# USP mRNA Vaccine Chapter

## <XXX> ANALYTICAL PROCEDURES FOR mRNA VACCINES

For decades, messenger RNA (mRNA) technology has been explored for vaccines for infectious diseases like flu, Zika, rabies, and cytomegalovirus. The COVID-19 pandemic accelerated research and development of this technology as a vaccine platform, leading to mRNA vaccines becoming the first modality to receive emergency use authorization and then approval for SARS-CoV-2 in the U.S. The mRNA vaccines for the prevention of COVID-19 proved to be a successful application of the technology, however, there is still limited guidance for testing the quality attributes of these vaccines. A standard set of analytical methods would support vaccine developers, manufacturers, regulatory agencies, and national control laboratories worldwide by providing tools that help accelerate the development of safe and effective vaccines using this platform and guard against substandard and falsified vaccine products.

Based on this need identified by various stakeholders, USP and our BIO3 Expert Committee developed a draft general chapter for mRNA vaccines as a first step towards developing a procedural chapter on testing of mRNA vaccines. The chapter includes analytical procedures and best practices to support the assessment of common quality attributes of mRNA vaccines. This draft chapter also builds on best practices described in general chapters *<1235> Vaccines for Human Use—General Considerations and <1239> Vaccines for Human Use—Viral Vaccines*. Methods in the draft chapter have been adapted from publicly available sources and have not been verified or validated by USP.

USP and our BIO3 Expert Committee are releasing the draft chapter early for public comment. By pursuing the early release, **USP would like to solicit feedback from stakeholders on the methods described in the referenced document and encourage the submission of any alternative methods and any additional supporting documentation, including validation documents, related to the methods presented in the draft chapter.**

### INTRODUCTION

Naturally occurring mRNA is produced in eukaryotic cells by the transcription of the DNA in the nucleus by RNA polymerase to produce mRNA. The mRNA molecules are transported out of the nucleus to the cytoplasm, where they serve as templates that are translated by the ribosomes to produce specific proteins. In this way the information stored in the nucleus is used to produce specific proteins. This mRNA cannot create any protein other than the protein for which it is coded. The estimated half-life for mRNA after injection is approximately 8–10 h before it quickly degrades and is broken down by native RNase in the body. mRNA does not need to enter the nucleus to be functional.

Typically, mRNA vaccine drug substance can be prepared by amplifying the starting DNA plasmid in a host (e.g., *Escherichia coli*). The plasmid is enzymatically linearized and purified before it is used in large scale manufacture of the mRNA intermediate, in a cell-free system via in vitro transcription from the linearized plasmid DNA template. Depending on the specific manufacturing process, the constructs are optimized with nucleosides to form the sequence, and the transcribed mRNA is enzymatically capped at the 5′ end of 7-methylguanosine and/or enzymatically tailed at the 3' end by poly (A). The mRNA drug substance is then purified and formulated to make drug product. The mRNA vaccine drug product can be a formulation of the mRNA embedded in a lipid nanoparticle (LNP). The LNP protects the mRNA from degradation and aids with the mRNA entering the cell through endocytosis. Once in the endosome, the mRNA vaccine molecules escape the endosome into the cytoplasm (depending on the molar ratio of the ionizable lipids and mRNA nucleotides) and provide a template to produce multiple copies of a viral protein. The viral protein serves as an antigen to stimulate an immunological response, which is the desired outcome of the vaccination.

There are two main forms of mRNA vaccines that have been developed: non-replicating mRNA vaccines (conventional) and the self-amplifying mRNA (SAM) vaccines as shown in **Figure 1** below. The conventional non-replicating mRNA vaccine construct commonly consists of a 5′ 7-methylguanosine cap structure, a 5′ untranslated region (UTR), the open reading frame (ORF) encoding the protein, a 3′ UTR, and a 3′ poly (A) tail. The SAM mRNA vaccines are derived from alphaviral genomes, where the mRNA molecule encodes additional replicase components that can direct intracellular mRNA amplification. In both forms of mRNA vaccines, the UTR regions are important for maximizing protein expression, the 5′ capping of the mRNA molecule, blocking exonuclease-mediated degradation and enhancing translation efficiency. The UTRs, 5′ cap, and poly(A) tail also help stabilize

the synthetic mRNA molecule inside the cell.

**Figure 1: Two Main Forms of mRNA Vaccines**


A) Non-replicating mRNA B) Self-amplifying mRNA (SAM)

When mRNA is used as a vaccine, chemical modifications can be introduced into the mRNA molecule to improve efficacy and enhance translation efficiency (e.g., nucleoside modification and codon optimization). The exogenous mRNA can activate innate-immune cells via Toll-like receptors (TLR) 3, 7, and 8. TLR ligation leads to the production of cytokines which results in the generation of adaptive T and B cell responses. Signaling of TLR7 augments production of proinflammatory cytokines, increases antigen presentation, and improves memory B cell survival.

The quality of mRNA drug substance is determined by their design, development, and specifications applied to them during the development and manufacturing process. This chapter provides methods for assessment of quality attributes for identity, purity, quantity, physical state (integrity) and safety of the bulk purified mRNA drug substance, as listed in **Table 1** below. These methods can also be applied for drug product following extraction of the mRNA from LNP.

**Table 1. Quality Attributes for mRNA Drug Substance**

| Quality | Attribute | Method |
|---|---|---|
| Identity | Sequence confirmation | Next generation sequencing (NGS) |
| | | Sanger sequencing |
| | | Reverse Transcriptase – PCR |
| Content | RNA content | RT-qPCR and RT–dPCR, Ultraviolet Spectroscopy |
| Integrity | Percentage of intact mRNA and fragment mRNA | Capillary gel electrophoresis |
| | 5' cap | IP–RP–HPLC |
| | 3' poly(A) | RP–HPLC |
| | mRNA Integrity | Gel electrophoresis |
| Purity | Product related impurities - dsRNA | Immunoblot |
| | Residual DNA template | qPCR |
| Safety | Endotoxin | USP <85> |
| | Bioburden | USP <61>, <62>, <1115> |
| | Sterility | USP <71> |
| Other | Appearance | USP<1>, <790> |
| | pH | USP <791> |

**IDENTITY**

- **METHOD A: IDENTITY OF ENCODED RNA SEQUENCE by NGS**

Multiple commercial instruments are available for mRNA sequencing. A common form of this technique involves library preparation, cluster generation, sequencing, and bioinformatic data analysis, including quality control determinations. Library preparation involves mRNA enrichment and isolation through the hybridization of the mRNA poly(A) tail to a poly(T) oligomer attached to a solid support, typically a magnetic bead. The isolated mRNA is fragmented in the presence of divalent cations and at high temperature, or through other appropriate mechanical cleavage methods. The mRNA fragments are then used as the templates to make double-stranded (ds) complementary DNA (cDNA) using reverse transcriptase and random primers. DNA adapters and indexes are then ligated onto the ends of the ds cDNA that are in preparation for amplification. The constructed library of cDNA

fragments is then subjected to amplification using specific primer sets that are complementary to those used during library construction along with fluorescent labeled deoxynucleoside triphosphates (dNTPs) and dideoxynucleotides triphosphates (ddNTPs). The ddNTPs act as terminators that prohibit any further attachment of nucleotides at the 3' end. Once completed, most sequencing instruments use optical detection to determine nucleotide incorporation during DNA synthesis, while others may use electrical detection. Appropriate software and bioanalytical tools are then used to determine the sequence of the starting mRNA molecule.

