

mRNA research

Generating high-quality mRNA for *in vivo* delivery

mMESSAGE mMACHINE T7 mRNA Kit with CleanCap Reagent AG works with LNP technology for effective *in vivo* delivery of mRNA

Highlights

- The mMMESSAGE mMACHINE T7 mRNA Kit with CleanCap Reagent AG can generate high yields of mRNA (>5 mg/mL) with over 95% capping efficiencies.
- Modified mRNA is associated with improved performance, higher cell viability, and lack of inflammatory response when used for transfection of the BJ skin fibroblast cell line. Modified mRNA can be easily synthesized using the mMMESSAGE mMACHINE T7 mRNA Kit with CleanCap Reagent AG.
- Lipid nanoparticle (LNP) technology can effectively and safely deliver mRNA to the liver in mice.
- Crude mRNA preparations lead to transient inflammatory responses and elevation of liver enzymes in BALB/c mice regardless of mRNA modification. HPLC purification significantly reduces these effects.
- HPLC-purified modified mRNA helps ensure exceptional performance and safety, and is associated with longer luciferase protein expression *in vivo*.

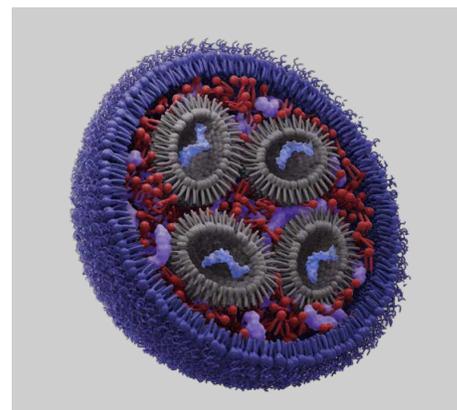
Keywords

in vitro transcription, IVT, mRNA, mRNA synthesis, lipid nanoparticle, LNP, *in vivo* delivery, mRNA therapeutics, mRNA capping technology

Introduction

In vitro transcribed (IVT) messenger RNA (mRNA) has emerged as one of the newest and most effective therapeutic modalities to prevent and treat various diseases ranging from infectious diseases to rare genetic disorders [1]. To function successfully, the mRNA needs to be of high quality and requires a safe and effective delivery system that can protect it from degradation and allow cellular uptake and release. Lipid nanoparticles (LNPs) have become one of the most effective modalities for mRNA delivery [2]. Currently, mRNA-LNP vaccines are in clinical use against COVID-19, which marks a milestone for mRNA therapeutics. Here we describe a workflow using the Invitrogen™ mMMESSAGE mMACHINE™ T7 mRNA Kit with CleanCap™ Reagent AG to produce high-quality mRNA that can be complexed with lipids to form mRNA-LNP and be used for *in vivo* delivery.

The mMMESSAGE mMACHINE T7 mRNA Kit with CleanCap Reagent AG can create capped mRNA in a simple and flexible workflow. It contains the CleanCap analog to give high mRNA yields (>5 mg/mL) with high capping efficiencies (>90%) compared to legacy cap analogs such as the anti-reverse cap analog (ARCA), which is provided in the Invitrogen™ mMMESSAGE™ mMACHINE™ T7 ULTRA Transcription Kit and produces mRNA yields of 1–2 mg/mL with 70–80% capping efficiencies. CleanCap Reagent AG contains the Cap 1 structure that is found in humans and other mammals [3]. ARCA has the Cap 0 structure found in lower eukaryotes such



as the budding yeast *Saccharomyces cerevisiae* [3]. ARCA-capped mRNAs can be immunostimulatory in many mammals, including humans, because of antiviral pathways that become activated when a Cap 0 mRNA is detected [3,4]. Thus, mRNAs with the CleanCap analog can provide better translation than mRNAs with ARCA.

To achieve high yields and capping efficiencies when using CleanCap Reagent AG, a modified T7 RNA polymerase promoter with an AG initiation sequence is required (Figure 1A). If the wild-type promoter (which has a GG initiation sequence; Figure 1A) is used with CleanCap Reagent AG, capping efficiencies will be low (e.g., ~50%), negatively impacting mRNA performance *in vivo*. A DNA template with the wild-type promoter can be easily changed to have the modified promoter with the AG initiation sequence, by performing PCR with a primer containing the base change.

The introduction of IVT mRNA *in vivo* can activate toll-like, MDA5, and/or RIG-I receptors, which in turn triggers the innate immune response, leading to inflammation and decreased mRNA efficiency [5,6]. The quality of the mRNA is therefore crucial to its performance and immunogenicity. Literature suggests that modifying mRNA through the substitution of uridine with a modified uridine such as pseudouridine [5] or removing double-stranded RNA (dsRNA) by-products formed during mRNA synthesis [7,8] can significantly improve mRNA performance and reduce its immunogenicity. Here we show how modified mRNA can be easily synthesized using the mMESSAGE mMACHINE T7 mRNA Kit with CleanCap Reagent AG. We also demonstrate how ion-paired reverse phase high-performance liquid chromatography (HPLC) can be utilized to remove dsRNA for increased mRNA efficiency [6,8,9].

