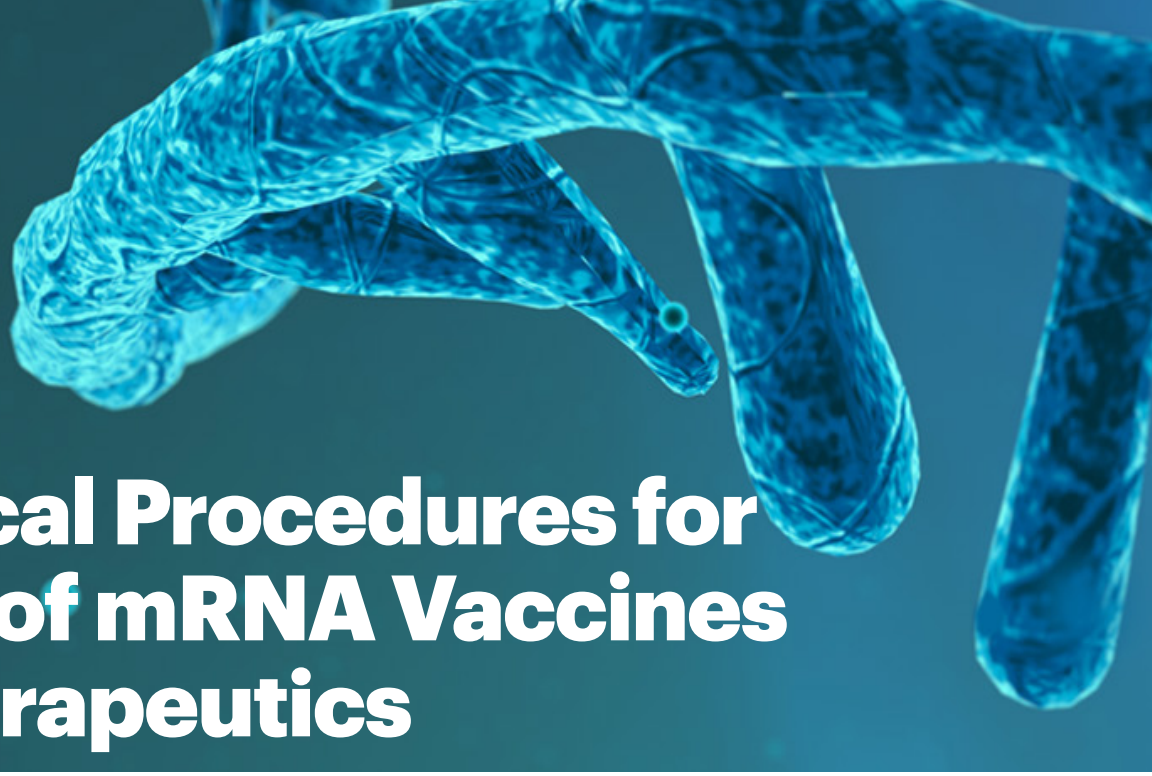


Analytical Procedures for Quality of mRNA Vaccines and Therapeutics

Draft Guidelines: 3rd Edition

Accelerating product development through a common understanding of quality

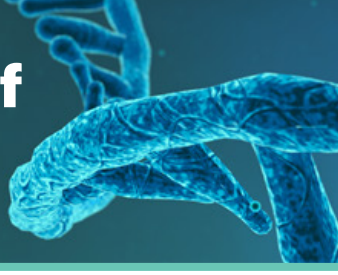
Vast potential of mRNA technology. The development of prophylactic vaccines to combat COVID-19 has shown the potential of mRNA technology, particularly in infectious diseases, and paved the way for a range of novel vaccines and therapies to come. Developing and manufacturing vaccines based on this modality have proven faster than other platforms, making mRNA a promising solution for addressing future pandemics, as well as other infectious diseases, such as rabies, Zika, and cytomegalovirus infection. Several mRNA-based therapeutic products are also in clinical pipelines for cystic fibrosis and various cancers.

