[00544] The editing percentage (e.g., the "editing efficiency" or "percent editing") is defined as the total number of sequence reads with insertions or deletions over the total number of sequence reads, including wild type.

Cas9 protein measurement

[00545] Cas9 protein levels were determined by ELISA assay. Briefly, total protein concentration are optionally determined by bicinchoninic acid assay. An MSD GOLD 96-well Streptavidin SECTOR Plate (Meso Scale Diagnostics, Cat. L15SA-1) was prepared according to manufacturer's protocol using Cas9 mouse antibody (Origene, Cat. CF811179) as the capture antibody and Cas9 (7A9-3A3) Mouse mAb (Cell Signaling Technology, Cat. 14697) as the detection antibody. Recombinant Cas9 protein was used as a calibration standard in Diluent 39 (Meso Scale Diagnostics) with 1X Halt™ Protease Inhibitor Cocktail, EDTA-Free (ThermoFisher, Cat. 78437). ELISA plates were read using the Meso Quickplex SQ120 instrument (Meso Scale Discovery) and data was analyzed with Discovery Workbench 4.0 software package (Meso Scale Discovery).

Serum TTR measurement

[00546] The total mouse TTR serum levels were determined using a Mouse Prealbumin (Transthyretin) ELISA Kit (Aviva Systems Biology, Cat. OKIA00111). Briefly, sera were serial diluted with kit sample diluent to a final dilution of 10,000-fold for 0.1 mpk dose and 2,500-fold for 0.3 mpk. This diluted sample was then added to the ELISA plates and the assay was then carried out according to directions.

Human Alpha 1-Antitrypsin (hA1AT) measurement

[00547] Human hA1AT levels were measured from media for *in vitro* studies. The total human alpha 1-antitripsin levels were determined using a Alpha 1-Antitrypsin ELISA Kit (Human) (Aviva Biosystems, Cat# OKIA00048) according to manufacturer's protocol. Serum hA1AT levels were quantitated off a standard curve using 4 parameter logistic fit and expressed as µg/mL of serum.

Example 2 - Characterization of Cas9 expression *in vitro*

[00548] Cas9 sequences using different codon schemes as described in Table 8 were designed to test for improved protein expression. Specifically, SEQ ID No: 3 and SEQ ID Nos: 15, 16, 17, 18, 19, 20, 21, 22, 23 and 24, comprising the ORFs of SEQ ID Nos. 5, 7, 9, 8, 10, 6, 11, 12, 13, and 14, respectively, were tested.

[00549] Translation efficiency was assessed *in vitro* by transfection of mRNA into HepG2 cells and measuring Cas9 protein expression levels by ELISA. HepG2 cells were transfected

Claims

1. A polynucleotide comprising an open reading frame (ORF) encoding a polypeptide, wherein
    (a) (i) at least 1.03% of the codon pairs in the ORF are codon pairs shown in Table 1; or (ii) at least 1% of the codon pairs in the ORF are codon pairs shown in Table 1 and the ORF does not encode an RNA-guided DNA binding agent; or
    (b) the ORF comprises a sequence with at least 95% identity to any one of SEQ ID NOs: 6-10, 29, 46, 71-73, 91, 97, 103, 109, 115, 121, and 127 optionally wherein identity is determined without regard to the start and stop codons of the ORF.
2. The polynucleotide of claim 1, wherein the repeat content of the ORF is less than or equal to 23.3%.
3. The polynucleotide of any one of claims 1-2, wherein the GC content of the ORF is greater than or equal to 55%.
4. The polynucleotide of any one of claims 1-3, wherein at least 1.03% of the codon pairs in the ORF are codon pairs shown in Table 1.
5. The polynucleotide of any one of claims 1-4, wherein
    (i) less than or equal to 0.9% of the codon pairs in the ORF are codon pairs shown in Table 2;
    (ii) at least 60%, 65%, 70%, or 75% of the codon in the ORF are codon shown in Table 3;
    (iii) less than or equal to 20% of the codons in the ORF are codons shown in Table 4;
    (iv) at least 1.05% of the codon pairs in the ORF are codon pairs shown in Table 1; and/or
    (v) less than or equal to 10% of the codon pairs in the ORF are codon pairs shown in Table 1.
6. The polynucleotide of any one of claims 1-5, wherein
    (i) the GC content of the ORF is greater than or equal to 56%
    (ii) the GC content of the ORF is less than or equal to 63%;
    (iii) the repeat content of the ORF is less than or equal to 23.2%; and/or
    (iv) the repeat content of the ORF is greater than or equal to 20%.

