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Design, Assembly, Production, and Transfection of Synthetic Modified mRNA

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Abstract

SHORT ABSTRACT—Proteins are drivers of cell functions and are targets of many therapies. Exogenous protein expression techniques, therefore, have been essential for research and medicine. The most common method for exogenous protein expression relies on DNA-based viral or non-viral vectors. However, DNA-based vectors have the potential to integrate into the host genome and cause permanent mutations. RNA-based vectors solve this shortcoming. In particular, synthetic modified mRNA provides non-viral, integration-free, zero-footprint method for expressing proteins. Modified mRNA can direct cell fate specification and cellular reprogramming faster and more efficiently than other methods. Furthermore, when simultaneously express multiple different proteins, mRNA vectors allow for greater flexibility and control over stoichiometric ratios, dose titrations, and complete silencing of expressions. Additionally, modified mRNAs have been shown to be viable and safe as therapeutic agents for gene therapy and vaccine, providing an alternative approach to address diseases. Despite these advantages, technical challenge, mRNA instability, and host immunogenicity have caused significant barriers to widespread use of this technology. The comprehensive method presented here addresses all of these shortcomings. This stepwise protocol describes every step necessary for the synthesis of modified mRNA from any coding DNA sequence of interest. The meticulously detailed protocol enables the users to make alterations to each component of modified mRNA for even greater customization, allowing the researchers to apply this technology to a wide range of uses. This non-cytotoxic synthetic modified mRNA can be used for protein expression, regulation of cell reprogramming or differentiation, and drug delivery.

LONG ABSTRACT—A variety of techniques for exogenous gene expression are widely used in research and hold potential for therapeutic applications. The most commonly used methods employ DNA-based viral and non-viral vectors. However, DNA-based vectors can integrate into the genome and cause permanent mutations in a cell.¹ This introduces a random variable in research and raises safety concerns for clinical application. RNA-based vectors provide a safer alternative class of tools. In particular, modified mRNA vectors are integration- and virus-free.² Despite many advantages, modified mRNA vectors are much less widely used and have had

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The authors have nothing to disclose.

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limited impact on the field, due in part, to technical challenges in synthesizing non-cytotoxic mRNA vectors.³ Furthermore, highly reproducible and validated protocols are sparse. The protocol presented here describes in detail every step necessary for the synthesis of modified mRNA from any coding DNA sequence of interest.

This protocol is comprised of four parts: design, assembly, production, and transfection. The design part specifies how to tailor DNA sequences for oligonucleotides for any gene of interest. Assembly describes how these oligonucleotides are combined through series of ligation steps to form DNA templates. Production shows how these DNA templates can generate stable and modified mRNAs. Lastly, in transfection, key steps for successful delivery to cells and expression of the modified mRNAs are detailed. Modified mRNAs can replace most DNA-based vector applications and have been successfully used for protein expression, regulation of cell reprogramming or differentiation, and drug delivery.⁴⁻⁹ Modified mRNAs are particularly useful for transient expression of proteins but also can be used for stable expression via repeated transfections.^{4,9}

Prior cellular reprogramming studies have shown that modified mRNAs can reprogram cells faster and more efficiently than DNA-based methods.^{4,10,11} Further, modified mRNAs also have been shown to be viable and safe therapeutic agents for gene therapy.^{5,6,12} However, detailed protocols are not available for users to create their own custom modified mRNAs for any genes of interest. We report a modified and improved protocol that can be used for any gene and that has sufficient detail to enable any user to use the protocol for a wide range of applications.

Keywords

messenger RNA; synthetic mRNA; modified mRNA; reprogramming factors; mRNA protocol; mRNA reprogramming; mRNA synthesis; mRNA transfection

INTRODUCTION

DNA-based viral vectors are the most commonly used method for expressing proteins in a cell. However, this approach has a number of significant drawbacks including the possibility of viral integration into the host genome, immunogenicity, and gene silencing. Non-viral transfection of plasmid DNA (pDNA) is associated with reduced immunogenicity and less chance of genome integration,^{13,14} but it generally has much lower efficiency and reduced protein production.¹⁵⁻¹⁷

RNA-based protein expression provides several key advantages when compared to DNA-based approaches. First, RNA-based vectors do not enter the cell nucleus, eliminating potential complications due to integration into the host genome. Second, the strength of protein expression is independent of promoter activity and thus avoids the gene silencing that is common with DNA-based techniques for gene expression. Third, RNA-mediated manipulation of cellular phenotype may have a higher efficiency than DNA-based methods.^{4,10} Similar to DNA-based methods, RNA-based methods have both viral and non-viral approaches. RNA viruses such as Sendai virus function exclusively in cytoplasm and are capable of producing large amounts of proteins.¹⁸ However, they are more difficult to

construct and produce, the cessation of gene expression is difficult to control accurately, and there are safety concerns related to persistent virus.

Synthetic modified mRNA is a non-viral RNA-based technology that solves many safety concerns present in other gene expression techniques. Being both non-integrative and non-viral, mRNA technology allows for direct clinical applications without the need for laborious and time-consuming screening of integrated oncogenes or viral components.^{2,10} In addition, unlike plasmid DNA-mediated expression, mRNA-mediated expression is effective for both mitotic and post-mitotic cells.² In contrast to retro- and lentiviral vectors which often drive multiple genes under a single promoter, each mRNA-mediated gene can be dosed independently from one another, resulting in more sophisticated stoichiometric expression of multiple genes.¹⁹ Furthermore, unlike other inducible systems that may have background leaky expression, the short half-life of mRNA allows good temporal control of silencing of exogenous expression. Despite these advantages, concerns with stability and immunogenicity of earlier versions of synthetic mRNAs prevented widespread use.²⁰ The protocol presented here improves upon these shortcomings.

The stability and immunogenicity of mRNA can be changed through transcript engineering.^{21–24} Five components make up the eukaryotic mature mRNA: 5' cap, 5' untranslated region (UTR), open reading frame (ORF), 3' UTR, and 3' poly adenosine (poly(A)) tail. Each component plays an important role in stability and immunogenicity.