Managing quality is critical. To build public trust and confidence in innovative products like mRNA vaccines and therapeutics, they must be, safe, effective, and of high quality. Like other biologics, manufacturing mRNA-based products is complex. As changes are made to raw materials, starting materials, processing steps, and formulation during the progression from early research and development to large-scale manufacturing, robust testing is needed to ensure the quality and safety of the final product. Methods for determining product quality that are suitable for preclinical or early clinical development phases may not be fit for purpose at commercial scale. Product quality attributes that are not properly identified and addressed can impact the integrity of the product, leading to poor clinical outcomes, causing costly delays, and threatening regulatory approval.

A common set of methods is needed. Since mRNA technology is relatively new, regulatory guidelines and industry standards to guide non-proprietary aspects of mRNA quality during development and manufacturing are still evolving. These include areas such as verifying identity, controlling impurities, and measuring content for dosing. Without a common set of methods for determining quality, developers and manufacturers must develop their own in-house methods and protocols, taking attention and resources away from a company's successful development and commercialization of mRNA technology unique to the medical product.

Analytical Procedures for Quality of mRNA Vaccines and Therapeutics

Draft guidelines



There are four main types of RNA therapeutic strategies:

mRNA, antisense RNA (asRNA), RNA interference (RNAi) and RNA aptamers. Of the four, only mRNA-based products involve delivery of *in vitro* transcribed mRNA into a target cell, where cellular machinery translates the mRNA into a protein. There are three different modalities that utilize mRNA.

1) **Vaccination** – where mRNA encoding specific target antigen(s) is administered to elicit protective immunity.
2) **Cell therapy** – where mRNA is transfected into the cells *ex vivo* to alter cell phenotype or function. These cells are then delivered into the patient.
3) **Replacement therapy** – where mRNA is administered to the patient to compensate for a defective gene or a protein.

The global mRNA therapeutic and vaccine market is expected to expand significantly in the next ten years due to the proven effectiveness of the platform (i.e., Covid-19 vaccines), speed to design and produce the product, as well as greater flexibility compared to traditional approaches. mRNA-based therapeutics and vaccines are in development for a variety of indications, including infectious diseases, metabolic diseases, cancer, and cardiovascular diseases.

Creating shared understanding is critical to advancing adoption of this technology. To address this need, USP is developing a set of analytical methods for mRNA quality to support developers, manufacturers, regulatory agencies, and national control laboratories worldwide. The goal is to create an overall shared understanding of mRNA quality attributes, with the aim of accelerating product development, guiding successful manufacturing scale-up, and bolstering regulatory confidence in the utilization of best practices and suitable quality controls when developing and manufacturing this new modality.

Based on needs identified by various stakeholders, USP and our BIO3 – Complex Biologics and Vaccines Expert Committee developed this draft guideline as a first step towards developing a procedural guideline for testing of mRNA vaccines. It includes analytical procedures and best practices to support the assessment of common quality attributes of mRNA vaccines and therapeutics. This draft guideline builds on best practices described in General Chapters *<1235> Vaccines for Human Use—General Considerations and <1239> Vaccines for Human Use—Viral Vaccines*.

In February 2022, USP and our BIO3 Expert Committee released the first edition of the draft guideline to solicit feedback from global stakeholders on the included methods and to encourage submission of any alternative methods and additional supporting documentation, including validation packages related to the methods presented in the draft guidelines. USP received over 300 comments, edits, and several donations of methods. In the second edition, stakeholder feedback was addressed, and the donated methods were incorporated.

In April 2023, the second edition of the guidelines was launched by USP to gather further feedback from stakeholders worldwide. During this phase, USP actively sought public comments on the revised mRNA draft guidelines, receiving input from various stakeholders alongside several donated methods. With the release of the third version, USP has meticulously addressed public comments and integrated donated methods into the guidelines. Additionally, USP has conducted evaluations of methods for several critical quality attributes (CQA) and incorporated these methods into the draft guidelines.