7. The polynucleotide of any one of claims 1-6, wherein
    (i) less than or equal to 15% of the codons in the ORF are codons shown in Table 4;
    (ii) at least 76% of the codons in the ORF are codons shown in Table 3; and/or
    (iii) less than or equal to 87% of the codons in the ORF are codons shown in Table 3.
8. The polynucleotide of any one of claims 1-7, wherein
    (i) the ORF has a uridine content ranging from its minimum uridine content to 101%, 102%, 103%, 105%, 110%, 115%, 120%, 125%, 130%, 135%, 140%, 145%, or 150% of the minimum uridine content; or
    (ii) the ORF has an A+U content ranging from its minimum A+U content to 101%, 102%, 103%, 105%, 110%, 115%, 120%, 125%, 130%, 135%, 140%, 145%, or 150% of the minimum A+U content; or
    (iii) the ORF has a GC content in the range of 55%-65%, such as 55%-57%, 57%-59%, 59-61%, 61-63%, or 63-65%; or
    (iv) the ORF has a repeat content ranging from its minimum repeat content to 101%, 102%, 103%, 105%, 110%, 115%, 120%, 125%, 130%, 135%, 140%, 145%, or 150% of the minimum repeat content; or
    (v) the ORF has a repeat content of 22%-27%.
9. The polynucleotide of any one of claims 1-8, wherein the polypeptide has a length of at least 30 amino acids, optionally wherein the polypeptide has a length of at least 50 amino acids.
10. The polynucleotide of any one of claims 1-9, wherein the polynucleotide comprises a sequence with at least 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to any one of SEQ ID NOs: 6-10, 29, 46, 71-73, 91, 97, 103, 109, 115, 121, and 127.
11. The polynucleotide of any one of claims 1-10, wherein the polynucleotide comprises a sequence with at least 90%, 95%, 96%, 97%, 98%, 99%, 99.5%, or 100% identity to any one of SEQ ID NOs: 16-20, 78-80, 194-197, or 200-201.
12. The polynucleotide of any one of claims 1-11, wherein the ORF encodes an RNA-guided DNA binding agent.

13. The polynucleotide of claim 12, wherein the RNA-guided DNA-binding agent has double-stranded endonuclease activity or nickase activity or comprises a dCas DNA binding domain.
14. The polynucleotide of any one of claims 1-13, wherein the ORF encodes an endonuclease.
15. The polynucleotide of any one of claims 1-14, wherein the polynucleotide further comprises a 5' UTR with at least 90% identity to any one of SEQ ID NOs: 177-181 or 190-192; and/or a 3' UTR with at least 90% identity to any one of SEQ ID NOs: 182-186 or 202-204.
16. The polynucleotide of any one of claims 1-15, wherein the encoded polypeptide comprises a nuclear localization signal (NLS).
17. The polynucleotide of claim 16, wherein the NLS comprises a sequence having at least 80%, 85%, 90%, or 95% identity to any one of SEQ ID NOs: 163-176.
18. The polynucleotide of any one of claims 1-17, wherein at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, at least 99%, or 100% of the uridine is substituted with a modified uridine, optionally, wherein the modified uridine is one or more of N1-methyl-pseudouridine, pseudouridine, 5-methoxyuridine, or 5-iodouridine.
19. The polynucleotide of any one of claims 1-18, wherein the polynucleotide is an mRNA.
20. The polynucleotide of any one of claims 1-19, wherein the polynucleotide is an expression construct comprising a promoter operably linked to the ORF.
21. A plasmid comprising the polynucleotide of claim 20.
22. A host cell comprising the polynucleotide of claim 20 or the plasmid of claim 21.
23. An *in vitro* or *ex vivo* method of preparing an mRNA comprising contacting the polynucleotide of claim 20 or the plasmid of claim 21 with an RNA polymerase under conditions permissive for transcription of the mRNA.
24. An *in vitro* or *ex vivo* method of expressing a polypeptide, comprising contacting a cell with the polynucleotide of any one of claims 1-20.
25. A composition comprising a polynucleotide according to any one of claims 1-20 and at least one guide RNA, wherein the polynucleotide encodes an RNA-guided DNA binding agent.

26. A lipid nanoparticle comprising a polynucleotide according to any one of claims 1-20.
27. A pharmaceutical composition comprising a polynucleotide according to any one of claims 1-20 and a pharmaceutically acceptable carrier.
28. An *in vitro* or *ex vivo* method of genome editing or modifying a target gene comprising contacting a cell with the polynucleotide according to any one of claims 1-20, the composition according to claim 25, the lipid nanoparticle according to claim 26, or the pharmaceutical composition according to claim 27, wherein the polynucleotide encodes an RNA-guided DNA binding agent comprising a Cas nuclease.
29. *In vitro* or *ex vivo* use of the polynucleotide according to any one of claims 1-20 for genome editing or modifying a target gene, wherein the polynucleotide encodes an RNA-guided DNA binding agent comprising a Cas nuclease.
30. The method of claim 28 or the use of claim 29, wherein the Cas nuclease comprises a Cas9 nuclease.
31. The polynucleotide according to any one of claims 1-20 for use in therapy.
32. The method of claim 28, wherein the genome editing or modification of the target gene occurs in a liver cell, optionally wherein the liver cell is a hepatocyte.
33. The use of claim 29, wherein the genome editing or modification of the target gene occurs in a liver cell, optionally wherein the liver cell is a hepatocyte.