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Using Lipid Nanoparticles for the Delivery of Chemically Modified mRNA into Mammalian Cells

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TITLE:

Using Lipid Nanoparticles for the Delivery of Chemically Modified mRNA into Mammalian Cells

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SUMMARY:

The protocol presents in vitro transcription (IVT) of chemically modified mRNA, cationic liposome preparation, and functional analysis of liposome enabled mRNA transfections in mammalian cells.

ABSTRACT:

In recent years, chemically modified messenger RNA (mRNA) has emerged as a potent nucleic acid molecule for developing a wide range of therapeutic applications, including a novel class of vaccines, protein replacement therapies, and immune therapies. Among delivery vectors, lipid nanoparticles are found to be safer and more effective in delivering RNA molecules (e.g., siRNA, miRNA, mRNA) and a few products are already in clinical use. To demonstrate lipid nanoparticle-mediated mRNA delivery, we present an optimized protocol for the synthesis of functional me1Ψ-UTP modified eGFP mRNA, the preparation of cationic liposomes, the electrostatic complex formation of mRNA with cationic liposomes, and the evaluation of transfection efficiencies in mammalian cells. The results demonstrate that these modifications efficiently improved the stability of mRNA when delivered with cationic liposomes and increased the eGFP mRNA translation efficiency and stability in mammalian cells. This protocol can be used to synthesize the desired mRNA and transfect with cationic liposomes for target gene expression in mammalian cells.

INTRODUCTION:

As a therapeutic molecule, mRNA offers several advantages due to its non-integrative nature and its ability to transfect non-mitotic cells when compared to plasmid DNA (pDNA)[1]. Although mRNA delivery was demonstrated in the early 1990s, therapeutic applications were limited due to its lack of stability, its lack of immune activation, and poor translational efficiency[2]. Recently

identified chemical modifications, such as pseudouridine 5'-triphosphate (Ψ-UTP) and methyl pseudouridine 5'-triphosphate (me1Ψ-UTP) on mRNA, helped to overcome these limitations, revolutionized mRNA research, and in turn, made mRNA a promising tool in both basic and applied research. The range of applications covers the generation of iPSCs to vaccination and gene therapy[3,4].

In parallel to advancement in mRNA technology, significant advances in non-viral delivery systems made the delivery of mRNA effective, making this technology feasible for multiple therapeutic applications[5]. Among the non-viral vectors, lipid nanoparticles have been found to be effective in delivering nucleic acids[6,7]. Recently, Alnylam has received FDA approval of lipid-based siRNA drugs for treating liver diseases, including Patisiran for hereditary transthyretin-mediated amyloidosis (hATTR amyloidosis) and Givosiran for acute hepatic porphyrias (AHP)[8]. During the COVID19 pandemic, lipid encapsulated mRNA based vaccines from Pfizer-BioNtech and Moderna demonstrated their efficacy and received FDA approvals[9,10]. Thus, lipid enabled mRNA delivery has a great therapeutic potential.

Here, we describe a detailed protocol for the production of chemically modified, in vitro transcribed eGFP mRNA, cationic liposome preparation, mRNA-lipid complex optimization and transfections into mammalian cells (**Figure 1**).

PROTOCOL:

1. Production of me1Ψ-UTP modified mRNA

1.1. In vitro transcription (IVT) DNA template preparation

NOTE: For IVT DNA template (T7 promoter- open reading frame (ORF) of the gene) preparation, design a gene-specific primer set for the gene of interest. Add the T7 promoter (5'-NNNNNNTAATACGACTCACTATAGGGNNNNNN-3') sequence before gene-specific forward primer.

1.1.1. Prepare PCR reaction mixture as described in **Table 1**.

NOTE: Run at least four PCR reactions to increase the IVT DNA template concentration and quality for IVT.

1.1.2. Completely mix the reaction mixture with a micropipette and spin down using a microfuge.

1.1.3. Run the PCR cycling protocol given in **Table 2** on a thermocycler.

1.2. Purification of IVT DNA template by organic extraction/ethanol precipitation

1.2.1. Adjust the amplified PCR reaction mixture to 200 µL total using DEPC-treated water in a 1.5 mL microfuge tube (nuclease-free).

1.2.2. Add 200 µL of TE-saturated phenol/chloroform, pH 8.0. Vortex vigorously for 10 seconds.

1.2.3. Centrifuge at 12,000 x g for 5 minutes to separate the phases and transfer the aqueous upper phase (approximately 200 µL) to a new 1.5 mL microfuge tube.

1.2.4. Add 1/10th (20 µL) volume of 3 M sodium acetate, pH 5.5 and two-volumes (400 µL) of 99-100% ethanol. Mix well and then incubate for at least 30 minutes at -20 °C.

1.2.5. Pellet the DNA template by centrifugation at 12,000 x g for 15 minutes at 4 °C.

1.2.6. Remove the supernatant completely without disturbing the pellet using a micropipette.

1.2.7. Add 0.5 mL of 75% ethanol to the pellet and invert 5-10 times.

1.2.8. Centrifuge for 2 minutes at 12,000 x g at 4 °C. Then remove the ethanol completely with a pipette without disturbing the DNA pellet.

1.2.9. Allow the pellet to dry at room temperature until the pellet becomes a little translucent.

1.2.10. Add 20 µL of nuclease-free water and re-suspend thoroughly for few seconds.

1.3. Quality control for the purified IVT DNA template

1.3.1. Quantification

1.3.1.1. Measure purified IVT DNA template concentration and quality using a micro-spectrophotometer.

NOTE: The expected DNA concentration will be around 300-600 ng/µL. Store the IVT DNA template at -20 °C for the long term.

1.3.2. DNA agarose gel electrophoresis

NOTE: This experiment is to verify whether the purified IVT DNA template is the correct size and devoid of non-specific product contamination.

1.3.2.1. To prepare a 1% agarose gel, add 0.5 g of agarose and 50 mL of 1x TAE in a conical flask. Microwave it until the agarose dissolves completely. Cool the agarose at room temperature for 5 minutes.

1.3.2.2. Add 1 µL of nucleic acid stain (SafeView dye) for a 50 mL agarose solution.

1.3.2.3. Pour the agarose solution into a gel casting tray with a comb and leave it until the gel becomes solidified.

1.3.2.4. Take out the comb from the gel and keep the gel in the 1x TAE buffered tank.

1.3.2.5. Mix 10 µL of 100-10000 bp DNA ladder and 100-200 ng of PCR purified template product with 2 µL of 6x DNA loading buffer to a total volume of 12 µL.

1.3.2.6. Load each sample on respective wells and run at 100 V for at least 45-60 minutes.

1.3.2.7. Visualize the DNA bands on a gel documentation instrument (**Figure 2**).

1.4. Synthesis of me1Ψ-UTP modified RNA

NOTE: Before starting this experiment, the working area (laminar airflow) should be cleaned with 70% ethanol in DEPC-treated water. Use sterile nuclease and endotoxin free, low retention tubes and filter barrier tips. Frequently apply 70% ethanol to gloved hands.

1.4.1. Prepare the IVT reaction mixture as given in **Table 3** at room temperature in a 0.2 mL tube and mix it thoroughly using a micropipette.

1.4.2. Spin the tube for 10 seconds in a microfuge.

1.4.3. Incubate at 37 °C for 3 hours in a thermocycler.

1.5. Degradation of IVT DNA template by DNase 1 treatment

1.5.1. Add 1 µL of 1 U/µL DNase 1 (RNase free) into the IVT reaction mix and incubate at 37 °C for 30 minutes.

1.6. Purification of RNA by organic extraction/ammonium acetate precipitation

1.6.1. Adjust the volume of the IVT reaction mix to 200 µL with 179 µL of DEPC-treated water.

1.6.2. Add 200 µL of TE-saturated phenol/chloroform pH 8.0. Vortex it for 10 seconds.

1.6.3. Centrifuge at 12,000 x g for 5 minutes at 25 °C to separate the two phases.

1.6.4. Transfer the aqueous upper phase (200 µL) to a 1.5 mL tube and add 200 µL of 5 M ammonium acetate. Mix well and then incubate for 15 minutes on ice to precipitate the RNA.