Major updates in this version include the following:

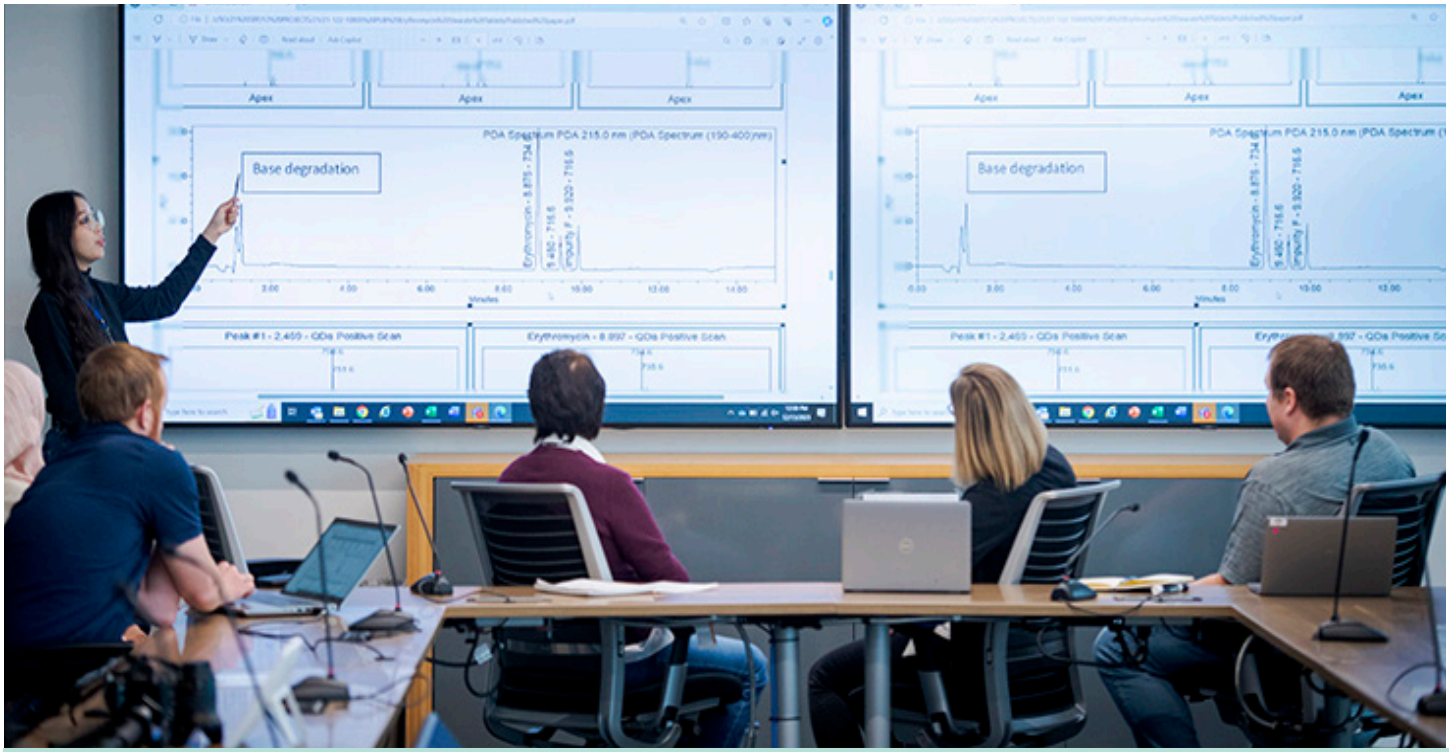
- Replaced Table 1 with a new version adapted from proposed *USP Chapter <1040>* which has been published in Pharmacopeial Forum.
- Added donated LC-MS method for 5′ capping efficiency
- Added donated LC-MS method for 3′ poly(A) tail length
- Updated immunoblot method
- Added residual NTPs and capping agent in mRNA method by AEX-HPLC
- USP has evaluated the following methods
  - LC-MS method for 3′ poly(A) tail length
  - Immunoblot method for dsRNA impurity
  - AEX-HPLC method for residual NTPs and capping agent in mRNA
  - CGE method for mRNA integrity
  - IP-RP-HPLC method for mRNA purity

Analytical Procedures for Quality of mRNA Vaccines and Therapeutics

Draft guidelines

To further support the quality and consistency of analytical methods used for mRNA-based vaccines and therapies, USP has initiated evaluation of several methods in USP laboratories, with the goal of qualifying and/or validating these methods. In some cases, where the method is applicable to a broad range of mRNA products, methods may be advanced as documentary standards (for example general chapters with validated methods).