**Purification and fragmentation of mRNA:** One of the key processes in NGS is the enrichment of mRNA for the subsequent library construct.

**SDS lysis buffer**: 1% SDS, 10 mM of EDTA

**RNA fragmentation buffer (10X)**: 1M Tris, pH 8.0 and 100 mM of $MgCl_2$

**Stop solution**: 200 mM of EDTA, pH 8.0

For the RNA purification step, for each reaction, add the following mixture in each well of the 96 well plate. Mix 14.5 µL of SDS lysis buffer, 48 µL of 6M GuHCl and 7.25 µL of proteinase K (20mg/mL). Add 1 – 10 µg of mRNA sample. Mix well and incubate at room temperature for 10 min and then heat at 65° for 10 min prior to the addition of 145 µL of RNA clean-up beads.[1] Wash beads twice in 70% ethanol using a magnetic bead stand and then elute RNA into the 30 µL resuspension buffer. Assess the quality of RNA by using Agilent Fragment Bioanalyzer system or CGE method (integrity methods provided below).

Alternatively, mRNA-sequencing (mRNA-Seq) protocol can be applied using the poly(A)-selection strategy for purifying mRNA by filtering RNA with 3' polyadenylated (poly(A)) tails to include only mRNA. All other non-polyadenylated transcripts such as rRNA, tRNA, and degraded RNA all gets washed away in the final step.

For mRNA fragmentation, mix 1–18 µL of purified mRNA, 2 µL of RNA fragmentation buffer (can be prepared fresh or purchased) and nuclease-free water to final volume of 20 µL in a sterile PCR tube.[2] Incubate in a preheated thermal cycler for 5 min at 94°. Transfer the tube to ice and add 2 µL of Stop solution. Clean fragmented RNA using ethanol precipitation. Mix 22 µL of fragmented RNA, 2 µL of 3M sodium acetate at pH 5.2, 1-2 µL of 10 mg/mL linear acrylamide and 60 µL of 100% ethanol in a sterile 1.5 mL microcentrifuge tube. Mix well and incubate at −80° for 30 min. Centrifuge the tube in a microcentrifuge at 14,000 rpm for 25 min at 4°. Carefully remove ethanol and wash the pallet with 300 µL of 70% ethanol. Repeat the wash step and remove 70% ethanol. Air dry the pellet for up to 10 min at room temperature to remove residual ethanol and resuspend in 14.5 µL of nuclease-free water.

**Synthesis of first strand cDNA:** RNA fragments are reverse transcribed to cDNA because the DNA is more stable and allows for amplification using DNA polymerases. mRNA can be transcribed from the coding strand (has the same sequence as mRNA) or template strand (used for transcription). This process will use the cleaved RNA fragments into the first stand of cDNA using random primers and reverse transcriptase.

**First strand buffer (5X)**: Mix 250 mM of Tris-HCl at pH 8.3, 375 of mM KCl, and 15 mM of $MgCl_2$

**Second strand buffer (2X):** Mix 0.2 M of HEPES at pH 6.9, 20 mM of $MgCl_2$, 5 mM dithiothreitol and 0.14 M KCl

**10 mM dNTP mix**: Mix 10 mM of each nucleotide (dATP, dCTP, dGTP and dTTP) in 0.6 mM of Tris-HCl.

In a 200-µL PCR tube, add 1 µL of gene specific primers and 11.1 µL of mRNA.[3] Incubate the sample in a PCR thermal cycler at 65° for 5 min and then on ice immediately. Set the thermal cycler to 25°. Per reaction, mix the following reagents in the order listed in a separate PCR tube. Add 4 µL of First strand buffer prepared fresh or from a kit,[4] 2 µL of 100 mM DTT, 0.4 µL of 25 mM dNTP mix (prepared fresh or from a kit), 0.5 µL RNase Inhibitor to final volume of 6.9 µL per reaction. Add 6.9 µL of mixture to the PCR tube and mix well. Heat the sample in the preheated PCR thermal cycler at 25° for 2 min. Add 1–2 µL of reverse transcriptase enzyme (1 µL for less than 5 kb cDNA and 2 µL for longer) to the sample and incubate the sample in a thermal cycler with programed at 25° for 10 min, 42° for 50 min, 70° for 15 min then hold at 4°. Then place the tube on ice.

**Synthesize second strand cDNA:** This process removes the RNA template and generates double-stranded cDNA. To the first stand of cDNA mix, add 62.8 µL of ultra-pure water. To this mixture, add 10 µL of Second strand buffer, and 1.2 µL of 25 mM dNTP mix.[5] Mix well and incubate on ice for 5 min. Add 1.0 µL of RNaseH, and 5.0 µL of DNA Polymerase I. Mix well and incubate at 16° in a thermal cycler for 2.5h. Purify the sample using a PCR purification kit, following the instructions provided by the manufacturer, and elute in 50 µL of elution buffer supplied in the kit.

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[1] RNAclean XP beads can be obtained from Beckman, Product Code A66514 or equivalent

[2] RNA Fragmentation Reagents can be obtained from Thermo Fisher, Product Code AM8740 or equivalent

[3] Random Primers can be obtained from Illumina, Product Code 1004784 or equivalent

[4] SuperScript II can be obtained from Invitrogen, Product Code 18064-014 or equivalent

[5] 25 mM dNTP mix can be obtained from Thermo, Product Code R1122 or equivalent

Final product will be in the form of double-stranded DNA. Here, samples can be stored at –15° to –25° or on ice before moving on to performing end repair protocol.

**End repair:** This process removes 3′ overhangs into blunt ends.

Preheat two heat blocks, one at 20° and the other 37°. In a 1.5 mL RNase-free tube, add 50 µL of eluted DNA, 27.4 µL of RNase–free water, 10 µL of 10X end repair buffer ,[6] 1.6 µL of 25 mM dNTP mix, 5 µL T4 DNA Polymerase , 1 µL of Klenow DNA Polymerase, and 5 µL T4 PNK to a total volume of 100 µL. Incubate the sample in a heat block at 20° for 30 min. Purify the sample using PCR purification kit, following the instructions provided by the manufacturer, and elute in 50 µL of elution buffer supplied in the kit. Final product will be in the form of double-stranded DNA. Here, samples can be stored at –15° to –25° or on ice before moving on to performing end repair protocol.

**Adenylate 3′ ends:** This process adds an "A" base to the 3′ end of the blunt phosphorylated DNA fragments.

In a 1.5 mL RNase–free tube add 32 µL of eluted DNA, 5 µL of A-tailing buffer,[7] 10 µL of 1 mM dATP, and 3 µL of Klenow exo (3′ to 5′ exo minus) to a total volume of 50 µL.[8] Incubate the sample in a 37° heat block for 30 min. Purify the sample using PCR purification kit following the instructions provided by the manufacturer,[9] and elute in 23 µL of elution buffer. Final product will be in the form of double-stranded DNA. Here, samples can be stored at –15° to –25° or on ice before moving on to performing end repair protocol.