1.6.5. Pellet the precipitated RNA by centrifugation at 12,000 x g for 15 minutes at 4 °C and remove the supernatant completely with a micropipette.

1.6.6. Wash the RNA pellet by using 70% ethanol and invert 5-10 times. Centrifuge at 12,000 x g for 5 minutes at 4 °C.

1.6.7. Remove the supernatant completely with a micropipette without disturbing the RNA pellet.

1.6.8. Allow the pellet to dry at room temperature till the pellet become semi-translucent. Then re-suspend the pellet in 60-75 µL of RNase-free water.

1.7. Quality control for purified RNA

1.7.1. Quantification

1.7.1.1. Measure the purified RNA concentration and quality using a micro-spectrophotometer.

NOTE: The expected RNA yield will be 140-180 µg for unmodified RNA and 100-150 µg for me1Ψ-UTP modified RNA per reaction, depending on the size of the gene of interest and quality of IVT DNA template. The optimal quality should be a OD_{260}/OD_{280} ratio around 1.9-2.0 and OD_{260}/ OD_{230} ratio >2.0. Store the RNA at -20 °C for a short time.

1.7.2. Denaturing RNA agarose gel electrophoresis

NOTE: This experiment is performed to verify whether the synthesized RNA is of the correct length and devoid of IVT by-product contamination.

1.7.2.1. To prepare a 1% agarose gel, add 0.5 g of agarose and 50 mL of 1x TAE in a conical flask. Microwave the solution until the agarose gets dissolved completely. Keep the agarose solution in a room temperature for 5 minutes to cool down.

1.7.2.2. Add 1 µL of nucleic acid stain for 50 mL of 1% agarose solution.

1.7.2.3. Pour the agarose solution into the gel casting tray with a comb and leave it until the gel becomes solidified.

1.7.2.4. Take out the comb from the gel and keep the gel in the 1x TAE buffered tank.

1.7.2.5. Prepare the RNA loading dye sample as described in **Table 4**.

1.7.2.6. Heat the samples at 65 °C for 10 minutes and then keep samples on ice.

1.7.2.7. Load each sample on the respective wells and run at 100 V for at least 45-60 minutes.

1.7.2.8. Visualize the RNA bands on a gel documentation instrument (**Figure 3**).

1.8. Synthesis of me1Ψ-UTP modified mRNA by enzymatic based capping & poly-A tailing

NOTE: The capping efficiency of IVT RNA using the enzymatic method is 100%. Hence, we used enzymatic capping of Cap-1 in mRNA synthesis in this protocol. We added poly-A tails at a length of >150 A bases per molecule to improve the translational efficiency of mRNA.

1.8.1. Add 55-60 µg of purified IVT RNA and make it up to 72 µL with the RNase free water in a 1.5 mL tube.

1.8.2. Denature the RNA at 65 °C for 10 minutes in a thermomixer and then immediately place the tube on ice for 5 minutes.

1.8.3. Meanwhile, prepare the capping reaction mixture as shown in **Table 5**.

1.8.4. Add the capping reaction mixture and 4 µL of capping enzyme to the denatured RNA and mix well by micropipette. Spin the tube for 10 seconds in a microfuge.

1.8.5. Incubate the reaction mixture at 37 °C for 2 hours.

1.8.6. After 2 hours, keep the tube on ice and prepare the Poly A tailing master mix as given in **Table 6**.

1.8.7. Add the Poly A tailing master mix to the capped RNA solution and mix well by micropipette. Spin the tube for 10 seconds in a microfuge.

1.8.8. Incubate the reaction mixture at 37 °C for 2 hours.

NOTE: mRNA can be further subjected to purification immediately, or crude mRNA can be stored at -20 °C overnight.

1.9. IVT mRNA purification

1.9.1. Purify the mRNA by organic extraction/ammonium acetate precipitation as given in protocol section 1.6.

1.9.2. Re-suspend the mRNA pellet with 60 µL of RNase-free water.

1.10. Quality control for the purified mRNA

1.10.1. Follow quality control protocol as described in protocol section 1.7.

NOTE: The mRNA band should appear above the RNA band due to the addition of Poly-A tailing in the denaturing RNA agarose gel electrophoresis (**Figure 3**). Also, Poly-A tailing increases the mRNA yield (should be > RNA concentration). After quantification, put multiple aliquots of mRNA at 1 µg/µL concentration and store immediately at -80 °C. Avoid multiple freeze and thaw cycles of RNA to prevent the degradation of synthesized mRNA.

2. Preparation of cationic liposomes and evaluation of in vitro mRNA transfection properties

2.1. Liposome preparation

2.1.1. For the preparation of 1 mM cationic liposome, use cationic lipid: DOPE: cholesterol in the molar ratio of 1:1:0.5.

2.1.2. Dissolve appropriate molar ratios of the cationic lipid, cholesterol, and DOPE (1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine) in chloroform (200 µL) in a glass vial.

2.1.3. Use a thin flow of moisture-free nitrogen gas for solvent removal.

2.1.4. Keep dried lipids under a high vacuum for further drying for 2 hours.

2.1.5. Add 1 mL of sterile deionized water to dried lipids after vacuum-drying and allow the mixture to swell overnight.

2.1.6. Vortex the vial at room temperature to make multi-unilamellar vesicles (MUVs).

2.1.7. Use bath sonication followed by probe sonication at 25 W power to make small unilamellar vesicles (SUVs) from multi unilamellar vesicles (MUVs).

NOTE: The SUVs should look like a translucent liposome solution. If not, increase the number of 30-second pulses on and off with an interval of 1 min. Hydrodynamic diameters and surface potentials are measured (**Figure 4**) in a particle size analyzer.

2.2. mRNA/liposomes complex formation and gel retardation assay

2.2.1. For the preparation of different lipid–RNA charge ratios from 1:1 to 8:1, dilute the mRNA and cationic liposome in deionized water separately as given in **Table 7**.

2.2.2. Mix the diluted mRNA to liposome solution as indicated in **Table 7** and incubate it for 10 minutes at room temperature for lipoplex formation.

2.2.3. Add 20 µL of 2x RNA loading dye into the complex and load onto the well. mRNA alone serves as a control.

2.2.4. Load the samples on a 1% agarose gel in 1x TAE buffer and run at 100 V for 45 minutes.

2.2.5. Visualize the RNA bands on a gel documentation instrument (**Figure 5**).

2.3. In vitro mRNA transfection

2.3.1. Seed 45,000 mammalian cells per well of 48 well plates in complete media and then incubate at 37 °C for 16 to 20 hours of transfection.

2.3.2. After 16 to 20 hours, check the cell density. At the time of transfection, the cell confluence should be around 80%.

2.3.3. To 0.5 mL tubes, add 150 ng of GFP protein-encoding mRNA complex with a 1:1 charge ratio of cationic liposomes and mRNA in DMEM medium without serum. The total volume makes up to 20 µL.

2.3.4. Incubate at room temperature for 10 minutes.

2.3.5. Add lipoplex into the cells and incubate it for 4 hours in a 37 °C and 5% CO_2 incubator.

2.3.6. Remove the media without disturbing the cells. Add 250 µL of complete media with 10% FBS (**Table 8**) into each well.

2.3.7. After 72 hours of transfection, view GFP expression under a fluorescent microscope (**Figure 6, 7**).

2.3.8. To quantify the GFP expression, process the cells for flow cytometer analysis.

2.3.9. Remove the media and wash with 1x PBS twice. Trypsinize the cells and process the cells to quantifying the percentage of GFP positive cells in a flow cytometer.

2.3.10. Acquire the cells in a flow cytometer using Laser 488. Gate the live population from that and analyze the percentage of GFP positive cells. Quantify the mean fluorescent intensity (MFI) (**Figure 6, 7**).