We explore the effect of mRNA modification and purity on performance and safety in a mouse model. An LNP platform* is employed to generate mRNA-LNPs. The composition consists of a proprietary ionizable lipid, a phospholipid, cholesterol, and a PEGylated lipid in ethanol that form a stable LNP upon mRNA encapsulation. The results show that mRNA modification and purity have a significant impact on performance, indicating that those factors should be considered when preparing mRNA for *in vivo* use.

Materials and methods

DNA template synthesis

A plasmid containing the firefly luciferase (*fLuc*) coding region flanked by 5' and 3' untranslated regions (UTRs) was ordered from Invitrogen™ GeneArt™ services (sequence available upon request). Immediately upstream of the 5' UTR–*fLuc*–3' UTR sequence is a T7

RNA polymerase promoter with an AG initiation sequence (Figure 1A). This AG initiation sequence is essential for obtaining the high yields and capping efficiencies with the mMESSAGE mMACHINE T7 mRNA Kit with CleanCap Reagent AG. An *fLuc* DNA template with a 120-nucleotide poly(A) tail was created by PCR using Invitrogen™ Platinum™ SuperFi™ II PCR Master Mix, the *fLuc* plasmid, a forward primer with the modified T7 RNA polymerase promoter sequence, and a reverse primer containing a 120-nucleotide poly(T) tract (Integrated DNA Technologies; sequence available upon request). The PCR product was purified using the Invitrogen™ PureLink™ PCR Purification Kit and diluted to 100 ng/μL in Invitrogen™ TE Buffer prior to *in vitro* transcription.

Firefly luciferase (*fLuc*) mRNA synthesis

The mMESSAGE mMACHINE T7 mRNA Kit with CleanCap Reagent AG was used for mRNA synthesis. Reactions were run following the 1 mg/200 μL protocol in the user guide. Modified mRNA was synthesized by substituting for UTP with a modified UTP. The mRNA was purified by lithium chloride (LiCl) precipitation as described in the user guide. Note that the LiCl precipitation protocol in the user guide is for the standard 20 μL *in vitro* transcription reaction; therefore, the protocol was scaled up 10-fold to purify the 1 mg transcription reactions. The mRNA pellets were dissolved in Invitrogen™ THE RNA Storage Solution. Concentrations were determined using a Thermo Scientific™ NanoDrop™ Eight Spectrophotometer.

Agarose gel

A 1.2% agarose gel was prepared using Invitrogen™ UltraPure™ Agarose in diluted UltraPure™ DNA Typing Grade 50X TAE Buffer with Invitrogen™ SYBR™ Safe DNA Gel Stain. The mRNA samples were

prepared by mixing 50 ng mRNA with Thermo Scientific™ RNA Gel Loading Dye (2X) and heating at 70°C for 10 min. Samples were chilled on ice for at least 5 min and then loaded onto the agarose gel. Samples were run in the gel in 1X TAE buffer at ~5 V/cm until the bromophenol blue dye had migrated ~3/4 of the length of the gel. The gel was visualized on an Invitrogen™ iBright™ CL1500 Imaging System. The Thermo Scientific™ RiboRuler™ High Range RNA Ladder was included for RNA sizing and quantification.

Capping efficiency assay

A DNazyme 10–23 was designed to cut the mRNA transcripts 24 nt from the expected transcription start site (sequence available upon request). DNazyme reactions contained 1 μg mRNA with 0.5 μM DNazyme in 50 mM Tris-HCl, pH 7.5, in a 20 μL volume. Reaction mixtures were preheated at 85°C for 30 sec and equilibrated at 37°C for 5 min. Magnesium chloride was added to 10 mM and incubated at 37°C for 1 hr. Reactions were stopped by adding 2 μL Invitrogen™ TURBO™ DNase and incubating at 37°C for 30 min. Reaction mixtures were mixed with Invitrogen™ Novex™ TBE-Urea Sample Buffer (2X), heated at 70°C for 5 min, and chilled on ice for 5 min. Samples were loaded onto a 15% Invitrogen™ Novex™ TBE-urea polyacrylamide gel and run at 180 V until the bromophenol blue dye migrated to the bottom of the gel. Gels were stained with Invitrogen™ SYBR™ Gold Nucleic Acid Gel Stain for 10 min and washed in water for 2 min, and then visualized on an iBright instrument. Capping efficiencies were calculated by measuring band intensities of the capped and uncapped transcripts (Figure 2A). The uncapped and ARCA-capped *fLuc* mRNAs were included as controls. These mRNAs were synthesized using a similar DNA template but with a GG initiation sequence at the promoter.