Standard *in vitro* transcription (IVT) of RNAs results in 5' triphosphate which is vulnerable to exonuclease degradation.^{25,26} In contrast, eukaryotic cells post-transcriptionally modify mRNA in the nucleus with a methylated cap structure at the 5' end. In addition to stabilizing the mRNA, the 5' cap increases protein production through recognition by eukaryotic initiation factor 4E (eIF4E) which leads to ribosome recruitment and translation.^{23,25,27,28} The 5' cap is also involved in other mRNA processes such as cellular transport, splicing into substrate RNAs, and translational repression via microRNA.^{29–31} The protocol in this paper acknowledges this importance and uses the RNA cap structure analog, m7G(5')ppp(5')G, which shares the uncommon 7-methylguanosine and 5'-5' triphosphate bridge found in eukaryotic mRNA.

Eukaryotic UTR sequences have variety of regulatory functions and influence the degree of stability of the mRNA transcript. For example, AU-rich sequences in the 3' UTR induce degradation by promoting the removal of poly(A) tail while iron response elements present in both 5' and 3' UTR regulate stability and translation.^{24,32,33} UTRs of the globin gene are known to be stable and therefore are used in this protocol.³⁴

The poly(A) tail also plays an important role in enhancing mRNA stability and translation.³⁵ Most actively translated eukaryotic mRNAs have a poly(A) tail of 100–250 bases.³⁶ While exogenous protein synthesis can occur with a much shorter tail, stability and translation efficiency are positively correlated with length.^{37,38} The poly(A) tail binds to polyadenosyl-binding proteins (PABP) which interact with the N-terminal region of eukaryotic initiation factor 4G (eIF4G) which then interacts with the 5' cap and eIF4E.³⁹ This cap-eIF4E-eIF4G-PABP-poly(A) interaction creates a circularized closed loop mRNA structure, which may

increase stability by protection from nucleolytic degradation and increase translation efficiency by ribosome reutilization.^{40,41} Since the poly(A) tail plays an important role in stability and translation, reproducible results require a consistent and standardized number of poly(A) bases. This protocol attaches the same length of 120 bases of poly(A) to the 3' UTR of each mRNA vector.

Immune responses also can be reduced by modifying the bases of mRNA.^{2,4,42} Exposure to non-self nucleic acid activates the mammalian innate immune system by activating Toll-like receptor (TLRs).⁴³ Different TLRs recognize different types of nucleic acids.^{42,44–47} TLR3 in particular recognizes mRNA and induces a strong cascading immune response.⁴⁷ The use of modified nucleosides in place of naturally occurring ones in mammalian cells dramatically reduces immunogenicity.^{2,4,42} Thus, this protocol substitutes cytidine and uridine with 5-methylcytidine and pseudouridine, respectively. Further reduction in immunogenicity is achieved by removing 5' triphosphates and adding interferon inhibitor B18R into the cell culture media.⁴

The use of synthetic modified mRNA for gene expression is very promising because of its superior safety profile and higher efficiency.^{4,10} When compared against other methods, mRNA-mediated reprogramming of somatic cells to pluripotent stem cells resulted in 10–100 times more efficient and faster emergence of colonies while still maintaining the lowest aneuploidy of the treated cells.^{10,48,49} Despite many advantages, mRNA stability and immunogenicity have been the greatest barriers for wide spread use.²⁰ This protocol addresses these shortcomings. The protocol is meticulously detailed so that any user, experienced or inexperienced, can readily apply this technique.

METHODS

1. Design

Note: Refer to Figure 1 for the schematic overview of the primers and oligonucleotides interactions.

1.1 ORF-specific Primers and Oligonucleotides

1.1.1 Synthesize the forward primer (ORF-FW) for the gene of interest.

Note: Do not include the start codon. The 5'UTR contains ATG.

1.1.2 Synthesize the reverse primer (ORF-RV) for the same ORF of interest.

Note: Ensure that the ORF-RV contains the stop codon.

1.1.3 Synthesize the 5'Bridge as follows: reverse complement of ORF-FW + CATGGTGGCTCTTATATTTCTTCTT

1.1.4 Synthesize the 3'Bridge as follows: CCCGCAGAAGGCAGC + ORF-RV

1.2 Common Primers and Oligonucleotides

1.2.1 Synthesize the Clone-FW/RV as follows:

GCGTCGACACTAGTTCTAGACCCTCA (26 bp)

- 1.2.2** Synthesize the IVT-FW as follows: TTGGACCCTCGTACAGAAGCTAATACG (27 bp)
- 1.2.3** Synthesize the IVT-RV as follows: T₁₂₀
CTTCCTACTCAGGCTTTATTCAAAGACCA (149 bp)
- 1.2.4** Synthesize the 5'UTR as follows:
GCGTCGACACTAGTTCTAGACCCTCATTGGACCCTCGTACAGAAGCTA
ATACGACTCACTATAGGGAAAT
AAGAGAGAAAAGAAGAGTAAGAAGAAATATAAGAGCCACCATG (113 bp)
- 1.2.5** Synthesize the 3'UTR as follows:
GCTGCCTTCTGCGGGGCTTGCCTTCTGGCCATGCCCTTCTTCTCTCCCT
TGCACCTGTACCTCTTGGTCTTT
GAATAAAGCCTGAGTAGGAAGTGAGGGTCTAGAACTAGTGTGCGACGC (119 bp)

Note: Fluorescent reporter should be included for internal control and transfection optimization. Destabilized and nuclear-localizing fluorescent protein with excitation and emission ranges that will not interfere with the downstream immunohistochemistry is highly recommended. Starting with just the open reading frame (ORF) of fluorescent protein may be beneficial when going over this protocol for the first time. Refer to Figure 2 for details of the components of the UTRs.

2. Assembly

- 2.1** Stock Solutions and Working Concentrations
- 2.1.1** Add TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) to make 100 μ M stock solution for each primer and oligonucleotide.
- 2.1.2** Further dilute each ORF-RV, 5'UTR, Clone-FW/RV, 5'Bridge, and 3'Bridge to achieve 1 μ M working concentration.
- 2.2** ORF-FW and 3'UTR N-Terminal Phosphorylation
- 2.2.1** Prepare phosphorylation reaction for ORF-FW and 3'UTR as shown below:

Reagent	Amount (μ L)	Final Concentration
ORF-FW or 3'UTR (100 μ M)	3	6 μ M
ATP (from MEGAscript T7 Kit, 75 mM)	0.7	1 mM
Polynucleotide Kinase Buffer (10X)	5	1X
Polynucleotide Kinase (10 U)	1	0.2 U μ L ⁻¹
Nuclease-free Water	40.3	50 μ L total volume

Mix and centrifuge briefly.

