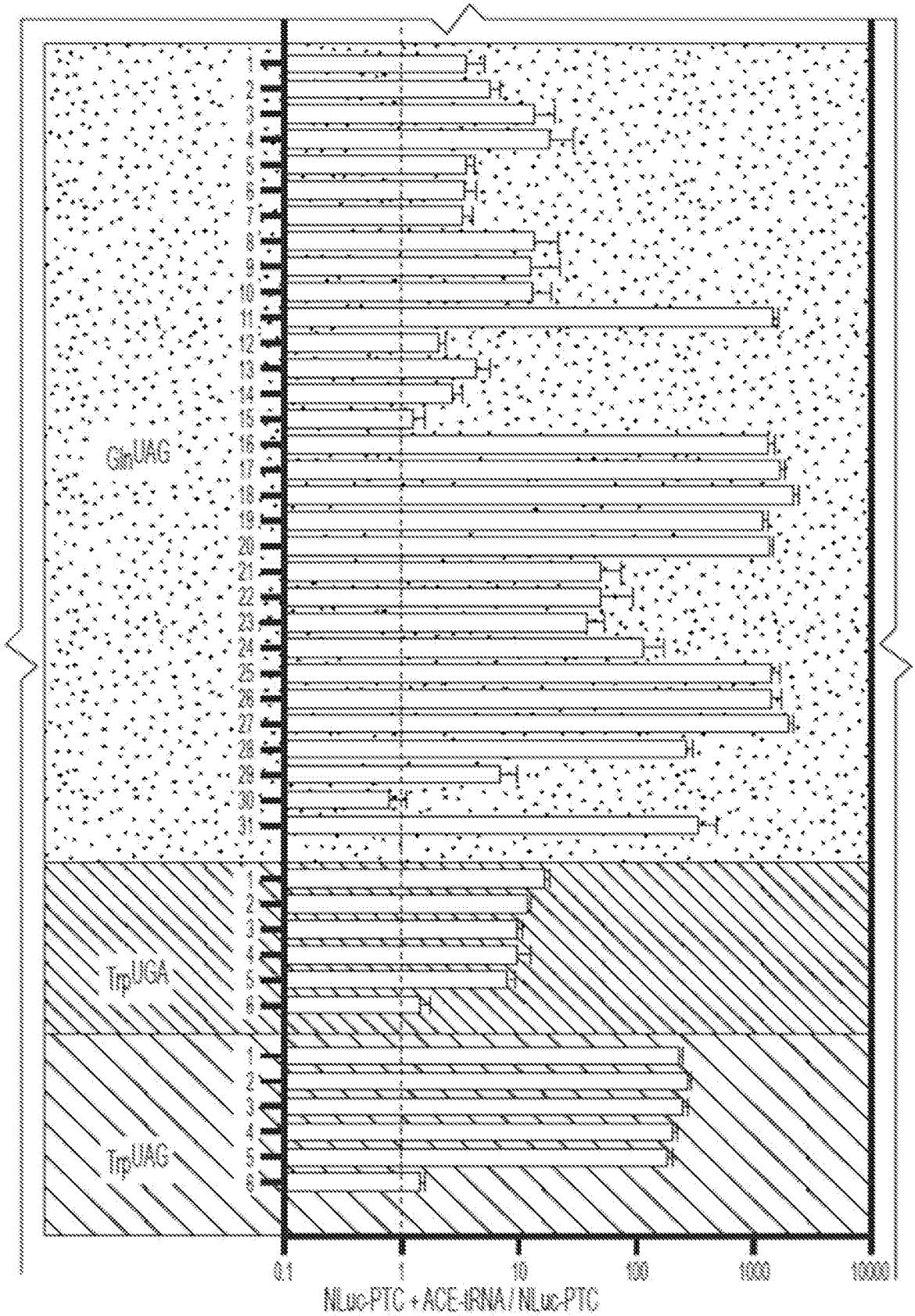


Figure 26
CONTINUED

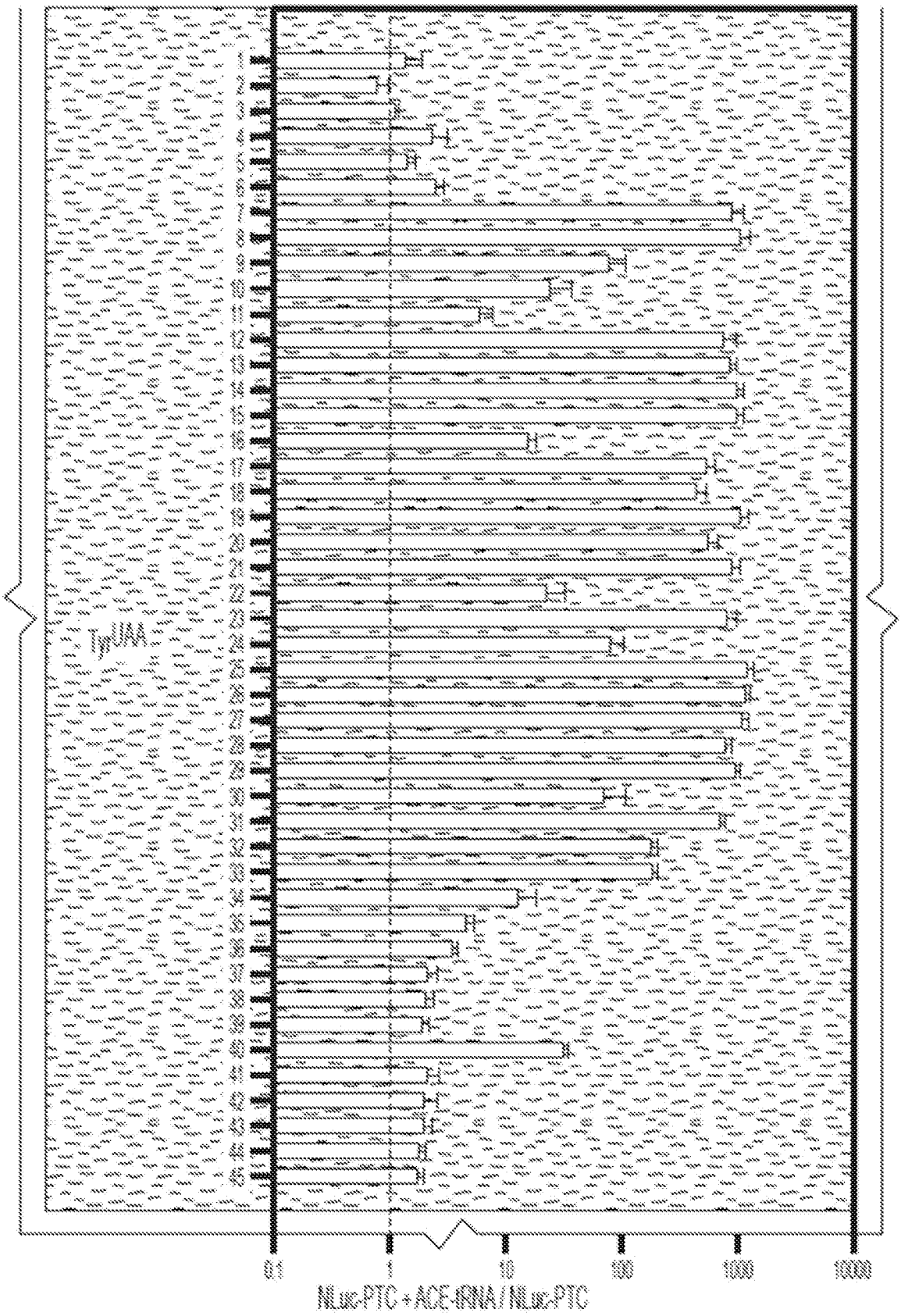


Figure 26
CONTINUED

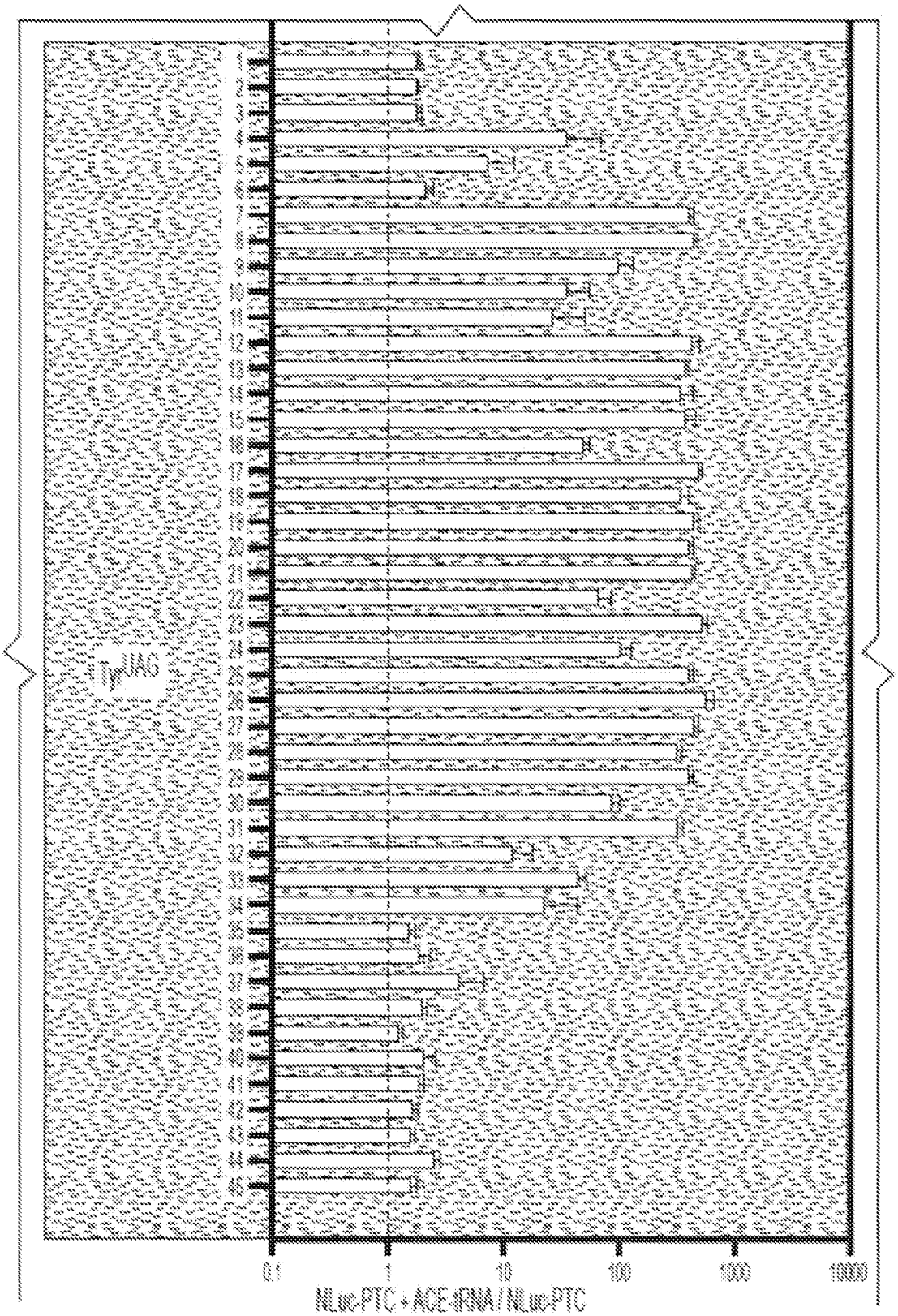


Figure 26
CONTINUED

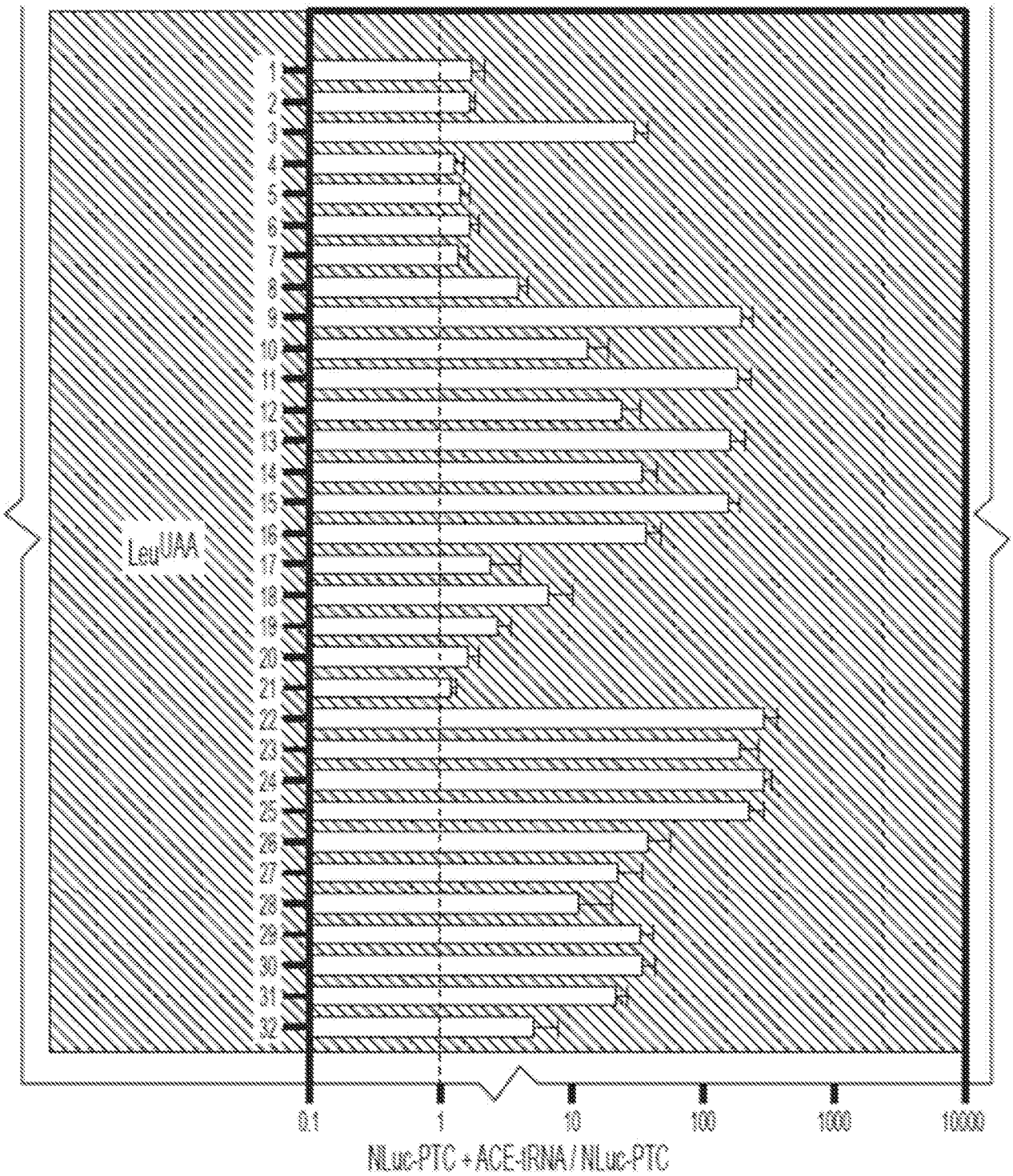


Figure 26
CONTINUED

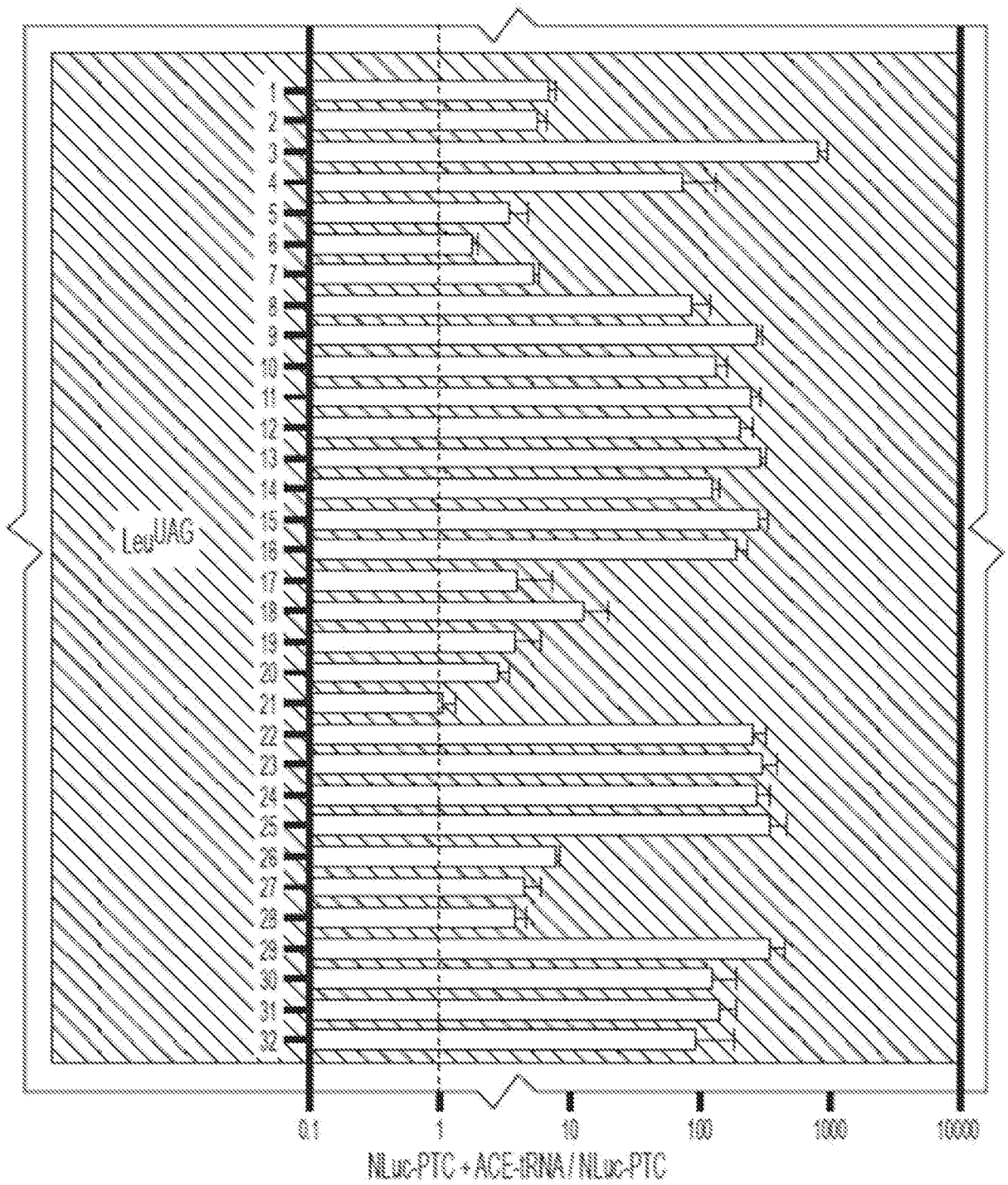


Figure 26
CONTINUED

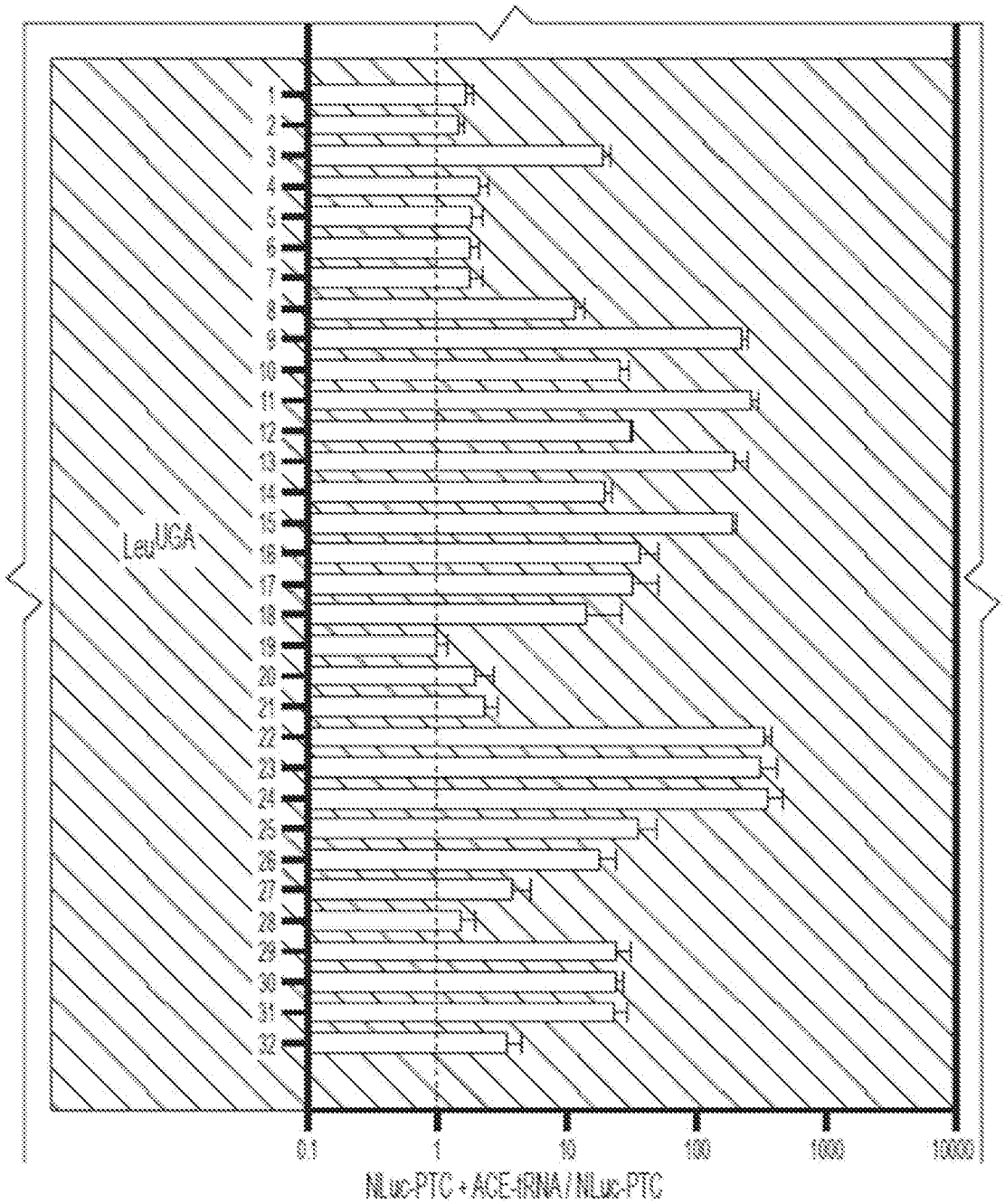


Figure 26
CONTINUED

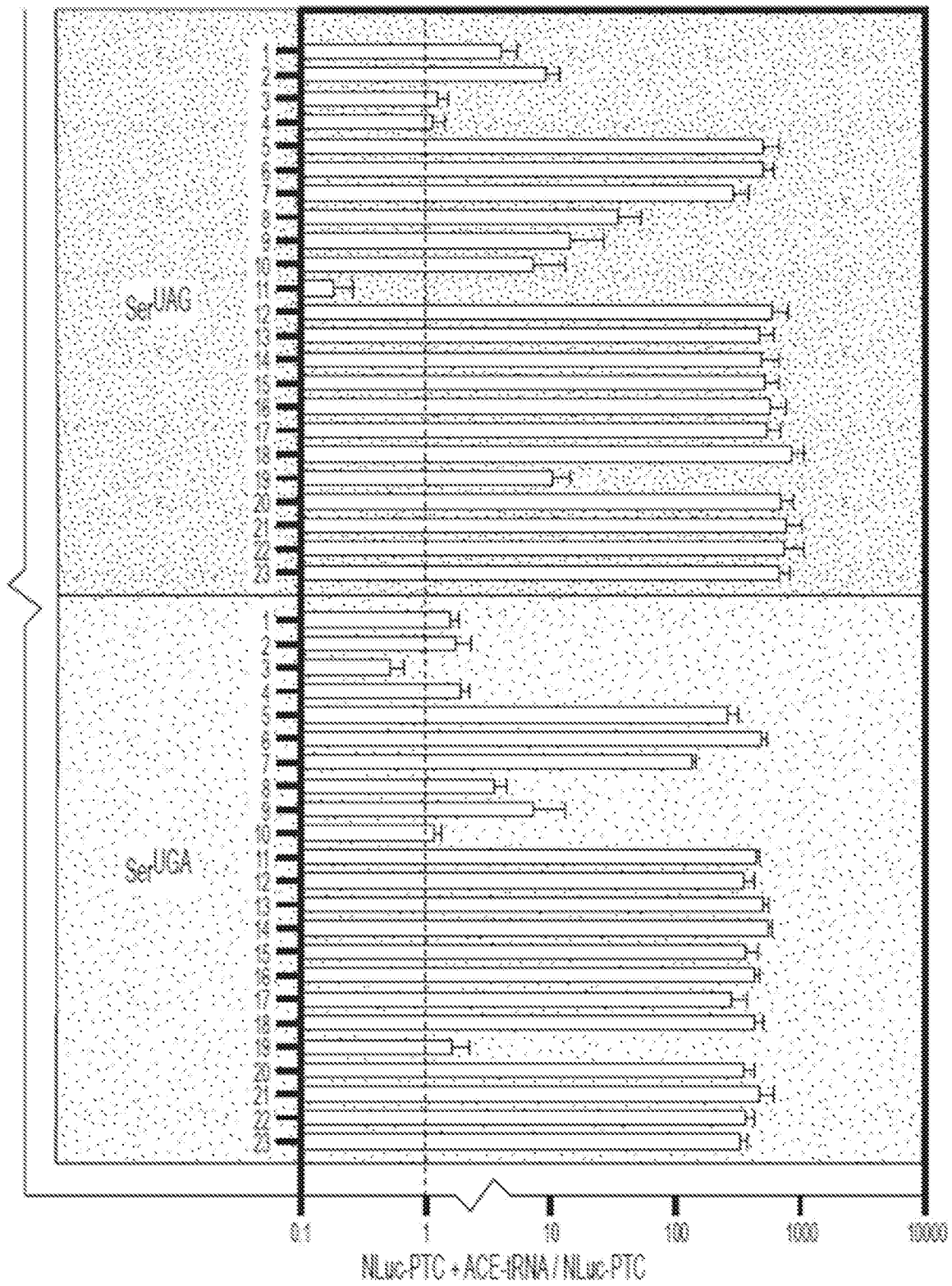


Figure 26
CONTINUED

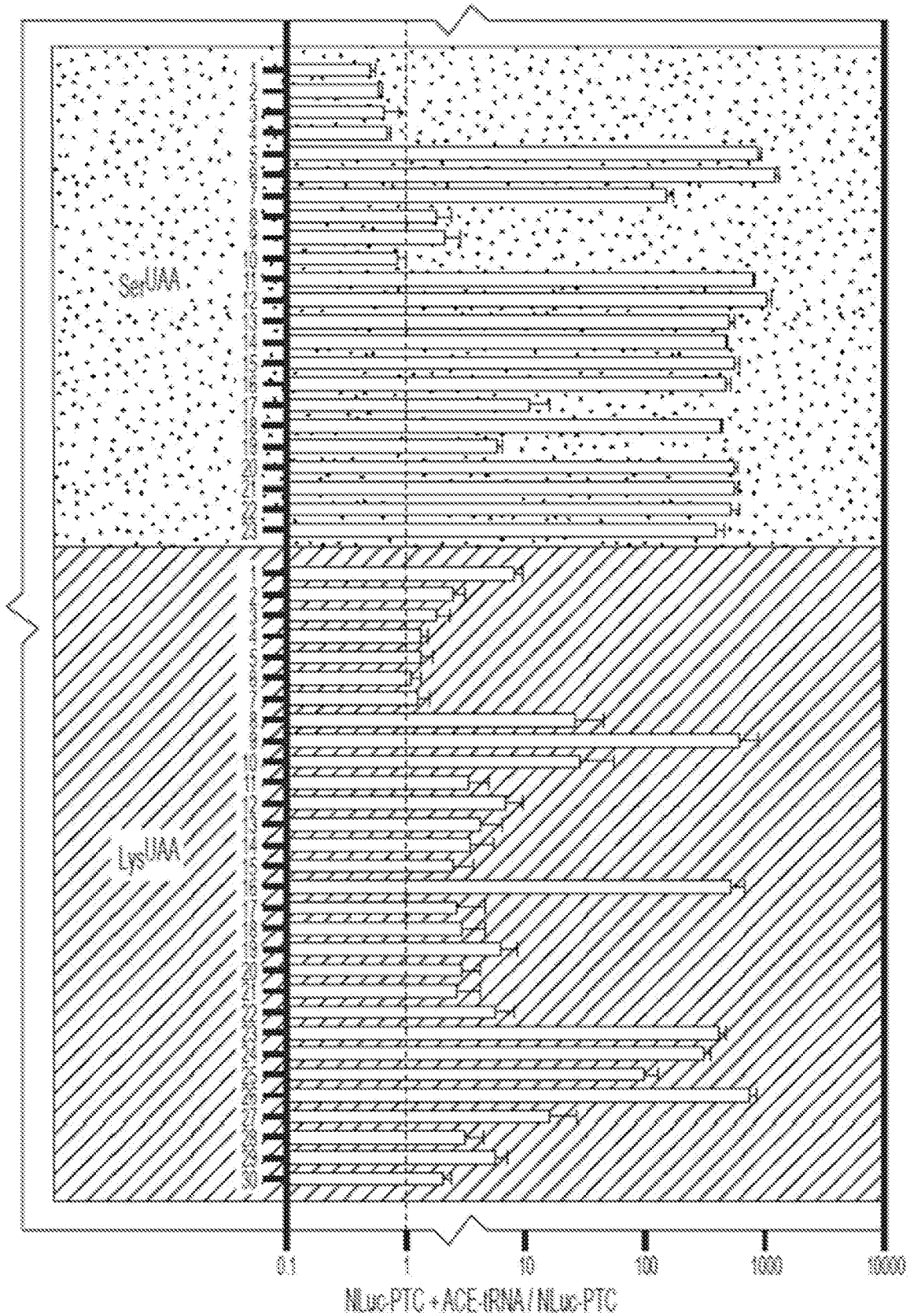


Figure 26
CONTINUED