These proposed compendial methods would be published in the Pharmacopeial Forum for public comment prior to publication as Chapters in USP-NF. In other cases, where standardization is more challenging due to the variety of mRNA products in development, methods and other associated information may be published outside of the compendial process (e.g., draft guidelines, technical notes, or scientific publications).



Additional collaboration is needed

To advance these draft guidelines, we invite industry, academic and government experts with experience or interest in mRNA vaccines and technology to provide feedback on the methods detailed in these draft guidelines and to recommend additional information supporting the understanding of mRNA quality. We encourage the submission of any alternative analytical methods as well as supporting documents (e.g., validation documentation). By collaborating with USP, participants play an active role in shaping standards and solutions that contribute to building the supply of safe, effective, quality medicines that people around the world can trust.

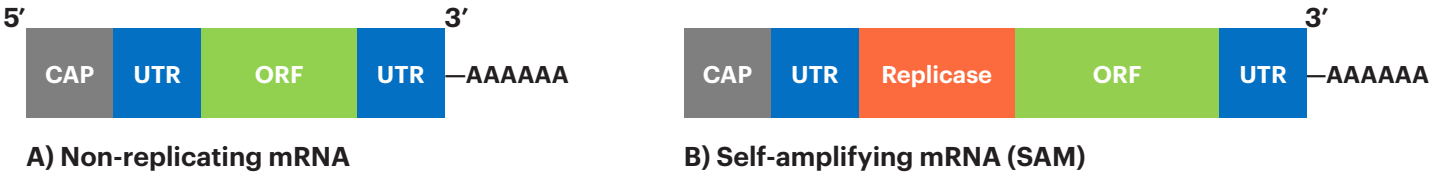
To provide feedback on this proposed draft or inquire about other aspects of this work, contact USPVaccines@usp.org. **More information can be found on our website at** http://www.usp.org/mrna-quality.

Introduction

Naturally occurring mRNA is produced in eukaryotic cells by the transcription of DNA in the nucleus by RNA polymerase. The mRNA molecules are transported out of the nucleus to the cytoplasm, where they serve as templates for translation by ribosomes to produce proteins. In this way, the information stored in the genome is used to express specific proteins. An mRNA cannot create any protein other than the protein for which it is coded.

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

Figure 1: Two Main Forms of mRNA Vaccines



There are several ways mRNA drug substances can be manufactured including with the use of PCR-generated template or non-linearized plasmid with the terminator sequence. The mRNA drug substance template can be prepared by amplification by host cells (e.g., *Escherichia coli*). In either case, each lot of the plasmid DNA that is used in manufacturing of the mRNA vaccines, must be tested to confirm its identity, purity, and quality before release. Plasmid DNA is considered a GMP-level starting material and must be manufactured in accordance with the principles of GMP from the initial cell bank stage onward. Best practices to manage cell banks and testing recommendations is described in General Chapters <1042> Cell Banking Practices for Recombinant Biologics.

Table 1 below summarizes the attributes and suggested methods which are described in more detail in the proposed General Chapter currently in PF <1040> Quality Considerations of Plasmid DNA As a Starting Material for Cell and Gene Therapies. The goal of this general chapter is to describe considerations for the manufacture and release testing of plasmids for use as starting materials. The document was published in the Pharmacopeial Forum (PF) 49(6) in November 2023 for 90-day public comment period. Currently, the public comments received are being reviewed by the expert panel followed by the expert committee.

