Chapter 3

HPLC Purification of In Vitro Transcribed Long RNA

Drew Weissman, Norbert Pardi, Hiro Muramatsu, and Katalin Karikó

Abstract

In vitro transcription of DNA with phage RNA polymerases is currently the most efficient method to produce long sequence-specific RNA. While the reaction can yield large quantities of RNA, it contains impurities due to various unwanted activities of the polymerases. Here, we described an easily performed HPLC purification that removes multiple contaminants from in vitro transcribed RNA and is scalable. The purified RNA is translated at much greater levels, especially in primary cells and in vivo. HPLC purification of RNA containing modified nucleosides that suppress RNA-mediated activation of innate immune sensors leads to a non-immunogenic RNA with superior translational capacity.

Key words: RNA, In vitro transcription, Nucleoside modification, HPLC

1. Introduction

The first in vivo delivery of mRNA encoding a therapeutic protein was reported in 1992 (1), but only recently has the delivery of mRNA for scientific and therapeutic purposes gained expanded interest. Potential uses include; delivery of mRNA encoding transcription factors to generate induced pluripotent stem (iPS) cells (2–4), in vivo administration to express therapeutic proteins (1, 5, 6), ex vivo delivery to expanded cells as a cancer therapeutic (7–11), as the vector for vaccines (12–14), and in vitro delivery to express protein at a high efficiency (15–17). The recognition that the immunogenicity of RNA could be reduced by the incorporation of modified nucleosides with an associated increase in translation (16, 18) potentially allows efficient expression of proteins in vivo and ex vivo without activation of innate immune receptors. Unfortunately, modified nucleoside-containing RNA transcribed by phage RNA polymerases still retains a low level of activation of such pathways (4, 16, 19, 20). This remaining activation of RNA sensors could be due to modified nucleosides that do not completely

Peter M. Rabinovich (ed.), *Synthetic Messenger RNA and Cell Metabolism Modulation: Methods and Protocols*,
Methods in Molecular Biology, vol. 969, DOI 10.1007/978-1-62703-260-5_3,

suppress the RNAs ability to activate sensors (5) or dsRNA contaminants that activate even in the presence of nucleoside modification (15). It is well established that RNA transcribed in vitro by phage polymerase contains multiple aberrant RNA, including short RNAs as a result of abortive transcription initiation events (21) and double stranded (ds)RNAs generated by RNA-dependent RNA polymerase activity (22), RNA-primed transcription from RNA templates (23), and self-complementary 3′ extension (24).

Multiple methods of purification from transcription or associated reactions (capping and poly(A)-tailing) to produce efficiently translated mRNA have been described, including precipitation either with alcohol (isopropanol, ethanol) in the presence of monovalent cations or with high concentration of LiCl that are supplied in commercial in vitro transcription kits, size exclusion columns, and matrices based on silica and other compounds that bind RNA. Each of these procedures removes free nucleotides, proteins, salt, and short RNA oligos with varying efficiencies, but none are capable of removing long RNA contaminants or contaminants bound to the RNA of interest. Preparative denaturing polyacrylamide gel electrophoresis is commonly used to purify in vitro transcribed RNA; however, this method is suitable only for short RNAs (reviewed in ref. 25). Long RNAs can be separated on denaturing agarose gels, but they are not translatable due to covalent modifications introduced by the denaturants glyoxal and formaldehyde (26).

The continued development of mRNA as a research tool or for therapeutics requires the RNA to have high translatability, and in many instances, to avoid RNA sensor activation. The continued development of mRNA as a vector for vaccines, which is currently in human trials, and other therapeutic approaches also requires the highest levels of translation for both cost and potency. The HPLC approach described here allows efficient and scalable purification yielding RNA that can produce up to 1,000-fold more protein in primary cells and in vivo compared to unpurified in vitro transcribed RNA through the removal of dsRNA contaminants that activate RNA sensors that inhibit protein translation directly (reviewed in ref. 27, 28) or indirectly (reviewed in ref. 29). In addition, when such purified RNA contains certain modified nucleosides, it can also ablate activation of innate immune RNA sensors resulting in a highly translatable non-immunogenic RNA (15).