**Ligate adapters:** This procedure ligates multiple indexing adapters to the ends of the ds cDNA, preparing them for hybridization onto a flow cell.

In a 1.5 mL RNase–free tube, add 23 µL of eluted DNA, 25 µL of 2X Rapid T4 DNA Ligase Buffer, 1 µL of PE Adapter Oligo Mix,[10] and 1 µL of T4 DNA Ligase to a total volume of 50 µL. Incubate the sample at room temperature for 15 min, then purify the sample using a PCR purification kit following the instructions provided by the manufacturer and elute in 10 µL of elution buffer. Ensure complete removal of ethanol. Here, samples can be stored at –15° to –25° or on ice before moving on to performing end repair protocol.

**Purification of cDNA templates:** This process purifies the products of the ligation reaction on a gel to select a size for enrichment.

Prepare a solution with 2% agarose gel in distilled water and 1X TAE buffer (final concentration) to a final volume of 50 mL. Load the samples onto the gel. On the first and the third well load 2 µL 100 bp DNA ladder, and on second well load 10 µL DNA elute from the ligation step mixed with 2 µL of 6X DNA Loading Dye.[11] Run the gel at 120 V for 60 min. Remove the gel slice by using a clean gel excision tip before following instructions in the Gel Extraction Kit, to purify the sample and elute in 30 µL of elution buffer. Here, samples can be stored at –15° to –25° or on ice before moving on to performing end repair protocol.

**Enrichment of purified cDNA templates:** This procedure uses PCR to selectively enrich those DNA fragments that have adapter molecules on both ends, and to amplify the amount of DNA in the library.

In a 200-µL PCR tube, per reaction add 10 µL of 5X phusion buffer,[12] 1.0 µL of PCR primer PE 1.0,[13] 1.0 µL of PCR Primer PE 2.0,[14] 0.5 µL of 25 mM dNTP mix, 0.5 µL of Phusion DNA Polymerase,[15] and 7.0 µL of nuclease–free water to a total volume of 20 µL per reaction. Add 30 µL of the purified ligation mixture to the PCR tube before amplification. PCR amplification can be done by 30s at 98°, then 15 cycles of 10s at 98°, 30s at 65°, 30s at 72°, 5 min at 72° and hold at 4°. Purify the sample using a PCR purification kit,[16] following the instructions provided by the manufacturer, and elute in 30 µL of elution buffer. Here, samples can be stored at –15° to –25° or on ice.

**Validation of library:** Quantify your libraries using qPCR, ddPCR, Bioanalyzer, or Micro Chip. Check the size and purity of the sample.

**Analysis of the sequencing data:** Vendor supplied software is used to analyze the run data files and determine the sequence of the starting mRNA molecule. Alternatively, tools such as Sailfish, RSEM, and BitSeq can also help

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[6] Fast DNA End Repair Kit can be obtained from ThermoFisher, Product Code K0771 or equivalent

[7] A-Tailing Buffer can be obtained from Illumina, Product Code 1002105 or equivalent

[8] Klenow Exo can be obtained from Illumina, Product Code 11318090 or equivalent

[9] MinElute PCR Purification Kit can be obtained from QIAGEN, Product Code 28004 or equivalent

[10] PE Adapter Oligo Mix can be obtained from Illumina, Product Code 1001782 or equivalent

[11] 6X DNA Gel Loading Dye can be obtained from ThermoFisher, Product Code R0611 or equivalent

[12] 5X Phusion Buffer (Finnzymes Oy) can be obtained from Illumina, Product Code 1000585

[13] PCR Primer PE 1.0 can be obtained from Illumina, Product Code 1001783

[14] PCR Primer PE 2.0 can be obtained from Illumina, Product Code 1001784

[15] Phusion DNA Polymerase (Finnzymes Oy) can be obtained from Illumina, Product Code 1000584

[16] QIAquick PCR Purification Kit can be obtained from QIAGEN, Product Code 28104 or equivalent

quantify the expression levels, while MISO can help quantify spliced genes. There are three steps to NGS analysis. First is the FASTQ "raw" data file generation using the vendor supplied software. Second, using the trimming and alignment tool for BAM/SAM files which have reads that are aligned to genome, and finally, identification of mutations/variants.

- **METHOD B: IDENTITY by SANGER SEQUENCING**

  Sanger sequencing is a standard sequencing technique that yields information about the identity and order of the four nucleotide bases in a segment of DNA. It is a technique that uses dye-labeled chemical analogs that are missing the hydroxyl group required for extension of the DNA chain called dideoxyribonucleotides (ddNTPs) of the nucleotide bases.

  **TE buffer solution**: 10 mM of Tris-Cl, 1 mM of EDTA, pH 8.0

  **cDNA Synthesis (prior to Sanger Sequencing)**: Combine 10 µL of master mix (containing hexamer and oligo-dT primers, dNTPs, RNase Inhibitor and reverse transcriptase),[17] 1 – 15 µL of sample (15 µL sample expected to have low titer), and water to final volume of 50 µL. Vortex the mixture briefly and centrifuge for 5–10 s at 1,000 x *g*. Put samples in the thermal cycler and run the program detailed in Table 2. Samples can be held at 4° for up to 8 h or freeze at –20° for longer storage.

**Table 2. Thermal Cycler Conditions (Prior to Sanger Sequencing)**

| | Steps | | | |
|---|---|---|---|---|
| | **Annealing** | **Polymerase Extension** | **Polymerase Inactivation** | **Hold** |
| **Temperature (°)** | 25 | 50 | 80 | 4 |
| **Time (min)** | 10 | 15 | 10 | Indefinitely |

**PCR amplification**: Primers should be in pairs consisting of forward primer and reverse primer, which focus on specific regions of the target gene. Resuspend dried and desalted primers to final concentration of 100 µM with TE buffer solution. Next, add 492 µL of TE buffer solution to each labeled microcentrifuge tubes for each primer pair. Add 4 µL each of both the forward and reverse primer pairs to the appropriate microcentrifuge tubes. Each one should be 0.8 µM in this amplification primer mix.

In each well of a 96-well PCR plate, combine 1.5 µL of amplification primer mix in duplicate, 5 µL of PCR dye mix,[18] 1 µL of cDNA sample (20-40 ng of cDNA), and water to final volume of 10 µL. Make sure to include a positive and a negative control (no-template). Seal the plate, vortex the mixture briefly and centrifuge for 5–10s s at 1,000 x *g.* Put samples in the thermal cycler and run the program detailed in Table 3.

**Table 3. Thermal Cycler Conditions (Amplification)**

| | Steps | | | | |
|---|---|---|---|---|---|
| | | Cycling (40 cycles) | | | |
| | **Polymerase Activation** | **Denaturation** | **Annealing** | **Extension** | **Hold** |
| **Temperature (°)** | 95 | 96 | 62 | 68 | 4 |
| **Time** | 10 min | 3 s | 15 s | 30 s | Indefinitely |

**Cycle sequencing:** Remove the seal from the plate and add 2 µL dye sequencing master mix, 1 µL of dye tagged forward, and tagged reverse primer.[19]

[NOTE—Add tagged forward primer to one of the duplicate PCR reactions, and the tagged reverse primer to the other reaction.]