REPRESENTATIVE RESULTS:

We optimized the protocol for me1Ψ-UTP modified mRNA production, liposome preparation,

and mRNA transfection experiments with cationic liposomes into multiple mammalian cells (**Figure 1**). To synthesize mRNA, the mammalian codon-optimized eGFP IVT template was amplified from the mEGFP-N1 mammalian expression vector and purified by organic extraction/ethanol precipitation method (**Figure 2**). Later, me1Ψ-UTP modified RNA and mRNA were produced by the IVT process. The denaturing RNA agarose gel electrophoresis data showed that these synthesized RNAs had good integrity and the correct length (750 base RNA, and ~1000 base mRNA with respect to the RNA ladder) (**Figure 3**).

To prepare cationic liposomes, a thin-film hydration method and sonication were used to form small unilamellar vesicles (SUVs). Physico-chemical characterization of the liposomes revealed that the hydrodynamic diameters were observed around 65 nm and the surface potentials were around +20 meV (**Figure 4**). A gel retardation assay was performed with liposome-mRNA complexes at varying charge ratios of lipid/base from 1:1 to 8:1. The cationic liposomes showed a high binding efficiency to mRNAs even at a 1:1 charge ratio (**Figure 5**). Hence, we used a 1:1 charge ratio for mRNA transfection experiments. Liposome mediated eGFP mRNA transfection experiments were performed in HEK293 and NIH/3T3 cell lines. eGFP expression was analyzed with flow cytometry. me1Ψ-UTP modified mRNA showed superior and stable eGFP protein expression when compared to unmodified mRNA in HEK-293T and NIH/3T3 cells on the 3rd-day post-transfection (**Figure 6, 7**).

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic presentation of me1Ψ-UTP modified mRNA production, liposome preparation and transfection protocol. The IVT DNA Template (T7 promoter-Gene ORF) is amplified by PCR and purified. me1Ψ-UTP modified mRNAs are generated by the IVT process using the IVT DNA template and purified. The cationic liposome is prepared and complexed with me1Ψ-UTP modified mRNA (Lipoplex) and can be transfected into mammalian cells.

Figure 2: Determination of IVT DNA template quality in agarose gel electrophoresis. The purified eGFP IVT DNA template was run on a 1% agarose gel and visualized by gel.

Figure 3: Analysis of size and quality of me1Ψ-UTP modified IVT RNA by denaturing RNA electrophoresis in agarose gel. The purified me1Ψ-UTP modified RNAs were denatured and loaded on a 1% TAE-agarose gel, and the size and quality of RNAs were determined by gel.

Figure 4: Physico-chemical characterization of the liposomes: Surface potentials (**A**) **and hydrodynamic diameters** (**B**)**.**

Figure 5: mRNA binding ability of liposome was determined by denaturing agarose gel retardation assay. Liposome-mRNA complexes (Lipoplexes) were prepared at different lipid/base charge ratios and loaded on a 1% agarose gel and gel documented.

Figure 6: Protein expression efficiency of me1Ψ-UTP modified eGFP mRNA in human HEK-293T cells. (**A**) Fluorescence images of unmodified eGFP mRNA and me1Ψ-UTP-modified eGFP mRNA transfected with cationic liposomes into HEK-293T cells obtained on 3rd-day post-transfection

(100x Magnification). % of eGFP protein expression (**B**) and mean fluorescent intensity (MFI). (**C**) of the transfected cells were analyzed using flow cytometry. (N=3)

Figure 7: Translation efficiency of me1Ψ-UTP modified eGFP mRNA in mouse NIH/3T3 cells. (**A**) Fluorescence images of unmodified eGFP mRNA and me1Ψ-UTP-modified eGFP mRNA transfected into NIH/3T3 cells obtained on the 3rd-day post-transfection (100x Magnification). % of eGFP protein expression (**B**) and mean fluorescent intensity (MFI). (**C**) of the transfected cells were analyzed using flow cytometry. (N=3)

Table 1: PCR reaction mixture preparation

Table 2: PCR cycling conditions

Table 3: IVT reaction mixture preparation

Table 4: RNA loading dye preparation

Table 5: Enzymatic Cap-1 synthesis reaction mixture

Table 6: Poly A tailing reaction mixture

Table 7: Preparation of lipoplex based on charge ratios

Table 8: Preparation of buffer and media

DISCUSSION:

Therapeutic applications of unmodified mRNAs have been limited due to their shorter half-life and their ability to activate intracellular innate immune responses, which in turn lead to poor protein expression in transfected cells[11]. Katalin et al. demonstrated that RNA containing modified nucleosides such as m5C, m6A, ΨU, and me1Ψ-UTP could avoid TLR activation[12]. More importantly, incorporation of ΨU or me1Ψ-UTP in IVT mRNA showed superior translational efficiency of target proteins, improved stability at room temperature, and prevented degradation from nucleases[13, 14].

In this video, we demonstrated the protocol for lipid enabled me1Ψ-UTP modified mRNA delivery into multiple cultured cells. The protocol includes production of me1Ψ-UTP modified mRNA, cationic liposome preparation, transfection into cells, and evaluation of protein expression. We used the mammalian codon-optimized eGFP reporter gene for transfection experiments to analyze protein expression levels by measuring fluorescent intensity. Cationic liposomes were prepared to complex mRNA, and their electrostatic complexation was analyzed at varying lipid/base charge ratios from 1:1 to 8:1. Since at 1:1, the cationic liposomes could completely complex mRNA, we used a 1:1 charge ratio for transfection. We demonstrated that transfection of mRNA with cationic liposomes could efficiently deliver both modified and unmodified eGFP mRNAs with 90% transfection efficiency in HEK293 cells, whereas there were 80% efficiency with

modified and 60% efficiency with unmodified mRNAs in NIH373 cells. More importantly, me1Ψ-UTP modified mRNA showed superior eGFP protein expression for 3 days in mammalian cells compared to unmodified mRNA (>6 fold in HEK-293T and >2 fold in NIH/3T3 cells). These studies demonstrated that modification of me1Ψ-UTP on mRNA could improve translation and stability of mRNA in mammalian cells.

The transfection efficiency of cationic liposome and translation efficiency of synthesized mRNA vary with different cell types. Hence, it is important to optimize mRNA concentration for each different cell type. Using the protocol, we synthesized functional me1Ψ-UTP modified mRNA, size up to 6 kb but the IVT DNA template concentration and time could be optimized to get good mRNA yield and correct length.

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DISCLOSURES:

No disclosures

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Figure 1

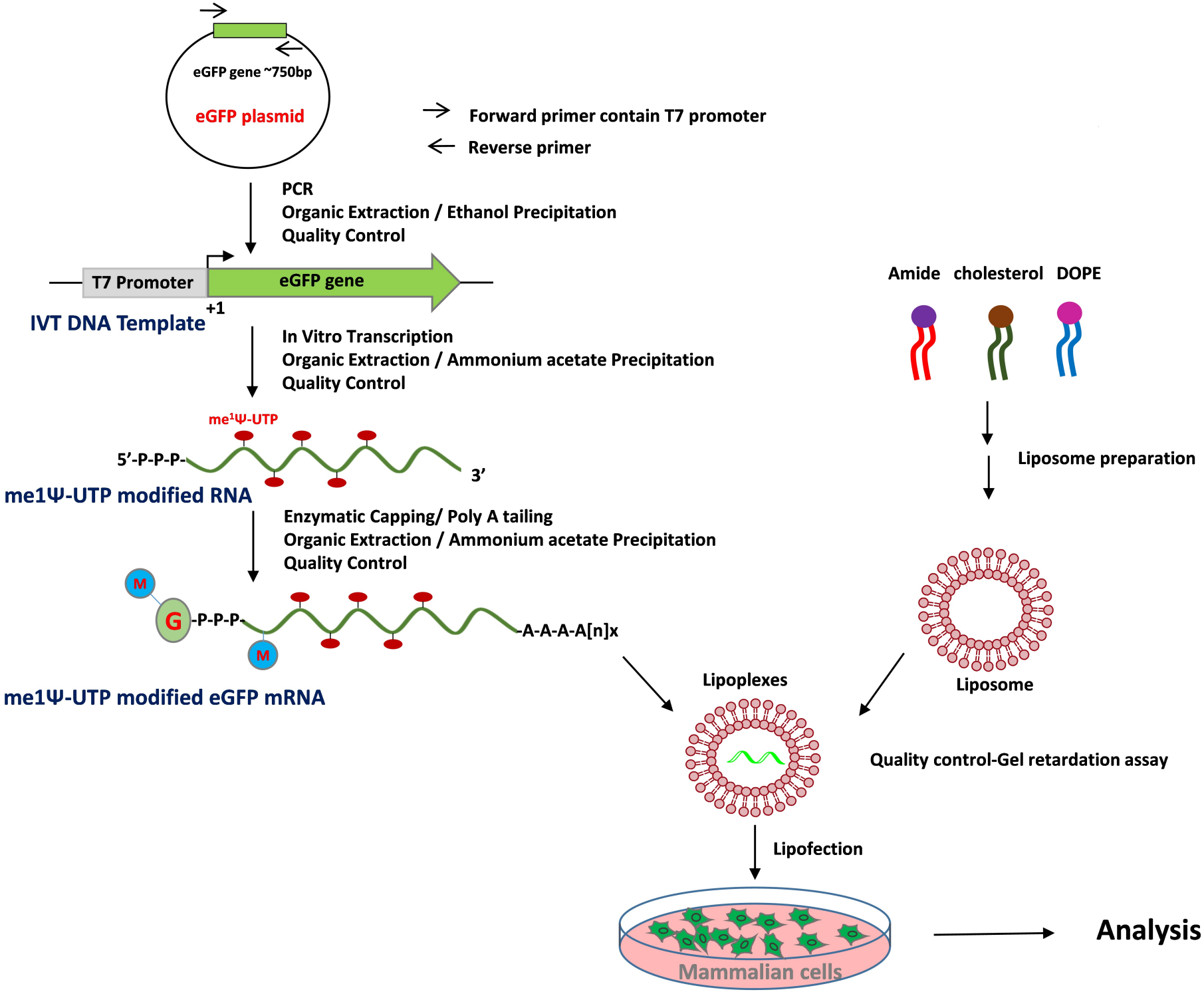


Figure 2

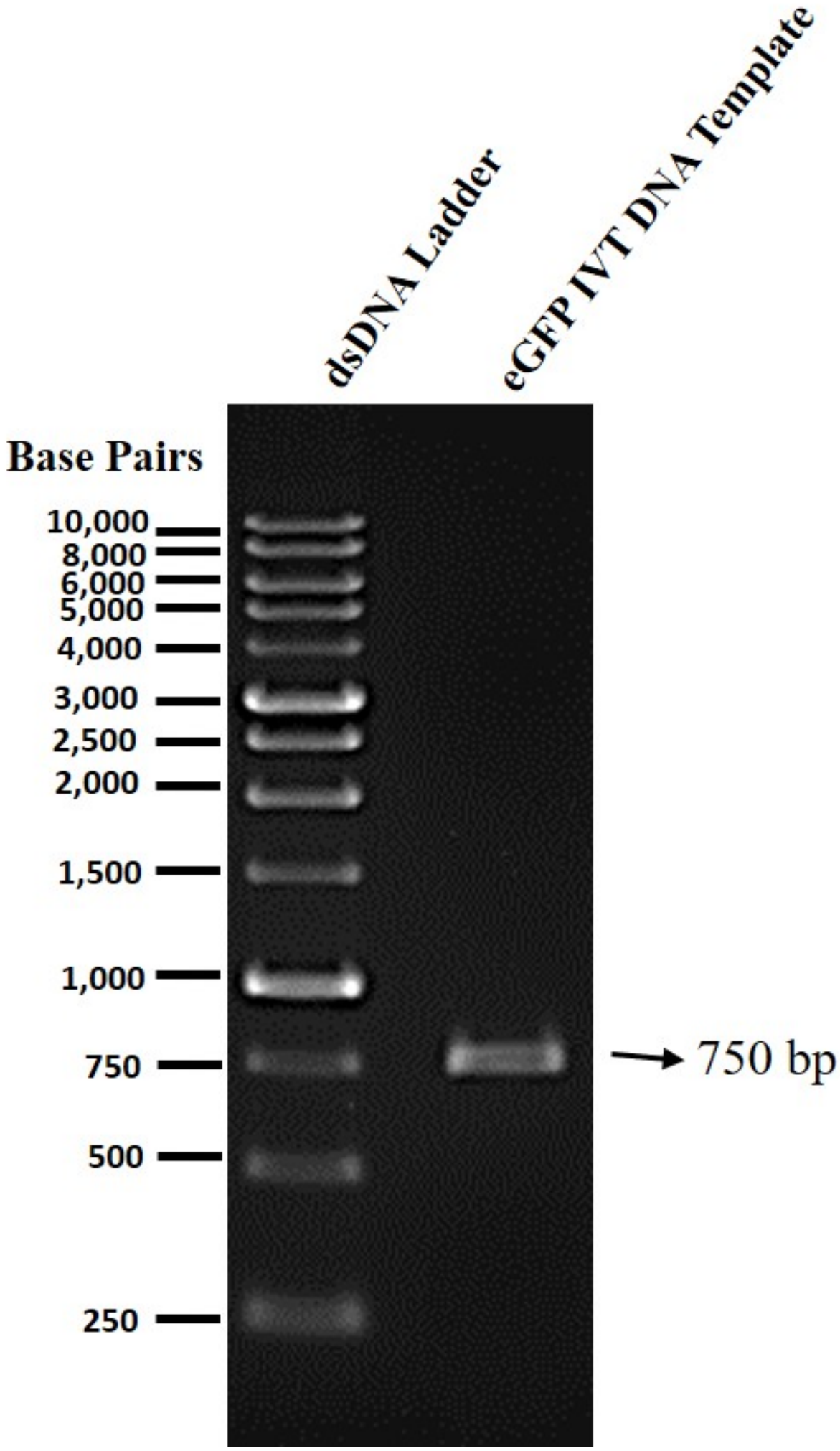


Figure 3

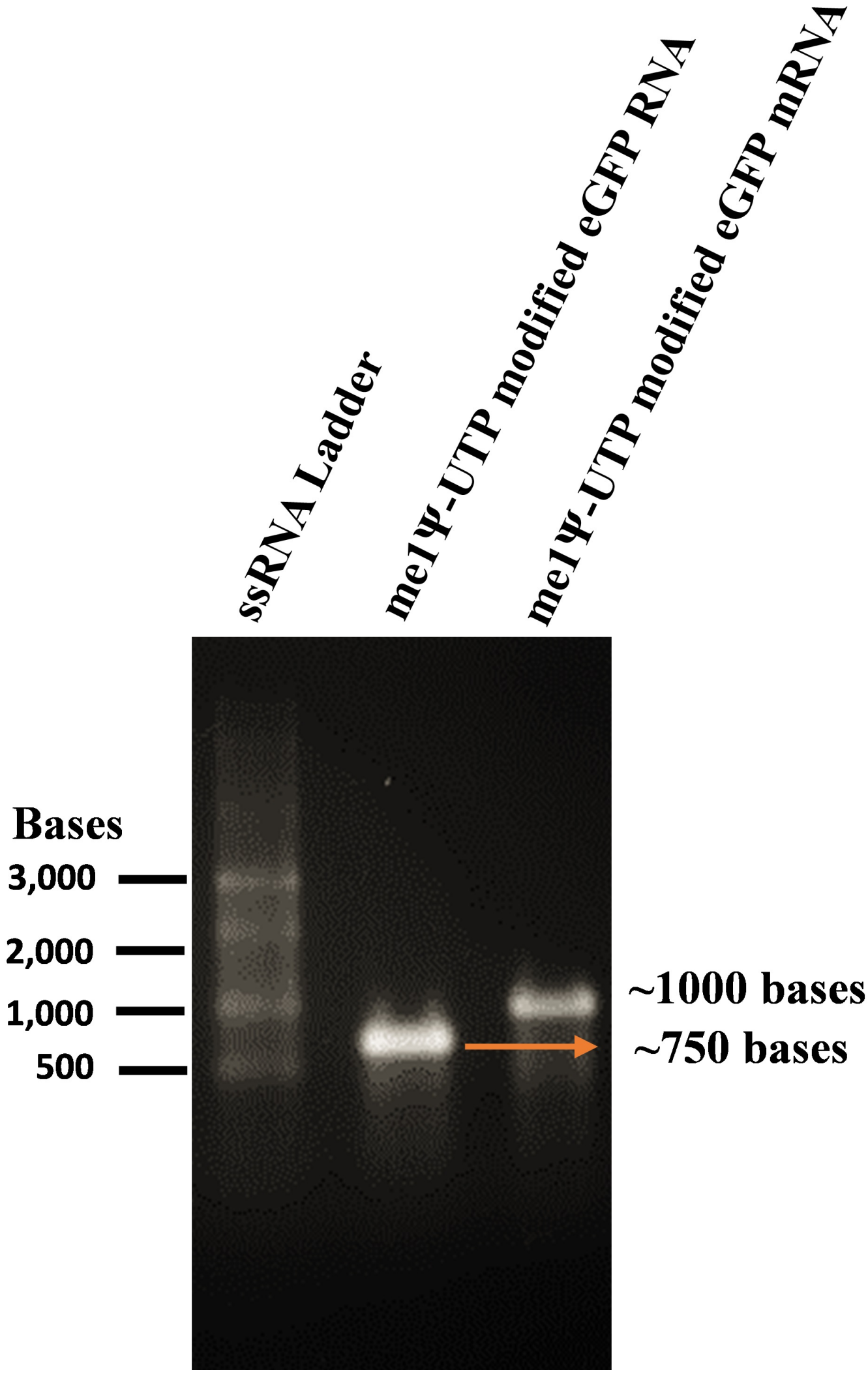
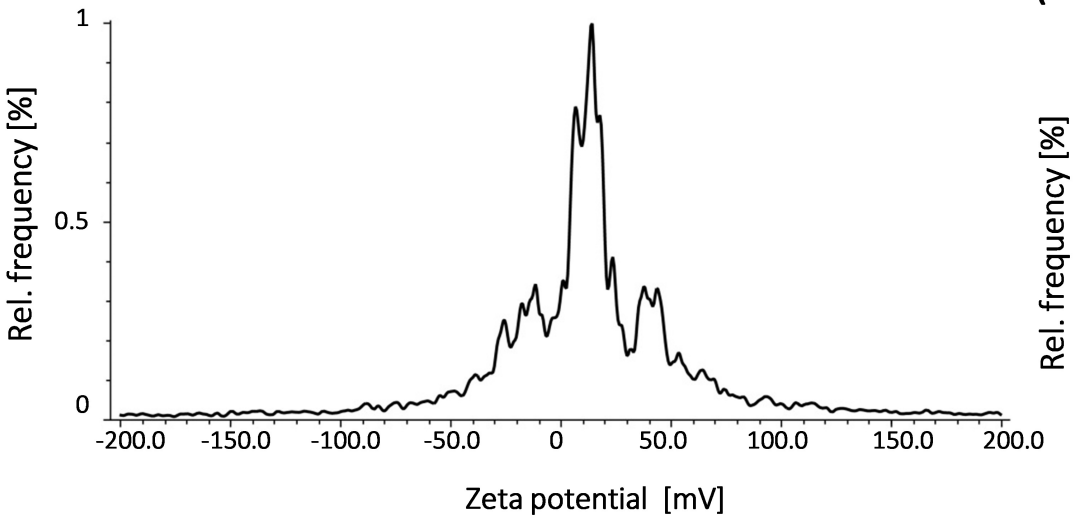


Figure 4

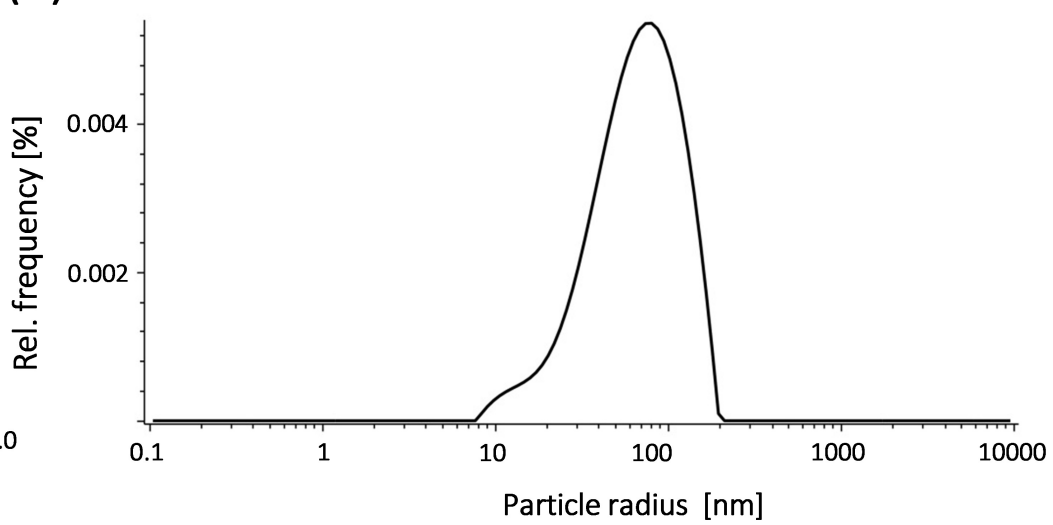
(A)