* For more information, please email vivofectamine@thermofisher.com

HPLC purification to deplete dsRNA

dsRNA by-products were removed from mRNA samples using a 1260 Infinity HPLC system (Agilent Technologies) equipped with a Clarity™ 5 µm Oligo-RP 150 x 4.6 mm column (Phenomenex). The column oven was set to 65°C, and 100 µg mRNA was loaded onto the column. A linear gradient of buffer B (0.1 M triethylammonium acetate, pH 7.0, 25% acetonitrile) from 38% to 70% in buffer A (0.1 M triethylammonium acetate, pH 7.0) over 10 min at 1 mL/min was applied. Fractions were collected using an Agilent Technologies 1260 Infinity II Fraction Collector and desalted with Thermo Scientific™ Pierce™ Protein Concentrators (PES, 30K MWCO) using three 500 µL washes with THE RNA Storage Solution. Concentrations of the purified mRNA samples were determined by a NanoDrop spectrophotometer.

dsRNA dot blot assay

A dot blot assay to check for dsRNA was performed by blotting 200 ng mRNA onto Thermo Scientific™ Biotodyne™ B Pre-Cut Modified Nylon Membranes, 0.45 µm. The membrane was allowed to dry for 1 hr before blocking with reagents from the Invitrogen™ WesternBreeze™ Chemiluminescent Kit. The mouse J2 anti-dsRNA primary antibody (Jena Biosciences) was diluted 1:5,000 into the Primary Antibody Diluent from the kit. The wash and secondary antibody incubations were performed as described in the user guide. The membrane was visualized on an iBright instrument.

Cell culture and transfection

The BJ human fibroblast cell line CRL-2522 was obtained from the American Type Culture Collection (ATCC). The cells were grown in Gibco™ MEM supplemented with 10% Gibco™ FBS in a 37°C incubator with 5% CO₂.

Cells were transfected using the Invitrogen™ Lipofectamine™ MessengerMAX™ Transfection Reagent. A day prior to transfection, 20,000 cells in 0.1 mL of medium were seeded in each well in a 96-well flat-bottom culture plate. For each transfection, 100 ng of *fLuc* mRNA was mixed with 0.3 µL Lipofectamine MessengerMAX Transfection Reagent in Gibco™ Opti-MEM™ Reduced Serum Medium in a total volume of 10 µL. This 10 µL mixture was added directly to cells in a well. As a positive control for innate immune activation, 12.5 mg of low molecular weight poly(I:C) (InvivoGen) was added to cells. The negative control was Opti-MEM medium mixed with Lipofectamine MessengerMAX reagent only. Cells were placed at 37°C after adding the transfection mixtures.

Cell assays

After 24 hours, the cell medium was transferred to a 96-well plate, sealed, and stored at –80°C until the interferon beta (IFN-β) ELISA. Cells were washed with 50 µL DPBS, and a cell viability assay was performed by adding to each well a 100 µL solution of Invitrogen™ PrestoBlue™ HS Cell Viability Reagent diluted 1:10 into prewarmed cell medium. The cells were incubated at 37°C for an hour before fluorescence was recorded on a Thermo Scientific™ Varioskan™ LUX Multimode Microplate Reader (560 nm excitation, 590 nm emission). After the viability assay, cells were washed twice with 50 µL DPBS. Next, the Thermo Scientific™ Pierce™ Firefly Luciferase Glow Assay Kit was used to assay for fLuc. Luminescence was measured on a Varioskan LUX Multimode Microplate Reader using 1,500 ms readings.

The frozen cell medium was thawed to room temperature prior to performing the IFN-β ELISA. The Invitrogen™ IFN-β Human ELISA Kit was used to measure the amount of IFN-β in the thawed medium.

Formulation of lipid nanoparticles

LNP technology was used for delivery. mRNA was diluted in 30 mM sodium acetate buffer (pH 5.2) to a final concentration of 0.215 mg/mL. Diluted mRNA was encapsulated in LNPs using a microfluidic instrument using a 3:1 aqueous to organic ratio at a flow rate of 12 mL/min. The mRNA-LNPs were dialyzed using a SpectraPor™ Float-A-Lyzer™ device (Repligen) in PBS (pH 7.4) for 4 hr at room temperature. Dialyzed mRNA-LNPs were diluted to 0.05 mg/mL mRNA using PBS.

In vivo experimentation

All mouse experiments were approved by the Institutional Animal Care and Use Committee (IACUC). A 0.5 mg/kg dose of mRNA-LNPs was injected into 8-week-old female BALB/c mice (Jackson Laboratories) intravenously via the tail vein. Control mice were injected with 200 µL of PBS. The mice were weighed prior to and 24 hr after injection to detect weight loss that could indicate toxicity.

In vivo imaging

Four hours after mRNA-LNP injection, the mice were injected intraperitoneally with 100 µL of IVISbrite™ D-Luciferin BIOLUMINESCENT Substrate in RediJect™ Solution (PerkinElmer). Mice were anesthetized using isoflurane, and the luciferase signal was analyzed 10 min after injection of the substrate, using an IVIS™ Lumina LT Series III *In Vivo* Imaging System (PerkinElmer).

Cytokine expression in serum

Mice were anesthetized and blood serum was collected from the retroorbital plexus into a serum separator collecting tube (Greiner Bio-One). The serum separator tube was centrifuged at 2,500 x g for 15 min at 4°C, and the supernatant was collected and stored at –80°C prior to analysis. Levels of inflammatory cytokines were analyzed using the Invitrogen™ ProcartaPlex™ Cytokine & Chemokine 36-Plex Mouse Panel and Luminex® 200™ instrument.