2. Materials

Use RNase-free and HPLC grade reagents, whenever possible. Acetonitrile is toxic, ensure proper protection is used. Its major

toxicity is due to metabolism to hydrogen cyanide. The onset of toxic effects typically occurs 2–12 h after exposure, due to the requirement for the metabolism to cyanide. Follow all waste disposal regulations when disposing waste materials.

2.1. HPLC Purification

1. HPLC system capable of monitoring optical density at 260 nm fitted with a fraction collector and a column heater. We use an ÄKTApurifier 10 FPLC with a Frac-920 fraction collector (GE Healthcare Biosciences, Piscataway, NJ) and a TL105 column heater (Timberline Instruments, Boulder, CO).
2. HPLC columns contain a nonporous matrix consisting of polystyrene-divinylbenzene (PS-DVB) copolymer beads (2 μm) alkylated with C-18 chains (Transgenomic, Omaha, NE) (see Note 1).
3. Buffer A: 0.1 M triethylammonium acetate (TEAA), pH 7.0 (Cat. number 553401) (Transgenomic).
4. Buffer B: 0.1 M TEAA, 25% acetonitrile, pH 7.0 (Cat. number 553402) (Transgenomic).
5. Acetonitrile: 100% for HPLC system cleaning.
6. NaOH: 0.1 M for HPLC system cleaning.
7. HPLC grade water.
8. Ethanol: 20% for long-term storage of HPLC system.

2.2. Concentration of RNA from Column Fractions

1. Amicon Ultra-15 centrifugal filter units (30K membrane) (Cat. number UFC903096) (Millipore, Billerica, MA).
2. Sorvall ST16R centrifuge (Thermo Scientific, Asheville, NC) or similar.
3. Nuclease-free water.
4. NaOAc: 3.0 M, pH 5.5.
5. Isopropyl alcohol.
6. Glycogen (Cat. number 10901393001) (Roche Applied Science, Indianapolis, IN) (see Note 2).

2.3. Analysis of RNA

2.3.1. dsRNA Dot Blot

1. Super charged Nytran membranes.
2. TBS-T buffer: 50 mM Tris–HCl, 150 mM NaCl, 0.05% Tween-20, pH 7.4.
3. Blocking buffer: 5% nonfat dried milk in TBS-T buffer.
4. Incubation buffer: 1% nonfat dried milk in TBS-T buffer.
5. dsRNA-specific mAb J2 (English & Scientific Consulting, Szirák, Hungary).
6. dsRNA-specific mAb K1 (English & Scientific Consulting).
7. HRP-conjugated donkey anti-mouse IgG (Cat. number 715-035-150) (Jackson ImmunoResearch, West Grove, PA).

8. ECL Plus Western blot detection reagent (Cat. number RPN2109) (Amersham/GE Healthcare Biosciences).
9. Fujifilm LAS1000 digital imaging system or similar.

2.3.2. RNA Translation

1. TransIT-mRNA transfection reagent containing TransIT-mRNA transfection and boost reagents (Cat. number MIR 2225) (Mirus Bio, Madison, WI) (see Note 3).
2. Human monocyte derived dendritic cells (DCs) for measuring RNA translation and immunogenicity (see Note 4).
   (a) Plate human primary monocytes at 1×10^6/ml, 3 ml per well in a 6-well plate in Aim V medium (Cat. number 12055-091) (Invitrogen, Grand Island, NY) supplemented with 50 ng/ml GM-CSF (Cat. number 215-GM-050) and 100 ng/ml IL-4 (Cat. number 204-IL-050) (R&D Systems, Minneapolis, MN).
   (b) On day 3 and 6 of culture, remove 1 ml of media from each well and add 1.5 ml of fresh Aim V medium supplemented with 50 ng/ml GM-CSF and 100 ng/ml IL-4.
   (c) On day 7–9, collect non-adherent cells and plate in a 96-well flat bottom plate in RPMI medium with 10% FCS and glutamine for transfection (see Note 5).
3. Luciferase Cell Culture Lysis 5× Reagent (Cat. number E1531) (Promega, Madison, WI) (see Note 6).
4. Firefly luciferase assay buffer (Cat. number E1500) (Promega) (see Note 6).
5. Luminometer, LUMAT LB 950 (Berthold/EG&G, Wallac/PerkinElmer, Waltham, MA) or similar.