Data output

Mean zeta potential	19.4 mV	**Electrophoretic mobility**	1.5124 µm*cm/Vs
Distribution peak value	14.0 mV	**Filter optical density**	2.194
Processed runs	200	**Conductivity**	0.075 mS/cm
Adjusted voltage	200.000 V	**+/- Standard deviation**	1.2 mV
Mean intensity	621.8 kcounts/s		

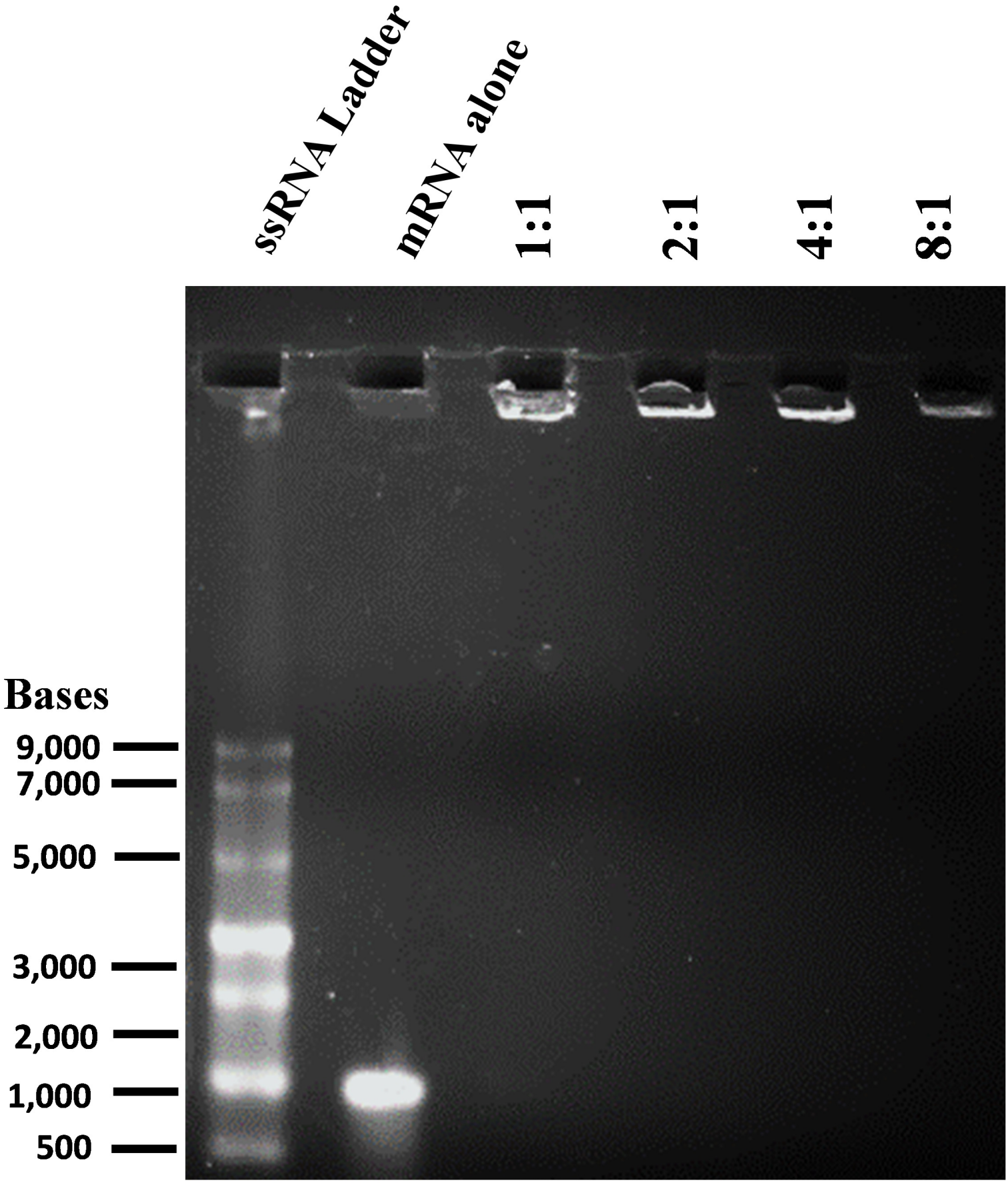
(B)