Results and discussion

mRNA synthesis and purification

Firefly luciferase (*fLuc*) was chosen as our reporter as it allows quantification of protein expression and visualization of biodistribution in mice, making it an attractive reporter for LNP testing. Figure 1A shows the schematic of the *fLuc* mRNA production workflow. We were able to obtain >1 mg of high-quality unmodified or modified mRNA using the 1 mg/200 μ L scale protocol for the mMMESSAGE mMACHINE T7 mRNA Kit with CleanCap Reagent AG (Figure 1B, 1C). Next, a capping efficiency assay was performed to determine what percentage of the *fLuc* mRNAs were capped (Figure 2A). The assay showed >95% capping (Figure 2B), which is expected for the mMMESSAGE mMACHINE T7 mRNA Kit with CleanCap Reagent AG.

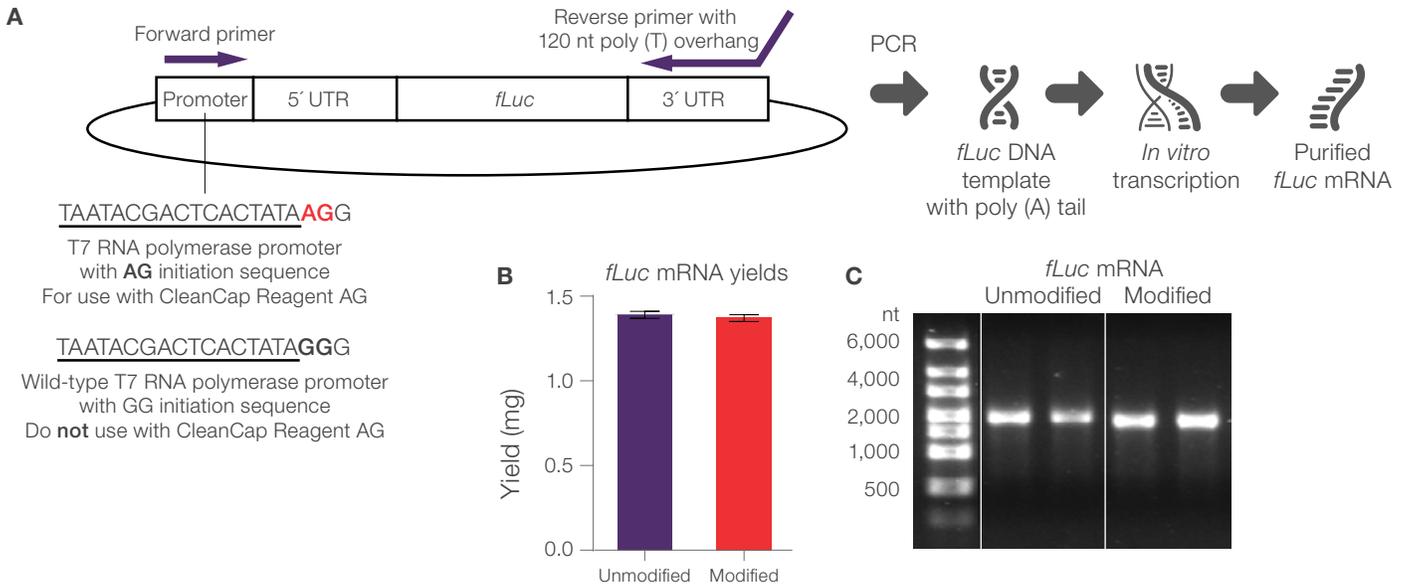


Figure 1. Firefly luciferase (*fLuc*) mRNA synthesis. (A) Schematic of the workflow for *fLuc* mRNA synthesis. The DNA template was prepared by PCR with a reverse primer containing a 120 nt poly(T) overhang to create an IVT template with a 120 nt poly(A) tail. The template was *in vitro* transcribed to generate *fLuc* mRNA, which was purified by lithium chloride (LiCl) precipitation. (B) The process yielded >1 mg of unmodified- or modified-uridine *fLuc* mRNA ($n = 2$; error bars denote standard deviation). (C) Agarose gel showing the *fLuc* mRNAs. The expected size was 2 kb.