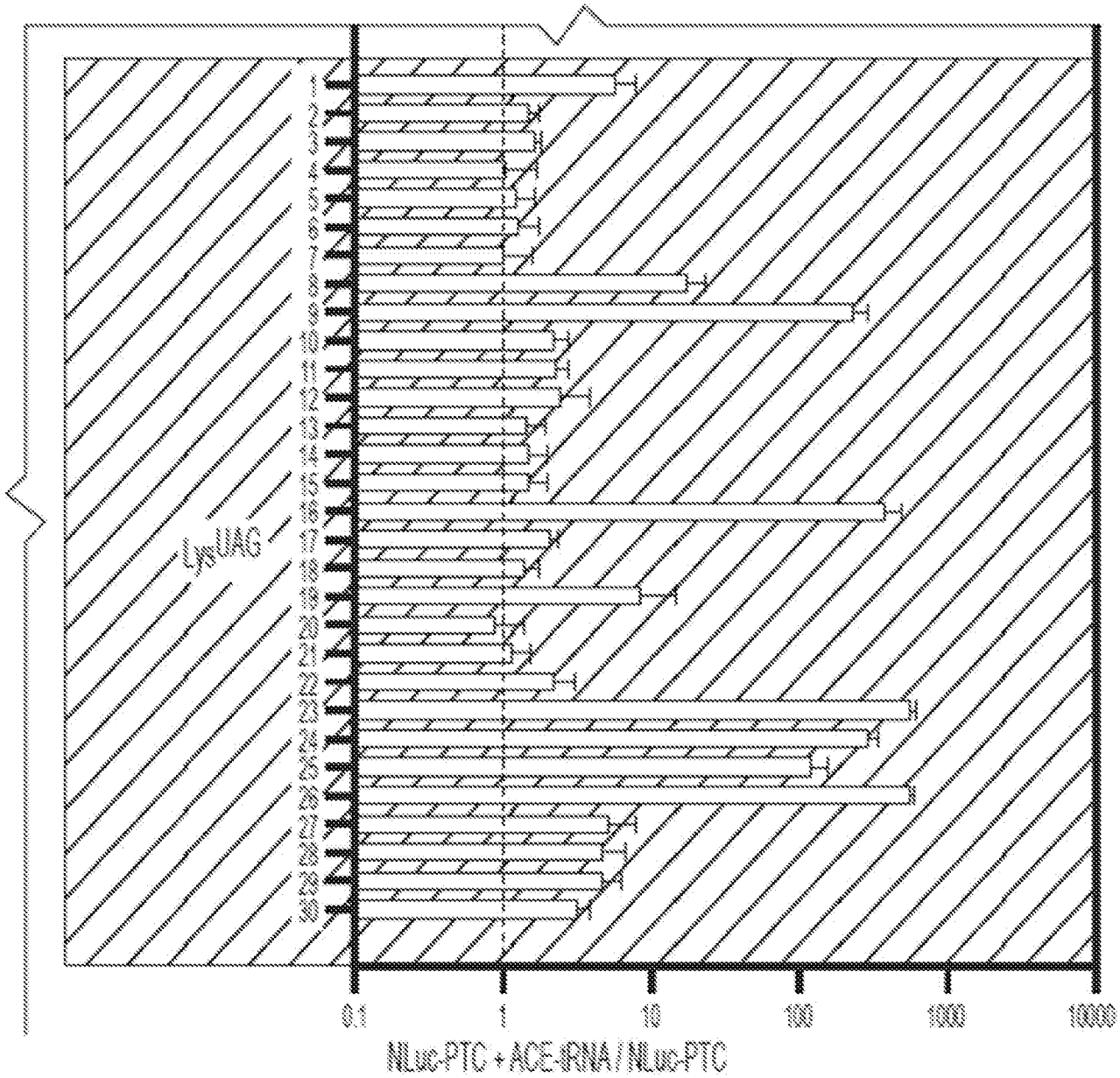


Figure 26
CONTINUED

ACCEPTOR STEM
TψC LOOP
D LOOP
ANTICODON LOOP
glychr1.trna122
glychr2.trna25
glychr17.trna11
glychr1.trna120
glychr1.trna2
glychr1.trna63
glychr2.trna1
glychr1.trna2*
glychr1.trna102
glychr1.trna16
glychr1.trna34
glychr1.trna61
glychr16.trna25
glychr1.trna42
glychr16.trna10
glychr6.trna80
glychr19.trna2
glychr1.trna107
glychr17.trna9
glychr1.trna75
glychr1.trna75-650-1

Figure 27

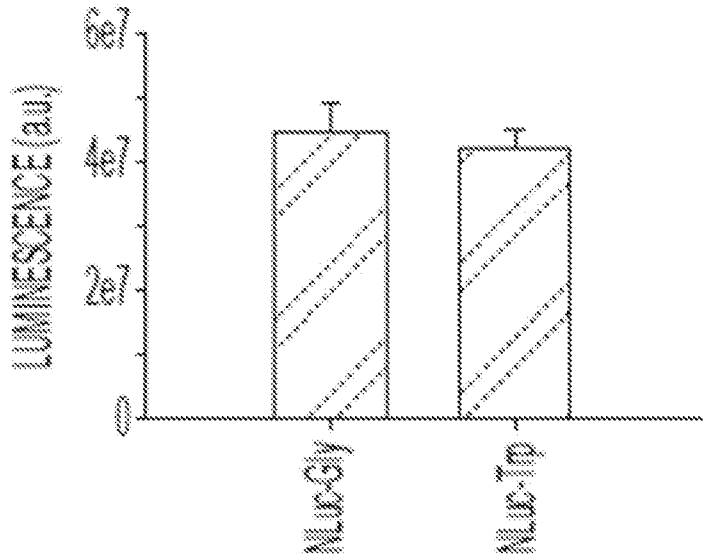


Figure 28