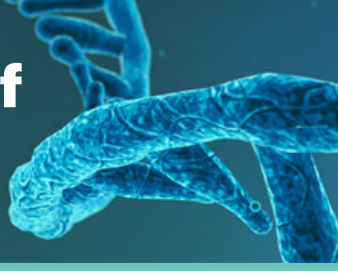


Table 1: Plasmid DNA Quality Attributes, USP Chapter <1040>

Quality Attribute	Analytical Procedures	Example Acceptance Criteria[a]
Appearance—color and clarity	Visual Inspection from ⟨631⟩[b] or Ph. Eur. 2.2.2	Clear, colorless solution but may be molecule and/or process specific
Visible particulates	⟨790⟩,[c]⟨1790⟩[d], Ph. Eur. 2.9.20, Ph. Eur. 2.9.19	Free of visible particulates
pH	⟨791⟩[e]	End use-specific
Concentration	A_{260}	End use-specific
	Size exclusion HPLC	
Identity	Restriction map	Identical to theoretical or identical to reference map
	Sequencing	Identical to reference sequence
Identity of homologous regions including long terminal repeats (LTRs), inverted terminal repeats (ITRs), and poly(A) tails	Restriction map	The integrity of these regions should be understood as part of the overall control strategy.
	PCR	
	Sequencing	
Plasmid DNA topology	Agarose gel electrophoresis	Criteria should be end use-specific. Here are some possible attributes of interest: Control for appropriate percentage total supercoiled with low percentage of open circular, linear, and other forms
	Anion exchange (AEX) HPLC	
	Capillary gel electrophoresis (CGE)	
	Analytical ultracentrifugation	
	Electron microscopy	
Residual protein	Colorimetric [e.g., bicinchoninic acid (BCA), Lowry]	End use-specific; 1–10% (w/w)[f]
	Host cell protein enzyme-linked immunosorbent assay (ELISA)	
Residual host DNA	Quantitative polymerase chain reaction (PCR)	End use-specific; 1–10% (w/w)[f]
Residual host RNA	Agarose gel electrophoresis (AGE) with an appropriate stain	End use-specific; 1–10% (w/w)[f]
	Fluorescent labeling of RNA measured by fluorimetry	
	Reverse phase HPLC RT-PCR	
Residual antibiotic(s)	ELISA	End use-specific; consider both patient and environmental safety
	Mass spectrometry	
	Reverse phase HPLC	
Endotoxins	<85>[g]	End use-specific; ≤10–100 EU/mg
Bioburden[h]	<61>[i]	End use-specific; in general, ≤1 CFU/10 mL

Quality Attribute	Analytical Procedures	Example Acceptance Criteria[a]
Sterility[h]	<71>[j]	No growth [NOTE—Scale of manufacturing may make sampling per ⟨71⟩ difficult. See 5.1.12 Bioburden and Sterility for more details.]
Mycoplasma[k]	<63>[l]	Should not be present in microbial generated plasmid DNA. Testing is optional de- pending on application. May be a requirement to rule out human contamination.

a Example acceptance criteria are provided when available, unless it is so specific and specific to the process (end use-specific).
b Color and Acromicity <631>
c Visible Particulates in Injections ⟨790⟩.
d Visual Inspection of Injections ⟨1790⟩.
e pH <791>.
f May need to consider patient safety guidelines when setting these limits.
g Bacterial Endotoxins Test <85>.
h Testing of both bioburden and sterility are not needed. One or the other should be chosen based on application and agreement with health authorities. If sterility is the testing approach that is chosen, sampling per <71> might not be feasible due to manufacturing scale, etc. See 5.1.12 Bioburden and sterility for more details.
i Microbial Enumeration Tests <61>.
j Sterility Tests <71>.
k Mycoplasma does not infect prokaryotic cells, so by default it should not be present in plasmids prepared from microbial sources. However, this test may be required for entry of the DNA starting material into eukaryotic manufacturing facilities and by regulatory authorities.
l Mycoplasma tests <63>.