- 2.2.2** Incubate at 37 °C for 30 min.
- 2.2.3** Denature polynucleotide kinase by incubating at 60 °C for 25 min.
- 2.2.4** Add 250 µL TE buffer to make 1 µM 5' phosphorylated (p-) ORF-FW and 3' UTR.
- 2.3** p-ORF Amplification
- 2.3.1** Prepare PCR reaction as shown below:

Reagent	Amount (µL)	Final Concentration
Template DNA (1–10 ng µL ⁻¹ for a simple template such as a plasmid or 10–100 ng µL ⁻¹ for a more complex template such as genomic DNA)	1	50–500 pg µL ⁻¹ (simple template) 0.5–5 ng µL ⁻¹ (complex template)
p-ORF-FW (1 µM)	4	200 nM
ORF-RV (1 µM)	4	200 nM
KAPA HiFi HotStart ReadyMix (2X)	10	1X
Nuclease-free Water	1	20 µL total volume

Mix and centrifuge briefly.

- 2.3.2** Amplify the ORF using PCR program as shown below:

Step	Temperature	Time	Cycles
Initial Denaturation	95.0 °C	5 min	1x
Denaturation	98.0 °C	20 sec	25x
Annealing	60.0–75.0 °C	15 sec	
Extension	72.0 °C	15–60 sec per kb	
Final Extension	72.0 °C	5 min	1x

- 2.4** Isolation and Purification of p-ORF Product
- 2.4.1** Run the products on gel electrophoresis as shown in Figure 4a.
- 2.4.2** Cut and extract only the product bands and elute in 30 µL Elution Buffer (EB).
- Note: If there is no discrete band of expected size then perform gradient PCR from Step 2.3.2. Even though gel extraction is performed, obtaining clean ORF amplification needs to be prioritized in order to minimize potential non-specific downstream reactions.
- 2.4.3** Use Nanodrop microvolume spectrophotometer to verify reasonable amplification. Adjust to 24 ng µL⁻¹ per sample.
- Note: If large amounts of p-ORF products are lost during the extraction step, decrease the EB to 10 µL. Minimum of 5 µL at 24 ng µL⁻¹ for each sample of amplified products are required for the next step.

Pause Point: Extracted and purified p-ORF can be stored at -20°C for prolonged time.

2.5 5'UTR-ORF-3'UTR Double Ligation

2.5.1 Prepare double ligation reaction as shown below:

Reagent	Amount (μL)	Final Concentration
p-ORF ($24\text{ ng }\mu\text{L}^{-1}$)	1	$1.2\text{ ng }\mu\text{L}^{-1}$
5'UTR ($1\text{ }\mu\text{M}$)	4	200 nM
5' Bridge ($1\text{ }\mu\text{M}$)	2	100 nM
p-3'UTR ($1\text{ }\mu\text{M}$)	4	200 nM
3' Bridge ($1\text{ }\mu\text{M}$)	2	100 nM
Ampligase Reaction Buffer (10X)	2	1X
Ampligase DNA Ligase ($5\text{ U }\mu\text{L}^{-1}$)	1	$0.25\text{ U }\mu\text{L}^{-1}$
Nuclease-free Water	4	20 μL total volume

Mix and centrifuge briefly.

2.5.2 Run PCR double ligation program as shown below:

Step	Temperature	Time	Cycles
1 st Incubation	95.0°C	10 sec	5x
2 nd Incubation	45.0°C	1 min	
3 rd Incubation	50.0°C	1 min	
4 th Incubation	55.0°C	1 min	
5 th Incubation	60.0°C	1 min	

2.5.3 Purify the products using QIAquick PCR purification kit and elute with $10\text{ }\mu\text{L}$ elution buffer (EB).

2.6 5'UTR-ORF-3'UTR Amplification

2.6.1 Prepare PCR reaction as shown below:

Reagent	Amount (μL)	Final Concentration
5'UTR-ORF-3'UTR (unamplified)	5	---
Clone-FW/RV ($1\text{ }\mu\text{M}$)	10	200 mM
KAPA HiFi HotStart ReadyMix (2X)	25	1X
Nuclease-free Water	10	50 μL total volume

Mix and centrifuge briefly.

2.6.2 Amplify using PCR program as shown below:

Step	Temperature	Time	Cycles
Initial Denaturation	95.0 °C	5 min	1x
Denaturation	98.0 °C	5 sec	25x
Annealing	60.0–75.0 °C	15 sec	
Extension	72.0 °C	15–60 sec per kb	
Final Extension	72.0 °C	5 min	1x

2.6.3 Run the products on gel electrophoresis as shown in Figure 4b.

2.6.4 Cut and extract only the product bands and elute in 40 μ L EB.

Note: If the products are absent, ligate each UTR one at a time. Refer to Step 2.7.

Pause Point: Amplified 5'UTR-ORF-3'UTR can be stored at -20°C for prolonged time.

2.7 Optional: *5'UTR-ORF-3'UTR Single Ligation*

Note: This Step should be performed only when double ligation has failed.

2.7.1 Prepare the first single-ligation reaction as shown below:

Reagent	Amount (μ L)	Final Concentration
p-ORF (24 ng μL^{-1})	1	1.2 ng μL^{-1}
5'UTR (1 μM)	4	200 nM
5'Bridge (1 μM)	2	100 nM
Ampligase Reaction Buffer (10X)	2	1X
Ampligase DNA Ligase (5 U μL^{-1})	1	0.25 U μL^{-1}
Nuclease-free Water	10	20 μL total volume

Mix and centrifuge briefly.

2.7.2 Run the PCR program as shown in Step 2.6.2.

2.7.3 Purify the products using PCR purification kit and elute in 12 μL EB.

2.7.4 Prepare the second single-ligation reaction as shown below:

Reagent	Amount (μ L)	Final Concentration
5'UTR-ORF	10	
p-3'UTR (1 μM)	4	200 nM
3'Bridge (1 μM)	2	100 nM
Ampligase Reaction Buffer (10X)	2	1X
Ampligase DNA Ligase (5 U μL^{-1})	1	0.25 U μL^{-1}

Reagent	Amount (μL)	Final Concentration
Nuclease-free Water	1	20 μL total volume

Mix and centrifuge briefly.

2.7.5 Run the PCR program as shown in Step 2.6.2.

2.7.6 Purify the products using PCR purification kit and elute in 10 μL EB.

2.7.7 Amplify the purified ligated 5'UTR-ORF-3'UTR by following Step 2.6.

2.8 5'UTR-ORF-3'UTR Cloning

Note: The following Steps use TOPO TA cloning method that is sensitive to the size of the inserts. If inserts are large, or TOPO TA cloning was unsuccessful, perform the traditional cloning method of restriction endonucleases followed by T4 ligation instead. Refer to Figure 2 and ensure that the correct endonucleases are used for the given ORF and UTRs.