2.3.3. RNA Immunogenicity

1. DCs (5×10^4 cells/well) in 96-well plates (see Note 7).
2. Poly(I:C): 50 μg/ml as a positive control.
3. Human IFN-α ELISA assay.

3. Methods

HPLC purification of RNA can be performed before or after the posttranscriptional addition of a 5′ cap with or without methylation (ScriptCap m7G capping system and ScriptCap 2′-O-methyltransferase kit, CELLSCRIPT) (see Note 8) or addition of poly(A) tail with poly(A) polymerase. The in vitro transcription reaction with or without DNase treatment or associated posttranscriptional modification reactions can be directly added to the HPLC column or any standard method of initial purification can be used before application to the HPLC column.

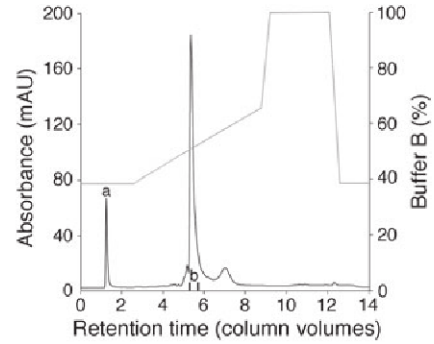

Fig. 1. HPLC purification of in vitro transcribed mRNA. RNA was applied to the HPLC column and eluted using a linear gradient of Buffer B (0.1 M TEAA, 25% acetonitrile, pH 7.0) in Buffer A (0.1 M TEAA, pH 7.0). The gradient spanned 38–65% Buffer B over 6 column void volumes (*gray line*). Absorbance at 260 nm was analyzed (*black line*), which demonstrated the expected sized RNA, as well as smaller and larger RNA species. A small peak is observed in the flow through (**a**). Column fraction selection noted by the *vertical black lines* on the *x*-axis (**b**) exclude RNA eluting before and after the expected major RNA peak.

Purify mRNA by LiCl sedimentation prior to HPLC.

The advantage of using a standard purification (LiCl or spin columns) prior to HPLC purification is that it helps to identify overloading of the HPLC column. When a standard purification is used first, a small peak measured by the OD at 260 nm representing residual nucleotides or other trace components of the transcription reaction will appear in the flow through (Fig. 1, noted as *a*). If the column is overloaded with RNA, a greater OD260 signal appears in the flow through fraction representing unbound RNA. In the absence of pre-purification, the flow through is overwhelmed by the constituents of the transcription reaction, namely nucleotide triphosphates, that give a signal 10–100-times greater than the purified RNA signal.

3.1. HPLC Purification of RNA Using a 35 ml Column

1. Set column oven to 45°C (see Note 9).
2. Set the flow rate to 5 ml/min.
3. Equilibrate column with 3 column void volumes (see Note 10) of 38% buffer B.
4. Load column with RNA in water (see Note 11) at one half the standard flow rate, 2.5 ml/min. Run 1.5 column void volumes of 38% buffer B after loading RNA.
5. Increase flow rate to 5 ml/min and run a 38–65% linear gradient of buffer B over 6 column void volumes.
6. Collect 5 ml column fractions corresponding to the major RNA peak excluding RNA eluting prior to and after (Fig. 1, noted as *b*).
7. Run a linear gradient from 65% to 100% buffer B over 0.2 column void volumes.

8. Run 100% buffer B for 3 column void volumes.
9. Run a linear gradient from 100% to 38% buffer B over 0.2 column void volumes.
10. Equilibrate with 38% buffer B for 1.5 column void volumes (see Note 12 for standard HPLC protocol).

3.2. RNA Contamination of HPLC Column

RNA contamination from previous purifications is found. For purifications where RNA immunogenicity is being reduced or the presence of contamination with the previously purified RNA is not acceptable, cleaning of the column and attention to the order that RNAs are purified is important. An unmodified RNA, which remains immunogenic after HPLC purification (15), should not be run prior to an RNA where no immunogenicity is required.

Run a cleaning program between RNA samples to reduce contamination.

1. Run 1 column void volume of 38% buffer B.
2. Increase to 100% buffer B over 0.2 column void volumes.
3. Run 1 column void volume of 100% buffer B.
4. Decrease to 38% buffer B over 0.2 column void volumes.
5. Repeat steps 1–4 two additional times for a total of 3 cycles (see Note 13 for HPLC cleaning protocol) (see Note 14).