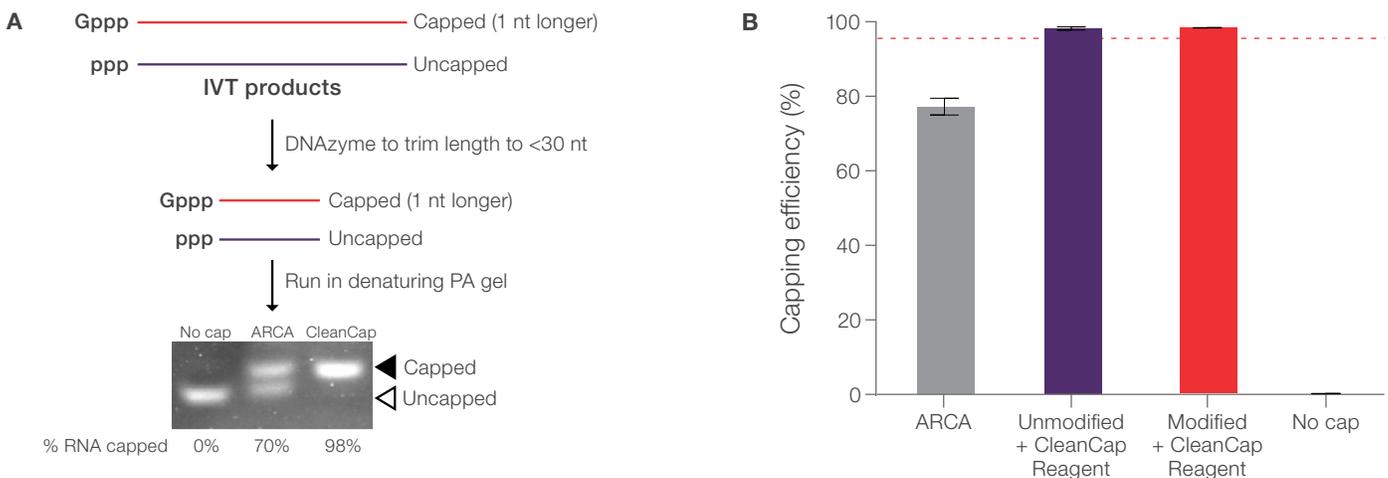


Figure 2. Capping efficiencies of *fLuc* mRNAs are >95%. (A) Schematic of the capping efficiency assay. The mRNAs are trimmed to <30 nucleotides long using a DNAzyme and run on a 15% denaturing TBE-urea polyacrylamide gel. Capping adds one nucleotide to mRNAs. Capping efficiency is calculated from the measured intensities of the capped band versus the uncapped band. (B) Quantification of capping efficiency. The mRNAs with CleanCap Reagent AG gave the expected >95% capping efficiencies (dotted red line). The mRNAs with ARCA gave the expected 70–80% capping efficiencies ($n = 2$; error bars denote standard deviation).

Ion-paired reverse phase HPLC was performed to remove the dsRNA by-product from the synthesized mRNA samples. Three separate fractions were collected from the main peak, as indicated in the chromatogram in Figure 3A. Assessment of mRNA quality by agarose gel and dsRNA levels by a J2-dsRNA dot blot assay (Figure 3B) indicates that the second fraction (HPLC-2) contains full-length mRNA depleted of dsRNA. The HPLC-1 fraction contains truncated mRNAs, and the HPLC-3 fraction contains most of the dsRNA by-products. The results also show that HPLC can eliminate almost all the dsRNA from the modified mRNA sample, as suggested by the J2-dsRNA dot blot assay. The HPLC-2 fraction was used for subsequent experiments.

***fLuc* mRNA performance evaluation by cell culture**

Prior to running *in vivo* experiments with the *fLuc* mRNAs, their performance was evaluated by cell culture to ensure that they can be translated into a functional luciferase protein and to assess how immunostimulatory they are. The cell line of choice is important for this type of evaluation, as some cell lines like HEK293 are immunocompromised [10], making them insensitive to mRNA purity. For these evaluations, we prefer to use the A549 human lung carcinoma epithelial cell line, the BJ human foreskin fibroblast cell line, or the JAWSII mouse immortalized immature dendritic cell line because they demonstrate innate immune response to dsRNA. For this study, the BJ fibroblast cell line was used.

BJ fibroblasts were transfected with unmodified or modified *fLuc* mRNA that was or was not HPLC purified (“crude” sample), using the Lipofectamine

MessengerMAX Transfection Reagent. Some cells were transfected with poly(I:C), a synthetic dsRNA analog, as a positive control for innate immune activation. Cells were assayed 24 hr later for viability, *fLuc* protein production, and secretion of interferon β (IFN- β), a cytokine that is part of the innate immune pathway.

The cell evaluation showed that HPLC purification of unmodified mRNA significantly increased cell viability, but not to the extent that the modified mRNA did (Figure 4A). Luciferase protein levels were the highest for the modified mRNA samples (Figure 4B). Activation of the innate immune system was the lowest for the HPLC-purified modified mRNA sample, as suggested by the IFN- β levels (Figure 4C). In all, the cell evaluation suggested that HPLC-purified modified mRNA will have the best performance *in vivo*.