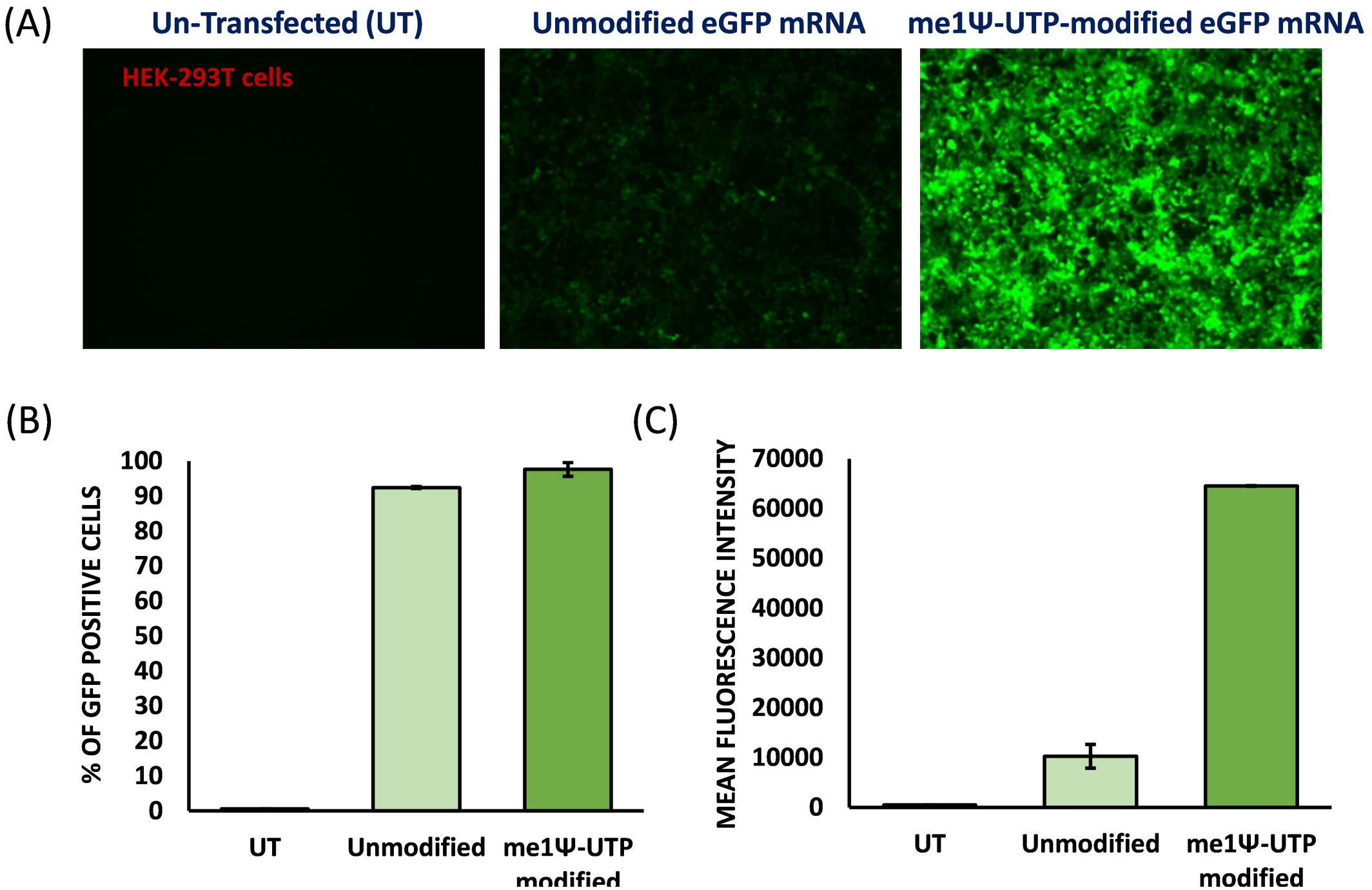


Data output

Hydrodynamic radius	64.73 nm	**Polydispersity index**	24.3 %
Intercept g1²	0.8059	**Mean intensity**	294.5 kcounts/s
Filter optical density	1.333	**Baseline**	1.001
Focus position	0.0 mm	**Angle used**	Back scatter
Processed runs	6	**Transmittance**	88.4 %

