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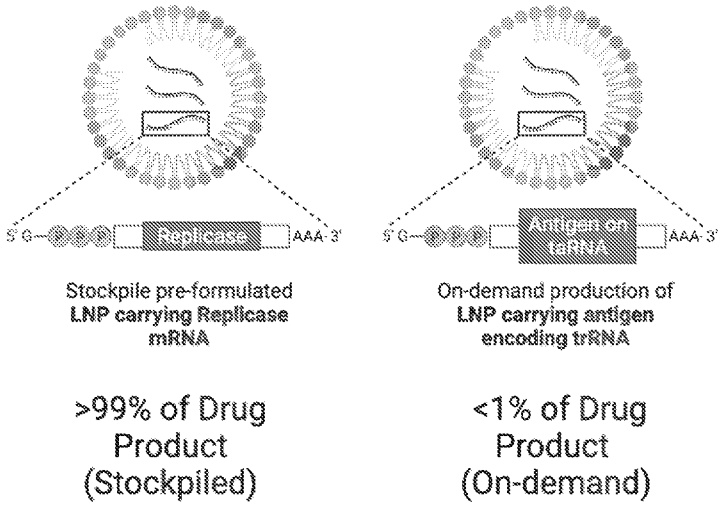

FIG. 1

(57) Abstract: This disclosure provides, in some aspects, multi-component trans-amplifying RNA (taRNA) vaccines that comprise a first nanoparticle (R-NP) comprising a polynucleotide encoding a replicase and a second nanoparticle (Tr-NP) comprising a polynucleotide comprising a nucleic acid encoding a payload (e.g., antigen), operably linked to a conserved sequence element (CSE) that is cognate to the replicase; methods of production thereof; and methods of use thereof.

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TWO-COMPONENT PLATFORM FOR TRANS AMPLIFYING RNA (taRNA) VACCINATION

RELATED APPLICATIONS

This application claims the benefit under 35 U.S.C. § 119(e) of US Provisional Application No. 63/610,087, filed December 14, 2023, entitled "TWO-COMPONENT PLATFORM FOR TRANS AMPLIFYING RNA (TARNA) VACCINATION," the content of which is hereby incorporated by reference herein in its entirety for all purposes.

REFERENCE TO AN ELECTRONIC SEQUENCE LISTING

The content of the electronic sequence listing (A141470003WO00-SEQ-ARM.xml; Size: 30,040 bytes; and Date of Creation: December 12, 2024) is herein incorporated by reference in its entirety.

BACKGROUND

The ability to quickly vaccinate a population is important to control and mitigate epidemic and pandemic disease spread. Ribonucleic acid (RNA) vaccines have proven useful in pandemic response, but distribution is delayed by the need to synthesize large quantities of RNA for the vaccine. For example, vaccinating 35,000 people requires about 4,200 mg of vaccine mRNA.

SUMMARY

Trans amplifying RNAs (taRNAs) comprise a first polynucleotide comprising a *trans* replicating RNA (trRNA) encoding a payload (e.g., a vaccine antigen) and a conserved sequence element (CSE); and a second polynucleotide comprising a replicase construct that is cognate to the CSE. Expression of the payload encoded by the taRNA by a cell requires the simultaneous presence of both the trRNA and the cognate replicase in the cell (e.g., expression of the replicase construct in the cell). Interaction of the replicase with the CSE of the trRNA, the replicase produces copies of the trRNA (e.g., amplifies the trRNA), which are in turn translated by the cell to generate the payload. When either the trRNA or replicase (e.g., expressed by the replicase construct) are absent in a cell, the payload (e.g., vaccine antigen) is unlikely to be produced in sufficient quantities to have a desired effect. Accordingly, when a taRNA is formulated for

delivery to a subject, the corresponding trRNA and replicase construct are typically comprised in the same nanoparticle (e.g., lipid nanoparticle), such that administration of the nanoparticle results in co-expression of the replicase and trRNA in the same cell.

The inventors of this disclosure have surprisingly discovered that a trRNA (comprising nucleic acids encoding a payload) and a corresponding replicase construct can be formulated in separate nanoparticles and concomitantly administered to produce a desired effect in the subject, for example, an immune response (e.g., antibody production against a vaccine antigen). The inventors have further surprisingly discovered that administration of a 25:1 molar ratio of replicase construct to trRNA induces an equivalent immune response in a subject compared to the typical administration of a 1:1 molar ratio of the same. Together, these findings indicate that production of vaccine formulations requires far less manufacture of trRNA compared to replicase construct; for example, preparation of vaccine doses for 35,000 people may require production of only about 42mg of trRNA, compared to 4148mg of the replicase construct. Based on these findings, the inventors have identified methods for improving RNA vaccine manufacture and distribution. In some aspects, generic replicase constructs are manufactured and stockpiled (e.g., stored long term) in nanoparticles, while antigen-encoding trRNA are designed and manufactured on an as-needed basis (e.g., in response to a pandemic, evolving variants of a virus, clinical trial data) and formulated in separate nanoparticles. These separate nanoparticles can then be concomitantly administered to subjects. Importantly, only relatively small amounts of trRNA (comprising nucleic acids encoding an antigen) would need to be synthesized to produce amounts required for population-scale vaccinations. These methods thus greatly enhance the rate at which an RNA vaccine can be produced, allowing for rapid turnover and mass vaccination, as well as significant reductions in manufacturing risk as the scale of material to be manufactured is lower. Moreover, these methods provide a strategy for rapid formulation and testing of multiple different candidate antigens to identify those with improved immunogenic properties.

In some embodiments, this disclosure provides a replicase nanoparticle (R-NP) comprising a replicase construct, wherein the replicase construct is a polyribonucleic acid comprising nucleic acids encoding a replicase, and wherein the R-NP does not comprise a polynucleotide comprising a gene operably linked to a conserved sequence element (CSE) that is cognate to the replicase.

In some embodiments, the replicase is an alphavirus replicase. In some embodiments, the alphavirus replicase is a Venezuelan equine encephalitis virus (VEEV), Semliki Forest virus (SFV), Sindbis Virus (SINV), or Chikungunya (CHIKV) replicase. In some embodiments, the alphavirus replicase is a SFV replicase.

In some embodiments, the replicase construct comprises: a 5'-UTR, the nucleic acids encoding the replicase, and a 3'-UTR. In some embodiments, the 5'-UTR comprises a human alpha-globin (5'-HBA-UTR) and the 3'-UTR comprises a human alpha-globin (3'-HBA-UTR). In some embodiments, the 5'-HBA-UTR comprises a nucleic acid sequence of SEQ ID NO: 5 and the 3'-HBA-UTR comprises a nucleic acid sequence of SEQ ID NO: 6. In some embodiments, the replicase construct comprises the sequence set forth in SEQ ID NO: 1.

In some embodiments, the R-NP is a lipid nanoparticle (LNP) (R-LNP). In some embodiments, the R-LNP comprises ionizable lipid (e.g., ALC-0315), distearoylphosphatidylcholine (DSPC), cholesterol, and a polyethylene glycol (PEG)-lipid. In some embodiments, the gene comprises nucleic acids encoding an antigen. In some embodiments, the ionizable lipid is ALC-0315. In some embodiments, this disclosure describes a composition comprising the R-NP and a trans-replicating RNA (trRNA) trRNA nanoparticle (Tr-NP), wherein the Tr-NP comprises a trRNA comprising nucleic acids encoding a payload operably linked to a CSE cognate to the replicase.

In some embodiments, the Tr-NP is a lipid nanoparticle (Tr-LNP). In some embodiments, the Tr-LNP comprises an ionizable lipid (e.g., ALC-0315), DSPC, cholesterol, and a PEG-lipid. In some embodiments, the R-NP and the Tr-NP comprise an ionizable lipid (e.g., ALC-0315), DSPC, cholesterol, and a PEG-lipid.

In some embodiments, the composition comprises a 1:1 molar ratio of the replicase construct to the trRNA. In some embodiments, the composition comprises a 1:5 molar ratio of the replicase construct to the trRNA. In some embodiments, the composition comprises a 10:1 molar ratio of the replicase construct to the trRNA. In some embodiments, the composition comprises a 25:1 molar ratio of the replicase construct to the trRNA. In some embodiments, the composition comprises a 50:1 molar ratio of the replicase construct to the trRNA. In some embodiments, the composition comprises a 100:1 molar ratio of the replicase construct to the trRNA. In some embodiments, the composition comprises a 500:1 molar ratio of the replicase construct to the trRNA. In some embodiments, the composition comprises a 1000:1 molar ratio of the replicase construct to the trRNA.

In some embodiments, the payload comprises an antigen. In some embodiments, the composition is a vaccine.

In some embodiments, the combined amount of replicase construct and trRNA in the composition is less than 2 μg. In some embodiments, the combined amount of replicase construct and trRNA in the composition is less than 1.5 μg. In some embodiments, the combined amount of replicase construct and trRNA in the composition is about 1 μg.

In some embodiments, this disclosure describes a kit comprising: a first vial comprising the R-NP; and a second vial comprising a trans-replicating RNA (trRNA) trRNA nanoparticle (Tr-NP), wherein the Tr-NP comprises a trRNA comprising nucleic acids encoding a payload operably linked to a CSE cognate to the replicase.

In some embodiments, this disclosure describes a method of producing a vaccine, the method comprising: (i) obtaining a plurality of the R-NP; (ii) determining a pathogen for which vaccination is desired; (iii) obtaining a plurality of Tr-NPs comprising a trRNA, the trRNA comprising nucleic acids encoding an antigen of the pathogen operably linked to a CSE that is cognate to the replicase of the R-NP.

In some embodiments, the method further comprises (iv) combining the plurality of R-NPs and the plurality of Tr-NPs. In some embodiments, this disclosure describes steps (i)-(iii) are performed in sequential order. In some embodiments, this disclosure describes after step (i) and before step (ii), the method comprises placing the plurality of R-NPs into long term storage. In some embodiments, this disclosure describes A method of expressing a replicase in a cell, the method comprising contacting the cell with the R-NP.

In some embodiments, this disclosure describes a method of expressing a payload in a cell, the method comprising contacting the cell with the R-NP, wherein the cell comprises an RNA polynucleotide comprising nucleic acids encoding the payload operably linked to a CSE that is cognate to the replicase of the R-NP. In some embodiments, this disclosure describes a method of vaccinating a subject, the method comprising administering the composition to the subject.

In some embodiments, this disclosure describes a method of vaccinating a subject, the method comprising administering a plurality of R-NPs and a tr-NP comprising a trRNA, the trRNA comprising nucleic acids encoding an antigen of the pathogen operably linked to a CSE that is cognate to the replicase of the R-NP.

In some embodiments, this disclosure describes a composition comprising: a replicase nanoparticle (R-NP) comprising a replicase construct, wherein the replicase construct is a ribonucleic acid (RNA) comprising nucleic acids encoding a replicase, and wherein the R-NP does not comprise a polynucleotide comprising a conserved sequence element (CSE) cognate to the replicase; and a trans-replicating RNA (trRNA) trRNA nanoparticle (Tr-NP), comprising a trRNA comprising nucleic acids encoding a payload operably linked to a CSE cognate to the replicase of the R-NP; wherein the R-NP and Tr-NP are formulated in separate nanoparticles relative to each other.

In some embodiments, the replicase construct of the R-NP is comprised in a lipid nanoparticle (LNP) (R-LNP) and wherein the trRNA of the Tr-NP is comprised in an LNP (Tr-LNP). In some embodiments, the composition comprises a 1:1 molar ratio of R-NP to the Tr-NP. In some embodiments, the composition comprises a 50:1 molar ratio of the R-NP to the Tr-NP. In some embodiments, the composition comprises a 100:1 molar ratio of the R-NP to the Tr-NP. In some embodiments, the composition comprises a 500:1 molar ratio of the R-NP to the Tr-NP. In some embodiments, the composition comprises a 1000:1 molar ratio of the R-NP to the Tr-NP. In some embodiments, this disclosure describes a vaccine comprising the composition.

In some embodiments, this disclosure describes a method of preparing a vaccine, the method comprising: (i) obtaining a replicase nanoparticle (R-NP) comprising a replicase construct, wherein the replicase construct is a ribonucleic acid (RNA) comprising nucleic acids encoding a replicase, and wherein the R-NP does not comprise a polynucleotide comprising a conserved sequence element (CSE) cognate to the replicase; and (iii) obtaining a trans-replicating RNA (trRNA) trRNA nanoparticle (Tr-NP), comprising a trRNA comprising nucleic acids encoding a payload operably linked to a CSE cognate to the replicase of the R-NP.

In some embodiments, this disclosure describes a system for vaccination of a population of human subjects against a pathogen, the system comprising: (i) a composition of replicase nanoparticles (R-NPs) comprising nucleic acids encoding a replicase; and (ii) a composition of pathogen specific trans-replicating RNA (trRNA) nanoparticles (Tr-NPs) that encode an immunogen, wherein the trRNA is operably linked to a CSE cognate to the replicase of (i).

BRIEF DESCRIPTION OF DRAWINGS

FIG. 1 show an example two-component *trans* amplifying ribonucleic acid (RNA) (taRNA) vaccine comprising: greater than 99% lipid nanoparticles (LNPs) comprising a replicase construct encoding a replicase (and not comprising a trRNA); and less than 1% LNPs comprising a *trans* replicating RNA (trRNA) encoding a vaccine antigen (and not comprising a replicase construct).

FIGs. 2A-2B show that trRNAs and replicase constructs can be separately formulated and still elicit immune responses in mice. **FIG. 2A** shows that a two-component vaccine composition having a first LNP comprising a replicase construct only (i.e., a "replicase nanoparticle" (R-NP)) and a second LNP comprising a trRNA encoding a vaccine antigen only (i.e., a "trRNA nanoparticle" (trRNA-NP)) can be co-administered to mice to produce an immune response equivalent to that induced by a single-component vaccine composition having a single LNP comprising both a replicase construct and a trRNA encoding the same vaccine antigen. **FIG. 2B** shows that the same immune response achieved by administering a 1:1 molar ratio of R-NP:trRNA-NP can be achieved by administering a 25:1 molar ratio of R-NP:trRNA-NP.

FIGs. 3A-3B show that trRNA and replicase constructs can be separately formulated and still elicit immune responses in mice. **FIG. 3A** shows that a two-component vaccine composition having a first LNP comprising a replicase construct only (i.e., a "replicase nanoparticle" (R-NP)) dosed at 1μg; and a second LNP comprising a trRNA encoding a vaccine antigen only (i.e., a "trRNA nanoparticle" (trRNA-NP)) at varying doses can elicit similar levels of binding antibody at >50x less antigen RNA dosed as compared to a single-component vaccine composition having an LNP comprising mRNA encoding the vaccine antigen only (and not taRNA). **FIG. 3B** shows that two-component vaccines comprising R-NP dosed at 1μg and trRNA-NPs at varying doses can elicit similar levels of neutralizing vaccine responses at >50x less antigen RNA dosed as compared to single-component vaccine compositions having an LNP comprising mRNA encoding the vaccine antigen only (and not taRNA).

FIGs. 4A-4B show that trRNA and replicase constructs can be separately formulated and still elicit immune responses in mice. **FIG. 4A** shows that administration of two-component vaccine compositions having a R-NP and one of various trRNA-NPs encoding vaccine antigens (e.g., having different sequences and chemical modifications), can elicit robust levels of binding antibody, even when the amount of trRNA-NP is 100x lower than the amount of R-NP. **FIG. 4B**

shows that two-component vaccine compositions having a R-NP and one of various trRNA-NPs encoding vaccine antigens (e.g., with different sequences and chemical modifications) can elicit robust levels of neutralizing vaccine response, even when the amount of trRNA-NP is 100x lower than the amount of R-NP.

FIG. 5 shows that a two-component taRNA vaccine composition having a first lipid nanoparticle (LNP) comprising a replicase construct (i.e., a replicase-nanoparticle (R-NP)) was dosed at 1μg and a second LNP comprising a trRNA encoding a vaccine antigen at varying doses can elicit similar levels of neutralizing vaccine response at 1000x less antigen RNA dosed as compared to a single-component vaccine composition comprising a LNP comprising mRNA encoding the vaccine antigen only (and not taRNA).

FIGs. 6A-6B show that trRNA and replicase constructs can be separately formulated and still elicit immune responses that are protective in mice after a single administration. **FIG. 6A** shows the binding antibody elicited from a single administration of antigen mRNA, a single-component taRNA vaccine composition comprising an lipid nanoparticle (LNP) having a co-formulated replicase and antigen trRNA, or a two-component taRNA vaccine composition having a first lipid nanoparticle (LNP) comprising a replicase construct (i.e., a replicase-nanoparticle (R-NP)) was dosed at 1μg and a second LNP comprising a trRNA encoding a vaccine antigen at varying doses. **FIG. 6B** shows the percentage of mice surviving challenge from influenza virus based on different doses of each vaccine condition.

DETAILED DESCRIPTION

In some aspects, this disclosure provides a replicase nanoparticle (R-NP) comprising a replicase construct, wherein the replicase construct is a polyribonucleic acid comprising nucleic acids encoding a replicase, and wherein the R-NP does not comprise a polynucleotide (e.g., RNA polynucleotide) comprising a nucleic acid payload or a nucleic acid encoding a payload (e.g., a gene) operably linked to a conserved sequence element (CSE) that is cognate to the replicase.

Replicase Nanoparticle (R-NP)

A "replicase nanoparticle (R-NP)" refers to a nanoparticle comprising a replicase construct and not comprising a polynucleotide (e.g., RNA polynucleotide) comprising a nucleic acid payload or a nucleic acid encoding a payload (e.g., a gene) operably linked to a conserved sequence element (CSE) that is cognate to the replicase. In some embodiments, an R-NP

comprises a replicase construct encapsulated by the nanoparticle. In some embodiments, the nanoparticle of R-NP is a polymeric nanoparticle. In some embodiments, the nanoparticle of the R-NP is a lipid nanoparticle (i.e., R-LNP). In some embodiments, the lipid nanoparticle of the R-LNP comprises an ionizable lipid, a phospholipid, a neutral sterol (e.g., cholesterol), and/or a lipid comprising polyethylene glycol (PEG) (i.e., a PEGylated lipid). Lipid nanoparticles for RNA polynucleotide delivery are known in the art e.g., as described in Jung HN Theranostics. 2022 Oct 24;12(17):7509-7531; Paunovska, K., Nat Rev Genet 23, 265–280 (2022); and by Han, X., et al., Nat Commun 12, 7233 (2021). Lipid nanoparticle components, production, formulations, and RNA delivery are also known in the art, e.g., as described by Jung.