2.8.1 Prepare dA-Tail reaction as shown below:

Reagent	Amount (μL)	Final Concentration
5'UTR-ORF-3'UTR (amplified)	40	---
dA-Tailing Reaction Buffer (10X)	5	1X
Klenow Fragment (3'→5' exo ⁻)	3	---
Nuclease-free Water	2	50 μL total volume

Mix and centrifuge briefly.

2.8.2 Incubate at 37.0 °C for 30 min.

2.8.3 Purify the products using PCR purification kit and elute in 30 μL EB.

2.8.4 Prepare TOPO TA cloning reaction as shown below:

Reagent	Vector Only (Negative Control)	Vector + Insert (Positive Control)	Vector + Insert (Experimental)
Positive Control PCR Insert	---	1 μL	---
5'UTR-ORF-3'UTR dA-tailed	---	---	4 μL
TOPO Vector	1 μL	1 μL	1 μL
Salt Solution (from TA Cloning Kit)	1 μL	1 μL	1 μL
Nuclease-free Water	4 μL	3 μL	---
Final Volume	6 μL	6 μL	6 μL

Mix and centrifuge briefly.

Note: Ensure to include reactions for positive and negative controls.

2.8.5 Incubate at room temperature for 30 min.

Pause Point: The cloned reactions can be stored at -20°C for prolonged time.

2.9 Cloned 5'UTR-ORF-3'UTR Transformation

2.9.1 Set up multiple transformations with varying amounts of cloned reactions per sample. Transform into competent cells according to the manufacturer's instructions.

Note: Be sure to include positive and negative transformation controls.

2.9.2 Compare against the positive and negative controls to confirm that the transformation was successful. Pick 4–8 colonies that are grown overnight on a selective plate and inoculate each colony into a culture of 1–5 mL LB medium containing the appropriate selective antibiotic. Incubate at 37.0°C for 12–16 hr with vigorous shaking.

Note: Pick larger colonies that tend to have correct inserts than the smaller colonies.

2.9.3 Extract and purify the plasmid DNA using a QIAprep Spin Miniprep kit according to the manufacturer's instructions.

Note: QIAprep Maxiprep kit is preferred for large scale experiments.

2.9.4 Adjust the plasmid concentration to $100\text{ ng }\mu\text{L}^{-1}$.

Pause Point: Extracted plasmids can be stored at -20°C for prolonged time.

2.10 Plasmid Linearization and Extraction

Note: RNA polymerases are very processive therefore even a small amount of undigested circular DNA templates can generate run-on heterogenous transcripts. Ensure that plasmids are completely linearized with appropriate restriction enzymes downstream of the insert.

2.10.1 Prepare digestion reaction as shown below:

Reagent	Amount (μL)	Final Concentration
Plasmid ($100\text{ ng }\mu\text{L}^{-1}$)	40	$20\text{ ng }\mu\text{L}^{-1}$
Restriction Enzyme	4	$0.1\text{--}1.0\text{ U }\mu\text{L}^{-1}$
NEBuffer (10X)	20	1X
Nuclease-free Water	136	200 μL total volume

Mix and centrifuge briefly.

2.10.2 Incubate at 37°C for 1 hr.

2.10.3 Run the products on gel electrophoresis.

Note: Ensure that the plasmids are completely digested as shown in Figure 4d. Run the undigested plasmid in another lane as a control as seen in Figure 4c.

2.10.4 Cut and extract only the linearized plasmid bands and elute in $50\text{ }\mu\text{L}$ EB.

Pause Point: Linearized plasmids can be stored at -20°C for prolonged time.

2.11 Attach Poly(T)-tail and Sequence Validation

2.11.1 Prepare the Poly(T) attachment master mix as shown below:

Reagent	Amount (μL)	Final Concentration
Digested Template DNA	16	---
IVT-FW (1 μM)	12	30 nM
IVT-RV (1 μM)	12	30 nM
KAPA HiFi HotStart ReadyMix (2X)	200	1X
Nuclease-free Water	160	400 μL total volume

Mix and centrifuge briefly.

2.11.2 Aliquot the master mix into 8 PCR tubes (50 μL each) and amplify by running the PCR program as shown below:

Step	Temperature	Time	Cycles
Initial Denaturation	95.0 $^{\circ}\text{C}$	3 min	1x
Denaturation	98.0 $^{\circ}\text{C}$	20 sec	15–25x
Annealing	60.0 $^{\circ}\text{C}$	15 sec	
Extension	72.0 $^{\circ}\text{C}$	15 sec per kb	
Final Extension	72.0 $^{\circ}\text{C}$	3 min	1x

2.11.3 Run the products on gel electrophoresis. Cut and pool all product bands. Extract and elute in 50 μL EB.

2.11.4 Adjust the final concentration to 100 ng μL^{-1} using EB.

2.11.5 Run an aliquot on gel electrophoresis to verify proper amplification. Verify clean extraction as shown in Figure 4e.

Note: If non-specific amplification was observed then reduce the number of PCR cycles on Step 2.11.2.

2.11.6 Sequence the templates prior to the next step.

Pause Point: Templates can be stored at -20°C for prolonged time.

3. Production

Note: Decontaminate all working surfaces and pipettes with RNaseZap. Ensure that all microcentrifuge tubes and pipette tips are RNase-free.

3.1 In Vitro Transcription

- 3.1.1** Spin down and reconstitute one vial of 3'-O-Me-m⁷G(5')ppp(5')G RNA cap analog with 16.7 μ L nuclease-free water to obtain 60 mM stock solution. Use GTP and ATP from MEGAscript T7 kit. Prepare the custom nucleotide triphosphates (NTPs) mix as shown below:

Reagent	Stock Concentration (mM)	Amount (μ L) per IVT reaction	NTP Mix Concentration (mM)	Final IVT Reaction Concentration (mM)
RNA cap analog	60	4.0	16.2	6.0
GTP	75	0.8	4.1	1.5
ATP	75	4.0	20.3	7.5
5-Me-CTP	100	3.0	20.3	7.5
Pseudo-UTP	100	3.0	20.3	7.5

Mix and centrifuge briefly.

Note: Scale up the amount of each reagent according to the desired number of IVT reactions.