Manufacturing of mRNA drug substance can be broken down to 2 essential steps: upstream enzymatic processes and downstream chromatography and ultrafiltration-based purification. The upstream process requires high quality template DNA for optimal in vitro transcription (IVT) yields. The DNA 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. Due to the degeneracy of the genetic code, the mRNA sequence can be optimized with respect to codon usage for the more efficient translation and stability. In addition, modified nucleosides can be used to enhance the function of the mRNA. The 5′-cap can be introduced co-transcriptionally by adding capping reagents to the IVT mix. Also, the 3′-poly (A) tail can be added enzymatically or can be encoded in the DNA template. This all depends on the specific manufacturing process. The mRNA drug substance is then purified and formulated to make the drug product. The mRNA-based therapeutics and vaccines require a delivery system such as polymers, polymer-based nanoparticles, lipids, or lipid nanoparticles for entry into recipient cells. While there are ranges of types of vehicles available to deliver mRNA, LNPs currently are the most advanced system that has been demonstrated to be safe and effective. LNPs protect mRNA from degradation and enable cell entry through endocytosis. Once in the endosome, mRNA molecules are released from the endosome into the cytoplasm providing templates to produce multiple copies of the expressed protein. In an mRNA-based vaccine, the expressed protein serves as an antigen to stimulate an immunological response, which is the desired outcome of vaccination.

When mRNA is used as a therapy, modifications can be introduced into the mRNA molecule to enhance functionality including translation efficiency. This is achieved through modifications which decrease nucleoside modification, inhibit nuclease resistance, and decrease immunogenicity of the mRNA itself. When mRNA is used as vaccines, the exogenous mRNA can activate innate immune cells via Toll-like receptors (TLR) 3, 7, and 8, other receptors like RIG-I, or effector proteins. 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 and drug product is determined by their design, development, and specifications applied to them during the development and manufacturing process (**Figure 2**). Quality control checks on starting materials such as nucleotides/ nucleosides, enzymes (for synthesis, linearization, and capping), solvents and buffers, polymers, nanoparticles, lipids/ lipid nanoparticles must be incorporated into the testing program. In cases where animal and/or human derived materials are used, they should be tested for adventitious agents. For a well characterized product, analytical methods for characterization, lot release and stability testing of drug substance and drug product must be developed and performed to monitor critical quality attributes and other product attributes. Stability studies must be performed to define and confirm

product shelf life, understand potential degradation pathways, and support comparability assessments. When scaling up or otherwise modifying the manufacturing process, comparability studies may include both routine (e.g., release) and non-routine (e.g. characterization) testing (ICH guideline Q5E). This guideline provides methods for assessment of quality attributes for characterization and release testing for bulk purified mRNA drug substance, as listed in **Table 2**. The mRNA drug substance requires formulation (e.g., LNPs) for delivery; suggested methods to support lot release and stability testing of LNP-mRNA encapsulated drug product characterization and release testing are listed in **Table 3** below. These tables define the quality profiles for drug substance and drug product with method options. These tests are examples and developers will need to evaluate and decide in conjunction with health authorities what is appropriate for a given product; alternative methods, technologies, equipment, and reagents may be selected on a case-by-case basis. Among these, stability-indicating parameters may include mRNA quantity, integrity, degree of encapsulation, potency, particle size, polydispersity and/or impurities associated with the mRNA and lipid components. Note however that stability-indicating methods must be assessed and defined on a case-by-case basis for each product and relevant stage, based on experience.