In some embodiments, a nanoparticle of an R-LNP comprises an ionizable lipid. In some embodiments, the ionizable lipid is an unsaturated ionizable lipid, a multi-tail ionizable lipid, a polymeric ionizable lipid, a biodegradable ionizable lipid and/or a branched-tail ionizable lipid. In some embodiments, the ionizable lipid is selected from Fig. 1 or Fig. 2 of Han, X., et al., Nat Commun 12, 7233 (2021). Figs. 1 and 2 of Han, X., et al., Nat Commun 12, 7233 (2021) are incorporated herein by reference in their entirety. In some embodiments, the unsaturated ionizable lipid comprises Dlin-MC3-DMA (i.e., MC3), OF-O2, A6, or A18-Iso2DC18. In some embodiments, the multi-tail ionizable lipid comprises $98N_{12}$-5, C12-200, CKK-E12, or 9A1P9. In some embodiments, the ionizable polymer-lipid comprises 7C1 or G0-C14. In some embodiments, the biodegradable ionizable lipid comprises L319, $304O_{13}$, C12-200, OF-Deg-Lin, or 306-O12B. In some embodiments, the branched-tail ionizable lipid is $306O_{i10}$ or FTT5. In some embodiments, the ionizable lipid comprises SM-102, ALC-0315, Acuitas A9, Lipid 2,2 (8,8) 4C CH3, Genevant CL1, LP000001, LP01, or MC3.

A “polynucleotide” refers to a polymer of nucleotides. A polynucleotide is generally composed of nucleotides that are naturally found in DNA or RNA (e.g., adenosine/deoxyadenosine (A), thymidine/deoxythymidine (T), guanosine/deoxyguanosine (G), cytidine/deoxycytidine (C) and uridine (U) joined by phosphodiester bonds. However, the term polynucleotide may also refer to polynucleotides comprising nucleotides or nucleotide analogs containing chemically or biologically modified bases, modified backbones, etc., whether or not these modifications are found in naturally occurring nucleic acids; indeed, such molecules may be preferred for certain applications.

A “replicase” is an RNA-dependent RNA polymerase capable of transcribing (*i.e.,* reading) an RNA template to produce an RNA (e.g., trRNA). A “replicase construct” is a

polyribonucleic acid comprising nucleic acids encoding a replicase from an RNA virus (e.g., an alphavirus). Once introduced to an environment comprising translational machinery (such as a cell), replicase constructs can be translated to generate the encoded replicase. In some embodiments, the replicase of the replicase construct is modified (*e.g.,* to comprise one or more mutations). In some embodiments, a replicase construct is a self-amplifying RNA (saRNA). A saRNA comprises a replicase construct. In some embodiments, a replicase does not comprise a gene (e.g., does not comprise a nucleic acid payload or a nucleic acid encoding a payload).In some embodiments, the replicase is an alphavirus replicase. The term "alphavirus" refers to an RNA virus belonging to the Togaviridae family. Alphaviruses generally comprise a single-stranded RNA genome encoding at least nsP1, nsP2, nsP3, nsP4, E1, E2, E3, 6K/TF and capsid proteins. An alphavirus may be any alphavirus known in the art; non-limiting examples include Semliki Forest virus (SFV), Sindbis virus SINV), Venezuelan equine encephalitis virus, and Chikungunya virus (CHIKV). Typically, an alphaviral replicase comprises a complex formed by the non-structural proteins nsP1, nsP2, nsP3, and nsP4. Once expressed, alphaviral replicase may interact with a RNA polynucleotide comprising one or more CSEs which are cognate to the replicase and generate mirrored copies of the RNA polynucleotide, which can be subsequently translated (e.g., by a host cell). A replicase that is "cognate" to a CSE refers to a replicase that is the capable of transcribing a portion of a polynucleotide comprising the CSE (e.g., capable of transcribing a polynucleotide comprising the payload). In some embodiments, a replicase construct encodes a replicase derived from an SFV (SFV replicase). In some embodiments, a replicase construct encodes a replicase derived from a SINV (SINV replicase). In some embodiments, a replicase and CSE from the same species are cognate; for example, an SFV replicase is assumed to be cognate to a CSE derived from an SFV. In some embodiments, a replicase and CSE from different species are cognate; for example, an SFV replicase may be cognate to a SINV and/or VEEV CSE.

In some embodiments, a polynucleotide encoding a replicase (e.g., a replicase construct) comprises one or more untranslated regions (UTRs). An "untranslated region," hereinafter referred to as "UTR," is a region in a polynucleotide which may be transcribed, but is not translated into a gene product. UTRs may act as stabilizing elements and/or provide regulation of transcription of a gene or transgene. Typically, UTRs are found upstream and/or downstream of a gene or transgene. A UTR located directly upstream of a start codon operably linked to a gene or transgene is referred to herein as a 5'-UTR. As a skilled artisan will understand, 5'-

UTRs may comprise sequence elements which play roles in regulation of expression (e.g., Kozak sequences) or structural elements which alter stability of the molecule (e.g., 5' cap structures). A UTR located directly downstream of a stop codon operably linked to a gene or transgene is referred to herein as a 3'-UTR. 3'-UTRs may comprise structural elements which alter the stability of a construct and/or provide transcriptional control, including, but not limited to AU-rich elements and polyA tails. A variety of 5'-UTRs and a 3'-UTRs are known to those of ordinary skill in the art. UTRs may be naturally occurring or synthetic. In some embodiments, a polynucleotide comprises a 5'-UTR and/or a 3'-UTR. In some embodiments, a replicase construct comprises a 5'-UTR. In some embodiments, a replicase construct comprises a 3'-UTR. In some embodiments, a replicase construct comprises a 5'-UTR and a 3'-UTR. In some embodiments, a replicase construct comprises a 5'-UTR derived from human alpha-globin (5'-HBA-UTR). An exemplary 5'-HBA-UTR is provided in SEQ ID NO: 5. In some embodiments, a replicase construct comprises a 3'-UTR derived from human alpha-globin (3'-HBA-UTR). An exemplary 5'-HBA-UTR is provided in SEQ ID NO: 6. In some embodiments, a replicase construct comprises a 5'-HBA-UTR and a 3'-HBA-UTR. In some embodiments, a replicase construct comprises a 5'-HBA-UTR, a SFV replicase-encoding sequence, and a 3'-HBA-UTR (5'-HBA-UTR-SFV replicase-3'-HBA-UTR). An exemplary 5'-HBA-UTR-SFV replicase-3'-HBA-UTR construct is provided in SEQ ID NO: 1.

Trans-Replicating RNAs (trRNAs)

In some embodiments, an R-NP does not comprise a polynucleotide comprising a gene operably linked to a conserved sequence element (CSE) that is cognate to the replicase, i.e., does not comprise a *trans* replicon construct.

The terms "*trans* replicon construct" or "*trans* replicating RNA", used synonymously herein and hereinafter referred to as "trRNA," refer to an RNA construct capable of being replicated by a cognate replicase. A trRNA comprises at least a nucleic acid payload or a nucleic acid encoding a payload (e.g., a gene) and one or more CSEs. As used herein, the terms "gene" and "nucleic acid payload or nucleic acid encoding a payload" are used interchangeably, and refer to a DNA polynucleotide or RNA polynucleotide comprising a functional RNA (e.g., siRNA, miRNA, lncRNA), or encoding a polypeptide (e.g., a vaccine antigen or a replicase). As used herein, a "payload" refers to one or more gene products of interest (e.g., a functional RNA or a polypeptide) for delivery to or expression by an organism. A payload may be a functional nucleic acid (e.g., RNA), a protein, a peptide or protein fragment, or a fusion protein.

In some embodiments, a payload is an antigen. An antigen refers to a molecule against which a host immune response is produced. In some embodiments, an antigen is a nucleic acid (e.g., aptamer), glycoprotein (e.g., a protein comprising and/or conjugated to a glycan, polysaccharide, or oligosaccharide), a lipoprotein (e.g., a protein comprising and/or conjugated to a lipid), or cancer antigen (e.g., tumor antigen). In some embodiments, an antigen is a polypeptide derived from a pathogen. In some embodiments, the antigen is a vaccine antigen. A vaccine antigen induces an immunogenic response in a subject (e.g., as measured by antibody production against the vaccine antigen). In some embodiments, a payload is an infectious disease antigen (e.g., a protein from a pathogen). In some embodiments, an antigen is viral antigen (e.g., a spike or fusion protein). In some embodiments, an antigen is a respiratory virus antigen. In some embodiments, a respiratory virus antigen is a coronavirus antigen, for example, a severe acute respiratory syndrome (SARS) or Middle East respiratory syndrome (MERS) antigen. In some embodiments, a respiratory virus antigen is an influenza virus antigen. In some embodiments, an antigen is an Ebola virus antigen. In some embodiments, an antigen is a bacterial antigen. In some embodiments, an antigen is a plague antigen. In some embodiments, an antigen is a parasite antigen. In some embodiments, a payload is a cancer antigen. In some embodiments, a payload is a vaccine antigen (e.g., results in an immunogenic response in a subject).

In some embodiments, a payload is a selectable marker. As used herein, a "selectable marker" is a peptide or protein that can be used to screen cells by artificial selection. Non-limiting examples of selectable markers include antibiotic resistance proteins (e.g., ampicillin, puromycin) and negative selection markers (e.g., thymidine kinase). In some embodiments, a payload is a reporter. A "reporter" is a peptide or protein which alters the appearance of a cell such that cells can be visually or optically screened for presence or absence of the peptide or protein. In some embodiments, a reporter is an enzyme which alters the appearance of a cell, such as beta-galactosidase. In some embodiments, a reporter is a peptide or peptide fragment (e.g., secreted embryonic alkaline phosphatase (SEAP)) which can be detected in combination with additional reagents (e.g., assay-specific media). In some embodiments, a reporter is a fluorophore, such as, but not limited to, green fluorescent protein (GFP), red fluorescent protein (RFP), blue fluorescent protein (BFP), yellow fluorescent protein (YFP), or any derivative thereof.

In some embodiments, a gene is a naturally occurring gene operably linked to CSE. In some embodiments, a gene is a transgene (e.g., derived from a different organism). In some embodiments, a gene encodes an antigen (e.g., a vaccine antigen).

trRNA constructs comprise one or more conserved sequence elements (CSEs). A "conserved sequence element (CSE)", refers to a recognition site for an alphavirus replicase. Typically, a CSE functions as a core promoter or enhancer for initiation of replication of a downstream sequence, such that a 5'-CSE may initiate synthesis of a plus-strand and a 3'-CSE may initiate synthesis of a minus-strand. An RNA polynucleotide may comprise one or more 5'-CSEs and/or 3'-CSEs. CSEs may be comprised in a UTR, for example a 5'-UTR and/or a 3' UTR. In some embodiments, a CSE forms one or more secondary structure(s), such as one or more stem-loops. Non-limiting examples of CSEs include CSE1, CSE2, CSE3, CSE4, and variants or derivatives thereof. In some embodiments, the CSE is a CSE derived from SFV, SINV, VEEV, or CHIKV alphavirus. CSEs are known in the art, e.g., as described in Hyde JL, Virus Res. 2015 Aug 3;206:99-107. In some embodiments, a trRNA comprises one or more CSEs, wherein the CSEs are present in a payload-encoding sequence. In some embodiments, a trRNA comprises one or more CSEs, wherein the CSEs are present in one or more UTRs.

In some embodiments, a polynucleotide comprising a gene (e.g., transgene) operably linked to a CSE that is cognate to a replicase is a trans-replicating RNA (trRNA).

In some embodiments, the trRNA comprises (a) a 5' alphavirus untranslated region (UTR); (b) a CSE; (c) nucleic acids encoding a payload; (d) an RNA barcode; and (e) a 3' alphavirus UTR. trRNAs do not encode a replicase. Self-amplifying RNAs (saRNAs), unlike trRNAs, comprise both trRNA elements and nucleic acids encoding a replicase (e.g., a replicase construct) in the same polynucleotide. saRNAs are known, e.g., as described in Comes JDG et al., Trends Biotechnol. 2023 Nov;41(11):1417-1429. When using trRNAs to express a payload in a cell (e.g., when administering a taRNA to a subject), a cognate replicase must be provided by another source (e.g., by the same cell, or by a separate RNA polynucleotide encoding the replicase). The term "trans-amplifying RNA" (taRNA) is used herein to refer to a trRNA and a separate RNA encoding a replicase (i.e., a replicase construct).

In some embodiments, a trRNA comprises one or more UTRs. In some embodiments, a trRNA comprises a 5' UTR. In some embodiments, a trRNA comprises a 3' UTR. In some

embodiments, a trRNA comprises a UTR (e.g., a 5' UTR and/or a 3' UTR) derived from an alphavirus. In some embodiments, an alphavirus UTR comprises a CSE. In some embodiments, a trRNA comprises a 5'-UTR of an alphavirus (e.g., an alphavirus 5'-UTR). In some embodiments, a trRNA comprises a 3'-UTR of an alphavirus (e.g., an alphavirus 3'-UTR). Alphavirus 5'-UTR and alphavirus 3'-UTR sequences are described in the art, e.g., by Hyde JL et al., Virus Res. 2015 Aug 3;206:99-107. In some embodiments, the alphavirus 5'-UTR is a VEEV 5'-UTR, SFV 5'-UTR, SINV 5'-UTR, or CHIKV 5'-UTR. In some embodiments, the alphavirus 3'-UTR is a VEEV 3'-UTR, SFV 3'-UTR, SINV 3'-UTR, or CHIKV 3'-UTR.

In some embodiments, a trRNA comprises a 5'-UTR and/or 3'-UTR derived from a SFV hereinafter referred to as a "SFV-UTR". In some embodiments, the trRNA comprises a 5'-UTR and/or 3'-UTR derived from a SINV, hereinafter referred to as a "SINV-UTR". In some embodiments, the trRNA comprises a 5'-UTR and 3'-UTR from the same virus. Non-limiting examples include a trRNA comprising a 5'-UTR derived from a SINV (5'-SINV-UTR), and a 3'-UTR derived from a SINV (3'-SINV-UTR); a trRNA comprising a 5'-UTR derived from a SFV (5'-SFV-UTR), and a 3'-UTR derived from a SFV (3'-SFV-UTR); and a trRNA comprising a 5'-SINV-UTR and a 3'-SINV-UTR. In some embodiments, a trRNA comprises a 5'-UTR and 3'-UTR from different alphaviruses.

In some embodiments, an alphavirus 5'-UTR comprises one or more mutations relative to a wildtype alphavirus 5'-UTR. In some embodiments, an alphavirus 3'-UTR comprises one or more mutations relative to a wildtype alphavirus 3'-UTR.

In some embodiments, an alphavirus 5'-UTR comprises the CSE.

In some embodiments, a trRNA comprises, from 5' to 3', (a) a 5' alphavirus UTR comprising a CSE; (b) a payload; (c) an RNA barcode; and (d) a 3' alphavirus UTR.

In some embodiments, the trRNA comprises a nucleic acid sequence of SEQ ID NO: 7-9.

R-NP and trRNA Nanoparticle (Tr-NP) Compositions

In some aspects, this disclosure provides a composition comprising: (i) an R-NP and (ii) a trRNA nanoparticle (Tr-NP), wherein the Tr-NP comprises a nanoparticle and a trRNA comprising a nucleic acid payload or a nucleic acid encoding a payload, operably linked to a CSE cognate to the replicase of the R-NP.

A "trRNA nanoparticle (Tr-NP)" refers to a nanoparticle comprising a trRNA. In some embodiments, a Tr-NP comprises a trRNA construct encapsulated by the nanoparticle. In some

embodiments, the Tr-NP is a polymeric nanoparticle. In some embodiments, the nanoparticle of a Tr-NP is a lipid nanoparticle (i.e., Tr-LNP). In some embodiments, the lipid nanoparticle of a Tr-LNP comprises an ionizable lipid.

In some embodiments, the nanoparticle of a Tr-NP and the nanoparticle of a R-NP each comprise the same nanoparticle formulation, such that the Tr-NP and the R-NP each comprise different RNAs comprised in nanoparticles sharing the same components (e.g., the same polymers, the same lipids). In some embodiments, the nanoparticle of a Tr-NP and the nanoparticle of a R-NP comprise the same nanoparticle formulation wherein the components of each nanoparticle are present in the nanoparticles in similar amounts (e.g., ratios). In some embodiments, the nanoparticle of a Tr-NP and the nanoparticle of a R-NP comprise the same nanoparticle formulation wherein the components of each nanoparticle are present in the nanoparticles in different amounts (e.g., ratios).

In some embodiments, the lipid nanoparticle of a Tr-LNP and the lipid nanoparticle of a R-LNP each comprise the same lipid nanoparticle formulation, such that the Tr-LNP and the R-LNP each comprise different RNAs comprised in lipid nanoparticles sharing the same components (e.g., the same lipids). In some embodiments, the lipid nanoparticle of a Tr-LNP and the lipid nanoparticle of a R-LNP comprise the same lipid nanoparticle formulation wherein the components of each lipid nanoparticle are present in similar amounts (e.g., ratios). In some embodiments, the lipid nanoparticle of a Tr-LNP and the lipid nanoparticle of a R-LNP comprise the same lipid nanoparticle formulation wherein the components of each lipid nanoparticle are present in different amounts (e.g., ratios).

In some embodiments, a composition described herein comprises a plurality of R-NPs and a plurality of Tr-NPs. A "plurality" refers to at least 2. In some embodiments, a plurality comprises at least 5 (e.g., at least 10, at least 100, at least 1,000, at least $1x10^4$, at least $1x10^5$, at least $1x10^6$, at least $1x10^7$, at least $1x10^8$, at least $1x10^9$, or at least $1x10^{10}$) R-NPs and Tr-NPs.

In some embodiments, this disclosure provides two-component taRNA "vaccine compositions", here referring to a composition comprising an R-NP and a Tr-NP, wherein the Tr-NP comprises a nanoparticle and a trRNA comprising a nucleic acid encoding an antigen (e.g., a vaccine antigen). In some embodiments, a vaccine composition comprises an R-NP and two or more Tr-NPs, wherein each Tr-NP comprises a trRNA comprising a nucleic acid encoding a different antigen.

In some embodiments, this disclosure provides a composition comprising a plurality of vaccine compositions, each vaccine composition comprising the same R-NP but two or more different Tr-NPs, wherein each of the Tr-NPs comprises a nanoparticle and a trRNA comprising a nucleic acid encoding a different antigen. In some embodiments, the composition comprising the plurality of vaccine compositions is administered to the same subject. In some embodiments, each composition of the plurality of vaccine compositions is administered to a different subject.