- 3.1.2** Add the IVT reagents in a PCR tube at room temperature in the following order as shown below:

Reagent	Amount (μ L)	Final Concentration
NTP Mix (from Step 3.1.1)	14.8	Refer to Step 3.1.1
Tailed Poly(T) PCR Product, 100 ng μ L ⁻¹ (from Step 2.15)	16.0	40 ng μ L ⁻¹
T7 Buffer (10X)	4.0	1X
T7 Enzyme (10X)	4.0	1X
Nuclease-free Water	1.2	40 μ L total volume

Mix and centrifuge briefly.

Note: Multiple reactions should be set up based on scale of the experiment.

- 3.1.3** Incubate at 37 °C for 4–6 hrs in a thermocycler. Do not incubate in water bath in order to avoid contamination with RNases.

3.2 DNase Treatment

- 3.2.1** Add 2 μ L of Turbo DNase (from MEGAscript T7 kit) to each reaction. Mix gently and centrifuge briefly. Incubate at 37 °C for 15 min in a thermocycler.

- 3.2.2** Purify each reaction using MEGAclean kit. Follow the RNA elution option 2 by pre-heating 110 μ L of EB to 95 °C and eluting twice with 50 μ L for total of 100 μ L.

3.3 Phosphatase Treatment

- 3.3.1** Add 11 μL of 10X Antarctic Phosphatase Buffer and 2 μL of Antarctic Phosphatase to each reaction. Mix gently and centrifuge briefly.
- 3.3.2** Transfer each reaction to PCR tube and incubate at 37 °C for 1 hr in a thermocycler.
- 3.3.3** Purify using MEGAclean kit as described in Step 3.2.2.
- 3.4** Quality Control
- 3.4.1** Measure the concentration of each purified mRNA sample using a NanoDrop. The expected yield is between 300–800 ng μL^{-1} in 100 μL elution volume. The absorbance ratios of 260 nm / 280 nm should be greater than 1.8 and 260 nm / 230 nm should be close to 2.0 as shown in Figure 5b. Absorbance ratios outside of the expected values require repetition of the reaction from Step 3.1.2.
- 3.4.2** Adjust each reaction to 100 ng μL^{-1} by adding EB. Reactions with yields too low to achieve 100 ng μL^{-1} concentration need to be repeated from Step 3.1.2.
- Note: Higher concentrations may be necessary depending on the cell types, media, expression levels, and other experimental parameters. Save an aliquot of undiluted reaction in case a higher dose is preferred.
- 3.4.3** Analyze the integrity of the synthetic modified mRNA using a Bioanalyzer as shown in Figure 5c. Running a small aliquot on a denaturing agarose gel should be performed to check the integrity if a Bioanalyzer is inaccessible.
- Note: If the integrity analysis shows non-specific amplification and/or degradation, repeat the Production from Step 3.1.2.
- Pause Point: Purified synthetic modified mRNA can be stored at –80 °C for several months.

4. Transfection

- 4.1** Synthetic Modified mRNA Cocktail Preparation
- Note: Spike each cocktail with an equimolar mRNA fluorescent reporter to monitor transfection efficiency. We recommend using destabilized fluorescent protein that has half-life of hours. This allows for accurate monitoring of daily transfection. Addgene has pRVGP (plasmid 15377) that contains the gene for destabilized eGFP which can be used as a DNA template to generate mRNA fluorescent reporter. Thaw all synthetic modified mRNAs on ice.
- 4.1.1** Determine the nucleotide base length of each ORF. This value should be derived from the template DNA on which the ORF PCR was performed. If genomic DNA was used as the template, determine the base length via NCBI Gene database and confirm with the sequencing data.
- 4.1.2** To the determined value from the previous (Step 4.1.1), add 297 base pairs. This addition represents the Poly(T)-tail and parts of the 5'UTR and 3'UTR that are carried over to IVT.

- 4.1.3** Convert each determined nucleotide length from the previous (Step 4.1.2) to mole. Refer to table below for conversion:

Size (nt)	20	100	300	500	1000	1800
pmol	152.23	31.05	10.38	6.23	3.12	1.73

Note: Nucleotide lengths that are outside the range of this table can be converted to moles by scaling up or down proportionately to the values in the table.

- 4.1.4** Alternatively, NEBioCalculator from New England BioLabs website can be used. Go to ssRNA: Moles to Mass Converter. Input the RNA length as determined from Step 4.1.2 then input the arbitrary but same value for RNA moles, 1 for example, to obtain RNA mass for each sample.
- 4.1.5** Normalize the values of each mRNA product calculated from the previous steps by dividing by the smallest number (moles for Step 4.1.3 or RNA mass for Step 4.1.4) across all samples that will be mixed together in a cocktail.
- 4.1.6** Determine the total volume of cocktail mRNA needed for a given experiment, then add the normalized volumetric ratios of each mRNA product to meet this demand. Mix gently but thoroughly and centrifuge briefly.

Note: For situations where unequal molar ratios are desired, make the appropriate calculation changes on Step 4.1.3 or 4.1.4.

- 4.1.7** Aliquot the cocktail for single-use in order to minimize freeze/thaw cycle.

Note: Cocktail aliquots can be stored at -80°C for several months.

4.2 Identifying the Optimal Transfection Reagent

Note: Cell types, media, and other experimental designs can greatly affect the success of mRNA transfection. To address this, we have provided three transfection reagents that have previously shown success. Choose the reagent that results in most successful transfection for a given experimental design as determined by the fluorescent reporter. We find that $1\text{ }\mu\text{g}$ mRNA for one well of 12-well plate was optimal in most conditions. The optimal dose of mRNA for a given experiment may need to be determined by titration. The following reaction volumes should be scaled according to the number of wells to be transfected.

4.2.1 Lipofectamine RNAiMax

- 4.2.1.1** Add $1\text{ }\mu\text{g}$ ($10\text{ }\mu\text{L}$ of $100\text{ ng }\mu\text{L}^{-1}$) mRNA cocktail into $40\text{ }\mu\text{L}$ Opti-MEM I Reduced-Serum Medium. Gently pipet and mix thoroughly.
- 4.2.1.2** In a separate tube, add $5\text{ }\mu\text{L}$ Lipofectamine RNAiMax into $45\text{ }\mu\text{L}$ Opti-MEM I Reduced-Serum Medium. Gently pipet and mix thoroughly.
- 4.2.1.3** Combine the $50\text{ }\mu\text{L}$ diluted mRNA and the $50\text{ }\mu\text{L}$ diluted Lipofectamine RNAiMax. Gently pipet and mix thoroughly.