[17] Suitable cDNA synthesis master mix can be obtained from Thermo Fisher, Product Code 117565500 or equivalent.
[18] BigDye Direct Cycle Sequencing Kit can be obtained from Thermo Fisher, Product Code 4458688 or equivalent.
[19] BigDye Direct Cycle Sequencing Kit can be obtained from Thermo Fisher, Product Code 4458688 or equivalent.

Seal the plate, vortex the mixture briefly, and centrifuge for 5–10 s at 1000 x *g.* Put samples in the thermal cycler and run the program as detailed in Table 4.

**Table 4. Thermal Cycler Conditions (Cycle Sequencing)**

| | Steps | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | Cycling (25 cycles) | | | |
| | Post PCR Clean up | Post PCR Inactivation | Polymerase Activation | Denaturation | Annealing | Extension | Hold |
| **Temperature (°)** | 37 | 80 | 96 | 96 | 50 | 60 | 4 |
| **Time** | 15 min | 2 min | 1 min | 10 s | 5 s | 75 s | Indefinitely |

**Sequencing clean-up**: Centrifuge the reaction plate for 1 minute at 1,000 x *g.* Prepare a mixture according to the kit. There are several kits available to support removal of unincorporated terminators and salts.[20]
[NOTE—Make sure solutions are homogeneous with no particulates before using.]
Add 55 µL of this mixture to each well. Seal the plate, vortex the reaction plate for 40 min, and centrifuge the plate for 2 min at 1000 x *g.*
**Collection of data**: Load the plate into the genetic analyzer such as the capillary electrophoresis (see integrity methods below). Select or create an appropriate run module according to capillary length, number of capillaries, and polymer type on the instrument. The electrophoresis will separate the labeled chain-terminated fragments by length with single-nucleotide resolution. Once the run is finished, the instrument will generate a file that can be converted into a sequence.
**Data analysis:** Use a sequence scanner software to generate a report. Software should be able to call low frequency somatic variants at a detection level below 5%.

- **METHOD C: IDENTITY by RT-PCR**

Reverse transcription PCR (RT-PCR) can be used to identify and quantify mRNA and is performed in two steps: reverse transcription (first strand of cDNA synthesis), and PCR amplification.
**10 mM dNTP mix**: Mix 10 mM of each nucleotide (dATP, dCTP, dGTP and dTTP) in 0.6 mM Tris-HCl.
**First strand buffer (5X)**: Mix 250 mM of Tris-HCl at pH 8.3, 375 mM of KCl, and 15 mM of $MgCl_2$.
**PCR buffer (10X):** 200 mM of Tris HCl at pH 8.4 and 500 mM of KCl.
**First strand cDNA synthesis:** Prepare the following mixed solution.

**Table 5: First-Strand cDNA Solution**

| Component | Volume |
|---|---|
| Gene specific primer (2 pmole) | 1 µL |
| mRNA (1–500 ng) | X µL |
| 10 mM dNTP mixture | 1 µL |
| RNase-free water | Final volume to 12 µL |

Heat the mixture at 65° for 5 min and then quickly cool on ice for 2 min. Centrifuge for 5–10 s at 1,000 x *g*. Next, prepare a reverse transcription reaction system by combining the following solutions.

**Table 6: Reverse Transcription Reaction Solution**

| Component | Volume |
|---|---|

[20] BigDye XTerminator Purification Kit can be obtained from Thermo Fisher, Product Code 4376486 or equivalent.

| | |
|---|---|
| cDNA mixture from above | 12 µL |
| First strand buffer (5X) | 4 µL |
| RNase-free water | 20 µL |

Gently vortex the mixture for few minutes. If random primers are used, incubate at 25° for 2 min, then add 1 µL (200 U) of Reverse Transcriptase to the reaction tube and mix gently with pipette. Incubate at 42–50 ° for 50 min.
[NOTE—If reverse primer of PCR is used as a reverse transcription primer, it is recommended to perform the reaction at 45–50 °; otherwise, it is generally recommended to perform the reaction at 42°.]
Inactivate and stop the reverse transcription reaction by heating at 70° for 15 min. Sample can be used immediately for subsequent PCR reactions or can be stored at –20° for short-term storage and –80° for long-term storage.
**RT-PCR:** Using Table 7 prepare a 50 µL reaction solution.

**Table 7. RT-PCR Reaction Solution**

| Component | Volume |
|---|---|
| PCR buffer (10X) | 5 µL |
| 50 mM $MgCl_2$ | 1.5 µL |
| 10 mM dNTP mixture | 1 µL |
| Forward primer (10 µM) | 1 µL |
| Reverse primer (10 µM) | 1 µL |
| Taq DNA polymerase (5 Units/µL) | 0.4 µL |
| cDNA from first strand reaction | 2 µL |
| ddH2O | Final volume to 50 µL |

[NOTE—There are 4 different fluorescent DNA probes that are available for RT-PCR product detection. These products are SYBR Green, TaqMan, Molecular Beacons and Scorpions. All these probes allow the detection of PCR products by generating a fluorescent signal. Follow manufacturers' protocols for each.]

Gently mix the reaction and place it in the thermal cycler using the following program.

**Table 8. Thermal Cycler Conditions (First Strand Synthesis)**

| Temperature (°) | Time | Cycles |
|---|---|---|
| 94 | 2 min | 1 |
| 94 | 30 s | 15-40 |
| Tm - 5 | 30 s | |
| 72 | 1 min | |
| 72 | 5 min | 1 |
| 4 | Hold | 1 |

**Preparation of cDNA for standard curve:** Standard curve is necessary to quantitate the results. Dilute stock plasmid 1:1000 to a dilution of 1 ng/µL. Prepare standards as described below.
[NOTE—Avoid using a plasmid that contains a gene of interest to avoid contamination.]

**Table 9. cDNA Standard Curve Preparation**

| Concentrations | Dilutions |
|---|---|
| 1 ng | Dilute 1 ng/µL stock solution 1:8 (70 µL of stock DNA solution + 490 µL of PCR-grade water) |
| 0.1 ng | Dilute 1 ng/8 µL standard solution 1:10 (50 µL of standard + 450 µL of PCR-grade water) |

| | |
|---|---|
| 0.01 ng | Dilute 0.1 ng/8 µL standard solution 1:10 (50 µL of standard + 450 µL of PCR-grade water) |
| 0.001 ng | Dilute 0.01 ng/8 µL standard solution 1:10 (50 µL standard + 450 µL PCR-grade water) |

**QUANTITATION**

- **METHOD A: QUANTITATION by digital PCR**

Digital PCR can be used for mRNA quantification without a standard curve. Genomic RNA is reverse transcribed to cDNA and amplified, followed by quantitation on a digital or droplet digital PCR System.[21]

**10 mM dNTP mix**: Mix 10 mM of each nucleotide (dATP, dCTP, dGTP and dTTP) in 0.6 mM of Tris-HCl.
**First Strand buffer (5X)**: Mix 250 mM of Tris-HCl at pH 8.3, 375 mM of KCl, and 15 mM of $MgCl_2$.
**Primer/probe mix (20X):** Mix 10 µL of 100 µM of forward primer, 10 µL of 100 µM of reverse primer, 5 µL of 100 µM labeled probe and 75 µL of PCR-grade water.
**First strand cDNA synthesis:** Prepare the following mixed solution.
[NOTE—To increase the efficiency of cDNA synthesis, the reverse transcription reaction should include a target gene-specific primer that is the same primer used as reverse primer for each target in the ddPCR reaction.]