Figure 5





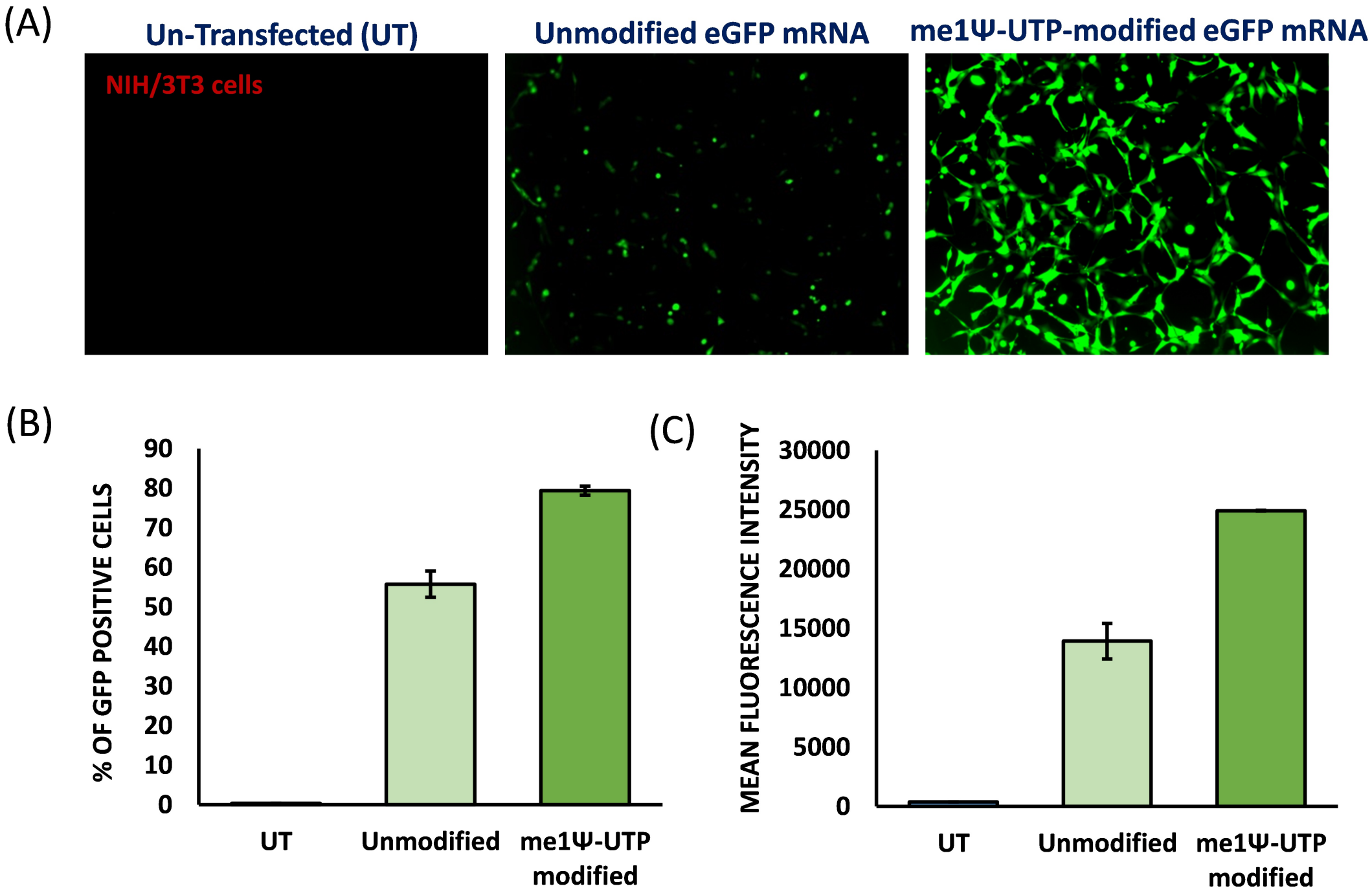


Table1: PCR reaction mixture preparation

Components	25 µL reaction	Final concentration
5x Q5 buffer	5 µL	1x
10 mM dNTP	0.5 µL	200 µM
10 µM Forward primer	1.25 µL	0.5 µM
10 µM Reverse primer	1.25 µL	0.5 µM
Q5 polymerase	0.25 µL	0.02 U/µL
Gene of interest in plasmid (template)	1-5 ng	variable
Nuclease free water	To 25 µL	

Table 2: PCR cycling condition

Steps	Duration	Temperature	Cycle number
Initial denaturation	30 seconds	98 °C	1
Denaturation	10 seconds	98 °C	
Annealing	20 seconds	variable	18-25
Extension	variable	72 °C	
Final extension	2 minutes	72 °C	1
Hold	∞	1 5°C	

Table 3: IVT Reaction mixture preparation

Components	Unmodified RNA
RNase-Free Water	variable
Linearized template DNA with T7 RNAP promoter	variable (1 µg)
10x T7 TranscriptionBuffer	2 µL
100 mM N1-methyl Pseudouridine	-
100 mM ATP	1.8 µL
100 mM UTP	1.8 µL
100 mM CTP	1.8 µL
100 mM GTP	1.8 µL
100 mM DTT	2 µL
40 U/µL RNase Inhibitor	0.5 µL
T7 Enzyme Solution	2 µL
Total Reaction Volume	20 µL

me1Ψ-UTPmodified RNA

variable

variable (1 µg)

2 µL

1.5 µL

1.8 µL

-

1.8 µL

1.8 µL

2 µL

0.5 µL

2 µL

20 µL

Table 4: RNA loading dye preparation

Components	RNA ladder	RNA sample
2x RNA loading dye (NEB)	6 µL	6 µL
RNA	2 µL	1 µL (0.5-1 µg)
DEPC treated water	4 µL	5 µL
Total volume	12 µL	12 µL

Table 5: Enzymatic Cap-1 synthesis reaction mixture

Components	Quantity
10x CappingBuffer	10 µL
20 mM GTP	5 µL
20 mM SAM	2.5 µL
RNase Inhibitor	2.5 µL
2'-O-Methyltransferase	4 µL
Total Volume	24 µL

Table 6: Poly A Tailing reaction mixture

Components	Quantity
5'-Capped IVT RNA	100 µL
RNase Inhibitor	0.5 µL
10x A-PlusTailingBuffer	12 µL
20 mM ATP	6 µL
4 U/µL A-Plus Poly(A) Polymerase	5 µL
Total Volume	123.5 µL

Table 7: Preparation of Lipoplex based on charge ratio

Charge ratio	Liposome	DI water	mRNA(500ng)	DI water
1:1	1.5 µL	8.5 µL	1 µL	9 µL
2:1	3 µL	7 µL	1 µL	9 µL
4:1	6 µL	4 µL	1 µL	9 µL
8:1	12 µL	-	1 µL	9 µL
Total volume (20 µL)	10 µL		10 µL	

Table 8: Preparation of Buffer and Medium

Name	Components
50x TAE buffer	Dissolve 50 mM EDTA sodium salt, 2 M Tris, 1 M glacial acetic acid in 1 L of water
HEK-293T and NIH/3T3 cell culture medium	DMEM with 4.5 g/L glucose, L-glutamine, 1% penicillin/streptomycin and 10% FBS

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Agarose	Lonza	50004	
Bath sonicator	DNMANM Industries	USC-100	
Cationic lipid	Synthesized in the lab		
Chlorofrom	MP biomedicals	67-66-3	"Caution"
Cholesterol	Himedia	GRM335	
DEPC water	SRL BioLit	66886	
DMEM	Lonza	12-604F	
DNA Ladder	GeneDireX	DM010-R50C	
DOPE	TCI	D4251	
EDTA sodium salt	MP biomedicals	194822	
Ethanol	Hayman	F204325	"Caution"
Fetal bovine serum	Gibco	10270	
Flow cytometry	BD	FACS Celesta	
Fluroscence Microscope	Leica	MI6000B	
Gel documentation system	Cell Biosciences	Flurochem E	
Glacial acetic acid	Fisher Scientific	85801	"Caution"
mEGFP-N1, Mammalian expression vector	Addgene	54767	
N1-Methylpseudo-UTP	Jena Bioscience	NU-890	
Phenol:chloroform:isoamyl alchol (25:24:1), pH 8.0	SRL BioLit	136112-00-0	"Caution"
Phosphate Buffer Saline (PBS), pH 7.4	CellClone	CC3041	
Probe sonicator	Sonics Vibra Cells	VCX130	
RNA ladder	NEB	N0362S	
RNase inhibitor	Thermo Scientific	N8080119	
SafeView dye	abm	G108	
Sodium acetate	Sigma	S7545	
Thermocycler	Applied biosystems	4375786	
Thermomixer	Eppendrof	22331	
Tris buffer	SRL BioLit	71033	
Trypsin	Gibco	25200056	

29th April, 2021

Dr. Vineeta Bajaj
Editor
JOVE

Dear Dr. Bajaj,

We sincerely thank the editorial team and reviewers for their constructive and insightful comments with suggestions on our manuscript "Lipid nanoparticle enabled delivery of chemically modified mRNA into mammalian cells" **(Manuscript ID: JoVE62407)**. Based on the comments, we have now revised the manuscript with the necessary modifications in the manuscript and proper explanations. The revised the manuscript has inclusion of additional text, figures and references wherever necessary. List of our itemized responses to all the comments raised by Reviewers are provided below immediately underneath the queries.

We thank you for giving us an opportunity to revise the manuscript. We hope that the present version meets the criteria.

Looking forward to receive your response.

Thanking you.

Sincerely,

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Our response to editorial comments:

Manuscript ID: JoVE62407

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
We have thoroughly checked spelling errors and grammar issues checked with grammarly software.

2. The current Abstract is under the minimum 150-word limit. Please rephrase the Abstract to meet this limit.
The abstract is below 150 word limit. Current abstract has 112 words

3. For in-text citations use E.g. "..therapy3,4." instead of "..therapy.3,4 ".
Now, we have changed referencing throughout the manuscript.

4. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Do not include "notes" as numbered steps of the protocol. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.
According to the suggestion, we have corrected the numbering as per JoVE guidelines and corrected the steps

5. Use appropriate symbols/abbreviations. Use "mL, µL, oC, s" instead of "ml, ul, uL, sec". Add a single space between the quantity and its unit: e.g. "5 mm" instead of "5mm". Follow this for the tables and figures as well.
Now we have corrected in the revised draft

6. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. E.g. GIVLAARI, FlowJo etc.
We have removed trademark symbols and provided the details in the Table of Materials.

7. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep, and note in the protocol section. Please use Calibri 12 points.
We have checked paragraph indentation, text font made it to Calibri 12 and line spacing made it to single.

8. Please check that the numbering of the figures corresponds to the legends.
We apologize for our mistake. Now, we have corrected that numbering of figures accordingly.

9. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Do not abbreviate journal names.
We used JoVE reference style in endnote to format the references, and verified that it was matching with the suggested format.

10. Please sort the Materials Table alphabetically by the name of the material.
Thank you for the suggestion. We have now corrected in alphabetical order.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The manuscript "Lipid Nanoparticle enable delivery of chemically modified mRNA into mammalian cells" describes a protocol of eGFP-mRNA production using in vitro transcription, and liposome production using sonication method. Following, formation of lipoplexes and transfection of two different types of cells with the lipoplexes. The manuscript should be carefully readed and corrected grammatically and in terms of content before publishing.

Major Concerns:
none

Minor Concerns:
1. The manuscript needs to be carefully reviewed for english grammer.
Thanks the reviewer for the comment corrected grammar errors with grammerly software.

2. In my oppinion the figures are not in the correct order. Please check the text and the figures, figure 1 and 2.
We apologize for our mistake and now corrected the order in the revised manuscript.

2. The numbering of the first section need to be checked. Point 1.2 exists two times.
We have corrected the numbering of sections in the revised version.

4. Figure3 and section 1.6.2 point 10: the note is not clear as well as the difference in the figure. The different terms in the descriptions are very confusing.
Note was given for suggestions. However, now we have removed the note in order to avoid confusion.

5. Section 2.1 point 1: is here the additional purification needed. The purification was described befor in section 1.8
We apologize for our mistake. That statement was repeated from the previous section. Now we have deleted in the revised manuscript.

6. Figure 6 and 7: You describe the transfection of different mRNA/Liposomes ratios. However you show only one figure of transfected mamalian cells without describing the ration in the figure description. Please add the pics and flow cytometry analysis of cells transfected with other ratios. or adjust the text section 2.3 and add a comment in the discussion why you choose this ratio to transfect the cells.
We thank reviewer for the suggestion. Now we have provided explanation in the discussion for choosing 1:1 lipid to base charge ratio.

7. You use the unmodified mRNA in transfections (see Figure 6 and 7). Is there no polyA tail and capping or is the no modified nucleotides. Please identify the modifications in this step and adjust the different terms for different modification in RNA.
We would like to clarify that both unmodified mRNA and Me1ψ-UTP modified mRNA contains poly A tail and Cap-1. We mentioned the Me1ψ-UTP modification of mRNA in figure 6&7.

8. Figure 6 and 7 and section 2.3 point 7: you describe that the cells were analysed 24 h post transfection. But in your results and figure dexription you write about analysis 3 days post transfection. So what is right?
We apologize for the confusion. We evaluated expression at 24 h and 72 h. In order to show the stability data, we showed the results at 72 h. Now we have corrected that in the description.

Reviewer #2:
Manuscript Summary:
This manuscript entitled "Lipid nanoparticle enabled delivery of chemically modified mRNA into mammalian cells" by Gokulnath Mahalingam et al., gives a full description of how: i) to produce a modified mRNA in vitro and cationic liposomes; ii) to assess the complexation of mRNA with liposomes and iii) the evaluation of the transfection efficiency of mRNA lipoplexes in 2 mammalian lines: NIH/3T3 cells and HEK 293 cells.
This protocol can be applied for any type of mRNA coding for a specific protein. It will be helpful for beginners who would like to perform mRNA delivery and is complementary for other Jove videos with the same subject as far as I know.

Major Concerns:
Below are different points that must be corrected to improve the description of the protocol.

A) in the part 1.5 Purification of RNA by Organic extraction/Ammonium acetate precipitation : For beginners or students, it would be good to add why some steps are dedicated for like precipitation, washing etc..
B) in the part 1.6.2 Denaturing RNA Agarose Gel Electrophoresis

We thank the reviewer for bringing this to our attention. In the revised version, we have provided the reasons in brief.

3. Please precise the nucleic stain used: Add 1µl nucleic acid stain for 50 ml of 1 % agarose solution.
We thank reviewer for bringing it to our attention. Now we provided the dye we used in the revised version.

C) 1.9 Quality control for Purified mRNAMake sure that you write mRNA instead of RNA for the sake of homogeneity

We agree with the reviewer and changed RNA to mRNA, where ever it is required.

We used term RNA, which is neither capped and nor added poly A tail to distinguish from mRNA.

D) In the part 2. Cationic Liposome preparation, This title has to be modified as this part includes as well how to evaluate the complexation of mRNA with liposomes as well as the transfection experiment-

We have modified the section title from "Cationic Liposome preparation" to "Preparation of cationic liposomes and evaluation of mRNA transfection properties in vitro"

In the part 2.1 Liposome preparation:

*** 1. Purify the mRNA by organic extraction/Ammonium acetate precipitation. This line must be taken out from this section as it concerns mRNA.**

We apologize for the mistake and deleted the sentence from the section.

*** in the part 2.2, the title must include mRNA/liposomes complexation and gel retardation assay.**

As per suggestion of the reviewer, we have now included the title for Part 2.2

- 2.3 In Vitro mRNA Transfection Then add lipoplex into cells and incubate it for 4 h at 37 oC/5 % CO2 incubator. Correct as: Then add lipoplex solution into cells and incubate it for 4 h at 37°C/5 % CO2 incubator.

The representative results must be thoroughly edited as there are some unclear sentences as for instance:

« The denaturing RNA Agarose Gel Electrophoresis data showed that synthesized these RNAs is good integrity and correct length (RNA, ~750 and mRNA, ~1000 bases with respect to RNA ladder)

« Post 24 hours of transfection, GFP expression viewed under fluorescent microscope (Figure 6, 7). »

We thank reviewer for the suggestion and corrected the sentences accordingly.

It would be interesting to show the data corresponding to the eGFP expression that can last up to 5 days in this protocol instead of putting it as "not shown".

We agree with the reviewer. But, we regret to inform that we cannot provide 5 day transfection data as it is being used in our other studies. Hence, we deleted that statement in the revised version.

Figures legends:

Figure 1 must be rewritten

As per the suggestion of reviewer, we have rewritten the figure legend in the revised version.

Figure 6 : Please correct the following sentence : The same (? ?) is subjected to quantification of eGFP protein expression by flow cytometry and the % of eGFP positive cells (B) and mean fluorescent intensity (MFI) (C) calculated by FlowJo software (N=3)
Now, we have corrected figure 6 legend.

Discussion: there are different sentences that are not well written and required to be edited Some examples are shown below:
*** « The critical limitations of In vitro transcribed, unmodified mRNA is: lesser stability, easilyrecognized and degraded by intracellular innate immune responses, which lead to poor proteinexpression in transfected cells.**

*** « We showed that single cationic lipoplex transfection, efficiently delivered eGFP mRNA (>80 transfection efficiency), and sufficient for high eGFP protein expression more than 3 days in mammalian cells ».**
Please correct > 80 to 80%.

*** « These studies revealed that modification of me1Ψ-UTP on mRNA, could be possible to maintain the target protein for longer periods in cells ».**

*** The transfection efficiency of cationic liposome and translation efficiency of synthesized mRNA are vary with different cell types. Hence the optimal mRNA concentration should be determined for each cell. Based on this protocol we synthesized functional me1Ψ-UTP modified mRNA, size up to 6Kb. But the IVT DNA template concentration and time could be optimized to get good mRNA yield and correct length.**

We humbly thank the reviewer for valuable suggestions. Now we have corrected these sentences in the revised version.