4.2.1.4 Incubate at room temperature for 15 min.

4.2.1.5 Add the complexes drop-wise to one well of a 12-well plate. Gently rock the plate back-and-forth and from side-to-side. Incubate for 4 hrs at 37 °C. Longer incubation may increase cytotoxicity.

4.2.2 *TransIT*-mRNA

4.2.2.1 Add 1 µg (10 µL of 100 ng µL⁻¹) mRNA cocktail into 100 µL Opti-MEM I Reduced-Serum Medium. Gently pipet and mix thoroughly.

4.2.2.2 Add 2 µL mRNA Boost Reagent to the above. Gently pipet and mix thoroughly.

4.2.2.3 Add 2 µL *TransIT*-mRNA Reagent to the above. Gently pipet and mix thoroughly.

4.2.2.4 Incubate at room temperature for 3 min. Do not incubate more than 5 min.

4.2.2.5 Add the complexes drop-wise to one well of a 12-well plate. Gently rock the plate back-and-forth and from side-to-side. Incubate for overnight at 37 °C.

4.2.3 Stemfect

4.2.3.1 Add 1 µg (10 µL of 100 ng µL⁻¹) mRNA cocktail into 60 µL Stemfect buffer. Gently pipet and mix thoroughly.

4.2.3.2 In a separate tube, add 4 µL Stemfect transfection reagent into 60 µL Stemfect buffer. Gently pipet and mix thoroughly.

4.2.3.3 Combine the 70 µL diluted mRNA and the 64 µL diluted Stemfect transfection reagent. Gently pipet and mix thoroughly.

4.2.3.4 Incubate at room temperature for 15 min.

4.2.3.5 Add the complexes drop-wise to one well of a 12-well plate. Gently rock the plate back-and-forth and from side-to-side. Incubate for overnight at 37 °C.

4.3 After the indicated incubation time, change the medium. If desired, mRNA transfection can be repeated daily or once every two days for continuous expression. Observe for fluorescent reporter and cell toxicity over the next few days. Confirm the expression of encoded protein by western blot or immunocytochemistry as shown in Figure 6.

Note: If high cytotoxicity was observed, supplement the media with B18R at 200 ng ml⁻¹. If low fluorescent reporter expression was observed, increase the dose of mRNA. Increasing the cell density and using low passage cells improve cell survival and transfection uptake. Additional optimization may be necessary.

REPRESENTATIVE RESULTS

The overall schematic of this protocol and its major steps are depicted in Figure 1. The ORF of gene of interest is amplified with N-terminal phosphorylated forward primer, which provides the necessary energy for the subsequent 5'UTR ligation. Energy necessary for the 3'UTR ligation is provided by N-terminal phosphorylation of 3'UTR. The 5'UTR and

3'UTR oligonucleotides are ligated to ORF with the assistance of 5' and 3'Bridge, respectively. The resulting 5'UTR-ORF-3'UTR construct is cloned into a vector which then transformed into ultra-competent *E. coli*. After subsequent bacterial amplification, the extracted clones are linearized in order to avoid producing run-on transcripts during the IVT reaction. The linearized templates, along with 5' cap analog and modified base pairs, are combined to generate the synthetic modified mRNA using *In Vitro* Transcription Kit. The newly synthesized mRNAs are transfected into the cells using one of three kits recommended in this protocol.

The details of 5'UTR and 3'UTR regions are shown in Figure 2. Special attention should be given to the three available restriction sites that are flanking both UTRs. Ensure that the restriction enzymes chosen are appropriate for both the UTRs and the gene of interest. If none of these three restriction sites can be used for a particular gene, the user can replace with the appropriate restriction sequence when synthesizing the UTRs. Ensure that the GC stability is present when making changes to the restriction sites. Other regions to note are the actual UTR sequences. The UTRs shown here are derived from alpha globin gene of *Mus. musculus* which have shown to have great stability and expression. UTRs are common sites of transcript regulation, therefore other UTRs may be more desirable depending on the need of particular experiment. In such situations, ensure that both BBS oligonucleotides are appropriately changed to match the new UTR sequences. Similarly, the length of poly(T)-tail, not shown in this figure, may be lengthened or shortened depending on desired degree of stability. Lastly, if users choose to perform IVT using other than the T7 RNA polymerase, the noted T7 Promoter sequence in this figure needs to be changed appropriately.

Key molecules used for IVT are shown in Figure 3. The four RNA bases used in this protocol are shown in Figure 3a. Other modified bases that have shown to be present in mammalian cells therefore may theoretically be used as substitute are shown in Figure 3b. Depending on the target mammalian cell types, other modified bases may be more appropriate. Please note that the substitution of other modified bases need to be validated. The red dashed circles note the modifications of each base pair in comparison to the generic ATCG. The Figure 3c shows the 5' anti-reverse cap analog (ARCA) used in this protocol and Figure 3d shows an interesting molecular modification called m⁶A_m that has shown to increase the half-life of mRNAs.^{50,51} Substituting ARCA with m⁶A_m, therefore, may change the decay rates of mRNAs which may provide for an interesting approach to investigating temporal dependent events. Modifications to this protocol need to be carefully validated for protein expression.

Gel electrophoresis of the major steps are shown in Figure 4. Ten ORFs (A to J) of varying sizes, 384 to 3336 nucleotides, and functions—histone variants (Th2b, Th2a, H1foo), embryogenesis (Npm2, Zar1), maternal-effect factors (Filia, Nlrp5), arginine-modifying enzyme (Padi6), oogenesis (Nobox)—have been successfully tested. The initial PCR products for each phosphorylated ORFs are shown in Figure 4a. The UTR double ligation of each sample shows expected upshift in the gel electrophoresis as shown in Figure 4b. The double ligated samples are subsequently cloned into vectors, which are then extracted and purified. The clones are linearized as seen in Figure 4d. Complete linearization is important for successful downstream IVT reaction therefore uncut clones should be ran in parallel for

comparison as shown in Figure 4c. The final DNA portion of the protocol concludes with attaching poly(T)-tail to 5'-ORF-3' template. As seen in Figure 4e, each sample shows the expected upshift—297 nucleotide increase to be exact—from their initial phosphorylated ORFs (Figure 4a) which would likely indicate successful 5'UTR, 3'UTR, and Poly(T) attachments.