**Table 10. First-Strand cDNA Solution**

| Component | Volume |
|---|---|
| Gene specific primer (2 pmole) | 1 µL |
| mRNA (1–500 ng) | X µL |
| 10 mM dNTP mixture | 1 µL |
| RNase-free water | Final volume to 12 µL |

Heat the mixture at 65° for 5 min, and then quickly cool on ice for 2 min. Centrifuge for 5–10 s at 1,000 x *g*. Next, prepare a reverse transcription reaction system by preparing the following mixed solution.

**Table 11. Reverse Transcription Reaction Solution**

| Component | Volume |
|---|---|
| cDNA mixture from above | 12 µL |
| First strand buffer (5X) | 4 µL |
| RNase-free water | 20 µL |

Gently vortex the mixture for few seconds. If random primers are used, incubate at 25° for 2 min then add 1 µL (200 U) of reverse transcriptase to the reaction tube and mix gently with pipette. Incubate at 42–50° for 50 min.
[NOTE—If reverse primer of PCR is used as a reverse transcription primer, it is recommended to perform the reaction at 45–50 °, otherwise, general recommendation is to perform the reaction at 42°.]
Inactivate and stop the reverse transcription reaction by heating at 70° for 15 min. Sample can be used immediately for subsequent PCR reactions or can be stored at –20° for short-term storage and –80° for long-term storage.
**Expression by dPCR:** Thaw all components including primer/probe mix. Additionally, primer/probe mix can also be purchased.[22] Mix thoroughly by vortexing each tube for 30s at maximum speed to ensure homogeneity. Centrifuge briefly to collect contents at the bottom. Prepare the following reaction mixture on ice.

**Table 12. dPCR Reaction Mixture**

| Component | Volume per Reaction (µL) | Final Concentration |
|---|---|---|

[21] QX200 or QX100 Droplet Digital PCR System from BioRad Product Code 186 or equivalent.
[22] One-Step RT-ddPCR Advanced Kit for Probes from Bio-Rad, Product Code 1864021.

| | | |
|---|---|---|
| Supermix | 5 | 1x |
| Reverse transcriptase | 2 | 20 U/ µL |
| 300 mM DTT | 1 | 15 mM |
| Target primers/probe | Variable | 900 nM/ 250 nM |
| RNA/Dnase–free water | Variable | NA |
| Total RNA | Variable | 100 fg – 100 ng per reaction |
| Total volume | 20 | NA |

Mix thoroughly by vortexing each tube for 10s at maximum speed. Centrifuge briefly and allow the reaction tubes to equilibrate to room temperature for no more than 10 min.
*Droplet generation*: Load 20 µL of each reaction mixture from above into a sample well of a DG8 cartridge.[23] Add 70 µL of Droplet Generator Oil to the bottom row of the cartridge designed for "oil". Fit rubber DG80 Gasket onto the Cartridge and place it on the Droplet Generator. This process should take about 1 min. Droplets are held in the top row. Using a multi-channel pipettor, transfer 45 µL droplets into 96-well PCR plate and cover the plate with foil sheet immediately. Seal the plate using the PCR Plate Sealer at 180° for 5s.

Run the plate on thermocycler using the following cycling conditions.

**Table 13. Thermal Cycler Conditions**

| Cycling Step | Temperature (°) | Time | Cycles |
|---|---|---|---|
| Reverse transcription | 42-63 | 60 min | 1 |
| Enzyme activation | 95 | 10 min | 1 |
| Denaturation | 95 | 30 sec | 40 |
| Annealing/extension | 52 | 1 min | 40 |
| Enzyme deactivation | 98 | 10 min | 1 |
| Hold | 4 | Infinite | 1 |

[ NOTE— To determine acceptable temperature ranges for reverse transcription, perform a thermal gradient from 42° to 51.5° while fixing the annealing/extension step at 52°. Using the optimized reverse transcription temperature, perform a thermal gradient from 50° to 63° to identify acceptable annealing/extension temperature ranges.]

**Data analysis**: Follow instructions for data acquisition and analysis based on the system used.

- **METHOD B: RNA CONCENTRATION by ULTRAVIOLET SPECTROSCOPY**
  (See *Ultraviolet-Visible Spectroscopy* <857>)
  This method is used to calculate RNA concentration in the bulk solution. The absorbance of a diluted RNA sample is measured at 260 nm and 280 nm, and the concentration is calculated using the Beer-Lambert Law equation. The A260/A280 ratio is used to assess RNA purity. The absorbance ratio of the *Sample solution* of 1.8/2.1 is indicative of highly purified RNA.
  [NOTE—The A260/A280 ratio is dependent on both pH and ionic strength. As pH increases, the A280 decreases while the A260 is unaffected.]

  **Buffer solution:** 0.01 M solution of Tris(hydroxymethyl)aminomethane and 0.001 M disodium ethylenediaminetetraacetic acid solution in water. Adjust with hydrochloric acid to a pH of 8.0.
  **Sample solution:** Mix RNA bulk solution with *Buffer solution* to obtain a solution with an absorbance value between 0.5 and 1.0 at the wavelength of maximum absorbance at 260 nm.

[23] DG8 Cartridges for QX200/QX100 Droplet Generator from BioRad Product Code 1864008 or equivalent.

Perform sample readings in quartz cuvettes. Perform a background correction by making readings from a blank (Buffer solution only) at 320 nm, 260 nm, and 280 nm.
[NOTE—Dirty cuvettes and dust particles cause light scatter at 320 nm which can impact absorbance at 260 nm.]
**Analysis:** Determine the absorbance of the *Sample solution* using Beer-Lambert Law equation, by calculating the concentration with the cell path length of 1 cm.

**Beer-Lambert Law equation**

$$A = \varepsilon b C$$

$A$ = absorbance
$\varepsilon$ = molar extinction coefficient
$b$ = path length, 1 cm
$C$ = concentration
[NOTE—The molar extinction coefficient of RNA is: 40 $(\mu g/mL)^{-1}cm^{-1}$ (absorbance max at 260 nm).]

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**RNA INTEGRITY**

A high-resolution analytical method that can measure the integrity of RNA molecules by size and length is crucial for quality assurance, understanding potency, and for optimization of manufacturing processes. Most commonly this evaluation is performed using capillary gel electrophoresis. A common form of this technique involves filling a capillary with a separation gel matrix with a fluorescent dye. A microliter size injection is made on the capillary using voltage injection and the RNA fragments bind the fluorescent dye as they migrate through the capillary by size using electrophoretic separation. Size comparison is performed against a reference ladder sample that has RNA fragments of defined size. The instrument software determines the size and concentration of the RNA fragments present in the sample. There are two common platforms, one using an Agilent system and the other using SCIEX system.