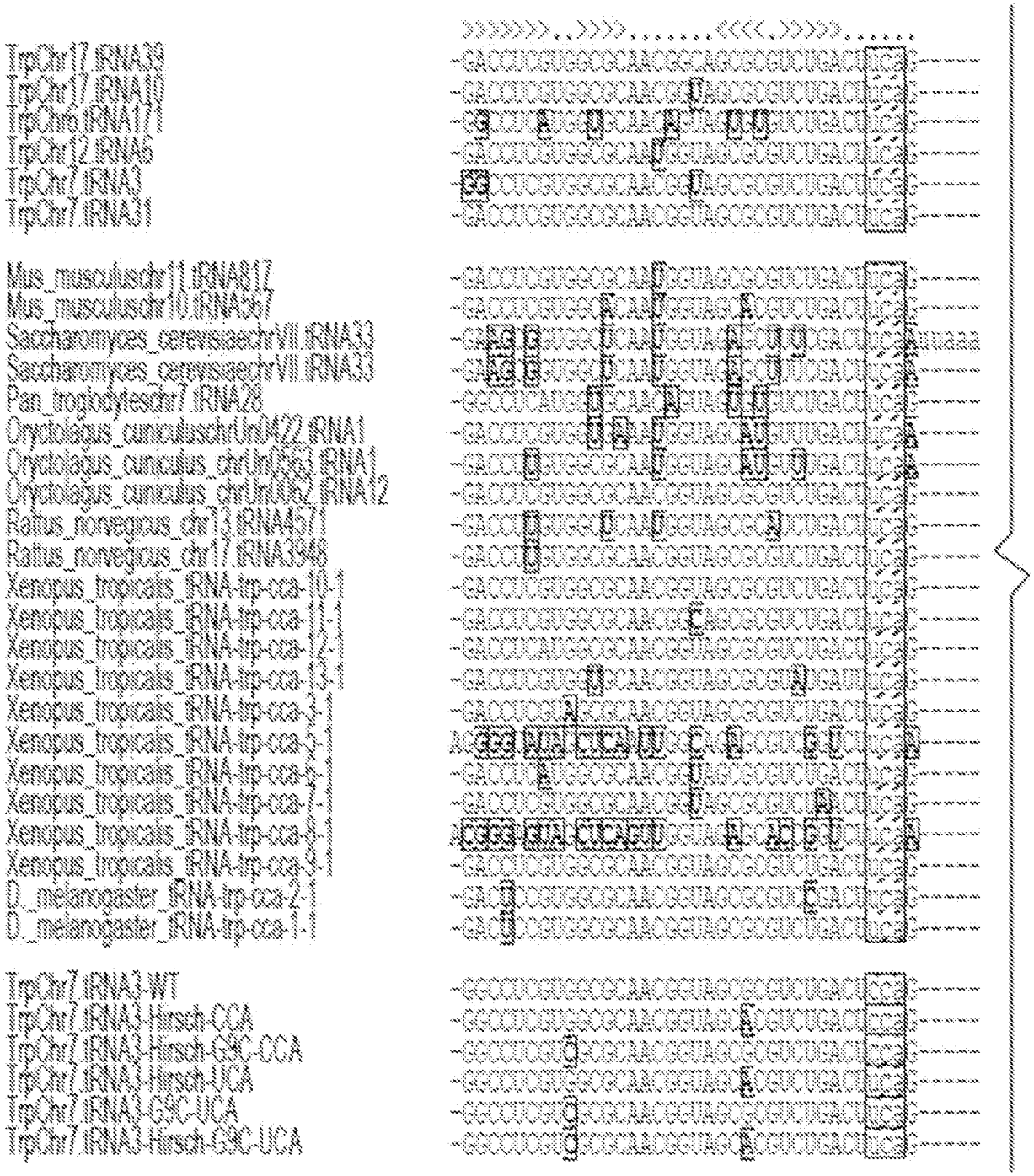

TrpChr17.tRNA39
TrpChr17.tRNA10
TrpChr6.tRNA171
TrpChr12.tRNA6
TrpChr7.tRNA3
TrpChr7.tRNA31
Mus_musculuschr11.tRNA817
Mus_musculuschr10.tRNA367
Saccharomyces_cerevisiaechrVII.tRNA33
Saccharomyces_cerevisiaechrVII.tRNA33
Pan_troglodyteschr7.tRNA28
Oryctolagus_cuniculuschrUnM22.tRNA1
Oryctolagus_cuniculus_chrUn(KB)_tRNA1
Oryctolagus_cuniculus_chrUn(KB)_tRNA12
Rattus_norvegicus_chr13.tRNA571
Rattus_norvegicus_chr17.tRNA3948
Xenopus_tropicalis_tRNA-trp-cca-10-1
Xenopus_tropicalis_tRNA-trp-cca-11-1
Xenopus_tropicalis_tRNA-trp-cca-12-1
Xenopus_tropicalis_tRNA-trp-cca-13-1
Xenopus_tropicalis_tRNA-trp-cca-3-1
Xenopus_tropicalis_tRNA-trp-cca-5-1
Xenopus_tropicalis_tRNA-trp-cca-6-1
Xenopus_tropicalis_tRNA-trp-cca-7-1
Xenopus_tropicalis_tRNA-trp-cca-8-1
Xenopus_tropicalis_tRNA-trp-cca-9-1
D_melanogaster_tRNA-trp-cca-2-1
D_melanogaster_tRNA-trp-cca-1-1
TrpChr7.tRNA3-WT
TrpChr7.tRNA3-Hirsch-CCA
TrpChr7.tRNA3-Hirsch-G9C-CCA
TrpChr7.tRNA3-Hirsch-UCA
TrpChr7.tRNA3-G9C-UCA
TrpChr7.tRNA3-Hirsch-G9C-UCA