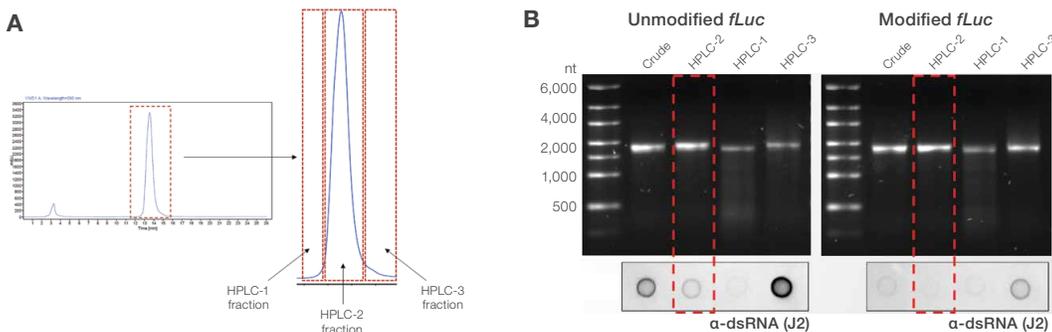


Figure 3. Ion-paired reverse phase HPLC to remove dsRNA from *fLuc* mRNAs. (A) Representative chromatogram for *fLuc* mRNA purification. The unmodified and modified mRNAs gave very similar chromatograms. Three fractions were collected from the main peak: the front tail end of the main peak (HPLC-1), the bulk of the main peak (HPLC-2), and the back tail end of the main peak (HPLC-3). (B) Agarose gels (top) and dsRNA dot blots (bottom) showing the quality of the HPLC-purified mRNAs. “Crude” are the input samples. The HPLC-2 fraction for both unmodified and modified mRNAs (red dashed boxes) contains the full-length mRNA that has been depleted of dsRNA. Equal amounts of mRNA were loaded for the agarose gels (50 ng) and the dot blots (200 ng).

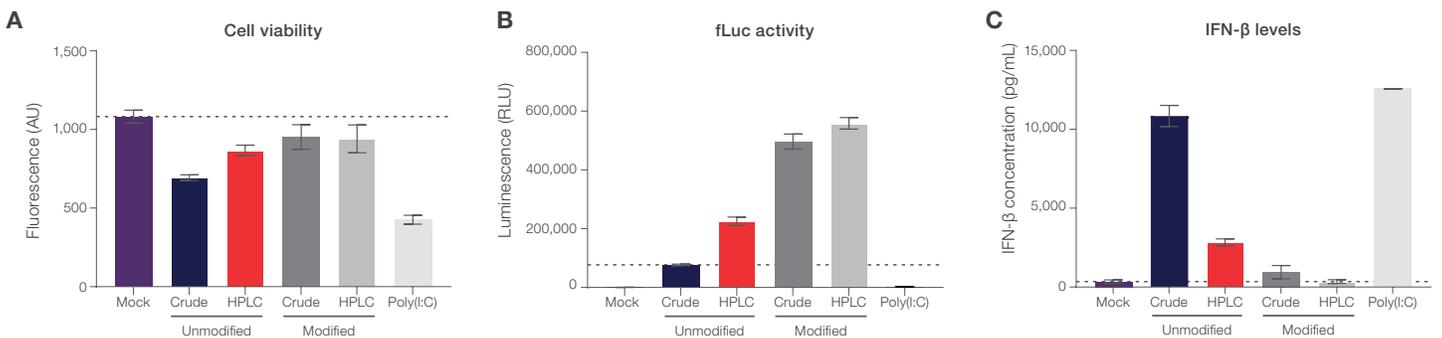


Figure 4. HPLC-purified modified *fLuc* mRNA has high protein expression with low immunogenicity. (A) Viability of cells transfected with the different *fLuc* mRNAs. The modified mRNAs demonstrated the highest cell viability. (B) *fLuc* protein activity. HPLC purification of mRNA significantly increased protein activity with the HPLC-purified modified mRNA showing the highest activity. (C) IFN- β levels in cell medium. HPLC-purified modified mRNA had very low levels, suggesting that it did not activate the innate immune system (n = 4; error bars denote standard deviation).

In vivo performance

To understand the effect of mRNA purity and modification on performance and safety of mRNA-LNPs *in vivo*, we encapsulated the 4 *fLuc* mRNA samples in LNPs using a microfluidic approach (Figure 5A). After formulation and dialysis, *fLuc* mRNA-LNPs were injected into mice intravenously.

Luciferase flux was analyzed *in vivo* 4 hr after injection (at the peak of expression) and 24 hr after injection to compare performance and duration of protein expression among the 4 groups. All groups showed high levels of expression in the liver 4 hr after delivery (Figure 5B). However, when the mice were imaged 24 hr after delivery, the HPLC-purified modified mRNA group demonstrated 10-fold higher expression than all three other groups, which is consistent with published data (Figure 5B, 5C) [11]. In the group of mice injected with HPLC-purified modified mRNA, we observed significantly better maintenance of expression between 4 and 24 hr (Figure 5B, 5C).