- **METHOD A: CAPILLARY GEL ELECTROPHORESIS USING AGILENT**

**RNA ladder**: Use a suitable RNA diluent marker solution.[24]
**RNA diluent marker solution:** Use a suitable **i**ntercalating dye solution.[25]
**Intercalating dye solution:** Use a suitable separation gel.[26]
**Separation gel:** Use a suitable 5X capillary conditioning solution.[27]
**Capillary conditioning solution (5X):** Use a suitable blank solution.[28]
**Capillary conditions solution (1X):** Mix 1-part *Capillary conditions solution* with 4 parts sub-micron filtered Type I water.
**Capillary gel solution:** Prepare by mixing *Intercalating Dye Solution*, *Separation Gel*, and *1X Capillary Conditioning Solution* in 0.1:1:1 proportion to create a sufficient volume depending on the number of samples to be analyzed.
**RNA ladder solution:** Prepare a 3 µL (25 ng/µL) *RNA ladder* aliquot and store on ice. Transfer 2 µL of the 25 ng/µL ladder to a 0.5 mL tube. Dilute to the working concentration of 2 ng/µL with RNase-free water. Heat-denature the ladder at 70° for 2 min, immediately cool to 4° and keep on ice.
**Blank solution:** Use a suitable blank solution
**mRNA sample preparation:** Heat-denature the RNA samples at 70° for 2 min and immediately cool to 4° and keep on ice before use. The mRNA input sample must be within a total concentration range of 250 pg/µL to 5000 pg/µL. If the concentration of the sample is above this range, dilute with RNase-free water. Prepare each sample in duplicate.

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[24] Suitable RNA Diluent Marker Solution can be obtained from Agilent, Product Code DNF-370-0004.
[25] Suitable Intercalating Dye Solution can be obtained from Agilent, Product Code DNF-600-U030.
[26] Suitable Separation Gel can be obtained from Agilent, Product Code DNF-265-0240.
[27] Suitable 5X Capillary Conditioning Solution can be obtained from Agilent, Product Code DNF-475-0050.
[28] Suitable Blank Solution can be obtained from Agilent, Product Code DNF-301-0008.

**Sample plate preparation:** Using a fresh RNase-free 96-well sample plate, pipette 18 µL of the *RNA diluent marker solution* to each well in a row that is to contain sample or *RNA ladder solution*. Fill any unused wells within the row of the sample plate with 20 µL of *Blank solution*. Pipette 2 µL of each denatured RNA sample into the assigned well on the plate containing 18 µL of *RNA diluent marker solution*. Mix the contents of the well by pipetting up and down. The *RNA ladder solution* must be run in parallel with the samples for each experiment to ensure accurate quantification. Pipette 2 µL of denatured *RNA ladder solution* into the 18 µL of *RNA diluent marker solution* in the designated ladder well. Mix the contents of the well by pipetting up and down. After mixing each well, centrifuge the plate to remove any air bubbles. Check the wells of the sample plate to ensure there are no air bubbles trapped in the bottom of the wells. The presence of trapped air bubbles can lead to injection failures.

**Separation procedure and analysis:** Run experiment in reverse polarity at −29 kv and 25° on an Agilent system equipped with DAD and 260 nm optical filter. Run current at 6.9 uA and introduce sample into the capillary inlet electrokinetically at −20 kV for 10 s with UV detection at 260 nm. The sample plate is loaded in the instrument. Capillary washes, and filling with *Separation gel* are performed automatically by the instrument. Samples and *RNA ladder solution* are injected onto the capillary using electrokinetic injection followed by electrophoretic separation through the capillary based on fragment size. Instrument software analyzes the samples by comparison to the *RNA ladder solution* to determine the size and quantitation of the RNA fragments present in the sample.

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- **METHOD B: CAPILLARY GEL ELECTROPHORESIS USING SCIEX**

A high-resolution analytical method that can measure the integrity of RNA molecules by size and length is crucial for quality assurance, understanding potency, and for optimization of manufacturing processes. Following method uses PA800 plus Pharmaceutical Analysis System from SCIEX with LIF detection to evaluate the total RNA integrity. The instrument software determines the size and concentration of the RNA fragments present in the sample.

**1X TBE buffer**: 89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3

**Separation buffer**: 1% Polyvinylpyrrolidone (PVP) at 1.3 MDa in 1X TBE buffer with 4 M Urea and 50,000x dilution or 0.002% SYBR green dye

**RNA ladder and marker**: Dilute RNA ladder in ddH20 to 25 ug/mL,[29] and spike with 1.2 K RNA marker,[30] then denature the solution for 5 min at 65° and cool on ice for 5 min before loading.

**Sample preparation**: Dilute sample in the separation buffer (RNA size ranging from 200 bases to 6500 bases)

**Cartridge**: EZ cartridge pre-assembled with bare fused-silica capillary (50 µm I.D., 30 cm total length, 20 cm effective length).[31]

**Capillary gel electrophoresis**: Carry out electrophoresis experiment with reverse polarity with 200V/cm electrical field (6 kV) at 25°. Introduce sample into the inlet of the capillary electrokinetically at 5kV for 3s. Sample tray temperature should be kept at 4° with LIF detector configured to 488 nm laser with an emission filter of 520 nm. Generate a calibration curve from the RNA ladder to estimate the size of an unknown sample peaks. Introduce the remaining of the samples into the inlet of the capillary. All samples should be analyzed in duplicate.

**Data analysis**: Process data using the 32 Karat software.

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- **QUANTITATION OF mRNA 5'- CAP by IP–RP–HPLC**

A cap is required at the 5' end of the mRNA molecule to protect the molecule from degradation and to facilitate successful protein translation. Capping efficiency is a critical quality attribute for a therapeutic mRNA vaccine. Capped and uncapped mRNA fragments can be separated and quantitated using ion pair reversed-phase high performance liquid chromatography (IP RP-HPLC). It may be necessary to perform site-specific cleavage of the

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[29] RNA ladders RNA 6000 ladder with 6 transcripts from Thermo Fisher Product Code AM7152 or RNA marker from Promega with 9 transcripts product code G3191.

[30] 1.2 kb Kanamycin Positive Control RNA from Promega product code C1381

[31] EZ cartridge from SCIEX product code A55625

mRNA molecule using ribonuclease H to produce smaller specific mRNA fragments in the sample that can be adequately resolved using IP RP-HPLC.

**Solution A:** 100 mM of triethylammonium acetate buffer, pH 7.0, is prepared by mixing 2.21 mL of glacial acetic acid in 350 mL of water. While mixing, 5.58 mL of triethylamine is added slowly. The pH is adjusted to pH 7.0 by addition of either triethylamine or acetic acid.