In some embodiments, a composition (e.g., a vaccine composition) described herein comprises a 1:1 molar ratio of a replicase construct to a trRNA. In some embodiments, a composition (e.g., a vaccine composition) described herein comprises a 5:1 molar ratio of replicase construct:trRNA. In some embodiments, a composition (e.g., a vaccine composition) described herein comprises a 10:1 molar ratio of replicase construct:trRNA. In some embodiments, a composition (e.g., a vaccine composition) described herein comprises a 25:1 molar ratio of replicase construct:trRNA. In some embodiments, a composition (e.g., a vaccine composition) described herein comprises a 50:1 molar ratio of replicase construct:trRNA. In some embodiments, a composition (e.g., a vaccine composition) described herein comprises a 100:1 molar ratio of replicase construct:trRNA. In some embodiments, a composition (e.g., a vaccine composition) described herein comprises a 500:1 molar ratio of replicase construct: trRNA. In some embodiments, a composition (e.g., a vaccine composition) described herein comprises a 1000:1 molar ratio of replicase construct:trRNA. In some embodiments, a composition (e.g., a vaccine composition) described herein comprises a 10000:1 molar ratio of replicase construct to trRNA. In some embodiments, a composition (e.g., a vaccine composition) described herein comprises a 20000:1 molar ratio of replicase construct to trRNA. In some embodiments, a composition (e.g., a vaccine composition) described herein comprises a 50000:1 molar ratio of replicase construct to trRNA. In some embodiments, a composition (e.g., a vaccine composition) described herein comprises a 10:1 to a 20000:1 molar ratio of replicase construct to trRNA. In some embodiments, a composition (e.g., a vaccine composition) described herein comprises a 100:1 to a 20000:1 molar ratio of replicase construct to trRNA. In some embodiments, a composition (e.g., a vaccine composition) described herein comprises a 100:1 to a 10000:1 molar ratio of replicase construct to trRNA.

In some embodiments, a composition (e.g., a vaccine composition) described herein comprises greater than or equal to 99% R-NP and less than or equal to 1% Tr-NP. In some embodiments, a composition (e.g., a vaccine composition) described herein comprises R-NPs

and Tr-NPs, wherein about 99%, about 99.1%, about 99.2%, about 99.3%, about 99.4%, about 99.5%, about 99.6%, about 99.7%, about 99.8%, about 99.9%, about 99.91%, about 99.92%, about 99.93%, about 99.94%, about 99.95%, about 99.96%, about 99.97%, about 99.98%, or about 99.99% of the composition comprises R-NPs and wherein about 1%, about 0.9%, about 0.8%, about 0.7%, about 0.6%, about 0.5%, about 0.4%, about 0.3%, about 0.2%, about 0.1%, about 0.09%, about 0.08%, about 0.07%, about 0.06%, about 0.05%, about 0.04%, about 0.03%, about 0.02%, about 0.01%, or less of the composition comprises Tr-NPs. In some embodiments, a composition (e.g., a vaccine composition) described herein comprises greater than 99% R-NP and less than 1% Tr-NP.

In some embodiments, the combined amount of replicase construct and trRNA in a composition (e.g., a vaccine composition) described herein is less than 2μg. In some embodiments, the combined amount of replicase construct and trRNA in a composition (e.g., a vaccine composition) described herein is less than 1.5μg. In some embodiments, the combined amount of replicase construct and trRNA in a composition (e.g., a vaccine composition) described herein is about 1μg. In some embodiments, a composition (e.g., a vaccine composition) described herein comprises a R-NP comprising a replicase construct and a Tr-NP comprising a trRNA encoding a payload, wherein the total amount of replicase construct in the composition is less than about 2μg (e.g., about 1.9μg, about 1.8μg, about 1.7μg, about 1.6μg, about 1.5μg, about 1.4μg, about 1.3μg, about 1.2μg, about 1.1μg, about 1.0μg, or less) and the total amount of trRNA in the composition is less than 1μg (e.g., about 0.9μg, about 0.8μg, about 0.7μg, about 0.6μg, about 0.5μg, about 0.4μg, about 0.3μg, about 0.2μg, about 0.1μg, about 0.09μg, about 0.08μg, about 0.07μg, about 0.06μg, about 0.05μg, about 0.04μg, about 0.03μg, about 0.02μg, about 0.01μg, about 0.009μg, about 0.008μg, about 0.007μg, about 0.006μg, about 0.005μg, about 0.004μg, about 0.003μg, about 0.002μg, about 0.001μg, about 0.0009μg, about 0.0008μg, about 0.0007μg, about 0.0006μg, about 0.0005μg, about 0.0004μg, about 0.0003μg, about 0.0002μg, about 0.0001μg). In some embodiments, the composition (e.g., the vaccine composition) comprises a total amount of replicase construct of about 1μg, and a total amount of trRNA between about 0.0001μg to about 0.2μg. In some embodiments, the composition (e.g., the vaccine composition) comprises a total amount of replicase construct of about 1μg and a total amount of trRNA of between about 0.0001μg to about 0.1μg. In some embodiments, the composition (e.g., the vaccine composition) comprises a total amount of

replicase construct of about 1μg and a total amount of trRNA of about 0.0001μg, about 0.001μg, about 0.01μg, or about 0.1μg.

"About" refers to ±5% of the numerical values cited. For example, "about 25% to about 75%" should be understood to include any value between 25% and 75% (inclusive), but also any value between 20-25% and 75-80%. About does not refer to percentages that are above 100% (e.g., above 100% of a composition).

Vaccines and Kits

In some aspects, this disclosure provides compositions comprising vaccines (i.e., "vaccine compositions"). A "vaccine" comprises a therapeutic or prophylactic material providing one or more antigens. In some embodiments, a vaccine composition elicits an immune response to one or more antigens. In some embodiments, a vaccine comprises a pharmaceutically acceptable carrier or excipient. In some embodiments, a vaccine comprises an adjuvant. Vaccine compositions provided herein are two-component vaccine compositions comprising a R-NP comprising a nanoparticle and a replicase construct and a Tr-NP comprising a nanoparticle and a trRNA comprising one or more nucleic acids encoding one or more antigens which, when administered to a subject (e.g., a human or mouse) and expressed therein, stimulate a host immune response (e.g., production of antibodies that bind to the antigen).

In some embodiments, this disclosure provides a kit or components of kits suitable for administering to a subject (e.g., as a vaccine). Notably, embodiments relating to the replicase constructs, trRNAs, R-NPs and Tr-NPs described herein may be understood as kits and/or components of kits suitable for administering to a subject.

In some embodiments, a kit comprises a first vial comprising an R-NP comprising a nanoparticle and a replicase construct encoding a replicase; and a second vial comprising Tr-NP, wherein the Tr-NP comprises a nanoparticle and a trRNA comprising a nucleic acid encoding a payload, operably linked to a CSE cognate to the replicase of the R-NP.

In some embodiments, this disclosure provides a kit comprising a first vial comprising a plurality of R-NPs, each R-NP comprising a nanoparticle and a replicase construct encoding a replicase; and a second vial comprising a plurality of Tr-NPs, wherein Tr-NPs of the plurality of Tr-NPs comprise a nanoparticle and a trRNA comprising a nucleic acid encoding a payload, operably linked to a CSE cognate to the replicase of R-NPs of the plurality of R-NPs.

In some embodiments, this disclosure provides a kit comprising a first vial comprising a plurality of R-NPs, each R-NP comprising a nanoparticle and a replicase construct encoding a

replicase; and a second vial comprising a plurality of Tr-NPs, wherein each most Tr-NP of the plurality of Tr-NPs comprises a nanoparticle and a trRNA comprising a nucleic acid encoding a payload, operably linked to a CSE cognate to the replicase of most R-NPs of the plurality of R-NPs.

In some embodiments, this disclosure provides a kit comprising a first vial comprising a plurality of R-NPs, each R-NP comprising a nanoparticle and a replicase construct encoding a replicase; and a second vial comprising a plurality of Tr-NPs, wherein each Tr-NP of the plurality of Tr-NPs comprises a nanoparticle and a trRNA comprising a nucleic acid encoding a payload, operably linked to a CSE cognate to the replicase of each R-NP of the plurality of R-NPs.

In some embodiments, the first vial and the second vial of a kit comprise two components of a vaccine.

In some embodiments, a kit described herein comprises a pharmaceutically acceptable carrier or excipient. In some embodiments, a kit described herein comprises a multidose container for administration of a composition described herein (*e.g.,* a vaccine). In some embodiment, a kit comprises instructions for administering a vaccine.

Methods of Production

In some embodiments, this disclosure provides a method of producing a two-component vaccine composition, the method comprising: (i) obtaining a plurality of R-NPs, each R-NP comprising a nanoparticle and a replicase construct encoding a replicase; (ii) determining a disease or disorder for which vaccination is desired; (iii) obtaining a plurality of Tr-NPs, each Tr-NP comprising a nanoparticle and a trRNA, the trRNA comprising: a nucleic acid encoding an antigen related to the disease or disorder, wherein the nucleic acids encoding the antigen are operably linked to a CSE that is cognate to the replicase of an R-NP of the plurality of R-NPs.

R-NPs may be obtained in any suitable way from any suitable source. In some embodiments, obtaining R-NPs comprises producing the R-NPs (e.g., using the methods described in the Examples). In some embodiments, obtaining R-NPs comprises obtaining R-NPs from a third party.

Determining a disease or disorder for which vaccination is desired may be performed using any suitable means. In some embodiments, determining comprises collecting a specimen from a subject and sequencing the specimen to determine a pathogen. In some embodiments, determining a disease or disorder for which vaccination is required comprises selecting a given

pathogen that has resulted in a pandemic or endemic or is expected to result in a pandemic or endemic. In some embodiments, determining a disease or disorder for which vaccination is required comprises obtaining a sample from a subject and identifying one or more mutated peptides against which an immune response is desired. In some embodiments, identifying am antigen comprises *in silico* identification of the antigen (e.g., vaccine antigen), for example, as described by Rawal, K. et al. Sci Rep 11, 17626 (2021).

Tr-NPs may be obtained in any suitable way from any suitable source. In some embodiments, obtaining Tr-NPs comprises produces the Tr-NPs (e.g., using the methods described in the Examples). In some embodiments, obtaining Tr-NPs comprises obtaining Tr-NPs from a third party.

In some embodiments, the method of producing a two-component vaccine composition further comprises (iv) combining the plurality of R-NPs and the plurality of Tr-NPs. Combining may be performed prior to administration to a subject (e.g., by combining the contents of two vials of a kit described herein into a single syringe) or in a subject (e.g., by separately administering the contents of two vials of a kit described herein to the same subject). In some embodiments, steps (i)-(iii) are performed in sequential order. In some embodiments, steps (i)-(v) are performed in sequential order.

In some embodiments, after step (i) and before step (ii), the method of producing a vaccine comprises placing a plurality of R-NPs into long term storage. In some embodiments, long term storage comprises storage at at least -20˚C. In some embodiments, long term storage comprises storage at at least -70 ˚C. In some embodiments, long term storage comprises storage at at least -80 ˚C. In some embodiments, long term storage comprises storage between about -20˚C and -70˚C. In some embodiments, long term storage comprises storage between at about -60˚C and -80˚C. In some embodiments, long term storage comprises storage for at least 3 months. In some embodiments, long term storage comprises storage for at least 6 months. In some embodiments, long term storage comprises storage for at least 1 year. In some embodiments, long term storage is at least 2 years. In some embodiments, long term storage is at least 3 years. In some embodiments, long term storage is at least 4 years. In some embodiments, long term storage is at least 5 years. In some embodiments, long term storage is 3 months to 1 year. In some embodiments, long term storage is 3 months to 2 years. In some embodiments, long term storage is 3 months to 3 years. In some embodiments, long term storage is 3 months to 4 years. In some embodiments, long term storage is 3 months to 5 years.

In some embodiments, the disclosure describes a plurality of compositions, each composition comprising a plurality of the same R-NPs and a plurality of Tr-NPs, wherein the plurality of Tr-NPs of each compositions is different (e.g., wherein each plurality of Tr-NPs comprises nucleic acids encoding an antigen that is different from the antigen encoded by another plurality of Tr-NPs).

In some embodiments, this disclosure provides a method of formulating and testing multiple different antigens to identify those with improved immunogenic properties. In some embodiments, the method comprises (i) producing two or more different compositions, wherein each composition comprises a plurality of the same R-NPs and a plurality of Tr-NPs, wherein the plurality of Tr-NPs of each compositions is different (e.g., wherein each plurality of Tr-NPs comprises nucleic acids encoding an antigen that is different from the antigen encoded by another plurality of Tr-NPs), (ii) administering the two or more compositions to different subjects, and (iii) measuring immune responses (e.g., antibody production against each antigen) induced by administration of the two or more compositions.

Methods of Expressing a Payload

In some aspects, this disclosure provides methods of expressing a replicase and/or payload (e.g., antigen) in a cell. A cell may be an isolated cell or a cell of a subject. In some embodiments, a cell is a derived from a subject. In some embodiments, a cell is a mammalian cell. In some embodiments, a cell is a human cell.

In some embodiments, expressing a replicase and/or payload in a cell comprises contacting the cell with an R-NP and/or Tr-NP of the disclosure. In some embodiments, contacting the cell with an R-NP and a Tr-NP comprises a physical interaction between the R-NP, Tr-NP, and the cell. In some embodiments, the physical interaction is transitory. In some embodiments, the physical interaction results in the trRNA and/or replicase construct entering the cell. In some embodiments, the physical interaction results in the trRNA and replicase construct entering the cell.

In some aspects, this disclosure provides methods of expressing a replicase, the method comprising contacting a cell (e.g., of a subject) with an R-NP.

In some aspects, this disclosure provides methods of expressing a payload in a cell (e.g., a cell of a subject).

A “subject” refers to an organism that has an adaptive immune system. In some embodiments, the subject is a mammal. In some embodiments, the subject is a rodent (e.g., a

mouse or rat). In some embodiments, the subject is a non-human primate. In some embodiments, the subject is a human.

In some embodiments, methods of expressing a payload in a subject comprise contacting a cell with a R-NP and a Tr-NP (e.g., with a composition comprising an R-NP and a Tr-NP, with a first composition comprising an R-NP and a second composition comprising a Tr-NP). In some embodiments, methods of expressing a payload in a cell comprise contacting the cell with a Tr-NP, wherein the cell comprises an RNA polynucleotide encoding a replicase (e.g., a replicase construct) cognate to the Tr-NP. In some embodiments, methods of expressing a payload in a cell comprise contacting the cell with a Tr-NP, wherein the cell comprises a replicase (e.g., a replicase construct) cognate to the Tr-NP. In some embodiments, methods of expressing a payload in a cell comprise contacting the cell with an R-NP, wherein the cell comprises an RNA polynucleotide comprising nucleic acids encoding the payload operably linked to a CSE that is cognate to the replicase of the R-NP. In some embodiments, methods of expressing a payload in a cell comprise contacting the cell with an R-NP, wherein the cell comprises nucleic acids encoding the payload operably linked to a CSE that is cognate to the replicase of the R-NP.

In some embodiments, a cell is comprised in a subject, such that contacting a cell comprises administering R-NPs and/or Tr-NPs to the subject in which the cell is comprised. R-NPs and Tr-NPs of the present disclosure may be administered to a subject in any manner known in the art; for example, an R-NP and/or Tr-NP may be administered to a subject via a route selected from the group consisting of subcutaneous, intradermal, intramuscular, intranasal, intravenous, and sublingual administration. In some embodiments, an R-NP and/or Tr-NP are administered to a subject as a vaccine.

Contact of R-NPs and/or Tr-NPs with a cell (e.g., a cell in a subject) may be determined by measuring trRNA amplification by the replicase or payload expression in a plurality of cells of the same type as the putatively contacted cell. Cells of the same "type" refer to a set of cells having similar characteristics (e.g., being of the same tissue). In some embodiments, cells of the same "type" are cells derived from the same tissue or organ of a subject. For example, in some embodiments, liver cells are the same cell type, regardless of whether they are derived from the same subject. In some embodiments, the cell type is a cell type of a specific organ (e.g., brain, eyes, skin, spinal cord, peripheral nervous system, lung, heart, spleen, kidney, liver, intestine, testis, and ovaries). In some embodiments, a cell type is a bodily fluid specific cell type (e.g.,

bone marrow cell or blood cell). In some embodiments, the cell type is specific cell type of specific organ or specific bodily fluid. For example, liver cells can be subdivided into different cell types (e.g., hepatocytes, hepatic stellate cells, Kupffer cells, and liver sinusoidal endothelial cells).