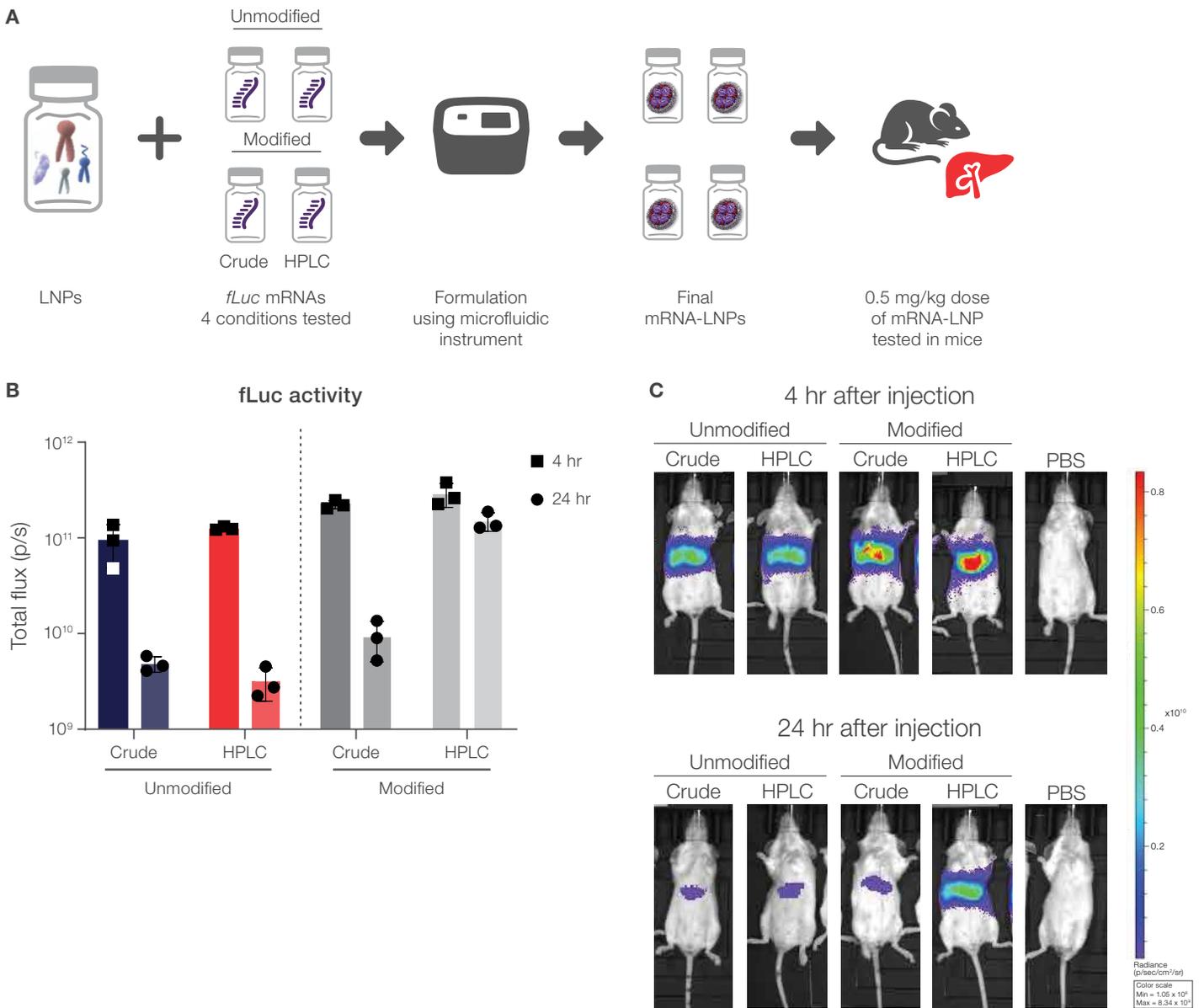


Figure 5. Changes in luciferase protein expression over time. (A) Experimental setup for encapsulation of *fLuc* mRNA with liver-specific VF232 formulation. (B) Quantification of luciferase expression in mice ($n = 3$, error bars denote standard deviation). (C) Visualization of luciferase expression in mice. One representative image per condition is shown for mice analyzed 4 hr after mRNA-LNP injection (top panel) and 24 hr after injection (lower panel).

To evaluate *in vivo* toxicity, we quantified body weight changes and liver enzymes. Liver enzymes (AST, ALT) are a known toxicity parameter for liver-directed delivery systems. Since the LNP formulations were used to deliver the mRNA predominantly to the liver, we evaluated liver enzymes as an early sign of any hepatocellular damage. All the groups showed less than 5% change in body weight over a 24 hr period, suggesting that neither mRNA was associated with substantial toxicity (Figure 6A). When quantifying AST and ALT 24 hr after injection, we observed that both crude mRNA groups showed substantial elevation in the liver enzyme levels (Figure 6B). The HPLC-purified mRNA groups had liver enzyme levels comparable to those of the PBS control mice.

We next assessed if a certain group was associated with an inflammatory response. Multiplex cytokine and chemokine profiling of mouse serum was employed using a 36-plex ProcartaPlex bead array. When comparing cytokine response 4 hr after injection, both crude mRNA groups showed a substantial inflammatory response (Figure 6C). HPLC-purified unmodified mRNA showed a low level of activation of inflammatory cytokines. The HPLC-purified modified mRNA group did not show any upregulation of cytokines, demonstrating that the response in other groups was driven by the payload, and not the LNP itself. This is consistent with the cell culture evaluation that indicated that the HPLC-purified modified mRNA had high protein expression with low immunogenicity. The levels of cytokines

in all groups returned to steady-state conditions after 24 hr (Figure 6D), indicating that this effect was transient. Interestingly, while mRNA modification was the main factor associated with cell viability and inflammatory response in *in vitro* culture, mRNA purification was more important for safety and inflammatory response *in vivo*, highlighting the differences in molecular responses *in vitro* and *in vivo*.