3.3. HPLC Column and System Storage and Cleaning

Standard HPLC protocols for short and long term storage of the HPLC columns and system should be followed.

1. For short-term storage, less than 4 days, wash the column and system with 10 column void volumes of HPLC grade water.
2. For longer-term storage, wash the column and system with 10 column void volumes of 20% ethanol after the HPLC grade water wash.

Weekly HPLC cleaning or at the completion of purifications prior to an extended period of nonuse.

1. Clean the HPLC system with 100% acetonitrile for 10 column void volumes.
2. Clean the HPLC system with HPLC-grade water for 10 column void volumes.

Complete HPLC cleaning. When RNA with no contaminating RNA from previous runs is needed or when purification efficiency drops.

1. Clean the column and system with 10 column void volumes of 0.1 M NaOH.
2. Clean the column and system with 20 column void volumes of HPLC-grade water.

3. Extensively re-equilibration with 38% buffer B after NaOH cleaning, run 38% buffer B at 5 ml/min for 10 column void volumes.
4. Run 38% buffer B at 0.5 ml/min continuously for 3–4 days.

3.4. Purification of RNA from Column Fractions

1. Concentrate and desalt RNA from the desired column fractions in Amicon Ultra-15 centrifugal filter units (30K membrane).
2. Add column fractions to the filter.
3. Centrifuged at 4,000×*g* for 10 min (25°C) in a ST16R centrifuge.
4. Dilute concentrated RNA with nuclease-free water.
5. Repeat concentration and dilution (steps 1–4) two more times or until below a desired concentration of acetonitrile.
6. Recover RNA with overnight precipitation at –20°C by adding 1/10th volume NaOAc, 1 volume of isopropanol, and 3 μl of glycogen.

3.5. Analysis of RNA

3.5.1. dsRNA Dot Blot

A major contaminant that is found in all RNA sequences and with all modified nucleosides studied thus far is dsRNA. Binding of dsRNA-specific mAb J2 occurs even when the dsRNA contains modified nucleosides, e.g., pseudouridine and/or 5-methylcytidine, while binding of the other dsRNA-specific mAb K1 is reduced when dsRNA contains such modifications (15) (see Note 15).

1. Blot RNA (200 ng) onto super charged Nytran membranes and dry (see Note 16).
2. Block membranes with blocking buffer for 1 h.
3. Incubate membranes with dsRNA-specific mAb J2 or K1 (5,000-fold dilution) in incubation buffer for 60 min.
4. Rinse membranes four times and then wash six times (5 min for each wash) with TBS-T buffer.
5. React membranes with HRP-conjugated donkey anti-mouse IgG (5,000-fold dilution) in incubation buffer for 60 min.
6. Rinse membranes four times and then wash six times (5 min for each wash) with TBS-T buffer.
7. Detect membranes with ECL Plus Western blot detection reagent.
8. Capture images on a Fujifilm LAS1000 or similar digital imaging system (see Note 17).

3.5.2. RNA Translation

1. Complex HPLC-purified and corresponding unpurified firefly luciferase encoding mRNA to TransIT-mRNA (see Note 3) according to the manufacturer by combining RNA (0.1 μg)

with 17 μl serum free RPMI and adding TransIT-mRNA transfection reagent (0.3 μl) and boost reagent (0.2 μl).

2. Add 17 μl of complexed RNA to a single well of DCs in a 96-well plate in 180 μl of RPMI 10% FCS medium (see Notes 4 and 5).
3. Three to 24 h later, remove medium and lyse cells in 25 μl of Luciferase Cell Culture Lysis Reagent (see Note 18).
4. Measure mRNA translation by adding 2 μl of cell lysate to 10 μl of Firefly luciferase assay buffer and measuring for 10 s in a luminometer (see Note 19).

3.5.3. RNA Immunogenicity

1. Treat DCs (5×10^4 cells/well) in 96-well plates with medium, poly(I:C) (50 μg/ml) not complexed to TransIT transfection reagent (positive control) (see Note 20), and TransIT transfection reagent-complexed RNA (0.1 μg/well), as prepared above in RNA translation (Subheading 3.5.2).
2. Harvest supernatant after 24 h.
3. Assay for IFN-α by ELISA on undiluted supernatant.