EXEMPLARY SEQUENCES

Rational Name	Sequence	SEQ ID NO:
5'-HBA-UTR-SFV replicase-3'-HBA-UTR Replicase Construct	AGGAGAAUAAACUAGUAUUCUUCUGGUCACAGACUCAGAGAGAACCCGCCACCAUGG CCGCCAAGGUGCAUGUUGACAUCGAGGCUGACUCGCCUUUUAUCAAGAGCCUGCAGA AGGCCUUCCCUAGCUUUGAAGUGGAGUCCCUUCAAGUGACACCCAAUGACCACGCCA ACGCGCGGGCAUUUAGCCACCUCGCUACUAAGCUUAUUGAGCAGGAGACAGACAAGG AUACGCUCAUCCUGGAUAUCGGCAGUGCUCCCUCCAGAAGGAUGAUGAGCACGCAUA AGUAUCACUGUGUGUGCCCAAUGAGGAGCGCCGAGGAUCCCGAACGGCUGGUUUGUU ACGCCAAAAAGUUAGCUGCCGCCAGCGGUAAGGUGCUUGACAGGGAAAUCGCGGGCA AGAUUACGGACUUGCAGACCGUGAUGGCCACCCCUGACGCCGAGAGCCCAACCUUCU GCCUCCACACCGAUGUCACUUGCAGAACCGCCGCUGAGGUCGCCGUGUACCAGGAUG UGUACGCUGUCCACGCUCCCACGAGCCUGUACCACCAGGCCAUGAAGGGUGUCAGAA CUGCCUACUGGAUUGGGUUCGACACGACCCCCUUCAUGUUCGACGCACUUGCAGGUG CCUACCCUACGUACGCGACUAACUGGGCCGAUGAGCAAGUGCUUCAAGCCAGAAAUA UAGGCCUGUGUGCCGCUAGUCUCACAGAGGGUCGGCUUGGAAAACUGUCCAUCCUGC GGAAGAAGCAGCUAAAGCCAUGCGACACCGUGAUGUUCUCCGUCGGCUCCACACUUU ACACAGAAAGCAGAAAGCUGCUGAGAUCCUGGCACCUACCUAGCGUAUUCCACCUGA AGGGCAAGCAGUCUUUCACGUGCCGCUGCGACACUAUCGUAAGCUGUGAGGGAUACG UUGUUAAGAAGAUCACCAUGUGUCCAGGCCUAUACGGCAAGACAGUGGGUUAUGCUG UGACCUACCAUGCCGAGGGGUUCCUGGUCUGUAAGACCACCGAUACCGUGAAGGGCG AACGGGUGAGCUUCCCUGUCUGCACCUAUGUCCCAAGUACGAUAUGUGAUCAGAUGA CCGGUAUUCUCGCAACAGAUGUGACACCCGAGGACGCCCAGAAACUGCUGGUUGGUC UAAACCAGAGAAUAGUUGUGAACGGCAGAACCCAACGAAACACCAAUACCAUGAAGA ACUAUUUAUUGCCUAUCGUGGCCGUCGCAUUCAGUAAGUGGGCCCGUGAGUACAAAG CGGACCUUGACGAUGAGAAACCUCUAGGAGUGAGAGAAAGAAGCCUGACCUGUUGCU GUCUCUGGGCGUUCAAGACCCGGAAAAUGCACACUAUGUAUAAGAAGCCUGACACCC AGACAAUAGUGAAGGUACCCUCUGAGUUCAACAGCUUCGUGAUCCCUAGCCUGUGGA GCACCGGACUGGCUAUUCCGGUGCGCUCUAGGAUAAAAAUGCUUCUGGCCAAGAAAA CCAAGCGAGAGUUAAUACCCGUGCUCGAUGCCUCCUCUGCAAGAGAUGCUGAGCAAG AAGAGAAAGAAAGAUUGGAGGCUGAAUUGACAAGAGAAGCCCUACCACCUCUGGUGC CAAUUGCACCCGCUGAAACAGGAGUUGUAGACGUGGACGUGGAGGAGCUGGAGUACC ACGCGGGAGCGGGCGUGGUUGAAACCCCUAGGAGCGCCCUCAAGGUCACCGCCCAGC CUAAUGAUGUGCUGCUCGGAAAUUACGUGGUUCUGUCCCCCCAGACCGUCCUGAAAU CUUCCAAGCUGGCCCCCGUGCACCCUCUGGCAGAGCAGGUGAAAAUUAUCACCCACA ACGGCCGGGCCGGACGUUACCAAGUCGACGGAUAUGAUGGUCGGGUGCUCCUUCCAU GUGGCAGUGCCAUUCCAGUACCAGAGUUCCAAGCUCUGAGCGAAAGCGCCACCAUGG UCUACAACGAGAGAGAGUUUGUCAACAGAAAGCUGUACCAUAUCGCCGUGCACGGCC CUAGCCUCAACACUGAUGAAGAGAACUAUGAGAAAGUCCGGGCUGAGAGAACAGACG CUGAAUAUGUGUUCGACGUGGAUAAGAAAUGUUGUGUGAAGCGUGAGGAGGCUAGUG GCCUGGUCCUAGUUGGAGAGCUAACAAAUCCUCCCUUUCAUGAAUUUGCUUAUGAAG GCCUUAAGAUUAGACCUUCCGCCCCUUACAAGACCACGGUAGUGGGGGUGUUCGGAG UACCCGGAUCUGGCAAGUCAGCGAUCAUCAAAAGCCUAGUGACCAAACAUGAUCUGG UGACCAGUGGCAAGAAAGAGAACUGCCAGGAAAUCGUGAAUGACGUCAAAAAGCACA GAGGCUUGGACAUUCAGGCCAAAACCGUGGAUUCUAUCCUGCUUAAUGGCUGCAGAC GGGCCGUUGACAUCUUGUACGUAGACGAGGCCUUCGCUUGCCAUUCAGGCACACUAU UGGCCCUGAUAGCCCUGGUGAAACCAAGAAGUAAAGUUGUACUGUGCGGGGAUCCUA	1

	AACAAUGUGGGUUCUUUAACAUGAUGCAGCUGAAGGUGAACUUCAACCAUAACAUCU	
	GCACCGAGGUCUGUCAUAAAUCCAUCUCCCGAAGAUGUACCAGGCCGGUGACGGCCA	
	UCGUGAGCACCCUGCACUAUGGCGGCAAAAUGAGGACCACCAACCCUUGCAACAAGC	
	CUAUCAUCAUCGAUACGACAGGGCAGACCAAGCCUAAGCCCGGCGACAUCGUCCUAA	
	CUUGCUUCAGGGGUUGGGUGAAGCAGCUGCAGCUCGAUUACCGCGGCCACGAAGUGA	
	UGACUGCUGCCGCCUCACAGGGGCUAACACGGAAGGGCGUGUACGCUGUGCGCCAGA	
	AGGUGAACGAAAAUCCCCUUUACGCCCCUGCUAGCGAGCACGUGAACGUUUUAUUGA	
	CACGGACCGAGGACCGACUCGUAUGGAAAACCCUGGCCGGGGACCCCUGGAUUAAAG	
	UUCUCUCUAACAUCCCGCAGGGUAACUUCACCGCCACACUGGAGGAGUGGCAGGAGG	
	AGCAUGACAAGAUCAUGAAAGUUAUCGAGGGACCCGCAGCUCCUGUUGAUGCUUUCC	
	AGAAUAAAGCUAACGUGUGCUGGGCGAAAUCUCUGGUGCCUGUGCUGGAUACGGCCG	
	GUAUCAGACUGACUGCCGAAGAAUGGAGUACAAUCAUCACAGCCUUUAAGGAGGAUA	
	GGGCUUAUUCGCCCGUGGUGGCCUUGAAUGAAAUUUGUACCAAGUACUACGGGGUGG	
	ACCUGGACAGCGGACUCUUCUCUGCCCCUAAAGUGUCUCUUUACUACGAGAACAACC	
	ACUGGGACAACCGCCCAGGCGGAAGAAUGUACGGCUUCAACGCCGCCACAGCCGCCA	
	GGCUGGAAGCCCGGCACACUUUUCUGAAAGGCCAGUGGCAUACUGGCAAGCAAGCCG	
	UGAUUGCCGAGAGGAAAAUCCAGCCACUGUCUGUGCUGGACAACGUGAUCCCCAUCA	
	ACAGACGGCUACCACACGCUCUGGUUGCUGAGUACAAGACCGUUAAAGGGAGCCGAG	
	UGGAGUGGCUGGUGAAUAAAGUACGGGGGUAUCACGUUUUGUUAGUGUCUGAGUACA	
	ACCUGGCCCUGCCCCGGAGACGCGUAACCUGGCUGUCCCCGCUCAACGUUACGGGCG	
	CCGACCGGUGCUAUGAUCUUUCUCUGGGCCUACCAGCUGACGCAGGUCGGUUCGACC	
	UCGUGUUCGUGAACAUUCAUACCGAGUUUAGAAUCCAUCACUACCAACAGUGCGUCG	
	ACCACGCUAUGAAACUGCAAAUGCUGGGCGGCGAUGCACUGAGGCUGUUAAAACCAG	
	GCGGCAGCCUGCUGAUGCGGGCCUAUGGUUAUGCUGAUAAGAUUUCCGAGGCCGUGG	
	UAUCUAGCCUCUCCCGCAAAUUUAGCAGCGCCAGAGUCCUGCGGCCUGACUGCGUGA	
	CUUCCAACACCGAAGUGUUCCUGCUGUUUAGCAAUUUCGACAAUGGAAAGCGGCCUA	
	GCACGCUUCACCAGAUGAACACCAAGCUGUCCGCGGUGUACGCCGGAGAAGCUAUGC	
	ACACCGCCGGCUGCGCCCCUUCAUACCGGGUGAAGCGAGCCGAUAUUGCCACGUGUA	
	CGGAGGCGGCGGUCGUCAACGCCGCAAAUGCUCGGGGAACUGUGGGCGAUGGAGUCU	
	GCCGGGCAGUUGCAAAGAAAUGGCCUUCAGCCUUCAAGGGCGAGGCGACACCAGUGG	
	GCACUAUCAAGACCGUGAUGUGCGGUAGCUACCCUGUGAUCCACGCGGUGGCUCCAA	
	AUUUUUCUGCCACCACGGAGGCCGAGGGUGACCGGGAGCUGGCCGCUGUCUACCGGG	
	CAGUGGCCGCCGAGGUCAAUAGACUCAGCCUGUCGUCAGUCGCUAUUCCUCUGCUGA	
	GCACCGGCGUGUUUUCUGGCGGCAGAGAUCGUCUCCAGCAGAGCCUGAACCAUCUGU	
	UCACCGCCAUGGACGCGACCGACGCCGAUGUCACAAUCUAUUGUAGGGACAAGUCUU	
	GGGAGAAGAAGAUCCAAGAAGCAAUCGAUAUGAGGACGGCCGUGGAGCUGCUGAACG	
	AUGAUGUUGAACUGACCACUGAUCUCGUGCGCGUGCACCCAGACUCUUCCCCUGGUCG	
	GCCGUAAGGGGUACUCUACCACUGACGGCAGCCUUUACUCAUAUUUCGAGGGUACAA	
	AAUUUAACCAGGCCGCCAUCGAUAUGGCGGAGAUCCUGACUCUCUGGCCCAGACUUC	
	AGGAAGCCAACGAACAGAUCUGCCUGUACGCGCUGGGCGAGACAAUGGAUAACAUCA	
	GAAGCAAGUGCCCUGUCAACGACAGCGACUCCUCCACCCCUCCUCGGACGGUUCCCU	
	GCCUGUGCAGAUAUGCCAUGACAGCUGAGCGAAUUGCCCGGCUGCGGUCCCACCAAG	
	UGAAGUCUAUGGUGGUAUGUUCCUCCUUUCCACUGCCCAAAUAUCAUGUGGAUGGUG	
	UGCAGAAGGUAAAGUGUGAAAAGGUGCUGCUGUUUGACCCUACGGUGCCCAGUGUGG	
	UGUCACCCAGAAAGUACGCCGCUUCCACAACUGACCACAGCGAUCGCUCUCUCCGGG	
	GUUUCGACCUUGACUGGACAACCGACUCGAGCUCGACUGCGAGCGAUACAAUGUCAC	
	UGCCCUCUCUGCAGUCCUGUGACAUCGAUAGCAUCUAUGAGCCCAUGGCCCCAAUCG	
	UGGUGACUGCGGAUGUCCACCCGGAACCUGCCGGCAUCGCUGACCUGGCCGCUGACG	
	UGCACCCAGAACCCGCCGAUCACGUUGAUCUGGAGAAUCCUAUCCCCCCUCCAAGAC	
	CUAAGCGGGCCGCUUACCUGGCCUCCAGAGCCGCUGAAAGGCCUGUGCCAGCCCCCC	
	GCAAACCCACGCCCGCUCCUCGGACAGCCUUCCGAAACAAGCUGCCCCUGACAUUCG	
	GCGACUUCGACGAACAUGAGGUGGAUGCCCUGGCUUCUGGCAUCACAUUUGGAGACU	
	UCGAUGAUGUCCUGCGGCUCGGCCGAGCCGGAGCCUACAUCUUCAGCUCUGACACAG	
	GAAGCGGGCACCUGCAGCAGAAGUCCGUGAGACAGCACAACCUCCAGUGCGCUCAGC	
	UGGAUGCUGUCGAAGAAGAGAAGAUGUAUCCUCCAAAGCUGGACACCGAAAGAGAGA	
	AGCUGCUGCUGUUGAAGAUGCAGAUGCAUCCUUCCGAGGCAAACAAGAGCAGAUAUC	
	AGAGCCGCAAGGUAGAGAAUAUGAAGGCUACGGUGGUGGAUAGACUGACCUCCGGCG	
	CCAGACUUUACACCGGGGCGGACGUGGGCCGAAUUCCCACCUACGCUGUGAGAUACC	

	CCAGACCUGUGUAUAGCCCUACAGUCAUCGAGAGAUUUAGCAGCCCAGACGUAGCUA UCGCAGCCUGUAACGAGUACCUGAGCCGGAAUUACCCAACAGUCGCAAGUUAUCAGA UCACCGACGAAUACGACGCAUAUUUGGAUAUGGUGGACGGCUCGGACAGCUGCUUAG ACAGAGCCACCUUCUGCCCGGCAAAACUACGGUGCUAUCCUAAGCACCACGCGUACC ACCAGCCUACCGUAAGAUCUGCCGUGCCAUCACCAUUCCAGAACACCCUCCAGAACG UCCUGGCUGCCGCCACUAAGAGAAACUGUAAUGUGACACAAAUGCGCGAACUGCCUA CAAUGGACUCAGCCGUGUUCAACGUUGAAUGUUUUAAACGAUACGCCUGCUCCGGCG AGUAUUGGGAAGAAUACGCAAAACAGCCGAUCAGAAUCACCACAGAAAACAUUACAA CGUACGUGACCAAGCUGAAAGGGCCUAAGGCUGCAGCGUUGUUUGCCAAGACUCACA ACCUGGUUCCUUUGCAGGAGGUCCCCAUGGACAGAUUUACAGUGGACAUGAAGAGGG ACGUAAAAGUCACCCCGGGCACCAAACACACGGAAGAACGGCCUAAGGUCCAGGUAA UCCAGGCUGCCGAGCCACUGGCCACAGCUUACCUGUGUGGGAUACACCGGGAACUUG UGAGACGGCUGAAUGCCGUUCUUCGACCUAACGUCCACACACUGUUCGACAUGUCCG CAGAGGAUUUCGACGCCAUAAUCGCCAGUCACUUCCACCCUGGGGACCCCGUGCUCG AGACAGACAUUGCUUCCUUCGACAAAAGCCAGGAUGAUAGCCUCGCCCUGACUGGCC UCAUGAUCCUCGAGGACCUGGGCGUUGACCAGUACCUGCUGGACCUGAUCGAAGCCG CCUUUGGAGAGAUUAGCAGCUGCCACCUGCCUACGGGGACAAGAUUCAAAUUUGGGG CAAUGAUGAAGUCUGGCAUGUUUCUUACUCUGUUCAUCAAUACAGUGCUGAAUAUUA CCAUCGCCAGUAGAGUGCUGGAACAGCGCCUGACGGACUCCGCAUGCGCCGCUUUUA UUGGAGAUGACAAUAUCGUGCACGGCGUCAUCAGUGAUAAAUUGAUGGCCGAAAGAU GUGCCAGCUGGGUCAAUAUGGAGGUCAAGAUUAUCGACGCUGUGAUGGGCGAAAAAC CCCCCUACUUCUGCGGCGGCUUUAUCGUUUUCGACUCUGUGACACAGACCGCCUGCA GGGUGUCUGAUCCCCUGAAGCGGCUGUUCAAACUGGGCAAGCCACUAACCGCUGAGG ACAAACAAGACGAGGACAGAAGGCGGGCCCUGAGCGAUGAGGUUUCUAAGUGGUUCC GGACCGGUCUGGGAGCCGAGCUCGAGGUUGCACUGACCUCCAGGUACGAGGUGGAGG GCUGCAAAAGCAUCCUGAUCGCCAUGGCUACCCUGGCGAGAGAUAUUAAAGCGUUCA AAAAGCUGAGAGGACCUGUUAUCCAUCUGUAUGGCGGUCCCAGACUCGUGCGGUGAU AAUAGGCUGGAGCCUCGGUGGCCAUGCUUCUUGCCCCUUGGGCCUCCCCCCAGCCCC UCCUCCCCUUCCUGCACCCGUACCCCCGUGGUCUUUGAAUAAAGUCUGAGUGGGCGG CAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	
5'-SINV-UTR-EGFP-3'-SINV-UTR trRNA	AUUGACGGCGUAGUACACACUAUUGAAUCAAACAGCCGACCAAUUGCACUACCAUCA CAACGGAGAAGCCAGUAGUAAACGUAGACGUAGACCCCCAGAGUCCGUUUGUCGUGC AACUGCAAAAAAGCUUCCCGCAAUUUGAGGUAGUAGCACAGGAGGUGACUCCAAAUC ACCAUCCUAAUCCCAGAGCAUUUUCGCAUCUGGGCAGUAAACUAAUCGAGCUGGAGG UUCCUACCACAGCGACGAUCUUGGACAUAGGCAGCGCACCGGCUCGUAGAACGAUAA ACCCCUAGUGCCACCAUGGUGUCCAAGGGAGAGGAGCUGUUCACCGGUGUGGUGCCC AUCCUGGUGGAGCUGGAUGGCGAUGUGAAUGGGCACAAGUUCUCUGUCAGCGGCGAA GGCGAGGGCGAUGCCACCUACGGGAAGCUGACACUGAAAUUCAUCUGCACCACAGGG AAACUUCCUGUUCCCUGGCCUACCCUGGUGACCACCCUCACCUAUGGCGUCCAGUGU UUCAGCCGCUACCCAGAUCACAUGAAGCAGCAUGACUUCUUUAAGUCUGCCAUGCCA GAGGGCUACGUGCAGGAGCGGACCAUCUUCUUCAAGGAUGACGGCAAUUAUAAGACA AGAGCUGAGGUGAAGUUUGAAGGAGACACACUGGUGAACAGAAUAGAACUGAAGGGC AUUGACUUCAAGGAAGAUGGAAACAUCUUAGGCCACAAACUGGAGUAUAACUACAAC UCCCACAAUGUCUACAUCAUGGCAGACAAACAAAAGAAUGGCAUCAAGGUGAACUUC AAAAUUAGGCACAACAUCGAGGACGGCUCUGUCCAACUGGCUGACCAUUACCAGCAG AACACACCCAUUGGUGAUGGCCCUGUGCUAUUGCCUGACAACCAUUACCUGAGCACC CAGAGUGCCCUCAGCAAGGACCCUAAUGAGAAGCGGGACCACAUGGUUUUGCUGGAG UUUGUGACAGCCGCCGGAAUCACUCUGGGCAUGGAUGAACUCUACAAAUGAUAAUAG CUCGAGGCGGCCGCCACGCAGCGUCUGCAUAACUUUUAUUAUUUCUUUUAUUAAUCA ACAAAAUUUUGUUUUUAACAUUUCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA AAAA	2
U3A-5'-SINV-UTR-EGFP-3'-SFV-UTR trRNA	AUAGACGGCGUAGUACACACUAUUGAAUCAAACAGCCGACCAAUUGCACUACCAUCA CAACGGAGAAGCCAGUAGUAAACGUAGACGUAGACCCCCAGAGUCCGUUUGUCGUGC AACUGCAAAAAAGCUUCCCGCAAUUUGAGGUAGUAGCACAGGAGGUGACUCCAAAUC ACCAUCCUAAUCCCAGAGCAUUUUCGCAUCUGGGCAGUAAACUAAUCGAGCUGGAGG UUCCUACCACAGCGACGAUCUUGGACAUAGGCAGCGCACCGGCUCGUAGAACGAUAA ACCCCUAGUGCCACCAUGGUGUCCAAGGGAGAGGAGCUGUUCACCGGUGUGGUGCCC AUCCUGGUGGAGCUGGAUGGCGAUGUGAAUGGGCACAAGUUCUCUGUCAGCGGCGAA	3