To conclude, we have shown that the mMESSAGE mMACHINE T7 mRNA Kit with CleanCap Reagent AG can be used to synthesize large quantities of high-quality capped mRNA for effective *in vivo* delivery with an LNP platform. Both mRNA modification and purity are important to ensure optimal expression and to avoid an immune response.

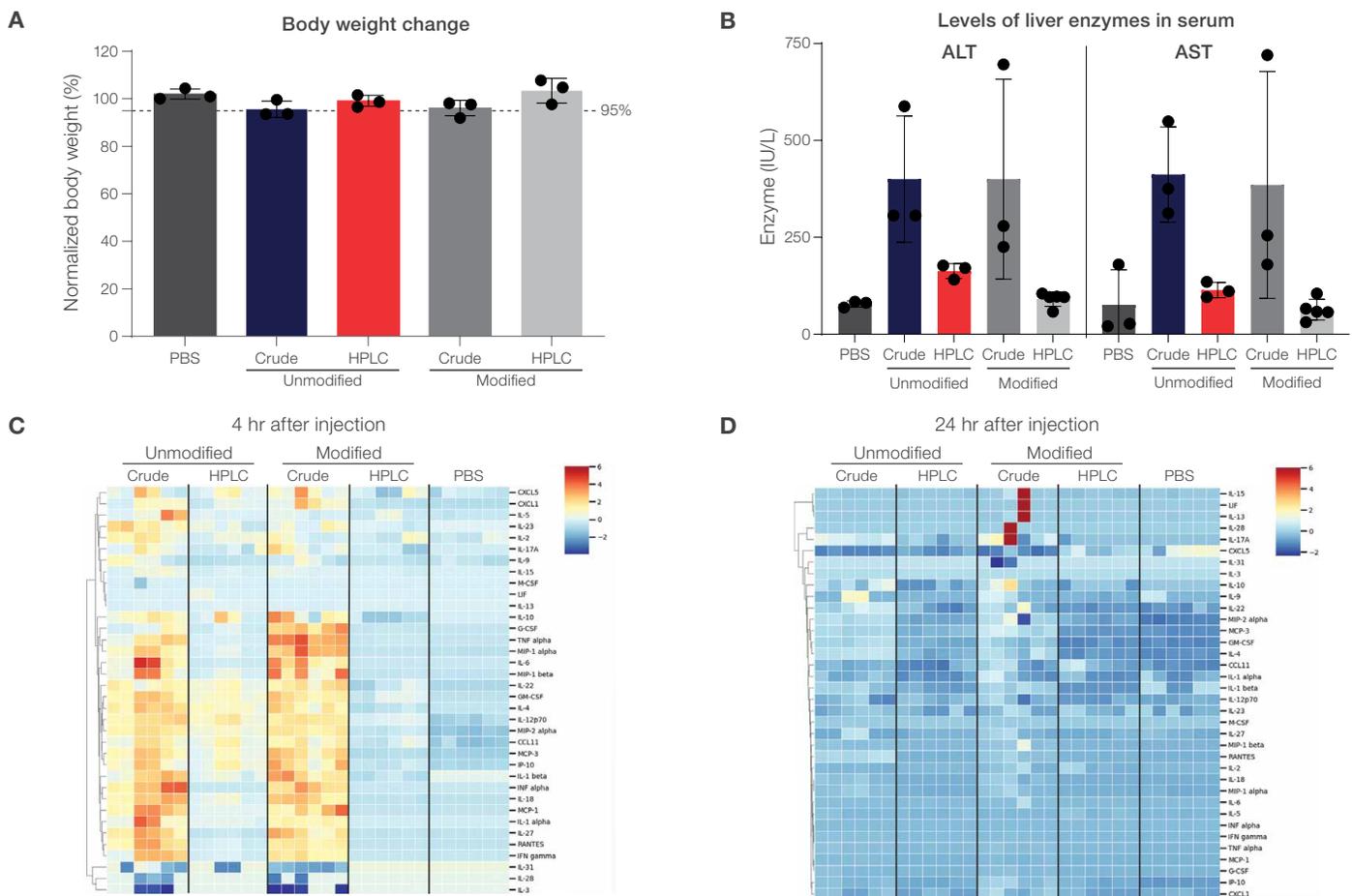


Figure 6. Crude mRNA drives elevation of liver enzymes in serum and is associated with inflammatory response. (A) Mice were weighed 24 hr after mRNA-LNP injection. Body weights were normalized to the initial weight at day 0. (B) Levels of liver enzymes ALT and AST were analyzed in serum 24 hr after injection with mRNA-LNPs or a PBS control (n = 3; error bars denote standard deviation). Serum cytokine levels (C) 4 hr and (D) 24 hr after mRNA-LNP injection. Three mice per time point per condition were analyzed, and technical duplicates were run.

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Ordering information

Description	Quantity	Cat. No.
mMESSAGE mMACHINE T7 mRNA Kit with CleanCap Reagent AG	50 reactions	A57620
	1,000 reactions	A57621
Lipofectamine MessengerMAX Transfection Reagent	0.1 mL	LMRNA001
	0.3 mL	LMRNA003
	0.75 mL	LMRNA008
	1.5 mL	LMRNA015
	15 mL	LMRNA150

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