**Solution B:** Solution A with 25% (v/v) acetonitrile

**Mobile phase:** See the gradient table below.

**Table 14. IP-RP-HPLC gradient table**

| Time (min) | Solution A (%) | Solution B (%) |
|---|---|---|
| 0 | 90.0 | 10.0 |
| 36 | 85.5 | 14.5 |

**RNase cleavage buffer:** Prepare a solution of 20 mM of HEPES-KOH 50 mM of KCl and 10 mM of $MgCl_2$, pH 9.0.

**Sample solution:** To increase the resolution, select a site-specific RNA cleavage probe with 2'-*O*-methyl modifications, except at the 3' end which has 4 to 6 deoxyribonucleic acids (DNA) at the cleavage site. The RNA cleavage probe is product specific and should be chosen to produce a 5'-cap fragment of sufficient size for the IP–RP–HPLC analysis. The RNA cleavage probe-RNA complex mixture should be between 0.5 and 2.0 mM in *RNase cleavage buffer*. Anneal the RNA cleavage probe to mRNA by heating to 90° and then cooling slowly to room temperature. The RNA cleavage probe concentration should be 120% of the mRNA concentration to ensure complete hybridization of the mRNA. Add RNase H to a final concentration of 20 units per 100 µL reaction volume. Incubate the reaction at 37° for 3 h.

**Chromatographic system**

(See *Chromatography* <621>*, System Suitability*.)

**Mode:** LC

**Detector:** UV 260 nm

**Column:** Xterra C18 4.6-mm × 7.5-cm; packing L1

**Column temperature:** 50°

**Flow rate:** 0.5 mL/min

**Injection volume:** 15 µL

**Analysis**

**Sample:** *Sample solution*

Measure the areas of the 5' capped mRNA peak and of the uncapped mRNA peaks.

Calculate the percentage of uncapped mRNA:

$$\text{Result} = [A_U/(A_U + A_C)] \times 100$$

$A_U$ = area of the uncapped mRNA peak

$A_C$ = area of the 5' capped mRNA peak

**System suitability requirements: (**See *Chromatography* <621>*, System Suitability.)*

**Acceptance criteria:** As determined by regulatory authorities.

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- **PERCENT POLY-A TAILED RNA by RP-HPLC**

A poly(A) tail is required at the 3' end of the mRNA molecule to protect the molecule from degradation and to facilitate successful protein translation. The presence of a poly(A) tail is a critical quality attribute for a therapeutic mRNA vaccine. mRNA molecules with and without a poly(A) tail (tailless) can be separated and quantitated using ion pair reversed-phase high performance liquid chromatography (IP RP-HPLC). It may be necessary to perform site-specific cleavage of the mRNA molecule using ribonuclease H to produce smaller

specific mRNA fragments in the sample that can be adequately resolved using IP RP-HPLC.
[NOTE— Poly(A) tail is dependent upon the manufacturing process and the design of the mRNA itself and could fall under characterization and not drug substance.]

**Solution A:** 100 mM of triethylammonium acetate buffer, pH 7.0, is prepared by mixing 2.21 mL of glacial acetic acid in 350 mL of water. While mixing, 5.58 mL of triethylamine is added slowly. The pH is adjusted to pH 7.0 by addition of either triethylamine or acetic acid.
**Solution B:** *Solution A* containing 25% acetonitrile.
**Mobile phase:** See the gradient table below.

**Table 15. RP-HPLC Gradient**

| Time (min) | Solution A (%) | Solution B (%) |
|---|---|---|
| 0 | 62 | 38 |
| 1 | 60 | 40 |
| 16 | 40 | 60 |
| 22 | 34 | 66 |
| 22.5 | 30 | 70 |
| 23 | 0 | 100 |
| 24 | 0 | 100 |
| 25 | 62 | 38 |
| 27 | 62 | 38 |

**RNase cleavage buffer:** Prepare a solution of 20 mM of HEPES-KOK, 50 mM of KCl, and 10 mM of $MgCl_2$, pH 9.0.
**Sample solution:** To increase the resolution, select a site-specific RNA cleavage probe with 2′-*O*-methyl modifications, except at the 3′ end which has 4 to 6 deoxyribonucleic acids (DNA) at the cleavage site. The RNA cleavage probe is product specific and should be chosen to produce a 5′-cap fragment of sufficient size for the IP–RP–HPLC analysis. The RNA cleavage probe-RNA complex mixture should be between 0.5 and 2.0 mM in *RNase cleavage buffer*. Anneal the RNA cleavage probe to mRNA by heating to 90° and then cooling slowly to room temperature. The RNA cleavage probe concentration should be 120% of the mRNA concentration to ensure complete hybridization of the mRNA. Add RNase H to a final concentration of 20 units per 100 µL reaction volume. Incubate the reaction at 37° for 3 h.
**Chromatographic system**
(See *Chromatography* <621>*, System Suitability*.)
**Mode:** LC
**Detector:** UV 260 nm
**Column:** DNASep, 7.8-mm × 5-cm; packing non-porous, alkylated polystyrene divinylbenzene matrix, packing LXX
**Column temperature:** 75°
**Flow rate:** 0.9 mL/min
**Injection volume:** 15 µL
**Analysis**
**Samples:** *Sample solution*
Measure the areas of the poly(A) mRNA peak and of the tailless mRNA peak.
Calculate the percentage of poly(A) mRNA:

$$\text{Result} = [A_U / (A_U + A_C)] \times 100$$

$A_U$ = area of the poly(A) mRNA peak
$A_C$ = area of the tailless mRNA peak

**System suitability requirements: (**See *Chromatography* <621*>, System Suitability.)*

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- **INTEGRITY OF mRNA by GEL ELECTROPHORESIS**

**HT stock solution (50X)**: Prepare a HEPES–triethanolamine (HT) solution by pouring 1.5M triethanolamine in a beaker placed on a balance, then add 35.7 g (1.5M) of HEPES. Add high quality deionized water to ~0.9% of the final volume. Dissolve reagents completely using a magnetic stirrer and bring to 100 mL final volume. The pH of the buffer should be 7.6 ± 0.2 without adjustment. Filter the solution through high-protein binding filter of 0.2 µm pore size.

**Electrophoresis buffer (5X):** Dissolve 54 g of tris base, 27.5 g of boric acid, and 20 mL of 0.5 M EDTA in water to final volume of 1000 mL. Place the solution in a hot water bather with a magnetic stir bar to dissolve.

**Running buffer (1X)**: Dilute *HT stock solution* with deionized water, 1:50.

**Loading dye**: Add *HT stock solution*, 0.5M EDTA (pH 8.0) and bromophenol blue to deionized water to the final concentration of 2.1X electrophoresis buffer, 1mM EDTA and 0.04% bromophenol blue. Add ethidium bromide for a final concentration of 10 µg/mL. Filter through a 0.2 µm syringe filter.

**Loading buffer (2X):** Prepare sufficient amount of the master mix by combining 14 volumes of loading dye with 1 volume of 37% formaldehyde

[NOTE— Loading dye mixed with formaldehyde is not stable upon storage and must be used within a few hours.]

Add the freshly prepared 2X master mix to each RNA sample (1:1 v/v). Close tubes tightly, mix the contents, and spin briefly in a microcentrifuge. Denature the sample by heating at 70° for 5 min, then cool to room temperature.

**mRNA sample preparation**: Dissolve 1–3 µg of mRNA in 50% formaldehyde.

**RNA markers (0.5–9 kb long):** Dilute 2 µL of the marker with 3 µL of nuclease-free water and mix with 15 µL of loading dye.

**Analysis**: Heat 1 g of agarose in 72mL of deionized water until dissolved. Cool agarose to 60°. Add 10 mL of *HT stock solution* and 0.4M formaldehyde. Pour the gel in the tank and add enough *Running buffer* to cover the gel by a few millimeters. Tightly cover the gel casting assembly with plastic wrap during agarose solidification to prevent formaldehyde losses from the gel. Remove the comb.