Figure 29A

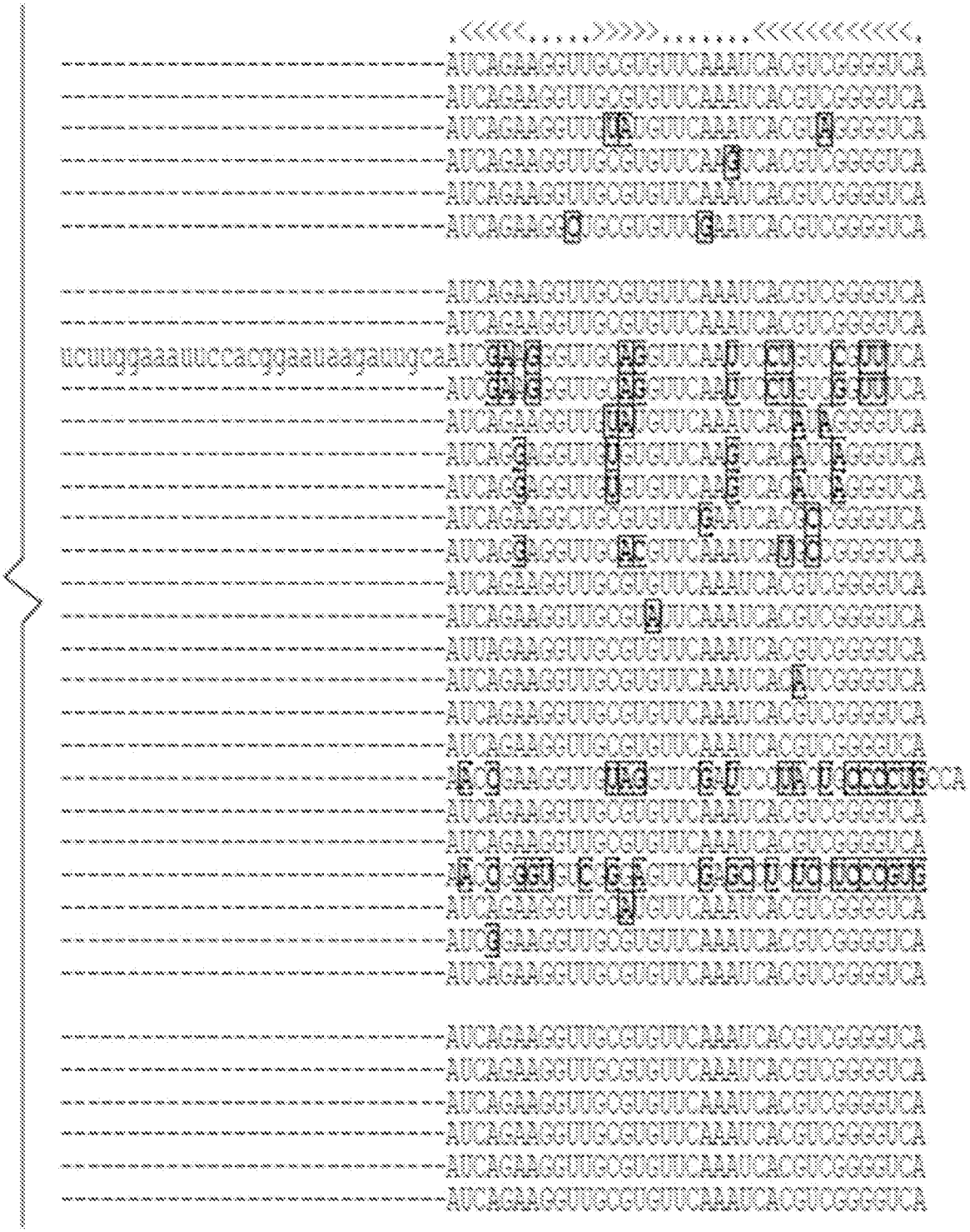


Figure 29A
CONTINUED

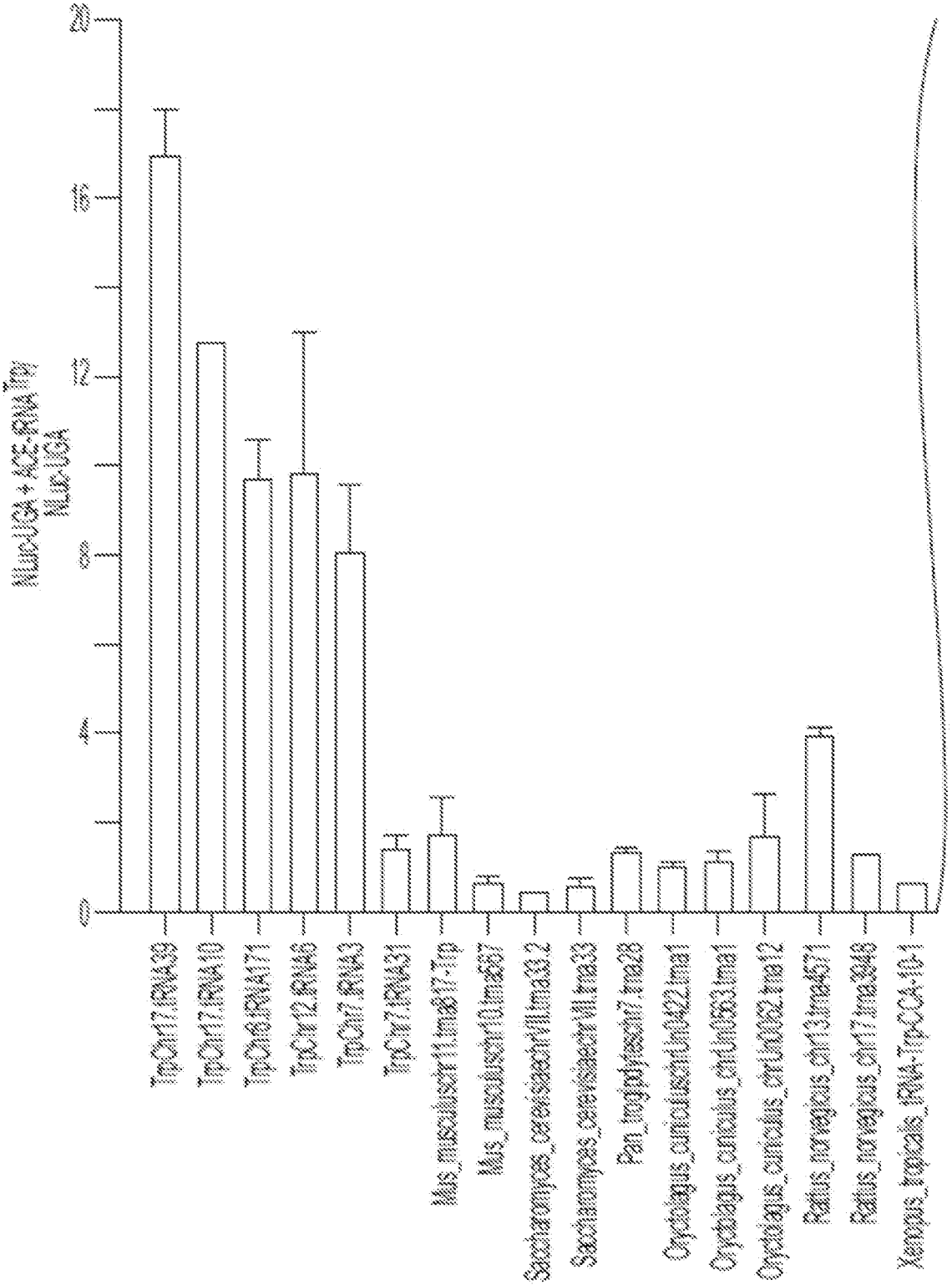

NLuc-UGA + ACE-tRNA Trp
NLuc-UGA
<0
4
8
12
16
20
TrpChr17.tRNA39
TrpChr17.tRNA10
TrpChr4.tRNA171
TrpChr12.tRNA6
TrpChr7.tRNA3
TrpChr7.tRNA31
Mus_musculus:chr11:ma617-Trp
Mus_musculus:chr10:ma267
Saccharomyces_cerevisiae:chrVIII:ma30.2
Saccharomyces_cerevisiae:chrVIII:ma33
Pan_troglodytes:chr7.ma28
Oryctolagus_cuniculus:chrUn0422.ma1
Oryctolagus_cuniculus_chrUn0063.ma1
Oryctolagus_cuniculus_chrUn0002.ma12
Rattus_norvegicus_chr13:ma4571
Rattus_norvegicus_chr17:ma2946
Xenopus_tropicalis_tRNA-TrpCCA-10-1