	GGCGAGGGCGAUGCCACCUACGGGAAGCUGACACUGAAAUUCAUCUGCACCACAGGG AAACUUCCUGUUCCCUGGCCUACCCUGGUGACCACCCUCACCUAUGGCGUCCAGUGU UUCAGCCGCUACCCAGAUCACAUGAAGCAGCAUGACUUCUUUAAGUCUGCCAUGCCA GAGGGCUACGUGCAGGAGCGGACCAUCUUCUUCAAGGAUGACGGCAAUUAUAAGACA AGAGCUGAGGUGAAGUUUGAAGGAGACACACUGGUGAACAGAAUAGAACUGAAGGGC AUUGACUUCAAGGAAGAUGGAAACAUCUUAGGCCACAAACUGGAGUAUAACUACAAC UCCCACAAUGUCUACAUCAUGGCAGACAAACAAAAGAAUGGCAUCAAGGUGAACUUC AAAAUUAGGCACAACAUCGAGGACGGCUCUGUCCAACUGGCUGACCAUUACCAGCAG AACACACCCAUUGGUGAUGGCCCUGUGCUAUUGCCUGACAACCAUUACCUGAGCACC CAGAGUGCCCUCAGCAAGGACCCUAAUGAGAAGCGGGACCACAUGGUUUUGCUGGAG UUUGUGACAGCCGCCGGAAUCACUCUGGGCAUGGAUGAACUCUACAAAUGAUAAUAG CUCGAGGCGGCCGCAGGAGCUUAAUUCGACGAAUAAUUGGAUUUUUAUUUUAUUUUG CAAUUGGUUUUUAAUAUUUCCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	
U3A-5'-SINV-UTR-SEAP-3'-SFV-UTR trRNA	AUAGACGGCGUAGUACACACUAUUGAAUCAAACAGCCGACCAAUUGCACUACCAUCA CAACGGAGAAGCCAGUAGUAAACGUAGACGUAGACCCCCAGAGUCCGUUUGUCGUGC AACUGCAAAAAAGCUUCCCGCAAUUUGAGGUAGUAGCACAGGAGGUGACUCCAAAUC ACCAUCCUAAUCCCAGAGCAUUUUCGCAUCUGGGCAGUAAACUAAUCGAGCUGGAGG UUCCUACCACAGCGACGAUCUUGGACAUAGGCAGCGCACCGGCUCGUAGAACGAUAA ACCCCUAGUGCCACCAUGCTGCUGGGGCCCUGCAUGCUGCUCCUGCUCCUGCUGCUG GGCCUGAGGCUACAGCUCUCCCUGGGCAUCAUCCCAGUUGAGGAGGAGAACCCGGAC UUCUGGAACCGCGAGGCAGCCGAGGCCCUGGGUGCCGCCAAGAAGCUGCAGCCUGCA CAGACAGCCGCCAAGAACCUCAUCAUCUUCCUGGGCGAUGGGAUGGGGGUGUCUACG GUGACAGCUGCCAGGAUCCUAAAAGGGCAGAAGAAGGACAAACUGGGGCCUGAGAUA CCCCUGGCUAUGGACCGCUUCCCAUAUGUGGCUCUGUCCAAGACAUACAAUGUAGAC AAACAUGUGCCAGACAGUGGAGCCACAGCCACGGCCUACCUGUGCGGGGUCAAGGGC AACUUCCAGACCAUUGGCUUGAGUGCAGCCGCCCGCUUUAACCAGUGCAACACGACA CGCGGCAACGAGGUCAUCUCCGUGAUGAAUCGGGCCAAGAAAGCAGGGAAGUCAGUG GGAGUGGUAACCACCACACGAGUGCAGCACGCCUCGCCAGCCGGCACCUACGCCCAC ACGGUGAACCGCAACUGGUACUCGGACGCCGACGUGCCUGCCUCGGCCCGCCAGGAG GGGUGCCAGGACAUCGCUACGCAGCUCAUCUCCAACAUGGACAUUGAUGUGAUCCUG GGUGGAGGCCGAAAGUACAUGUUUCGCAUGGGAACCCCAGACCCUGAGUACCCAGAU GACUACAGCCAAGGUGGGACCAGGCUGGACGGGAAGAAUCUGGUGCAGGAAUGGCUG GCGAAGCGCCAGGGUGCCCGGUAUGUGUGGAACCGCACUGAGCUCAUGCAGGCUUCC CUGGACCCGUCUGUGACCCAUCUCAUGGGUCUCUUUGAGCCUGGAGACAUGAAAUAC GAGAUCCACCGAGACUCCCACACUGGACCCCUCCCCUGAUGGAGAUGACAGAGGCUGCC CUGCGCCUGCUGAGCAGGAACCCCCGCGGCUUCUUCCUCUUCGUGGAGGGUGGUCGC AUCGACCACGGUCAUCACGAAAGCAGGGCUUACCGGGCACUGACUGAGACGAUCAUG UUCGACGACGCCAUUGAGAGGGCGGGCCAGCUCACCAGCGAGGAGGACACGCUGAGC CUCGUCACUGCCGACCACUCCCACGUCUUCUCCUUCGGAGGCUACCCCCUGCGAGGG AGCUCCAUCUUCGGGCUGGCCCCUGGCAAGGCCCGGGACAGGAAGGCCUACACGGUC CUCCUAUACGGAAACGGUCCAGGCUAUGUGCUCAAGGACGGCGCCCGGCCGGAUGUU ACCGAGAGCGAGAGCGGGAGCCCCGAGUAUCGGCAGCAGUCAGCAGUGCCCCUGGAC GAAGAGACCCACGCAGGCGAGGACGUGGCGGUGUUCGCGCGCGGCCCGCAGGCGCAC CUGGUUCACGGCGUGCAGGAGCAGACCUUCAUAGCGCACGUCAUGGCCUUCGCCGCC UGCCUGGAGCCCUACACCGCCUGCGACCUGGCGCCCCCCGCCGGCACCACCGACGCC GCGCACCCGGGGCGGUCCCGGUCCAAGCGUCUGGAUUGAUAAUAGCUCGAGGCGGCC GCAGGAGCUUAAUUCGACGAAUAAUUGGAUUUUUAUUUUAUUUUGCAAUUGGUUUUU AAUAUUUCCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	4
5'-HBA-UTR (Human Alphaglobin)	AGGAGAAUAAACUAGUAUUCUUCUGGUCACAGACUCAGAGAGAACCCGCCACC	5
3'-HBA-UTR (Human Alphaglobin)	GCUGGAGCCUCGGUGGCCAUGCUUCUUGCCCCUUGGGCCUCCCCCCAGCCCCUCCUC CCCUUCCUGCACCCGUACCCCCGUGGUCUUUGAAUAAAGUCUGAGUGGGCGGC	6
U3A-5'-SINV-UTR-	AUAGACGGCGUAGUACACACUAUUGAAUCAAACAGCCGACCAAUUGCACUACCAUCA CAACGGAGAAGCCAGUAGUAAACGUAGACGUAGACCCCCAGAGUCCGUUUGUCGUGC	7

PR8 HA -3'-SFV-UTR trRNA	AACUGCAAAAAAGCUUCCCGCAAUUUGAGGUAGUAGCACAGGAGGUGACUCCAAAUC ACCAUCCUAAUCCCAGAGCAUUUUCGCAUCUGGGCAGUAAACUAAUCGAGCUGGAGG UUCCUACCACAGCGACGAUCUUGGACAUAGGCAGCGCACCGGCUCGUAGAACGAUAA ACCCCUAGUGCCACCAUGAAAGCCAACCUUCUGGUACUACUGUGCGCCCUGGCGGCC GCUGAUGCAGACACGAUAUGCAUCGGCUACCACGCCAACAACAGCACAGACACCGUG GACACAGUGCUGGAGAAGAACGUUACUGUGACCCACAGUGUGAACCUCCUGGAGGAC AGCCACAAUGGGAAGCUCUGUCGGCUCAAGGGCAUCACACCCCUGCAGCUGGGGAAA UGCAACAUCGCCGGCUGGCUGUUGGGCAACCCAGAGUGUGAUCUGCUGCUACCAGUG AGAUCAUGGAGUUACAUUGUGGAGACACCUAACUCUGAGAAUGGAAUCUGCUACCCA GGAGACUUCAUCGACUAUGAGGAGCUGCGGGAGCAGCUGAGCUCUGUCAGUUCCUUU GAAAGAUUUGAGAUCUUCCCUAAAGAGAGCUCCUGGCCAAACCACACCACGACCAGA GGGGUGACAGCCGCCUGCAGCCACGCAGGCAAGAGCAGCUUCUACCGCAACCUGUUG UGGCUCACAGAGAAGGAAGGAUCCUAUCCUAAGUUGAAGAACAGCUACGUUAACAAG AAAGGAAAGGAGGUGCUGGUGCUCUGGGGCAUACAUCACCCUAGUAAUUCUAAGGAU CAGCAGAAGCUCUACCAAAACGAGAACGCCUACGUGUCUGUGGUAAGCUCCAACUAC AACAGGCGCUUCACUCCUGAAAUCGCUGAAAGACCUAAAGUCAGAGAUCAGGCAGGC CGAAUGAAUUACUACUGGACCCUGCUAAAGCCUGGUGACACCAUCAUCUUUGAGGCC AAUGGAAACUUAGUGGCUCCUAGGUAUGCUUUUGCCCUGAGCCGAGGUUUCGGCAGC GGCAUUAUUACCUCUAACGCUUCCAUGCAUGAAUGUAACACCAAGUGCCAGACACCU CUGGGCGCCAUCAACAGCAGCCUCCCCUUCCAGAACAUCCACCCUGUCACCAUAGGA GAGUGCCCGAAAUACGUGCGGAGUGCCAAACUGCGGAUGGUGACGGGCCUGAGAAAC AUUCCCUCCAUUCAGUCUAGAGGCCUGUUUGGGGCCAUUGCCGGGUUCAUUGAGGGA GGCUGGACUGGCAUGAUCGACGGGUGGUAUGGAUACCAGCACCAGAAUGAGCAAGGC AGUGGCUACGCCGCCGACCAAAAGUCAACCCAGAACGCUAUCAAUGGGAUCACAAAC AAAGUUAACUCCGUCAUCGAGAAAAUGAACACCCAAUUCACAGCUGUGGGCAAAGAA UUCAAUAAACUGGAGAAAAGAAUGGAAAAUCUCAACAAGAAGGUGGAUGAUGGCUUC CUGGACAUCUGGACAUAUAAUGCUGAGCUGCUGGUCCUGCUGGAAAAUGAACGGACU CUGGAUUUCCAUGACAGCAAUGUGAAGAACCUUUAUGAAAAGGUUAAGAGCCAGCUG AAGAACAAUGCCAAGGAAAUUGGCAAUGGCUGUUUUGAGUUCUAUCACAAGUGUGAC AAUGAAUGCAUGGAGUCUGUGAGGAAUGGUACCUAUGACUACCCUAAGUACUCAGAA GAAAGCAAACUCAAUCGGGAAAAGGUUGAUGGCGUUAAGCUGGAGAGCAUGGGUAUC UACCAAAUCUUGGCUAUAUAUAGCACCGUGGCCAGCUCUUUAGUGCUACUGGUGUCC CUGGGAGCCAUCUCCUUCUGGAUGUGUAGCAACGGCUCACUUCAGUGCAGAAUAUGU AUCUGAUAAUAGCUCGAGGCGGCCGCAGGAGCUUAAUUCGACGAAUAAUUGGAUUUU UAUUUUAUUUUGCAAUUGGUUUUUAAUAUUUCCAAAAAAAAAAAAAAAAAAAAAAA AAAAAAAAAAAA	
oeSTR NS1-iCVB3-PR8 HA	auagaagauggcggcguaguacacacuauugaaucaaacagccgaccaauugcacua ccaucacaacggagaagccaguaguaaacguagacguagacccccagaguccguuug ucgugcaacugcaaaaaagcuuccccgcaauuugagguaguagcacaggaggugacuc caaaucaccaauccuaaucccagagcauuuucgcaucugggcaguaaacuaaucgagc uggagguuccuaccacagcgacgaucuuggacauaggcagcgcaccggcucguagaa cgauaaaccccuagugccaccAUGGAUCCCAACACAGUCUCUUCCUUCCAGGUGGAC UGUUUCCUCUGGCAUGUAAGGAAGCGCGUAGCAGACCAGGAGUUGGGAGAUGCCCCC UUCCUGGAUCGGCUGAGACGGGACCAGAAAAGCCUGCGAGGCAGAGGCAGCACCCUG GGGCUGGACAUAGAAACAGCCACCCGGGCAGGGAAGCAGAUCGUGGAGCGCAUCCUU AAGGAGGAGUCUGAUGAGGCUCUCAAGAUGACCAUGGCCUCAGUGCCUGCCAGCAGG UACCUGACAGACAUGACACUGGAGGAAAUGUCAAGAGACUGGAGCAUGCUGAUCCCU AAACAGAAGGUGGCGGGCCCUCUUUGCAUCCGGAUGGAUCAAGCCAUCAUGGACAAG AACAUUAUCCUGAAAGCCAACUUCAGCGUCAUCUUUGACAGGCUGGAAACCCUCAUC CUUCUGCGGGCCUUCACAGAAGAAGGAGCCAUUGUGGGAGAGAUCAGCCCACUGCCC AGUCUGCCUGGCCACACUGCAGAAGAUGUGAAGAAUGCUGUGGGCGUCUUAAUUGGA GGCUUGGAGUGGAAUGACAACACUGUGAGAGUUAGUGAGACACUGCAGAGAUUUGCC UGGAGGUCCAGCAAUGAAAACGGCCGGCCUCCCCUUACCCCAAAGCAAAAACGGGAA AUGGCUGGCACCAUCCGCUCAGAGGUGugauaauaguuaaaacagccuguggguuga ucccacccacaggcccauugggcgcuagcacucugguaucacgguaccuuugugcgc cuguuuuauaccccccuccccaacuguaacuuagaaguaacacacaccgaucaacag ucagcguggcacaccagccacguuuugaucaagcacuucuguuaccccggacugagu aucaauagacugcucacgcgguugaaggagaaagcguucguuauccggccaacuacu	8

	ucgaaaaaccuaguaacaccguggaaguugcagaguguuucgcucagcacuacccca guguagaucaggucgaugagucaccgcauuccccacgggcgaccguggcgguggcug cguuggcggccugcccaugggaaacccaugggacgcucuaauacagacauggugcg aagagucuauugagcuaguugguaguccuccggccccugaaugcggcuaauccuaac ugcggagcacacacccucaagccagagggcagugugucguaacgggcaacucugcag cggaaccgacuacuuugggugucccguguuucauuuuauuccuauacuggcugcuuau ggugacaauugagagaucguuaccauauagcuauuggauuggccauccggugacuaa uagagcuauuauauauucccuuuguuggguuuauaccacuuagcuugaaagagguuaa aacauuacaauucauuguuaaguugaauacagcaaagccaccAUGAAAGCCAACCUU CUGGUACUACUGUGCGCCCUGGCGGCCGCUGAUGCAGACACGAUAUGCAUCGGCUAC CACGCCAACAACAGCACAGACACCGUGGACACAGUGCUGGAGAAGAACGUUACUGUG ACCCACAGUGUGAACCUCCUGGAGGACAGCCACAAUGGGAAGCUCUGUCGGCUCAAG GGCAUCACACCCCUGCAGCUGGGGAAAUGCAACAUCGCCGGCUGGCUGUUGGGCAAC CCAGAGUGUGAUCUGCUGCUACCAGUGAGAUCAUGGAGUUACAUUGUGGAGACACCU AACUCUGAGAAUGGAAUCUGCUACCCAGGAGACUUCAUCGACUAUGAGGAGCUGCGG GAGCAGCUGAGCUCUGUCAGUUCCUUUGAAAGAUUUGAGAUCUUCCCUAAAGAGAGC UCCUGGCCAAACCACACCACGACCAGAGGGGUGACAGCCGCCUGCAGCCACGCAGGC AAGAGCAGCUUCUACCGCAACCUGUUGUGGCUCACAGAGAAGGAAGGAUCCUAUCCU AAGUUGAAGAACAGCUACGUUAACAAGAAAGGAAAGGAGGUGCUGGUGCUCUGGGGC AUACAUCACCCUAGUAAUUCUAAGGAUCAGCAGAAGCUCUACCAAAACGAGAACGCC UACGUGUCUGUGGUAAGCUCCAACUACAACAGGCGCUUCACUCCUGAAAUCGCUGAA AGACCUAAAGUCAGAGAUCAGGCAGGCCGAAUGAAUUACUACUGGACCCUGCUAAAG CCUGGUGACACCAUCAUCUUUGAGGCCAAUGGAAACUUAGUGGCUCCUAGGUAUGCU UUUGCCCUGAGCCGAGGUUUCGGCAGCGGCAUUAUUACCUCUAACGCUUCCAUGCAU GAAUGUAACACCAAGUGCCAGACACCUCUGGGCGCCAUCAACAGCAGCCUCCCCUUC CAGAACAUCCACCCUGUCACCAUAGGAGAGUGCCCGAAAUACGUGCGGAGUGCCAAA CUGCGGAUGGUGACGGGCCUGAGAAACAUUCCCUCCAUUCAGUCUAGAGGCCUGUUU GGGGCCAUUGCCGGGUUCAUUGAGGGAGGCUGGACUGGCAUGAUCGACGGGUGGUAU GGAUACCAGCACCAGAAUGAGCAAGGCAGUGGCUACGCCGCCGACCAAAAGUCAACC CAGAACGCUAUCAAUGGGAUCACAAACAAAGUUAACUCCGUCAUCGAGAAAAUGAAC ACCCAAUUCACAGCUGUGGGCAAAGAAUUCAAUAAACUGGAGAAAAGAAUGGAAAAU CUCAACAAGAAGGUGGAUGAUGGCUUCCUGGACAUCUGGACAUAUAAUGCUGAGCUG CUGGUCCUGCUGGAAAAUGAACGGACUCUGGAUUUCCAUGACAGCAAUGUGAAGAAC CUUUAUGAAAAGGUUAAGAGCCAGCUGAAGAACAAUGCCAAGGAAAUUGGCAAUGGC UGUUUUGAGUUCUAUCACAAGUGUGACAAUGAAUGCAUGGAGUCUGUGAGGAAUGGU ACCUAUGACUACCCUAAGUACUCAGAAGAAAGCAAACUCAAUCGGGAAAAGGUUGAU GGCGUUAAGCUGGAGAGCAUGGGUAUCUACCAAAUCUUGGCUAUAUAUAGCACCGUG GCCAGCUCUUUAGUGCUACUGGUGUCCCUGGGAGCCAUCUCCUUCUGGAUGUGUAGC AACGGCUCACUUCAGUGCAGAAUAUGUAUCugauaauagcucgaggcggccgcagga gcuuaauucgacgaauaauuggauuuuuauuuuauuuugcaauugguuuuuaauauu uccaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa	
oeSTR NS1-iCVB3-Cal09 HA	auagaagauggcggcguaguacacacuauugaaucaaacagccgaccaauugcacua ccaucacaacggagaagccaguaguaaacguagacguagacccccagaguccguuug ucgugcaacugcaaaaaagcuucccgcaauuugagguaguagcacaggaggugacuc caaaucaccauccuaaucccagagcauuuucgcaucugggcaguaaacuaaucgagc uggagguuccuaccacagcgacgaucuuggacauaggcagcgcaccggcucguagaa cgauaaaccccuagugccaccAUGGAUCCCAACACAGUCUCUUCCUUCCAGGUGGAC UGUUUCCUCUGGCAUGUAAGGAAGCGCGUAGCAGACCAGGAGUUGGGAGAUGCCCCC UUCCUGGAUCGGCUGAGACGGGACCAGAAAAGCCUGCGAGGCAGAGGCAGCACCCUG GGGCUGGACAUAGAAACAGCCACCCGGGCAGGGAAGCAGAUCGUGGAGCGCAUCCUU AAGGAGGAGUCUGAUGAGGCUCUCAAGAUGACCAUGGCCUCAGUGCCUGCCAGCAGG UACCUGACAGACAUGACACUGGAGGAAAUGUCAAGAGACUGGAGCAUGCUGAUCCCU AAACAGAAGGUGGCGGGCCCUCUUUGCAUCCGGAUGGAUCAAGCCAUCAUGGACAAG AACAUUAUCCUGAAAGCCAACUUCAGCGUCAUCUUUGACAGGCUGGAAACCCUCAUC CUUCUGCGGGCCUUCACAGAAGAAGGAGCCAUUGUGGGAGAGAUCAGCCCACUGCCC AGUCUGCCUGGCCACACUGCAGAAGAUGUGAAGAAUGCUGUGGGCGUCUUAAUUGGA GGCUUGGAGUGGAAUGACAACACUGUGAGAGUUAGUGAGACACUGCAGAGAUUUGCC UGGAGGUCCAGCAAUGAAAACGGCCGGCCUCCCCUUACCCCAAAGCAAAAACGGGAA	9