Load the gel and electrophoresis at 6 V/cm until the bromophenol blue has migrated as far as two-thirds the length of the gel. Visualize the gel on a UV transilluminator. The bands can also be quantified by densitometry using the known RNA standards.

**Acceptance criteria:** Visual observation of the marker should show distinct bands and a single band for the intact RNA sample, similar to those of the in-house control standard.

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- **dsRNA by IMMUNOBLOT**

If dsRNA is present in the mRNA vaccine, it has the potential of being immunogenic. For that reason, dsRNA content should be determined and controlled.

[NOTE— dsRNA is dependent upon the manufacturing process and the design of the mRNA itself and could fall under characterization and not for drug substance.]

**TBS-T buffer:** Prepare a solution of 50 mM Tris–HCl, 150 mM NaCl, 0.05 % of Tween-20, pH 7.4.

**Blocking buffer:** Prepare a solution 5 % nonfat dried milk in *TBS-T buffer*.

**Incubation buffer:** Prepare a solution 1 % nonfat dried milk in *TBS-T buffer*.

**dsRNA Antibody Solution:** Dilute the reconstituted antibody[1] 1:5000 in *Incubation Buffer*.[32]

**Detection Antibody Solution:** Dilute the reconstituted HRP-conjugated donkey anti-mouse IgG 1:5000 in *Incubation Buffer*.[33]

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[32] Suitable dsRNA antibody can be obtained from SCICONS English & Scientific Consulting, Product Code 10010500.

[33] Suitable Detection Antibody can be obtained from Jackson ImmunoResearch, Product Code 715-035-151.

**Detection Reagent:** Chemiluminescent Western Blotting Detection Reagent[34]

**Procedure and Analysis:** Blot 200 ng of the mRNA test sample and a dsRNA reference sample at the limit of detection onto a positively charged nylon blotting membrane and dry for 30 min. Incubate membrane with *Blocking Buffer* for 1 h. Rinse membrane with *TBS-T buffer* twice. Incubate membrane with *dsRNA Antibody Solution* at room temperature for 1 h. Rinse membrane 4 times and wash 6 times, 5 min per wash, with *TBS-T buffer*. Incubate membrane with *Detection Antibody Solution* at room temperature for 1 h. Rinse membrane 4 times and wash 6 times, 5 min per wash, with *TBS-T buffer*. Detect membrane with *Detection Reagent*. Capture images with an appropriate digital imaging system.

- **RESIDUAL DNA TEMPLATE (qPCR)**

The following method is suitable for measurement of residual host cell DNA in mRNA vaccines drug substance. Extraction is not required for drug substance; therefore, a quantitative polymerase chain reaction (qPCR)-based method can be directly used for the measurement of residual host cell DNA. For discussion of the principles and best practices for this type of testing, see Nucleic Acid-Based Techniques—Approaches for Detecting Trace Nucleic Acids (Residual DNA Testing) ⟨1130⟩, which may be a helpful resource.

**Sample Preparation**: There are several procedures for nucleic acid extraction that may be appropriate for biopharmaceutical sample testing. One such procedure is described in detail below and validated for starting DNA concentrations ranging from 0.01 to 50 pg/µL.

**Resuspension solution**: Dissolve tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) and ethylenediaminetetraacetic acid (EDTA) to obtain a solution of 10 mM and 1.0 mM, respectively. Add hydrochloric acid or sodium hydroxide to adjust to a pH of 8.0.

**DNA standard stock solution**: Dilute reference material to a concentration of 1 µg/mL in *Resuspension solution*.

**Sample solutions**: Samples for testing may require dilution or reconstitution to 1) overcome matrix interference affecting the DNA recovery, 2) yield an appropriate starting volume, or 3) bring the analyte concentration within the quantitative range of the qPCR method. *Sample solutions* may be diluted in water or in *Resuspension solution* if necessary. For drug substance samples, *Sample solutions* should contain sufficient starting material to allow determination of the residual DNA content, if present at the specification limit.

**Positive control solution**: Prepare by spiking *DNA standard stock solution* to *Sample solutions* at a concentration appropriate for the *assay* (specification, or otherwise justified).

**Negative control solution**: Water or *Resuspension solution* is used in place of *Sample solutions* in the extraction procedures and will be extracted with any samples (if extraction is necessary). The *Negative control solution* is tested using the qPCR-based method to determine the DNA content contributed by the background and to demonstrate that there is no potential cross-contamination during the assay. This is also known as the no template control.

**qPCR Analysis**

**2X Master mix**: A suitable buffer containing magnesium chloride, deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxycytidine triphosphate, deoxyuridine triphosphate, deoxythymidine triphosphate, and highly purified DNA polymerase. Mix well immediately before use.

**DNA stock primers and probes**: Determine the fragment of the DNA template that needs to be amplified and design the forward and reverse primers.

Prepare individual 10-µM solutions of the primer pairs and probe specific to mRNA vaccines, using DNAse-free water.

**DNA probe solution**: Dilute *DNA stock probe* to 2.5 µM with DNAse-free water.

**Standard solutions**: Dilute the *DNA standard stock solution* to obtain 5 or more suitable standards within the concentration range of 0.001–100 pg/µL.

[34] Suitable Detection Reagent can be obtained from Cytiva, Product Code RPN2109.

**Analysis of Samples**: *Sample solutions, Positive control solution, Negative control solution*, and *Standard solutions*

[NOTE—If samples are extracted, then extracted *Sample solutions* and extracted *Control solutions* will be used.]

Transfer 25 µL of the 2X Master mix to each well of a 96-well qPCR plate. Add 5 µL each of the *DNA stock forward primer*, the *DNA stock reverse primer*, and the *DNA probe solution* of the appropriate species to each well. Add 10 µL of either (extracted) *Sample solutions, Standard solutions,* (extracted) *Negative control solution*, or (extracted) *Positive control solution* to their respective wells.

[NOTE—The qPCR reaction volume may be scaled as appropriate to accommodate different instruments.]

Mix, seal the plate tightly, and centrifuge for 1 min at 1000 × *g*. Place the plate in a suitable qPCR thermal cycler. Incubate for 2 min at 50°, then for 10 min at 95°, followed by 40 cycles, with each cycle consisting of 95° for 15s and 60° for 1 min.

[NOTE—Some instruments and reagents require a preincubation step. Carefully follow specific instrument/reagent recommendations.]

Monitor the signal of the labeled probe using a suitable fluorescence detector. Determine the threshold value using the instrument-specific recommendations. Record the cycle thresholds ($C_t$) for each sample.

**Calculations**:

Plot the log quantity of DNA of the Standard solutions versus the Ct.

Calculate the slope and the intercept.

Using these values and the following equation, calculate the quantity of DNA in each well:

$$\text{Result} = 10^{(Ct - b/m)}$$

| | |
|---|---|
| $C_t$ | = cycle threshold of the Sample solutions |
| b | = intercept of the line for the Standard solutions |
| m | = slope of the line for the Standard solutions |

Calculate the quantity of DNA in each of the Sample solutions. Correct for any dilution or concentration of the sample.