Figure 29B

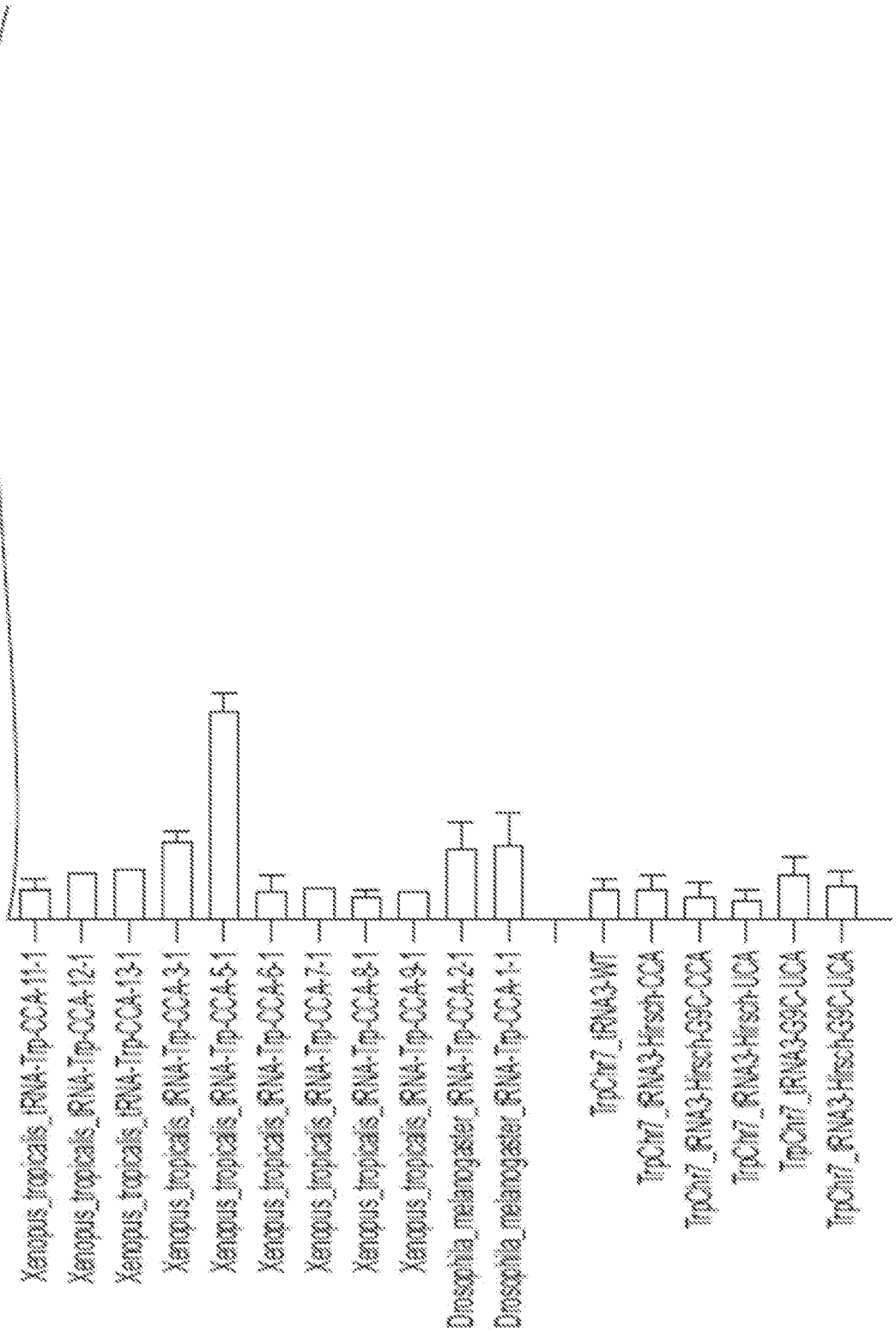


Figure 29B
CONTINUED

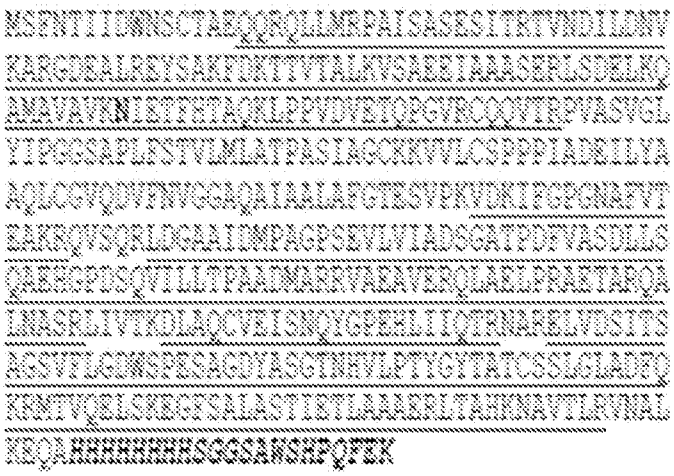


Figure 30A

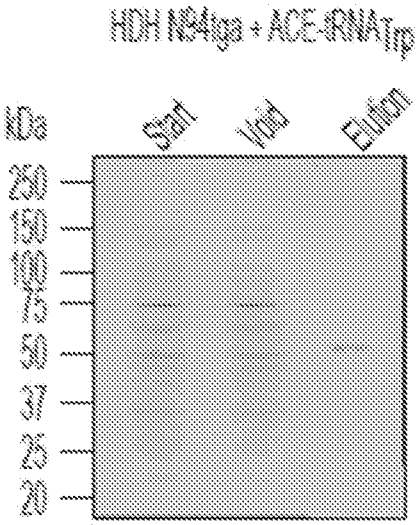


Figure 30B

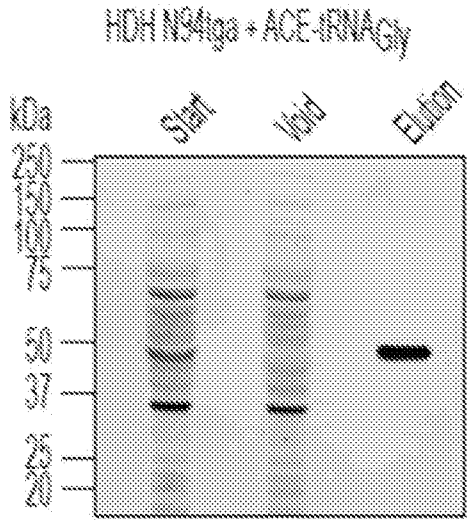


Figure 30C

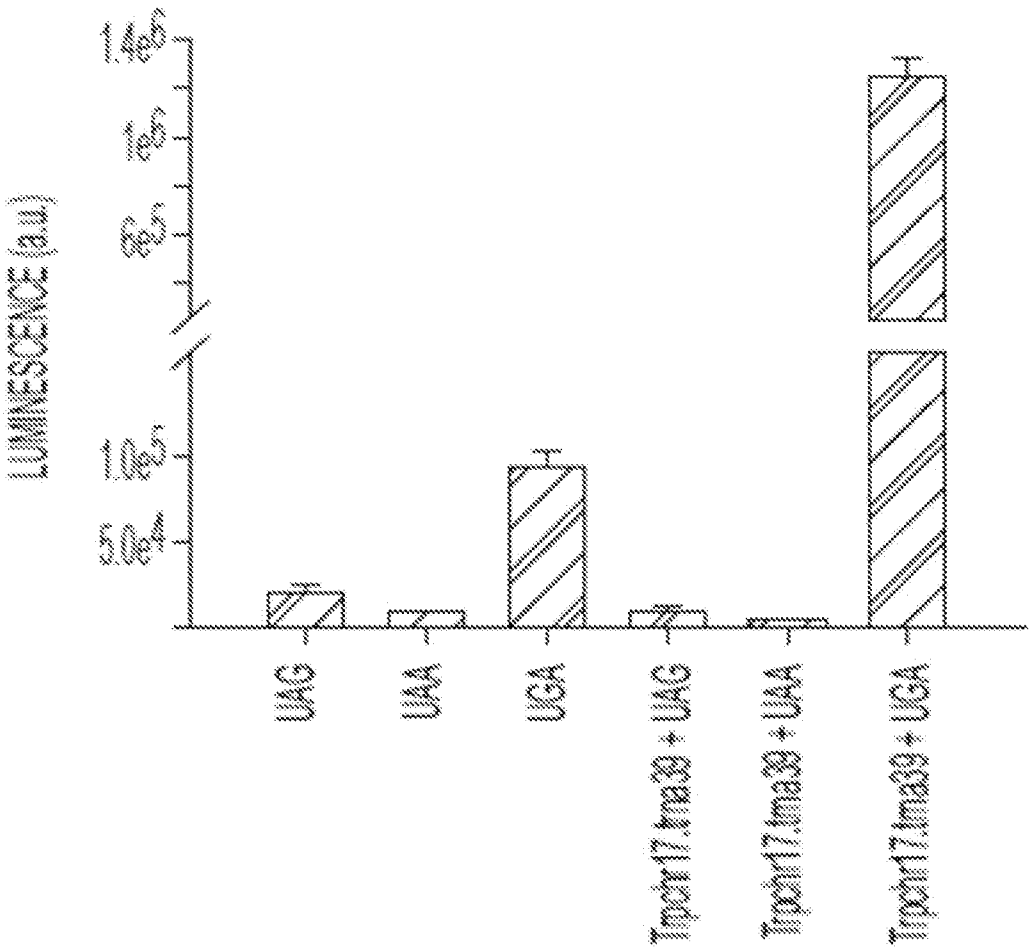

LUMINESCENCE (a.u.)
1.4e5
1e5
6e5
1.0e5
5.0e4
UAC
UAA
UGA
Tmchr17.tma39 + UAC
Tmchr17.tma39 + UAA
Tmchr17.tma39 + UGA