	AUGGCUGGCACCAUCCGCUCAGAGGUGugauaauaguuaaaacagccuguggguuga ucccacccacaggcccauugggcgcuagcacucugguaucacgguaccuuugugcgc cuguuuuauaccccccuccccccaacuguaacuuagaaguaacacacaccgaucaacag ucagcguggcacaccagccacguuuugaucaagcacuucuguuaccccggacugagu aucaauagacugcucacgcgguugaaggagaaagcguucguuauccggccaacuacu ucgaaaaaccuaguaacaccguggaaguugcagaguguuucgcucagcacuacccca guguagaucaggucgaugagucaccgcauucccccacgggcgaccguggcgguggcug cguuggcggccugcccaugggggaaacccaugggacgcucuaauacagacauggugcg aagagucuauugagcuaguugguaguccuccggcccccugaaugcggcuaauccuaac ugcggagcacacacccucaagccagagggcagugugucguaacgggcaacucugcag cggaaccgacuacuuuggguguccguguuucauuuuauuccuauacuggcugcuuau ggugacaauugagagaucguuaccauauagcuauuggauuggccauccggugacuaa uagagcuauuauauauccccuuuguuggguuuauaccacuuagcuugaaagagguuaa aacauuacaauucauuguuaaguugaauacagcaaagccaccAUGAAAGCAAUACUA GUAGUACUGCUAUACACAUUCGCAACCGCAAACGCAGACACAUUAUGCAUAGGCUAC CACGCGAACAACUCAACAGACACCGUAGACACAGUACUAGAAAAGAACGUAACAGUA ACACACUCCGUCAACCUCCUAGAAGACAAGCACAACGGGAAACUAUGCAAACUAAGA GGGGUAGCCCCAUUGCACUUGGGCAAAUGCAACAUCGCUGGCUGGAUCCUGGGAAAC CCAGAGUGCGAAUCACUCUCCACAGCAAGCUCAUGGUCCUACAUCGUGGAAACACCG AGCUCAGACAACGGAACGUGCUACCCAGGAGACUUCAUCGACUACGAGGAGCUAAGA GAGCAAUUGAGCUCAGUGUCAUCAUUCGAAAGGUUCGAGAUAUUCCCCAAGACAAGC UCAUGGCCCAACCACGACUCGAACAAAGGCGUAACGGCAGCAUGCCCGCACGCCGGA GCAAAAAGCUUCUACAAAAACUUAAUAUGGCUAGUGAAAAAAGGAAACUCAUACCCA AAGCUCAGCAAAUCCUACAUCAACGACAAAGGGAAAGAAGUCCUCGUGCUAUGGGGC AUCCACCACCCAUCGACCAGCGCCGACCAACAAAGCCUCUACCAGAACGCAGACGCA UACGUGUUCGUGGGGUCAUCAAGAUACAGCAAGAAGUUCAAGCCGGAAAUAGCAAUA AGACCCAAAGUGAGGGGCCAAGAAGGGAGAAUGAACUACUACUGGACACUAGUAGAG CCGGGAGACAAAAUAACAUUCGAAGCAACCGGAAACCUAGUGGUACCGAGAUACGCA UUCGCAAUGGAAAGAAACGCCGGAUCCGGCAUCAUCAUAUCAGACACACCAGUCCAC GACUGCAACACAACCUGCCAAACACCCAAGGGCGCGAUAAACACCAGCCUCCCAUUC CAGAACAUACACCCGAUCACAAUCGGAAAAUGCCCAAAAUACGUAAAAAGCACAAAA UUGAGACUGGCCACAGGAUUGAGGAAUAUCCCGUCCAUCCAAUCCAGAGGCCUAUUC GGGGCCAUCGCCGGCUUCAUCGAAGGGGGGUGGACAGGGAUGGUAGAUGGAUGGUAC GGUUACCACCACCAAAACGAGCAGGGGUCAGGAUACGCAGCCGACCUGAAAAGCACA CAGAACGCCAUCGACGAGAUCACGAACAAAGUAAACUCCGUCAUCGAAAAGAUGAAC ACACAGUUCACAGCAGUAGGCAAAGAGUUCAACCACCUGGAAAAAAGAAUAGAGAAC UUAAACAAAAAAGUCGACGACGGCUUCCUGGACAUCUGGACCUACAACGCCGAACUG UUGGUCCUAUUGGAAAACGAAAGAACCUUGGACUACCACGACUCAAACGUGAAGAAC UUAUACGAAAAGGUAAGAAGCCAGCUAAAAAACAACGCCAAGGAAAUCGGAAACGGC UGCUUCGAAUUCUACCACAAAUGCGACAACACGUGCAUGGAAAGCGUCAAAAACGGG ACGUACGACUACCCAAAAUACUCAGAGGAAGCAAAAUUAAACAGAGAAGAAAUAGAC GGGGUAAAGCUGGAAUCAACAAGGAUCUACCAGAUCUUGGCGAUCUACUCAACCGUC GCCAGCUCAUUGGUACUGGUAGUCUCCCUGGGGGCAAUCAGUUUCUGGAUGUGCUCC AACGGGUCCCUACAGUGCAGAAUAUGCAUCUGAUAAUAGCUCGAGGCGGCCGCAGGA GCUUAAUUCGACGAAUAAUUGGAUUUUUAUUUUAUUUUGCAAUUGGUUUUUAAUAUU UCCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	

EXAMPLES

Example 1. Replicase Lipid Nanoparticle (LNP) (R-LNP) and trans replicon (trRNA) LNP (Tr-LNP) Production

Experiments were performed to determine whether replicase constructs (e.g., as R-NPs) could be stockpiled in lipid nanoparticles and then later administered to a subject in combination

with different lipid nanoparticles comprising trRNAs (e.g., Tr-NPs) in order to induce an immune response to an antigen encoded by the trRNAs. Results in mice show that an immune response to an antigen encoded by a trRNA can be elicited using this system with a 25:1 molar ratio of replicase construct to trRNA. Accordingly, preparation of vaccine doses for 35,000 people would require about 4,158 mg of replicase construct and only about 42 mg of trRNA. In this system, replicase constructs could be formulated in LNPs (e.g., R-NPs, R-LNPs) and stockpiled in long term storage, and trRNAs could be synthesized on as-needed basis, for example, in response to a pandemic or epidemic. This 1000-fold reduction in RNA synthesis burden would expedite vaccination of a population.

RNA Production

trRNAs (T3A-5'-SINV-UTR-PR8 HA -3'-SFV-UTR (SEQ ID NO: 7)) encoding the Hemagglutinin (HA) protein for the Influenza A virus (strain A/Puerto Rico/8/1931 H1N1) were produced by *in vitro* transcription using a linearized DNA template. The final volume of each IVT reaction was 20 to 250uL. The standard IVT reaction mix was as follows:

Table 1: In Vitro Transcription Reaction

Linearized DNA Template	0.5-5µg
Cleancap AU (mM)	4mM
rATP (or analog)	5mM
rGTP (or analog)	5mM
rCTP (or analog)	5mM
rUTP (or analog)	5mM
Murine RNase Inhibitor (U/µl)	0.025
YIPP (U/µl)	0.002
10X Transcription Buffer	1X
T7 RNA Polymerase (U/µl)	1.25
Molecular Biology Grade Water (µl)	Remaining

This reaction mixture was incubated for 2-3 hours (37ºC), after which DNaseI and DNaseI buffer were added, and the mixture was incubated for an additional 30-minute period (37ºC). After the full incubation period, RNA was isolated using NEB Monarch RNA Cleanup kits according to manufacturer instructions, then eluted into 100µL RNase-free water. Total RNA produced (ng) was measured via Nanodrop for each construct. RNA integrity was assessed

via denaturing gel electrophoresis or Agilent Fragment Analyzer. RNA was stored at -20°C or 80°C.

mRNAs encoding the replicase of Semliki Forest Virus (SFV) were produced by *in vitro* transcription using a linearized DNA template and the standard IVT reaction. The final volume of each IVT reaction was 20 to 250uL.

This reaction mixture was incubated for 1.5 to 2 hours (37°C), after which DNaseI and DNaseI buffer were added, and the mixture was incubated for an additional 30-minute period (37°C). After the full incubation period, RNA was isolated using NEB Monarch RNA Cleanup kits according to manufacturer instructions, then eluted into 100µL RNase-free water. Total RNA produced (ng) was measured via Nanodrop for each construct. RNA integrity was assessed via denaturing gel electrophoresis or Agilent Fragment Analyzer. RNA was stored at -20°C or 80°C.

LNP Formulation

Lipid nanoparticles (LNP) were formulated with field standard practices. Briefly, ALC-0315, distearoylphosphatidylcholine (DSPC), cholesterol, and a polyethylene glycol (PEG)-lipid were solubilized in ethanol at a molar ratio of 46.3:9.4:42.5:1.6. LNPs were prepared at a total lipid to RNA weight ratio of approximately 20:1. The RNA for each formulation was diluted to 0.14mg/mL in 50mM Sodium Acetate (pH=5) buffer. Syringe pumps were used to mix the ethanolic lipid solution with the RNA-acetate solution at a ratio of about 1:3 with total flow rates of 15ml/min. Ethanol was removed and replaced by dialysis into PBS and sterile filtered using 0.22-micron filter. Sterilized, buffer exchanged LNPs were then concentrated in Amicon Spin concentrators and RNA concentration was adjusted to 200µg/mL with 10mM Tris Acetate such that final solution contained 10% sucrose. This LNP-RNA was then frozen at -80°C for future usage.

Example 2: *In vivo* Administration of R-LNPs and Tr-LNPs

Frozen LNPs encapsulating SFV replicase mRNA and, separately, LNPs encapsulating HA-encoding trRNAs were thawed on ice and diluted (1 volume LNP to 9 volumes sterile PBS), resulting in working LNP stocks of 20ng/uL RNA. The two LNP types were then mixed at specific molar ratios (Table 2) of replicase mRNA to trRNA from 1:1 to 25:1.

Table 2: Molar Ratios of replicase mRNA:trRNA mixtures

Molar Ratio (replicase to trRNA)	Mass Ratio (replicase to trRNA)
1:1	0.78:0.22
5:1	0.946:0.054
25:1	0.99:0.01

Replicase:trRNA LNP mixtures were administered to 6–12-week-old female Balb/C mice via intramuscular administration (50uL LNP injected into the quadricep) for a total RNA dose of 1µg. As controls, mRNA-LNPs encapsulating a non-replicase mRNA encoding PR8 HA and taRNA-LNPs formulated to co-encapsulate replicase mRNA and trRNA at a 1:1 molar ratio were also injected into separate cohorts of mice (1µg total RNA). Matched LNPs were administered to each cohort 3 weeks later as a vaccine boost. 4 weeks after first LNP administration and 1 week after boost, mice were bled and serum was collected for subsequent analysis.

Binding Titer determination with ELISA

An ELISA was conducted to determine the binding titer from the serum of immunized mice. Briefly, NUNC Maxisorp 96-well plates were coated 100uL per well of 2µg/ml recombinant PR8 HA (Sinobiological) and allowed to incubate overnight at 4C. The following day, each plate was washed three times with 1x TBS-Tween and 100uL per well of StartingBlock Blocking Buffer was added and allowed to incubate for 1 hour at room temperature. Assay samples were diluted in 1xBSA and 1xTBS to a starting dilution of 1:500 and serially diluted in 1:5 intervals seven times (to 1:39,062,500). Blocking buffer was removed and diluted serum was added to plates and allowed to incubate at room temperature for 2 hours. The plate was then washed four times with TBS before addition of Goat anti-Mouse IgG secondary antibody with conjugated HRP was added and allowed to incubate for 1 hour. The plates were again washed four times with TBS before addition of TMB substrate, which was incubated for 15 minutes before stop solution was added, at which time absorbance was measured on a plate reader at 450nm and 620nm wavelength. The 450-620nm absorbance for each group of mice was then plotted relative to the serum dilution of determine binding titer for each LNP treatment (FIGs. 2A-2B).

We observed all taRNA groups potently inducted anti-HA binding titers, comparable to the mRNA control. Surprisingly, groups receiving separately formulated replicase mRNA and trRNA showed a similar binding titer compared to taRNA-LNPs with the replicase mRNA and trRNA co-formulated (FIG. 2A), indicating that the formulation of replicase mRNA and trRNA into different LNPs did not impact vaccine results. Most surprising was the finding that all ratios of separately formulated replicase:trRNA LNPs (1:1, 5:1, and 25:1) showed comparable binding titers, despite the 25:1 group being administered with 22-fold less antigen encoding trRNA (FIG. 2A). Critically, this indicates that a relatively small amount of trRNA is needed to induce an immune response, and that separately formulated replicase:trRNA LNPs may drastically reduce the amount of trRNA needed for population-level vaccine production.

Example 3: Advantages of two-component taRNA influenza virus vaccine compositions

In this Example, BALB/c mice received one of three influenza virus vaccines comprising RNA formulated in ALC-0315 LNPs: single-component mRNA vaccines (i.e., comprising mRNAs formulated in LNPs), single-component "co-formulated" taRNA vaccines (i.e., comprising a replicase construct and trRNA formulated in the same LNP), or two-component "split-formulated" taRNA vaccines (i.e., comprising R-LNPs and Tr-LNPs). Mice were assessed for binding antibodies, neutralizing antibodies, and survival in response to varying dosages of each vaccine.

Split-formulated taRNA decreases amount of antigen-RNA needed

BALB/c mice were primed and boosted (day 21) with either single-component mRNA vaccines having mRNA encoding PR8 HA antigen and formulated in ALC-0315 LNPs; or two-component taRNA vaccines having R-LNPs, and Tr-LNPs encoding PR8 HA antigen. For mRNA, the specified amount of mRNA LNP was dosed. taRNA was split formulated, with mice receiving 1μg of total replicase construct RNA and an additional specified amount of trRNA. At day 28, PR8 HA binding antibodies (ELISA) and HAI were measured. As shown in FIGs. 3A-3B, two-component taRNA vaccines led to higher binding antibodies at >50x less antigen RNA than mRNA vaccines.

Split-formulated taRNA vaccines tolerate modified nucleosides

BALB/c mice were primed and boosted (day 21) with either single-component mRNA vaccines encoding PR8 HA antigen and formulated in ALC-0315 LNPs; or two-component

taRNA vaccines having R-LNPs, and Tr-LNPs encoding PR8 HA antigen. For the mRNA vaccines, the specified amount of mRNA was dosed. Mice receiving two-component taRNA vaccines received 1μg of total replicase construct RNA and an additional specified amount of trRNA. Tested taRNA vaccine groups included taRNA '1' (oeSTR-PR8 HA), taRNA '2' (oeSTR-NS1-IRES-PR8 HA (SEQ ID NO: 8)) with mod1 (m5C) or mod 2 (5moU) applied to the trRNA (replicase mRNA was modified with N1methyl-pseudouridine for all taRNA groups, while HA mRNA was also N1methyl-pseudouridine modified). At day 14 (post prime) and day 28 (post boost), PR8 HA binding antibodies were measured via ELISA. At day 28,HAI was measured. As shown in FIGs. 4A-4B, split-formulated taRNA with 0.1μg or 0.01μg trRNA (+1μg replicase mRNA) was more potent than 1μg mRNA. These tested trRNA modifications were tolerated for vaccines.

Split-formulated taRNA have enhanced potency at 1000x less antigen dose compared to mRNA

BALB/c mice were primed and boosted (day 21) with either single-component mRNA vaccines encoding Cal09 HA antigen and formulated in ALC-0315 LNPs; or two-component taRNA vaccines having R-LNPs, and Tr-LNPs encoding Cal09 HA antigen. For the mRNA vaccines, the specified amount of mRNA was dosed. Mice receiving two-component taRNA vaccines received 1μg of total replicase construct RNA and an additional specified amount of trRNA. Data shown is for oeSTR NS1-iCVB3-Cal09 HA trRNA (SEQ ID NO: 9). At day 28, HAI was measured. As shown in FIG. 5, taRNA generates a higher neutralizing antibody response than mRNA at 1000x lower antigen dose (0.1μg mRNA vs 0.0001μg trRNA). All taRNA groups were protected from lethal challenge, while only 1μg mRNA was protective (not shown).

Split-formulated taRNA is more potent than mRNA after 1 administration

BALB/c mice were vaccinated once with either single-component mRNA vaccines encoding Pr8 HA antigen and formulated in ALC-0315 LNPs; single-component ("co-formulated") taRNA vaccines having a replicase construct and trRNA encoding PR8 HA antigen formulated in the same LNP; or two-component ("split-formulated") taRNA vaccines having R-LNPs, and Tr-LNPs encoding PR8 HA antigen. For mRNA vaccines, the specified amount of mRNA was dosed. taRNA vaccines were either split formulated (mice received 1μg of replicase RNA and an additional specified amount of trRNA) or co-formulated (mice received specified

amount of total taRNA, containing replicase constructs and trRNA at a 10:1 mass ratio). At day 28, PR8 HA binding antibodies were measured via ELISA and mice were challenged with a lethal dose of PR8 influenza virus. As shown in FIGs. 6A-6B, split-formulated taRNA vaccines were protective against lethal doses of PR8 influenza virus even at low antigen-encoding trRNA doses (e.g., 0.0001μg).