4. Notes

1. A small column (7.8 mm diameter by 50 mm long (2.4 ml total volume)) can be directly obtained from Transgenomics (http://www.transgenomic.com/) (Cat # RNA-99-3810). It can purify up to 10 μg of RNA and runs at a flow rate of 1 ml/min. Larger columns can be special ordered from Transgenomic. We use a 21.2 mm diameter by 100 mm long column (35 ml total volume) that allows up to 600 μg of RNA per run to be purified.
2. Numerous commercially available glycogen preparations were found to be contaminated with RNA and DNA (30). We found that Roche Applied Science glycogen was free of DNA or RNA contamination.
3. Other transfection reagents compatible with long RNA can be used.
4. Human DCs are optimal for analyzing mRNA as they are primary cells and contain all known RNA sensors (31) and their ability to translate exogenously delivered mRNA is very sensitive to contaminants. Other primary cells or cell lines can be used.
5. TransIT-mRNA transfection reagent requires serum in the medium of cells when they are transfected.

6. Translation can be measured for any encoding mRNA with the appropriate quantitative assay.
7. Other cell types or cell lines can be used for measuring RNA immune activation. The appropriate assay to measure immunogenicity is needed and can include IFN-β, TNF-α, or IL-8 ELISAs depending on the cell used.
8. If 5′-cap is added enzymatically after HPLC purification, ensure that the RNA is precipitated with ½ volume of LiCl without adding alcohol to avoid S-adenosyl-methionine contamination that can reduce translation.
9. RNA purification can be optimized for maximum translation per RNA molecule transfected and, if it contains nucleoside modifications, for lack of immunogenicity. Each RNA has an optimal temperature for purification that results in the highest level of translation. For optimal performance, this should be determined for each RNA. We have observed differences of up to a twofold increase in translation comparing purifications at different temperatures. It is typically in the 45–55°C range.
10. The void volume of an HPLC column is the total volume of the liquid or mobile phase in a packed column. It is typically 50% of the total column volume for alkylated (PS-DVB) copolymer beads.
11. The column can be loaded with RNA in the constituents of the transcription reaction or post transcription reactions (cap or poly(A) tail addition) without any purification. Run 3 column void volumes of 38% buffer B after loading RNA in this fashion.
12. HPLC program for purifying RNA using a 35 ml column:
    (a) Column Pressure Limit 25.00 MPa
    (b) Averaging Time UV 1.30
    (c) Flow Rate 5 ml/min
    (d) Start 38% Buffer B
    (e) Equilibrate with 3 column void volumes
    (f) Flow Rate 2.5 ml/min
    (g) Inject sample
    (h) Empty loop with 6 ml
    (i) Wash column with 3 column void volumes
    (j) Flow Rate 5 ml/min
    (k) Start fraction collection for mAU>20 (dependent on amount and type of RNA), 1–5 ml fractions
    (l) Linear gradient to 65% Buffer B over 6 column void volumes
    (m) Stop fraction collection

(n) Linear gradient to 100% Buffer B over 0.2 column void volumes

(o) Equilibrate with 100% Buffer B for 3 column void volumes

(p) Linear gradient to 38% Buffer B over 0.2 column void volumes

(q) Equilibrate with 38% Buffer B for 1.5 column void volumes

13. HPLC program for cleaning between runs using a 35 ml column:
    (a) Column Pressure Limit 25 MPa
    (b) Flow Rate 5 ml/min
    (c) Start 38% Buffer B
    (d) Equilibrate with 38% Buffer B for 1 column void volume
    (e) Linear gradient to 100% Buffer B over 0.2 column void volumes
    (f) Equilibrate with 100% Buffer B for 1 column void volume
    (g) Linear gradient to 38% Buffer B over 0.2 column void volumes
    (h) Equilibrate with 38% Buffer B for 1 column void volume
    (i) Linear gradient to 100% Buffer B over 0.2 column void volumes
    (j) Equilibrate with 100% Buffer B for 1 column void volume
    (k) Linear gradient to 38% Buffer B over 0.2 column void volumes
    (l) Equilibrate with 38% Buffer B for 1 column void volume
    (m) Linear gradient to 100% Buffer B over 0.2 column void volumes
    (n) Equilibrate with 100% Buffer B for 1 column void volume
    (o) Linear gradient to 38% Buffer B over 0.2 column void volumes
14. For additional column cleaning to reduce contamination by RNA from previous runs, a single cycle of the above cleaning cycle (see Note 13) can be run prior to equilibration with buffer B in the standard RNA purification run (see Note 12).
15. The effect of other nucleoside modifications on binding by the dsRNA-specific mAbs will need to be determined.
16. dsRNA (25 ng) can be used as a positive control.