Measure the concentration and purity of each major step of protocol using a NanoDrop and a Bioanalyzer as shown in Figure 5. The poor result (left) and the expected result (right) for DNA in Figure 5a correspond to post-PCR steps and plasmid extraction. For gel extraction steps, the yield will be less than what is shown. While the yield and purity of DNA can be suboptimal and still be successful, the yield and purity of RNA require higher standards. Pay particular attention to the yield and purity of mRNA products as shown on the right in Figure 5b. Poor yield or purity as shown on the left in Figure 5b requires repeat IVT reaction. In addition to NanoDrop, mRNA products require additional analysis by a Bioanalyzer. Ensure that the mRNA products have clean and discrete expected bands as shown in electrophoresis and electropherogram. Representative successful (Figure 5c) and poor (Figure 5d) Bioanalyzer results are shown.

The results of modified mRNA transfections are shown in Figure 6. Protein expression was observed in transfected cells with modified mRNA encoding a number of different fluorophores in Figure 6a. A cocktail of modified mRNA composed of OCT4, SOX2, KLF4, c-MYC, and LIN28A was transfected daily into human fibroblasts for 10 days. As shown in Figure 6b, human fibroblasts started to undergo morphological changes over time during this reprogramming process. The transfection was stopped and two days later, the colonies that emerged show immunostaining for SSEA-4 and OCT-3/4.

DISCUSSION

The comprehensive and detailed protocol presented here provides a non-viral, integration-free method for expressing proteins using modified mRNA.² This protocol can be applied to any gene of interest and therefore can replace most other gene expression methods. The inherent properties of synthetic modified mRNAs provide some unique advantages such as the ability to express varying stoichiometric ratios of vectors, to adjust the dose of each gene accurately, and to rapidly and completely silence expression.⁴

Furthermore, as shown in Figure 6b, modified mRNA method results in faster protein expression and higher efficiency than the commonly used DNA-based viral approach for hard-to-transduce cell types. Transfection efficiency, expression kinetics, and degradation rates of mRNA for *in vitro* cell culture and *in vivo* animal model have been well characterized by others.^{52–55}

To fully realize all of the advantages of this method, some generic precautions and optimizations are needed. Modified mRNA must be handled carefully and often requires fresh synthesis prior to experiments. All cell types must be optimized for transfection. This includes the dose of mRNA, types of transfection reagents, the ratios of mRNA to transfection reagents, the health and cell density, types of media, and other necessary

supplements such as B18R or serum. All such optimizations should be monitored with modified mRNA encoding a fluorescent reporter to provide fast and convenient feedback. Such reporters should always be included in assays.

One of the challenges of this method is the labor-intensive and time-consuming work associated with it. In contrast to an integrating viral method, which relies on the host cells to continuously generate the desired proteins, modified mRNAs must be repetitively synthesized and transfected into cells to maintain gene expression. The modified mRNA approach thus is not ideal for exploratory experiments or early stages of projects where large sets of genes are tested on a wide variety of cell types. One solution to this limitation is construction of a plasmid that bears all parts of the IVT template minus the ORF. This would streamline some of the labor-intensive work of generating modified mRNAs encoding large numbers of different genes. However, this would limit the ability to change or customize UTR regions and potentially increase the chance of mutations in the amplification steps.

Another caveat when applying this method is that, despite careful UTR designs and incorporation of modified bases, synthetic modified mRNAs are still potentially recognized as non-self nucleic acids by mammalian cells.⁴³ This could potentially activate an innate immune response and cytokine signaling that alters the intended effects of the modified mRNAs. If such immune responses are encountered, B18R must be included in the culture medium to inhibit interferon secretion caused by the modified mRNA transfection, and stringent purification procedures by HPLC, instead of the standard silica-based spin column purification, would be necessary to further reduce immunogenic responses.³

Despite the above limitations, synthetic modified mRNAs provide a safe and clinically applicable method for delivering genetic information into a mammalian cell. Previous concerns about mRNA stability and translation efficiency are largely addressed by this protocol. Other nucleotide modifications can be incorporated (Figure 3) to optimize for the cellular background and increase the stability of mRNA. Importantly, synthetic modified mRNAs have been used successfully to reprogram and differentiate cells at a higher efficiency and faster kinetic than commonly used viral approaches.^{4,10} This suggests that modified mRNA can be uniquely applied in areas of cell biology that may have been previously inaccessible by other methods.

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MATERIALS

Name of Material/Equipment	Company	Catalog Number	Comments/Description
KAPA HiFi HotStart ReadyMix	KAPA Biosystems	KK2602	store at –20°C
Ampligase Enzyme and Buffer	Epicentre Biotechnologies	A32750	store at –20°C
T4 Polynucleotide Kinase	New England Biolabs (NEB)	M0201S	store at –20°C
NEBNext dA-Tailing Module	NEB	E6053S	store at –20°C
TOPO TA Cloning Kit	Invitrogen	K4500-01	store at –20°C
One Shot TOP10 Chemically Competent E. coli	Invitrogen	C4040-03	store at –80°C
S.O.C. Medium	ThermoFisher Scientific	15544034	store at RT
Difco LB Broth	BD	240230	store at RT
Ampicillin, sodium salt, irradiated	ThermoFisher Scientific	11593027	store at 4°C
Falcon® 100 mm × 15 mm Not TC-Treated Bacteriological Petri Dish, Sterile	Corning	351029	store at RT