Figure 31

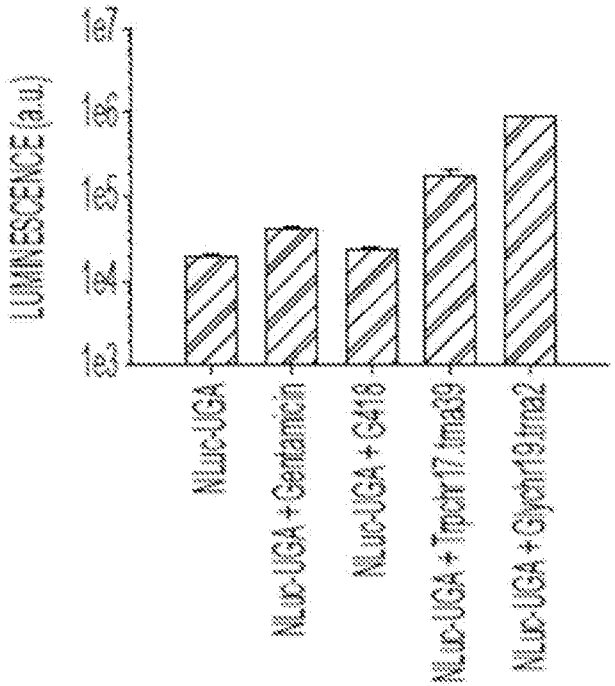


Figure 32A

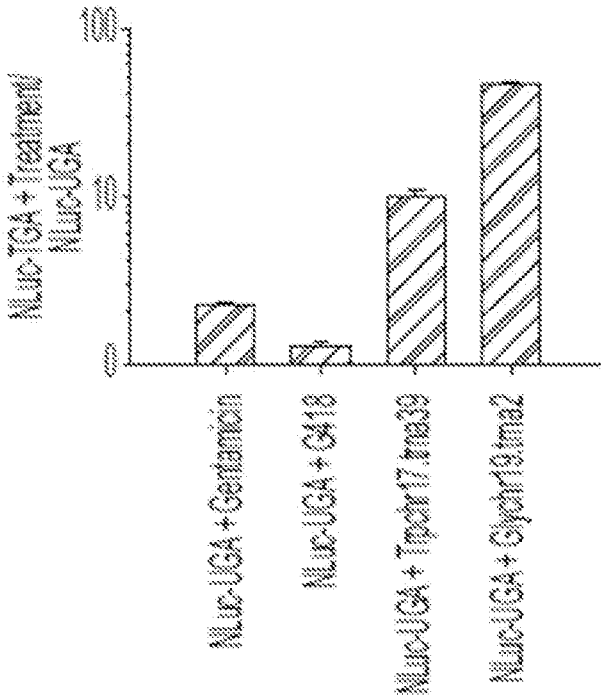


Figure 32B

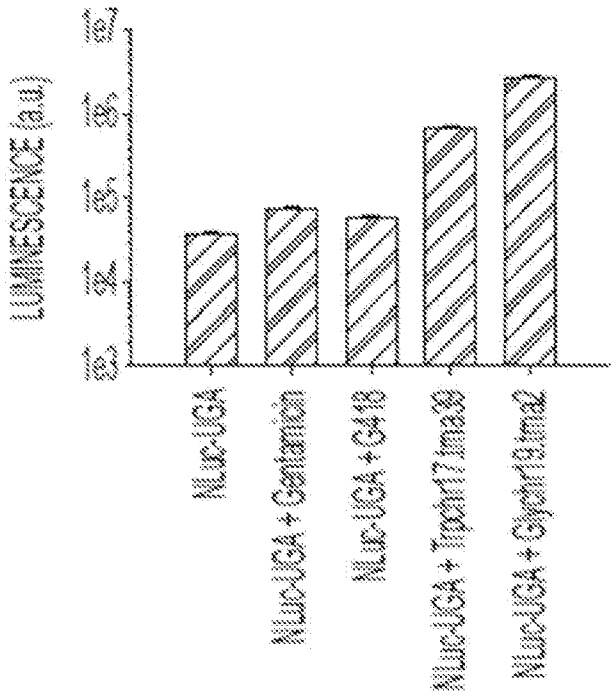


Figure 32C

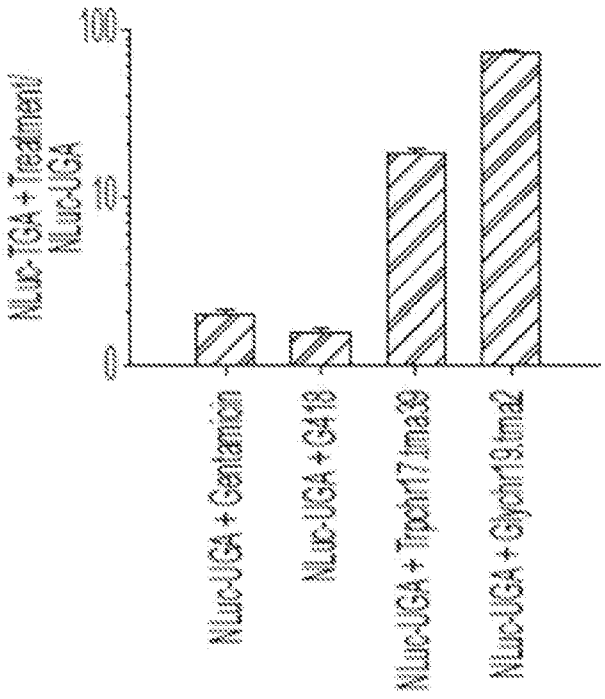


Figure 32D

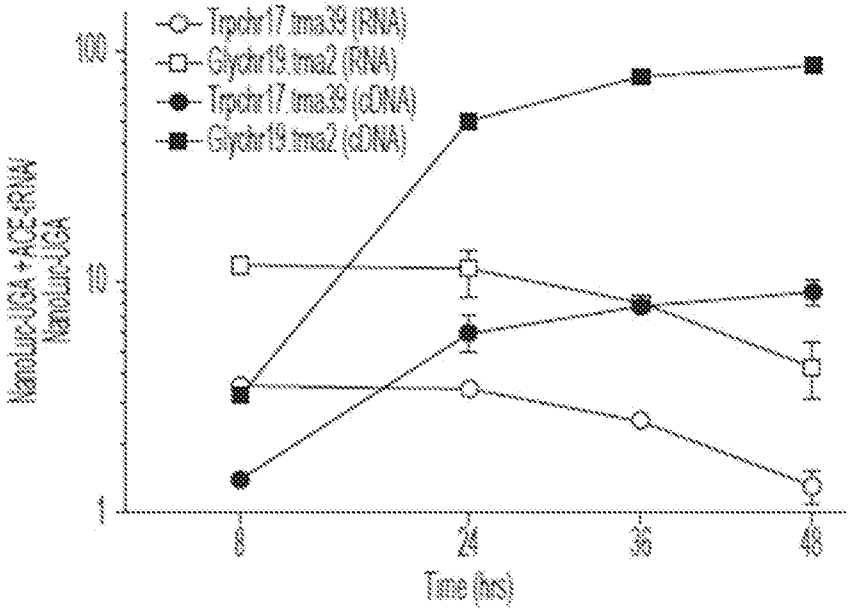

Tpcdr17.ima39 (RNA)
Glycdr19.ima2 (RNA)
Tpcdr17.ima39 (cDNA)
Glycdr19.ima2 (cDNA)
NanoLuc/UGA + ACE-tRNA
NanoLuc-UGA
100
10
1
0
24
36
48
Time (hrs)

Figure 33

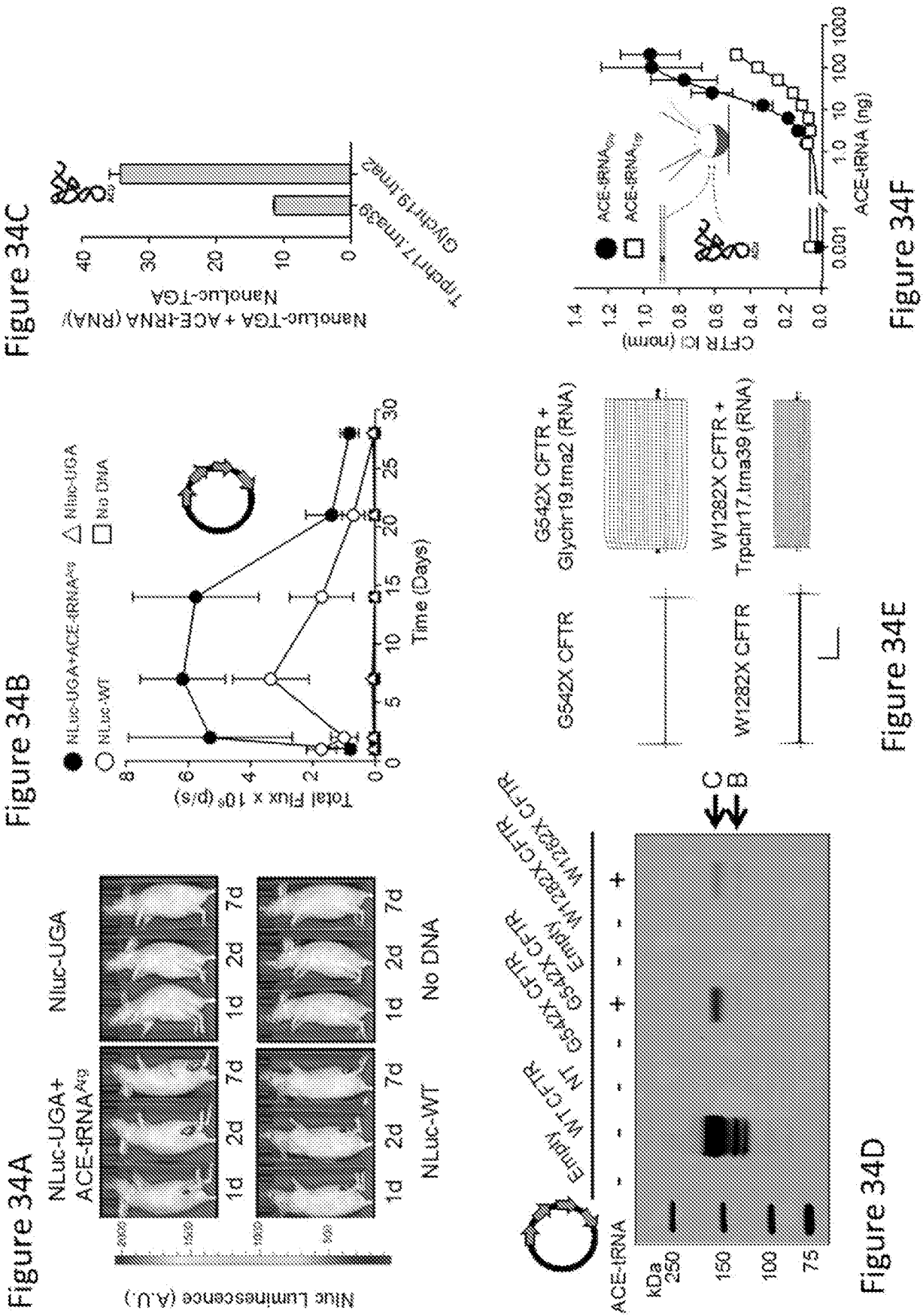


Figure 34A
NLuc-UGA+ ACE-tRNA^Arg
NLuc-UGA
1d 2d 7d 1d 2d 7d
NLuc-WT
1d 2d 7d 1d 2d 7d
No DNA
Nluc Luminescence (A.U.)
Figure 34B
NLuc-UGA+ACE-tRNA^Arg
NLuc-WT
NLuc-UGA
No DNA
Total Flux x 10^6 (p/s)
Time (Days)
Figure 34C
Nanoluc-TGA + ACE-tRNA (RNA)
Nanoluc-TGA
Tryptophan T.tma39
Glycine T.tma2
Figure 34D
ACE-tRNA
kDa
250
150
100
75
Empty
WT CFTR
NT
G542X CFTR
Empty
W1282X CFTR
W1282X CFTR
B
C
Figure 34E
G542X CFTR
G542X CFTR + Glychr19.tma2 (RNA)
W1282X CFTR
W1282X CFTR + Trpchr17.tma39 (RNA)
Figure 34F
CFTR IC (norm)
ACE-tRNA^Gly
ACE-tRNA^Arg
ACE-tRNA (ng)