17. Hybridization with labeled DNA complementary to the desired RNA can be performed to confirm RNA loading on the membrane.
18. Both translation and immunogenicity of RNA can be measured with TransIT-mRNA complexing at 24 h post transfection. The peak and duration of translation varies with other transfection reagents, which will have to be determined.
19. RNA encoding other proteins can be analyzed with the appropriate assay.
20. R-848 or other TLR7 or TLR7/8 agonists can be used as a positive control for DCs.

Acknowledgments

These studies were funded by the National Institutes of Health (grant numbers HL87688 to K.K. and AI050484, AI090788, and DE019059 to D.W).

References

1. Jirikowski GF, Sanna PP, Maciejewski-Lenoir D, Bloom FE (1992) Reversal of diabetes insipidus in Brattleboro rats: intrahypothalamic injection of vasopressin mRNA. Science 255:996–998
2. Angel M, Yanik MF (2010) Innate immune suppression enables frequent transfection with RNA encoding reprogramming proteins. PLoS One 5:e11756
3. Yakubov E, Rechavi G, Rozenblatt S, Givol D (2010) Reprogramming of human fibroblasts to pluripotent stem cells using mRNA of four transcription factors. Biochem Biophys Res Commun 394:189–193
4. Warren L, Manos PD, Ahfeldt T, Loh YH, Li H, Lau F, Ebina W, Mandal PK, Smith ZD, Meissner A, Daley GQ, Brack AS, Collins JJ, Cowan C, Schlaeger TM, Rossi DJ (2010) Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. Cell Stem Cell 7(5):618–630
5. Kormann MS, Hasenpusch G, Aneja MK, Nica G, Flemmer AW, Herber-Jonat S, Huppmann M, Mays LE, Illenyi M, Schams A, Griese M, Bittmann I, Handgretinger R, Hartl D, Rosenecker J, Rudolph C (2011) Expression of therapeutic proteins after delivery of chemically modified mRNA in mice. Nat Biotechnol 29:154–157
6. Kariko K, Muramatsu H, Keller JM, Weissman D (2012) Increased erythropoiesis in mice injected with sub-microgram quantities of pseudouridine-containing mRNA encoding erythropoietin. Mol Ther 20(5):948–953
7. Barrett DM, Zhao Y, Liu X, Jiang S, Carpenito C, Kalos M, Carroll RG, June CH, Grupp SA (2011) Treatment of advanced leukemia in mice with mRNA engineered T cells. Hum Gene Ther 22(12):1575–1586
8. Almasbak H, Rian E, Hoel HJ, Pule M, Walchli S, Kvalheim G, Gaudernack G, Rasmussen AM (2011) Transiently redirected T cells for adoptive transfer. Cytotherapy 13:629–640
9. Zhao Y, Moon E, Carpenito C, Paulos CM, Liu X, Brennan AL, Chew A, Carroll RG, Scholler J, Levine BL, Albelda SM, June CH (2010) Multiple injections of electroporated autologous T cells expressing a chimeric antigen receptor mediate regression of human disseminated tumor. Cancer Res 70:9053–9061
10. Rabinovich PM, Komarovskaya ME, Wrzesinski SH, Alderman JL, Budak-Alpdogan T, Karpikov A, Guo H, Flavell RA, Cheung NK, Weissman SM, Bahceci E (2009) Chimeric receptor mRNA transfection as a tool to generate antineoplastic lymphocytes. Hum Gene Ther 20:51–61
11. Yoon SH, Lee JM, Cho HI, Kim EK, Kim HS, Park MY, Kim TG (2009) Adoptive immunotherapy using human peripheral

blood lymphocytes transferred with RNA encoding Her-2/neu-specific chimeric immune receptor in ovarian cancer xenograft model. Cancer Gene Ther 16:489–497