Name of Material/Equipment	Company	Catalog Number	Comments/Description
50x Tris/Acetic Acid/EDTA (TAE), Nucleic Acid Electrophoresis Buffer	Bio-Rad	1610743	store at RT
Agarose	Denville Scientific Inc	GR140-500	store at RT
QIAquick Gel Extraction Kit	QIAGEN	28704	store at RT
QIAquick PCR Purification Kit	QIAGEN	28106	store at RT
QIAprep Spin Miniprep Kit	QIAGEN	27106	store at 4°C for P1 Buffer, rest at RT
MEGAscript T7 Kit	Ambion	AM1334	store at -20°C
MEGAclear Kit	Ambion	AM1908	store at 4°C
Antarctic Phosphatase	NEB	M0289S	store at -20°C
3'-O-Me-m7G(5')ppp(5')G RNA Cap Structure Analog	NEB	S1411S	store at -20°C
Pseudouridine-5'-Triphosphate	TriLink BioTechnologies	N-1019	store at -20°C
5-Methylcytidine-5'-Triphosphate	TriLink BioTechnologies	N-1014	store at -20°C
Sall	NEB	R3138S	store at -20°C
SpeI	NEB	R0133S	store at -20°C
XbaI	NEB	R0145S	store at -20°C
Lipofectamine RNAiMAX	Invitrogen	13778-075	store at 4°C
TransIT-mRNA Transfection Kit	Mirus Bio	MIR 2225	store at 4°C
Stemfect™ RNA Transfection Kit	Stemgent	00-0069	store at 4°C
B18R Recombinant Protein Carrier-free	eBioscience	34-8185-85	aliquot and store at -80°C
Stemfactor™ B18R Recombinant Protein, Carrier-free	Stemgent	03-0017	aliquot and store at -80°C
pRVGP (d2eGFP)	Addgene	Plasmid 15377	sequence verify and store at 4°C for short-term or -20°C for long-term
RNaseZap™ RNase Decontamination Solution	Life Technologies	AM9780	store at RT
TE, pH 7.0	ThermoFisher Scientific	AM9861	store at RT
Opti-MEM™ 1 Reduced Serum Medium	ThermoFisher Scientific	31985070	store at 4°C
Nucleases-free 0.5 mL Sterile Microcentrifuge Tubes	Capitol Scientific	022600001	store at RT
Nucleases-free 1.5 mL Sterile Microcentrifuge Tubes	Fisher Scientific	05-402-24B	store at RT
Falcon 15 mL Conical Centrifuge Tubes	Fisher Scientific	352097	store at RT
Falcon 50mL High Clarity PP Centrifuge Tube, Conical Bottom, Sterile	Corning	352098	store at RT
Falcon 24 Well Clear Flat Bottom TC-Treated Sterile	Corning	353047	store at RT
Falcon 12 Well Clear Flat Bottom TC-Treated Sterile	Corning	353043	store at RT
Falcon 6 Well Clear Flat Bottom TC-Treated Sterile	Corning	353046	store at RT

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Corning 100mm TC-Treated Culture Dish	Corning	430167	store at RT
Nucleases-free Sterile Aerosol-barrier Tips	Denville Scientific Inc		10, 20, 200, 1000 μ L
Nucleases-free Sterile Serological Pipettes	Fisher Scientific		1, 5, 10, 25 mL
Pipetman	Fisher Scientific		P2, P10, P20, P200, P1000
Pipet-Aid	Fisher Scientific		or equivalent
Gloves	Denville Scientific Inc		or equivalent
Gel Electrophoresis Equipment	Bio-Rad		or equivalent
Vortex-Mixers	VWR		or equivalent
Microcentrifuges	Eppendorf		or equivalent
ThermoMixer	Eppendorf		or equivalent
PCR Thermocycler	Bio-Rad		or equivalent
NanoDrop	ThermoFisher Scientific		or equivalent
Bioanalyzer	Agilent Technologies		or equivalent
Cell Incubator	ThermoFisher Scientific		or equivalent
Fluorescence Microscope	Nikon		or equivalent

Figure 1. Schematic overview of the method

Major steps are depicted with corresponding Steps on the left-hand side. Gaps between major steps, as indicated by the breaks in vertical lines, indicate possible pause points. MCS refers to SalI, SpeI, and XbaI restriction sites.

Figure 2. UTR maps and sequences

(A) 5'UTR and its restriction, binding, and promoter sites are shown. Note the ATG on the 3' end which obviates the need for an ORF-FW start codon. (B) 3'UTR and its restriction and binding sites are shown. Other UTR sequences are compatible with this protocol as long as the various corresponding sequences noted here also are changed appropriately.

Figure 3. Nucleosides and 5' caps of mRNA

(A) Canonical and modified base pairs used in this protocol. (B) Other nucleoside modifications for immune escape. Dashed red circles note base modifications. (C) The 5' cap used in this protocol. (D) Alternative 5' cap that has been shown to increase the half-life of eukaryotic mRNA.⁵⁰ Blue and orange indicate modifications.

Figure 4. Gel electrophoresis of all intermediate and final DNA products

(A) The beginning PCR products of the N-terminal phosphorylated ORF. (B) Double ligation of 5' and 3' UTRs. (C) Undigested cloned vectors. (D) Linearized cloned vectors. (E) Poly(T)-tail attached DNA templates for the IVT reaction. Note the corresponding increase in size at each step. A = Th2b (384 bp), B = Th2a (390 bp), C = Npm2 (624 bp), D = H1foo (915 bp), E = Zar1 (1086 bp), F = Filia (1323 bp), G = Nobox (1584 bp), H = Padi6 (2049 bp), I = Nlrp5 isoform b (3288 bp), J = Nlrp5 isoform a (3336 bp). Th2b, Th2a, H1foo (histone variants), Npm2, Zar1 (embryogenesis), Filia, Nlrp5 (maternal-effect factors), Padi6 (arginine-modifying enzyme), Nobox (oogenesis)

Figure 5. Quality control of DNA and RNA products

(A) Representative NanoDrop microvolume spectrophotometer measurement of a typical cloned DNA extraction (left) and of a typical IVT RNA result (right). **(B)** Analysis of successfully generated (left) and of unsuccessfully generated (right) modified mRNAs by Bioanalyzer automated electrophoresis instrument.

Figure 6. Modified mRNA protein expression and reprogramming of transfected cells
(A) A number of different cell lines were transfected with modified mRNAs encoding fluorophore variants: stabilized eGFP, destabilized eGFP, nuclear-localizing eGFP, or mCherry. HEK = human embryonic kidney, mNS = mouse neurosphere, hBJF = human foreskin fibroblasts, mESC = mouse embryonic stem cells, AD = human Alzheimer's fibroblasts. **(B)** Time-course comparison of eGFP expression on mESC by lentivirus (top) or by modified mRNA (bottom). Multiplicity of infection of 5 in the presence of polybrene at $8 \mu\text{g mL}^{-1}$ were used for lentiviral transduction. Lipofectamine RNAiMax was used for modified mRNA transfection. **(C)** Modified mRNA cocktails comprised of OCT4, SOX2, KLF4, c-MYC, and LIN28A were transfected into human fibroblasts every day. Daily morphological changes are shown. Characteristic mesenchymal-to-epithelial transitions typically observed in cellular reprogramming are marked with yellow dashed circles. An emerging colony with sharp boundary is marked with a solid yellow arrow. **(D)** Emerging colonies after several days post-transfection showing immunostaining of SSEA-4 and OCT-3/4. Scale bars, 100 μm .