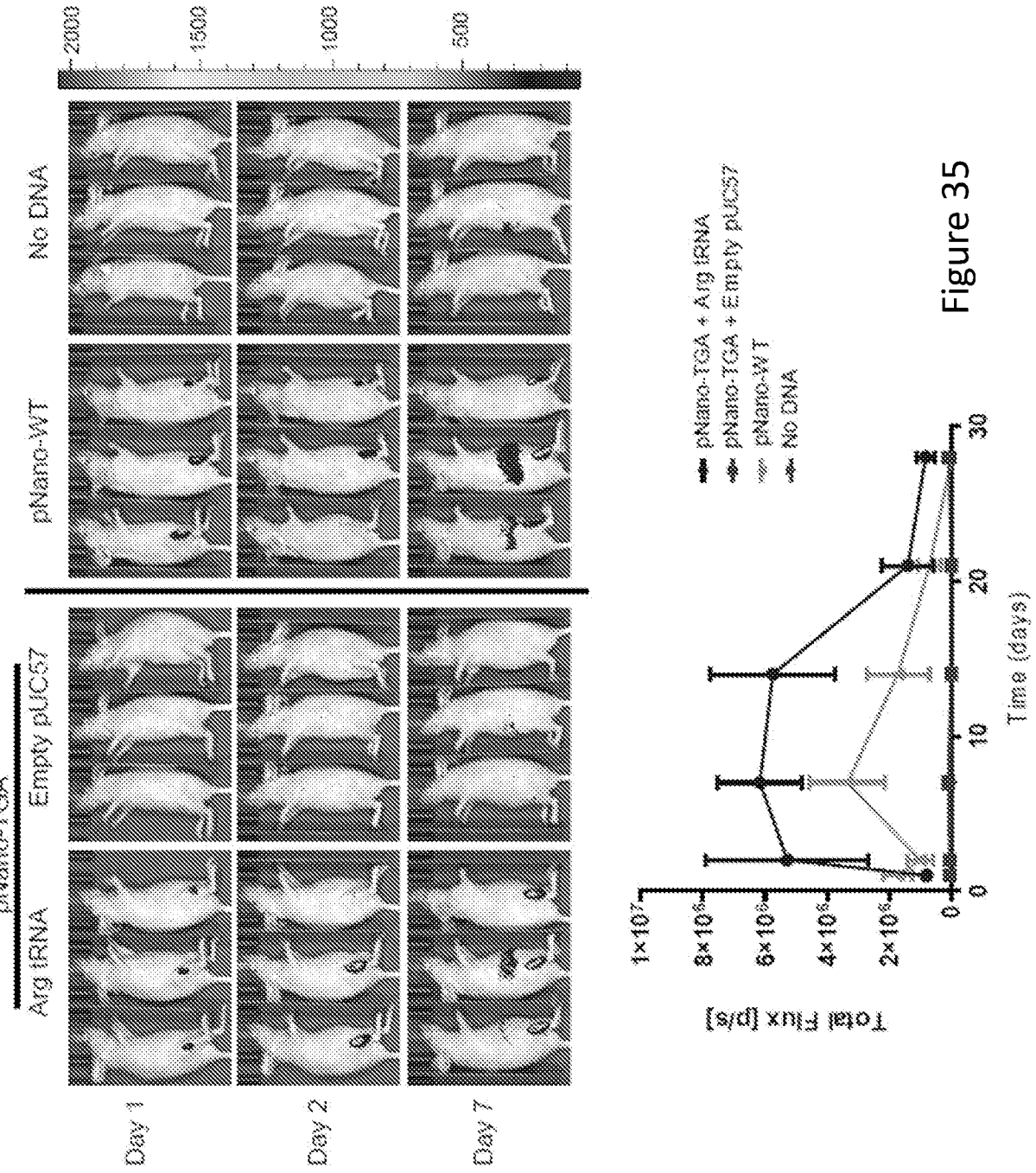
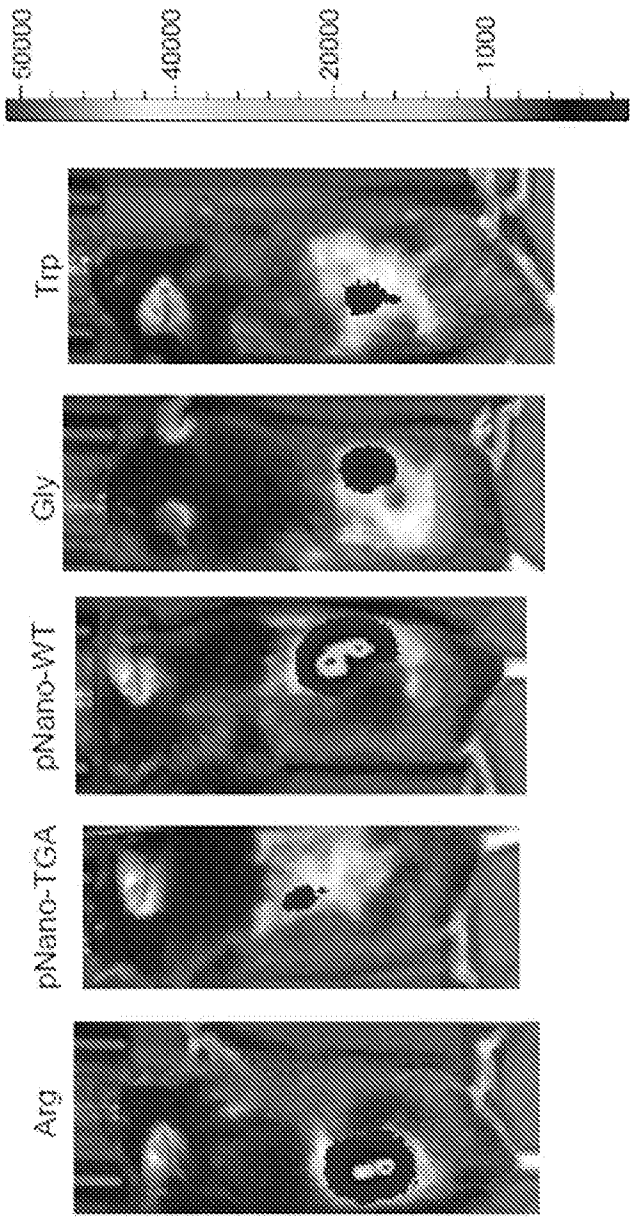

pNano-TGA
Arg tRNA
Empty pUC57
pNano-WT
No DNA
2000
1500
1000
500
Day 1
Day 2
Day 7
pNano-TGA + Arg tRNA
pNano-TGA + Empty pUC57
pNano-WT
No DNA
Total Flux [p/s]
1×10^7
8×10^6
6×10^6
4×10^6
2×10^6
0
0
10
20
30
Time (days)

Figure 35


60000
40000
20000
1000
Arg
pNano-TGA
pNano-WT
Gly
Trp

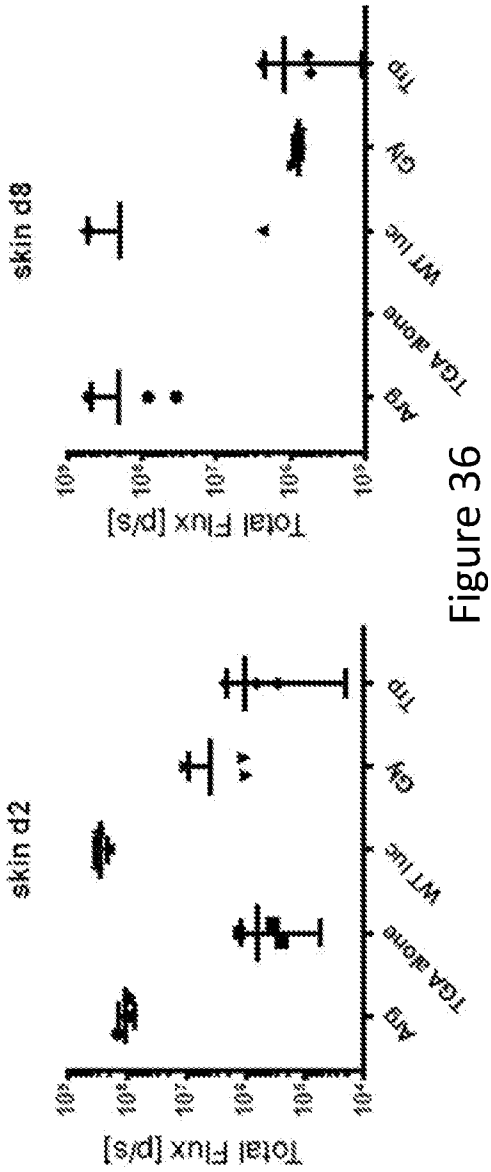

skin d8
skin d2
Total Flux [p/s]
Arg
TGA alone
WT luc
Gly
Trp

Figure 36

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/59085

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 48/00; C12N 15/00; C07H 21/04 (2019.01)

CPC - A61K 48/00; C12N 15/00; C12N 15/86; C12N 15/867; C12N 15/8645; C12N 15/861; C12N 2800/10; C12N 15/70; C12N 2800/102; C12N 15/63; C12N 15/111; C12N 15/85

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)

See Search History Document

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

See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LUECK et al., Engineered tRNA suppression of a CFTR nonsense mutation. bioRxiv preprint first posted. bioRxiv preprint first posted online 20 November 2016 [online]. [Retrieved on 2019.01.23]. Retrieved from the Internet: <URL: https://www.biorxiv.org/content/biorxiv/early/2016/11/20/088690.full.pdf> PDF File: pg 1-9. pg 1; pg 2, up para; pg 3, last para, and Fig 1; and pg 5, para 1, and para 2	1-2, 4/(1-2)
X --- Y	US 2009/0298920 A1 (DARDEL et al.) 03 December 2009 (03.12.2009), Abstract, para [0001], [0009], [0010], [0013], [0060], [0061], [0062], [0065], [0066], [0110], [0112], and [0113]	1-2, 4/(1-2) --- 3, 4/(3)
Y	SCHMID et al., A Versatile RNA Vector for Delivery of Coding and Noncoding RNAs. J Virol. 2014,Vol. 88(4), p. 2333-6. Abstract; pg 2333, col 1, para 1, and middle para; pg 2334, Fig 1, and Fig 2; and pg 2335, Fig 4	3, 4/(3)
A	TUORTO et al., Genome recoding by tRNA modifications. Open Biol. 2016, Vol. 6(12). PDF File: pg 1-9. Entire documentation, especially Abstract; pg 2, Fig 1; pg 3, col 2, para 1, and Fig 3	1-4
A	BIDDLE et al., Modification of orthogonal tRNAs: unexpected consequences for sense codon reassignment. Nucleic Acids Res. 2016, Vol. 44(21), p. 10042-10050. Epub 2016 Oct 23. Entire documentation, especially Abstract; pg 10042, col 1, para 2, middle para and last para; pg 10043, col 1, and col 2, last para 10044, col 2, middle para, and Fig 1; and pg 10046, col 1, top para, Fig 2, and Fig 3	1-4
A	BEDNAROVA et al., Lost in Translation: Defects in Transfer RNA Modifications and Neurological Disorders. Front Mol Neurosci. 2017 May, Vol. 10:135. PDF File: pg 1-8. Entire documentation, especially Abstract; pg 2, col 2, last para; pg 3, Fig 1; and pg 4, Table 1	1-4

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

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

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

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

"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

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 January 2019	21 FEB 2019
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/59085

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	LUECK et al., Engineered tra 1 nsfer RNAs for suppression of premature termination codons. bioRxiv preprint first posted online 27 August 2018. [online]. [Retrieved on 2019.01.23]. Retrieved from the Internet: <URL: https://www.biorxiv.org/content/biorxiv/early/2018/08/27/400127.full.pdf> PDF File: pg 1-25. Entire documentation, especially Abstract; ln 86-91; ln 113-116; ln 156-162; ln 198-248; ln 260-269; ln 675-682; Fig 1; and Fig 5	1-4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/59085

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 5-15
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.