12. Weide B, Pascolo S, Scheel B, Derhovanessian E, Pflugfelder A, Eigentler TK, Pawelec G, Hoerr I, Rammensee HG, Garbe C (2009) Direct injection of protamine-protected mRNA: results of a phase 1/2 vaccination trial in metastatic melanoma patients. J Immunother 32:498–507
13. Weide B, Carralot JP, Reese A, Scheel B, Eigentler TK, Hoerr I, Rammensee HG, Garbe C, Pascolo S (2008) Results of the first phase I/II clinical vaccination trial with direct injection of mRNA. J Immunother 31:180–188
14. Pascolo S (2006) Vaccination with messenger RNA. Methods Mol Med 127:23–40
15. Kariko K, Muramatsu H, Ludwig J, Weissman D (2011) Generating the optimal mRNA for therapy: HPLC purification eliminates immune activation and improves translation of nucleoside-modified, protein-encoding mRNA. Nucleic Acids Res 39:e142
16. Kariko K, Muramatsu H, Welsh FA, Ludwig J, Kato H, Akira S, Weissman D (2008) Incorporation of pseudouridine into mRNA yields superior nonimmunogenic vector with increased translational capacity and biological stability. Mol Ther 16:1833–1840
17. Weissman D, Ni H, Scales D, Dude A, Capodici J, McGibney K, Abdool A, Isaacs SN, Cannon G, Kariko K (2000) HIV gag mRNA transfection of dendritic cells (DC) delivers encoded antigen to MHC class I and II molecules, causes DC maturation, and induces a potent human in vitro primary immune response. J Immunol 165:4710–4717
18. Kariko K, Buckstein M, Ni H, Weissman D (2005) Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA. Immunity 23:165–175
19. Anderson BR, Muramatsu H, Nallagatla SR, Bevilacqua PC, Sansing LH, Weissman D, Kariko K (2010) Incorporation of pseudouridine into mRNA enhances translation by diminishing PKR activation. Nucleic Acids Res 38:5884–5892
20. Kariko K, Weissman D (2007) Naturally occurring nucleoside modifications suppress the immunostimulatory activity of RNA: implication for therapeutic RNA development. Curr Opin Drug Discov Devel 10:523–532
21. Milligan JF, Groebe DR, Witherell GW, Uhlenbeck OC (1987) Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. Nucleic Acids Res 15:8783–8798
22. Arnaud-Barbe N, Cheynet-Sauvion V, Oriol G, Mandrand B, Mallet F (1998) Transcription of RNA templates by T7 RNA polymerase. Nucleic Acids Res 26:3550–3554
23. Nacheva GA, Berzal-Herranz A (2003) Preventing nondesired RNA-primed RNA extension catalyzed by T7 RNA polymerase. Eur J Biochem 270:1458–1465
24. Triana-Alonso FJ, Dabrowski M, Wadzack J, Nierhaus KH (1995) Self-coded 3′-extension of run-off transcripts produces aberrant products during in vitro transcription with T7 RNA polymerase. J Biol Chem 270:6298–6307
25. Summer H, Gramer R, Droge P (2009) Denaturing urea polyacrylamide gel electrophoresis (Urea PAGE). J Vis Exp 32:e1485
26. Masuda N, Ohnishi T, Kawamoto S, Monden M, Okubo K (1999) Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples. Nucleic Acids Res 27:4436–4443
27. Nallagatla SR, Toroney R, Bevilacqua PC (2011) Regulation of innate immunity through RNA structure and the protein kinase PKR. Curr Opin Struct Biol 21:119–127
28. Chakrabarti A, Jha BK, Silverman RH (2011) New insights into the role of RNase L in innate immunity. J Interferon Cytokine Res 31:49–57
29. Boo KH, Yang JS (2010) Intrinsic cellular defenses against virus infection by antiviral type I interferon. Yonsei Med J 51:9–17
30. Bartram A, Poon C, Neufeld J (2009) Nucleic acid contamination of glycogen used in nucleic acid precipitation and assessment of linear polyacrylamide as an alternative co-precipitant. Biotechniques 47:1019–1022
31. Kramer M, Schulte BM, Eleveld-Trancikova D, van Hout-Kuijer M, Toonen LW, Tel J, de Vries IJ, van Kuppeveld FJ, Jansen BJ, Adema GJ (2010) Cross-talk between human dendritic cell subsets influences expression of RNA sensors and inhibits picornavirus infection. J Innate Immun 2:360–370