CLAIMS

What is claimed is:

1. A replicase nanoparticle (R-NP), comprising: a replicase construct and a nanoparticle, wherein the replicase construct is a polyribonucleic acid comprising a nucleic acid encoding a replicase, and

wherein the R-NP does not comprise a polynucleotide comprising a gene operably linked to a conserved sequence element (CSE) that is cognate to the replicase.

2. The R-NP of claim 1, wherein the replicase is an alphavirus replicase.

3. The R-NP of claim 2, wherein the alphavirus replicase is a Venezuelan equine encephalitis virus (VEEV), Semliki Forest virus (SFV), Sindbis Virus (SINV), or Chikungunya (CHIKV) replicase.

4. The R-NP of claim 3, wherein the alphavirus replicase is a SFV replicase.

5. The R-NP of claim 1, wherein the replicase construct comprises: a 5' untranslated region (UTR), the nucleic acids encoding the replicase, and a 3'-UTR.

6. The R-NP of claim 1, wherein the 5'-UTR comprises a human alpha-globin (5'-HBA-UTR) and the 3'-UTR comprises a human alpha-globin (3'-HBA-UTR).

7. The R-NP of claim 6, wherein the 5'-HBA-UTR comprises a nucleic acid sequence of SEQ ID NO: 5 and the 3'-HBA-UTR comprises a nucleic acid sequence of SEQ ID NO: 6.

8. The R-NP of any one of claims 1-7, wherein the replicase construct comprises the sequence set forth in SEQ ID NO: 1.

9. The R-NP of any one of claims 1-8, wherein the nanoparticle is a lipid nanoparticle (LNP) and the R-NP is a R-LNP.

10. The R-NP of claim 9, wherein the LNP comprises an ionizable lipid, distearoylphosphatidylcholine (DSPC), cholesterol, and polyethylene glycol (PEG)-lipid.

11. The R-NP of claim 10, wherein the ionizable lipid is ALC-0315.

12. A composition comprising the R-NP of any one of claims 1-11 and a trans-replicating RNA (trRNA) nanoparticle (Tr-NP),

wherein the Tr-NP comprises a trRNA and a nanoparticle, the trRNA comprising a nucleic acid encoding a payload, operably linked to a CSE cognate to the replicase.

13. The composition of claim 12, wherein the nanoparticle of the Tr-NP is an LNP and the Tr-NP is a Tr-LNP.

14. The composition of claim 13, wherein the Tr-LNP comprises an ionizable lipid, DSPC, cholesterol, and a PEG-lipid.

15. The composition of claim 14, wherein the ionizable lipid is ALC-0315.

16. The composition any one of claims 12-15, wherein the R-NP and the Tr-NP are an R-LNP and a Tr-LNP, and

wherein the R-LNP and Tr-LNP each comprise ALC-0315, DSPC, cholesterol, and PEG-lipid.

17. The composition of claim 12, comprising a 1:1 molar ratio of replicase construct:trRNA.

18. The composition of claim 12, comprising a 5:1 molar ratio of replicase construct:trRNA.

19. The composition of claim 12, comprising a 10:1 molar ratio of replicase construct:trRNA.

20. The composition of claim 12, comprising a 25:1 molar ratio of replicase construct:trRNA.

21. The composition of claim 12, comprising a 50:1 molar ratio of replicase construct:trRNA.

22. The composition of claim 12, comprising a 100:1 molar ratio of replicase construct:trRNA.

23. The composition of claim 12, comprising a 500:1 molar ratio of replicase construct:trRNA.

24. The composition of claim 12, comprising a 1000:1 molar ratio of replicase construct:trRNA.

25. The composition of any one of claims 12-24, wherein the payload comprises an antigen.

26. The composition of any one of claims 12-25, wherein the composition is a vaccine.

27. The composition of any of claims 12-26, comprising a combined amount of replicase construct and trRNA of less than 2μg.

28. The composition of any of claims 12-27, wherein the combined amount of replicase construct and trRNA is less than 1.5μg.

29. The composition of any of claims 12-28, wherein the combined amount of replicase construct and trRNA is about 1μg.

30. A kit comprising:

a first vial comprising the R-NP of any one of claims 1-11; and

a second vial comprising a trans-replicating RNA (trRNA) nanoparticle (Tr-NP),

wherein the Tr-NP comprises a trRNA comprising: a gene operably linked to a CSE cognate to the replicase.

31. A method of producing a vaccine, the method comprising:

(i) obtaining a plurality of the R-NP of any one of claims 1-11;

(ii) determining a pathogen for which vaccination is desired;

(iii) obtaining a plurality of Tr-NPs comprising a trRNA, the trRNA comprising a nucleic acid encoding an antigen of the pathogen, operably linked to a CSE that is cognate to the replicase of the R-NP.

32. The method of claim 31, further comprising

(iv) combining the plurality of R-NPs and the plurality of Tr-NPs.

33. The method of claim 31, wherein steps (i)-(iii) are performed in sequential order.

34. The method of any one of claims 31-33, wherein after step (i) and before step (ii), the method comprises a step of placing the plurality of R-NPs into long term storage.

35. A method of expressing a replicase in a cell, the method comprising contacting the cell with the R-NP of any one of claims 1-11.

36. A method of expressing a payload in a cell, the method comprising contacting the cell with the R-NP of any one of claims 1-11,

A wherein the cell comprises an RNA polynucleotide comprising a nucleic acid encoding a payload operably linked to a CSE that is cognate to the replicase of the R-NP.

37. A method of vaccinating a subject, the method comprising administering the composition of any one of claims 12-29 to the subject.

38. A method of vaccinating a subject against a pathogen, the method comprising administering to the subject:

a plurality of the R-NP of any one of claims 1-11, and

a Tr-NP comprising a trRNA, the trRNA comprising a nucleic acid encoding an antigen of the pathogen, operably linked to a CSE that is cognate to the replicase of the R-NP.

39. A composition comprising:

a replicase nanoparticle (R-NP) comprising a replicase construct and a nanoparticle,

wherein the replicase construct is a ribonucleic acid (RNA) polynucleotide comprising nucleic acids encoding a replicase, and wherein the R-NP does not comprise a polynucleotide comprising a gene operably linked to a conserved sequence element (CSE) cognate to the replicase; and

a trans-replicating RNA (trRNA) nanoparticle (Tr-NP), comprising a trRNA and a nanoparticle,

wherein the trRNA is an RNA polynucleotide comprising a nucleic acid encoding a payload, operably linked to a CSE cognate to the replicase of the R-NP;

wherein the nanoparticle of the R-NP and the nanoparticle of the Tr-NP are separate nanoparticles.

40. The composition of claim 39, wherein the replicase construct of the R-NP is comprised in a lipid nanoparticle (LNP) and the R-NP is a R-LNP; and wherein the trRNA of the Tr-NP is comprised in an LNP and the Tr-NP is a Tr-LNP.

41. The composition of claim 39 or 40, comprising a 1:1 ratio of R-NP:Tr-NP.

42. The composition of claim 39 or 40, comprising a 50:1 ratio of R-NP:Tr-NP.

43. The composition of claim 39 or 40, comprising a 100:1 ratio of R-NP:Tr-NP.

44. The composition of claim 39 or 40, comprising a 500:1 ratio of R-NP:Tr-NP.

45. The composition of claim 39 or 40, comprising a 1000:1 ratio of R-NP:Tr-NP.

46. A vaccine comprising the composition of any of claims 39-45.

47. A method of preparing a vaccine, the method comprising:

(i) obtaining a replicase nanoparticle (R-NP) comprising a replicase construct and a nanoparticle,

wherein the replicase construct is a ribonucleic acid (RNA) polynucleotide comprising a nucleic acid encoding a replicase, and wherein the R-NP does not comprise a polynucleotide comprising a encoding a vaccine antigen, operably linked to a conserved sequence element (CSE) cognate to the replicase; and

(ii) obtaining a trans-replicating RNA (trRNA) nanoparticle (Tr-NP), comprising a trRNA,

wherein the trRNA is a RNA polynucleotide comprising a nucleic acid encoding a vaccine antigen, operably linked to a CSE cognate to the replicase of the R-NP; and

(iii) combining the R-NP and the Tr-NP, thereby preparing the vaccine.

48. A system for vaccination of a population of human subjects against a pathogen, the system comprising:

(i) a composition comprising a replicase nanoparticle (R-NP), the R-NP comprising a replicase construct and a nanoparticle,

wherein the replicase construct is a ribonucleic acid (RNA) polynucleotide comprising a nucleic acid encoding a replicase, and wherein the R-NP does not comprise a polynucleotide comprising an encoding a vaccine antigen, operably linked to a conserved sequence element (CSE) cognate to the replicase; and

(ii) a composition comprising a pathogen specific trans-replicating RNA (trRNA) nanoparticle (Tr-NP), the Tr-NP comprising a trRNA and a nanoparticle,

wherein the trRNA is a RNA polynucleotide comprising a nucleic acid encoding an antigen of the pathogen, operably linked to a CSE cognate to the replicase of the R-NP of (i).

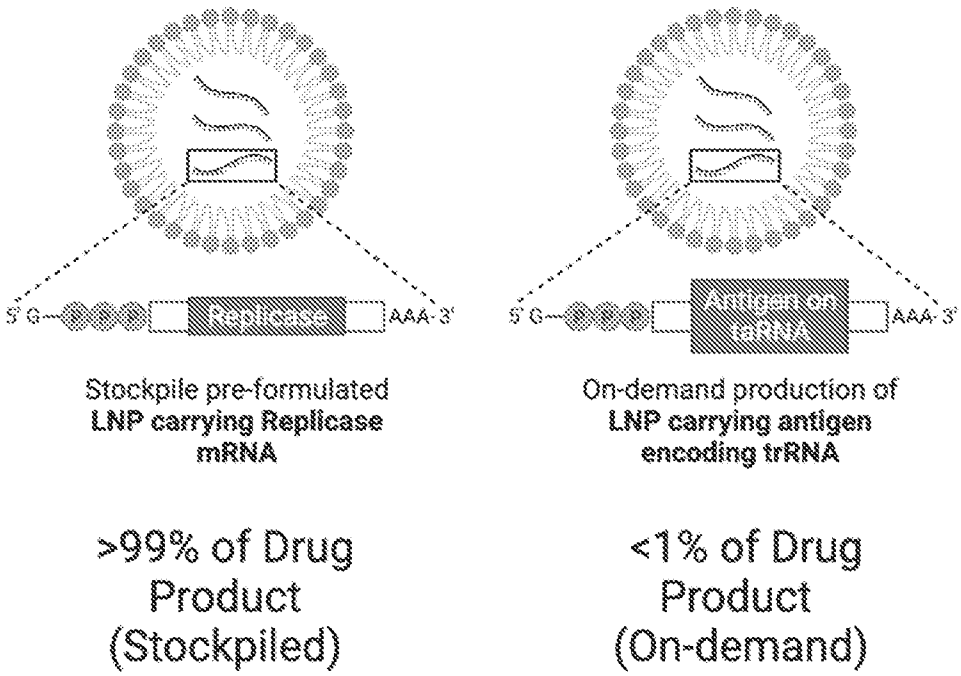
5' G
Replicase
AAA 3'
Stockpile pre-formulated
LNP carrying Replicase
mRNA
>99% of Drug
Product
(Stockpiled)
5' G
Antigen on
taRNA
AAA 3'
On-demand production of
LNP carrying antigen
encoding trRNA
<1% of Drug
Product
(On-demand)

FIG. 1

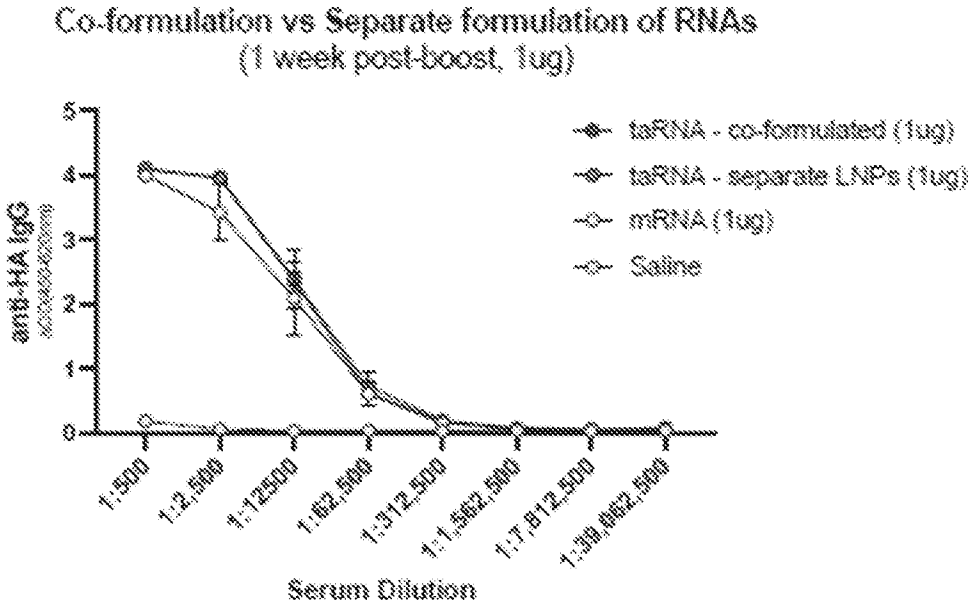
Co-formulation vs Separate formulation of RNAs
(1 week post-boost, 1ug)
taRNA - co-formulated (1ug)
taRNA - separate LNPs (1ug)
mRNA (1ug)
Saline
anti-HA IgG
0
1
2
3
4
5
1:500
1:2,500
1:12500
1:62,500
1:312,500
1:1,562,500
1:7,812,500
1:39,062,500
Serum Dilution

FIG. 2A

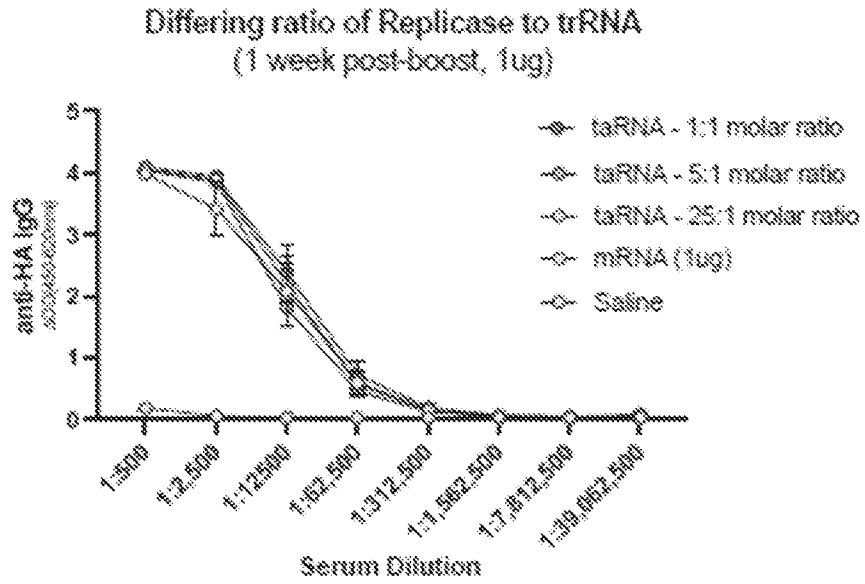
Differing ratio of Replicase to trRNA
(1 week post-boost, 1ug)
taRNA - 1:1 molar ratio
taRNA - 5:1 molar ratio
taRNA - 25:1 molar ratio
mRNA (1ug)
Saline
anti-HA IgG
0
1
2
3
4
5
1:500
1:2,500
1:12500
1:62,500
1:312,500
1:1,562,500
1:7,812,500
1:39,062,500
Serum Dilution

FIG. 2B

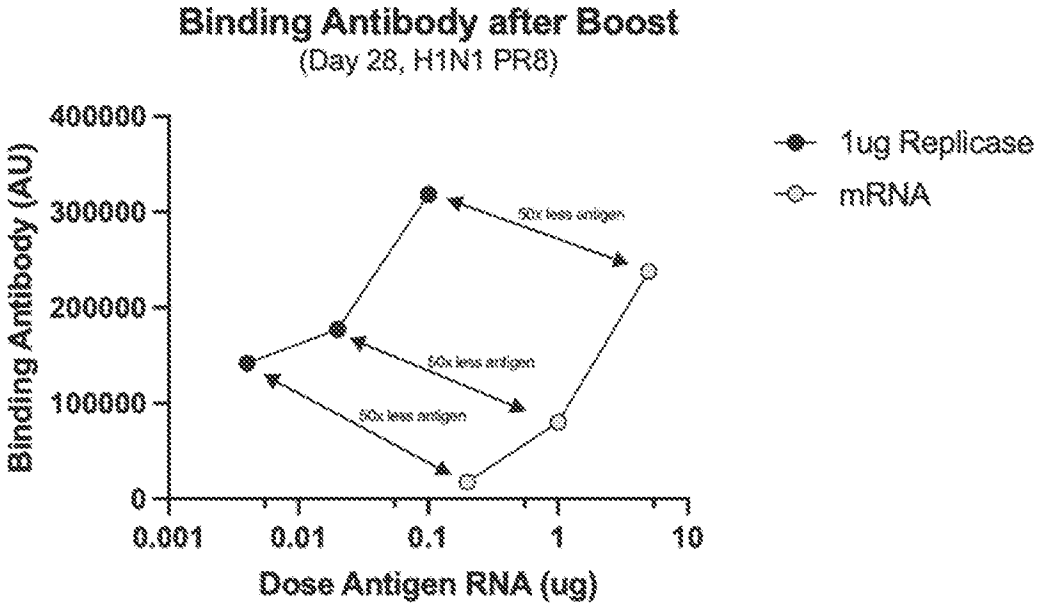
Binding Antibody after Boost
(Day 28, H1N1 PR8)
Binding Antibody (AU)
400000
300000
200000
100000
0
0.001
0.01
0.1
1
10
Dose Antigen RNA (ug)
50x less antigen
50x less antigen
50x less antigen
1ug Replicase
mRNA

FIG. 3A

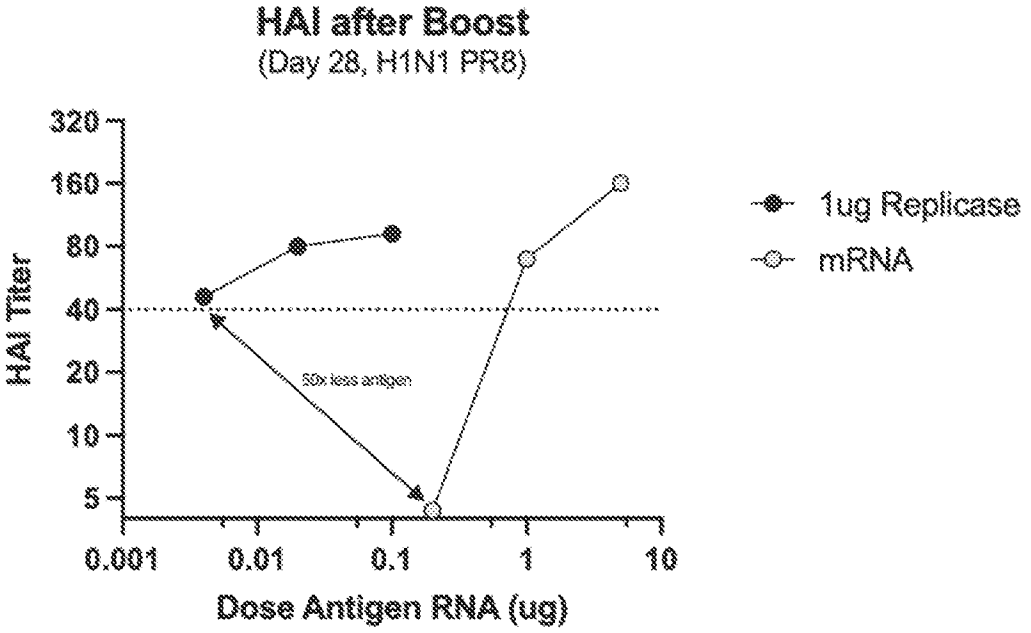
HAI after Boost
(Day 28, H1N1 PR8)
HAI Titer
320
160
80
40
20
10
5
0.001
0.01
0.1
1
10
Dose Antigen RNA (ug)
50x less antigen
1ug Replicase
mRNA

FIG. 3B

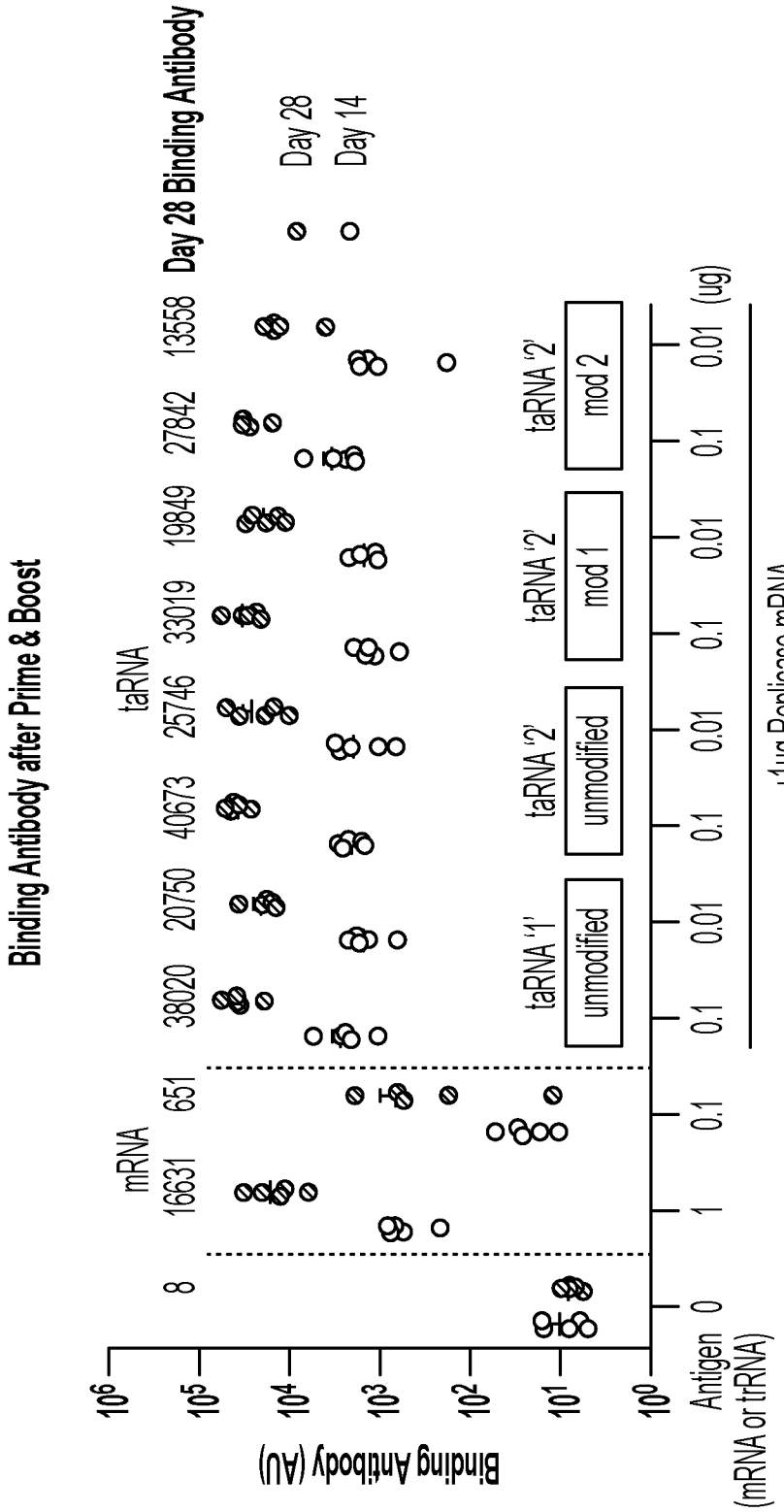
Binding Antibody after Prime & Boost
Day 28 Binding Antibody
Day 28
Day 14
Binding Antibody (AU)
Antigen 0
(mRNA or trRNA)
mRNA
8
16631
651
taRNA
38020
20750
40673
25746
33019
19849
27842
13558
taRNA '1'
unmodified
taRNA '2'
unmodified
taRNA '2'
mod 1
taRNA '2'
mod 2
0.1
0.01
0.1
0.01
0.1
0.01
0.1
0.01 (ug)
+1ug Replicase mRNA

FIG. 4A

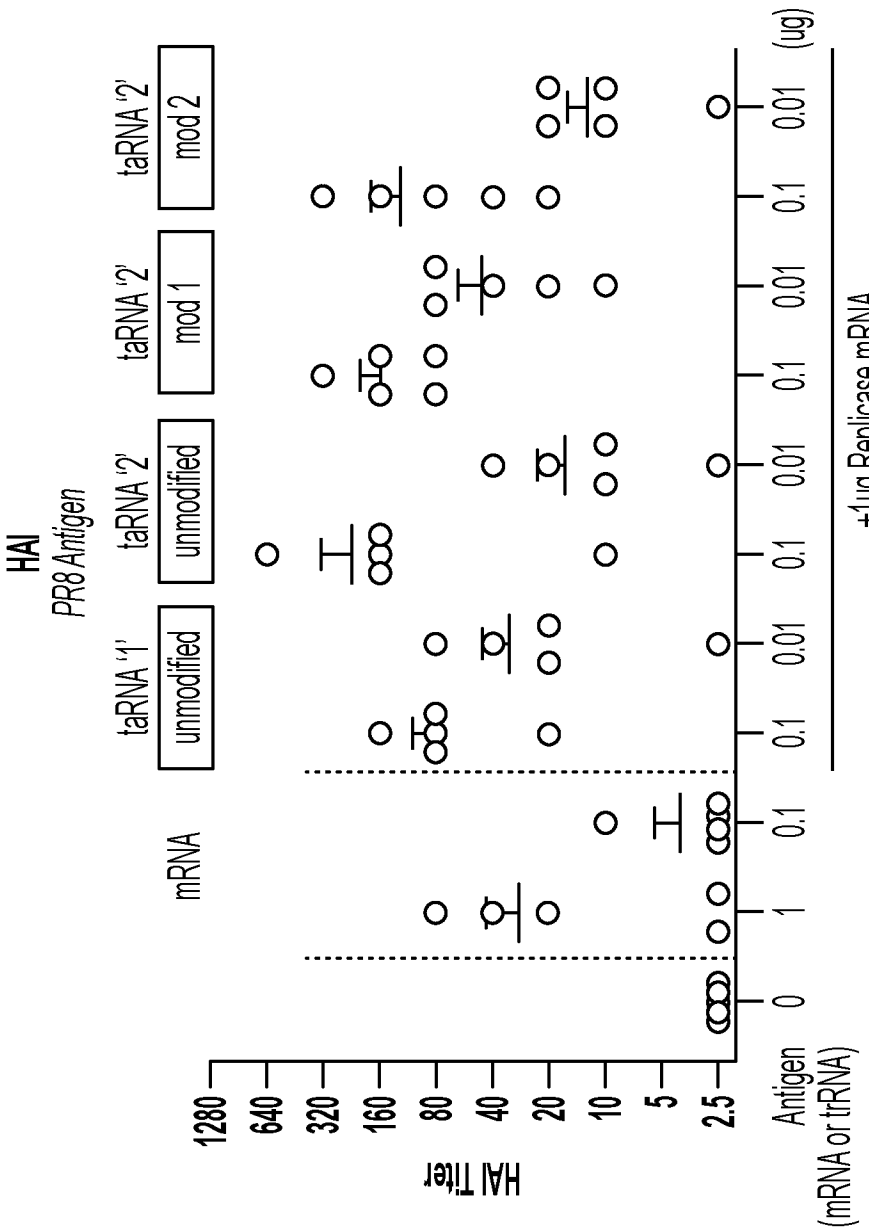
HAI
PR8 Antigen
mRNA
taRNA '1'
unmodified
taRNA '2'
unmodified
taRNA '2'
mod 1
taRNA '2'
mod 2
HAI Titer
1280
640
320
160
80
40
20
10
5
2.5
Antigen
(mRNA or trRNA)
0
1
0.1
0.01
0.1
0.01
0.1
0.01
0.1
0.01
(ug)
+1ug Replicase mRNA

FIG. 4B

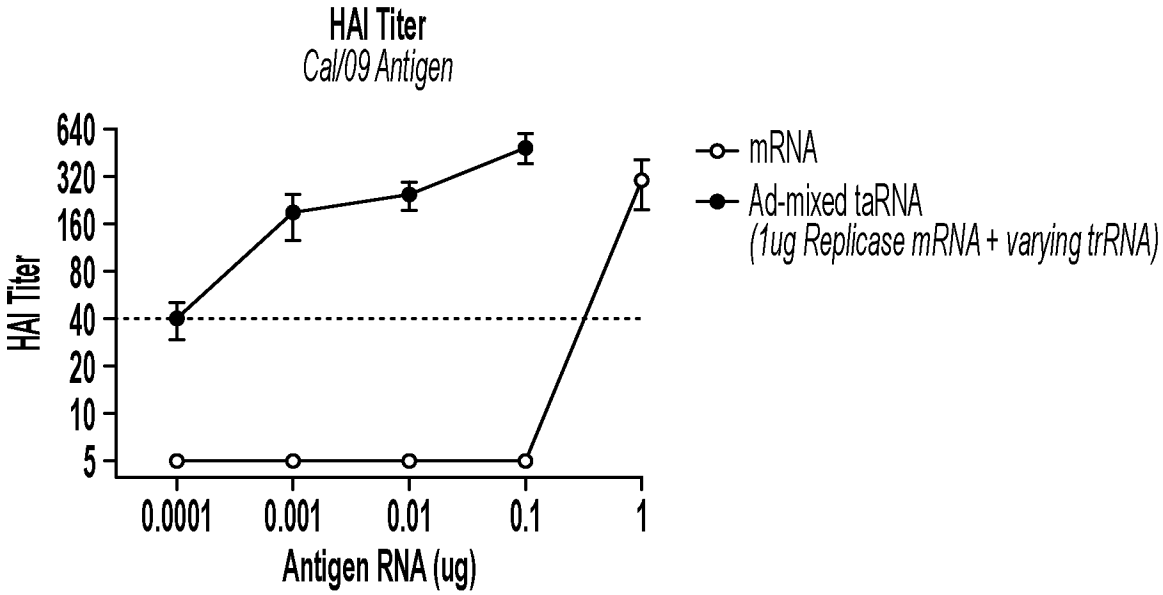
HAI Titer
Cal/09 Antigen
640
320
160
80
40
20
10
5
HAI Titer
0.0001
0.001
0.01
0.1
1
Antigen RNA (ug)
mRNA
Ad-mixed taRNA
(1ug Replicase mRNA + varying trRNA)

FIG. 5

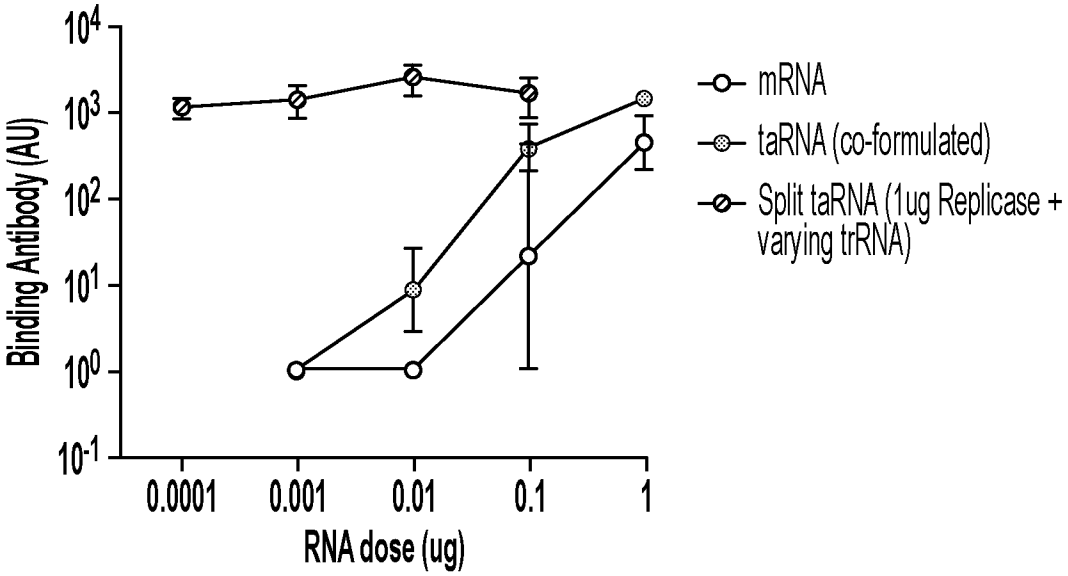
Binding Antibody (AU)
10^4
10^3
10^2
10^1
10^0
10^{-1}
0.0001
0.001
0.01
0.1
1
RNA dose (ug)
mRNA
taRNA (co-formulated)
Split taRNA (1ug Replicase + varying trRNA)

FIG. 6A

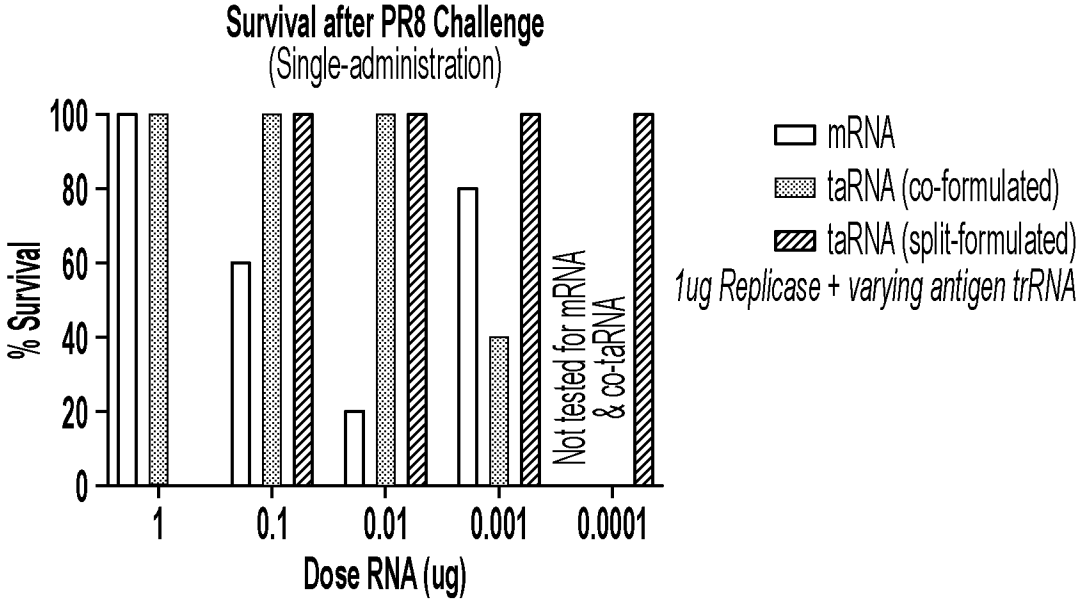
Survival after PR8 Challenge
(Single-administration)
% Survival
100
80
60
40
20
0
1
0.1
0.01
0.001
0.0001
Dose RNA (ug)
Not tested for mRNA & co-taRNA
mRNA
taRNA (co-formulated)
taRNA (split-formulated)
1ug Replicase + varying antigen trRNA

FIG. 6B

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2024/060231

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K39/12
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K C07K A61P C12N

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

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

EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BEISSERT TIM ET AL: "A Trans-amplifying RNA Vaccine Strategy for Induction of Potent Protective Immunity", MOLECULAR THERAPY, vol. 28, no. 1, 1 January 2020 (2020-01-01), pages 119-128, XP055847688, ISSN: 1525-0016, DOI: 10.1016/j.ymthe.2019.09.009 Retrieved from the Internet: URL:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6953774/pdf/main.pdf> *** Figure 1, 3, paragraph spanning pages 120/121, page 125 right col., page 126 *** ----- -/--	1-48

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

[x] See patent family annex.

* Special categories of cited documents :

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

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

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

"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
25 March 2025	08/05/2025

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

1

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2024/060231

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PERKOVIC MARIO ET AL: "A trans-amplifying RNA simplified to essential elements is highly replicative and robustly immunogenic in mice", MOLECULAR THERAPY, vol. 31, no. 6, 1 June 2023 (2023-06-01), pages 1636-1646, XP093242772, ISSN: 1525-0016, DOI: 10.1016/j.ymthe.2023.01.019 *** Figure 4 ***	1-48
Y	CA 3 234 214 A1 (BIONTECH SE [DE] ET AL.) 27 April 2023 (2023-04-27) *** Example 11, page 139 ***	1-48

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2024/060231

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

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

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

   b. [ ] furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

      [ ] accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. [ ] With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2024/060231

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
CA 3234214 A1	27-04-2023	AU 2022372325 A1	02-05-2024
		CA 3234214 A1	27-04-2023
		CN 118451194 A	06-08-2024
		EP 4419708 A1	28-08-2024
		JP 2024539089 A	28-10-2024
		WO 2023066874 A1	27-04-2023