Figure 2: mRNA Production Process and Testing

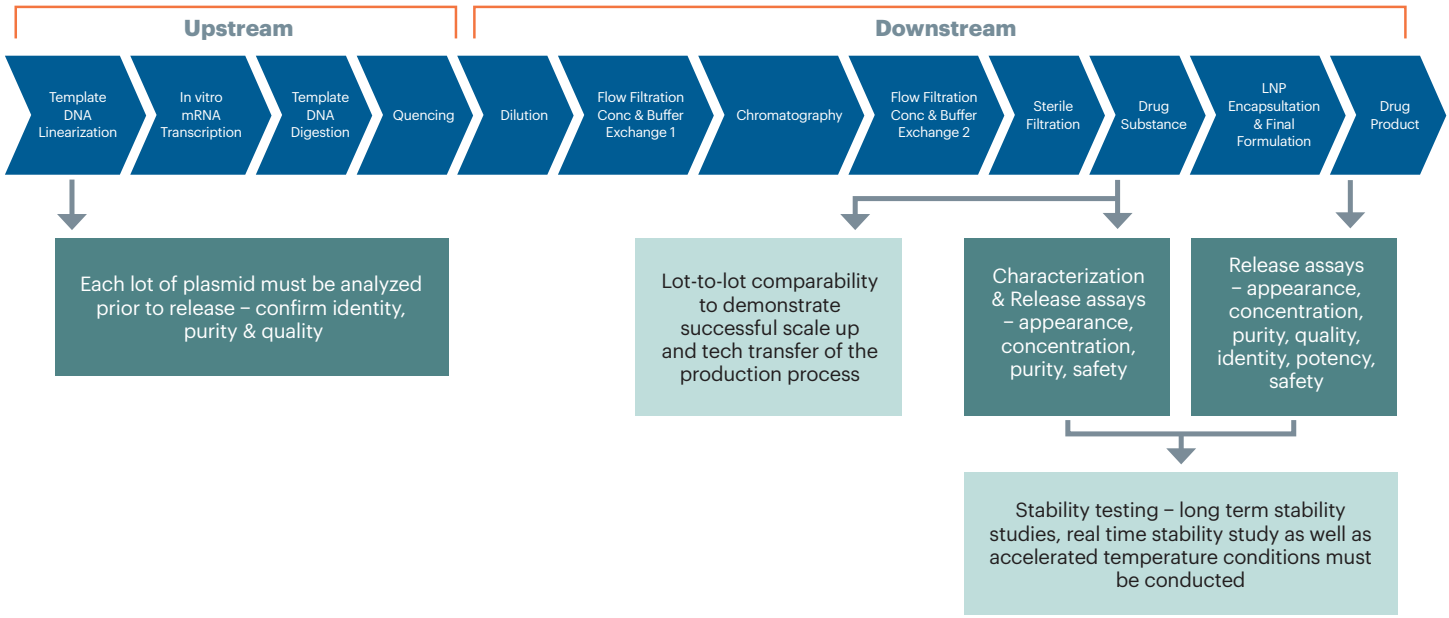


Table 2: Characterization and release testing for mRNA Drug Substance

Quality	Attribute	Method
Identity	mRNA sequence identity confirmation	High throughput sequencing (HTS)
		Sanger sequencing
		Reverse Transcriptase – PCR (RT-PCR)
Content	RNA concentration	Quantitative reverse-transcription PCR (RT -qPCR)
		Reverse-transcription digital PCR (RT–dPCR)
		Ultraviolet Spectroscopy (UV)

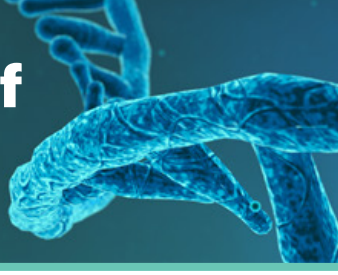


Table 2: Characterization and release testing for mRNA Drug Substance (Continued)

Quality	Attribute	Method
Integrity	mRNA intactness	Capillary electrophoresis [D]
		Capillary gel electrophoresis (CGE) [D]
		Agarose gel electrophoresis
Purity	mRNA purity	Ion pair reversed-phase high-performance liquid chromatography (IP-RP-HPLC)
	5′ capping efficiency	Reverse-phase liquid chromatography mass spectroscopy (RP–LC-MS/MS) [D]
		Ion pair reversed-phase high-performance liquid chromatography (IP-RP-HPLC)
		Liquid chromatography mass spectroscopy (LC-MS/MS) [D]
	3′ poly(A) tail length	Liquid chromatography mass spectroscopy (LC-MS/MS) [D]
		Ion pair reversed-phase high-performance liquid chromatography (IP-RP-HPLC)
	Product related impurities - dsRNA	Immunoblot
		Enzyme-linked immunosorbent assay (ELISA)
	Product related impurities - aggregate quantitation	Size exclusion-high-performance liquid chromatography (SEC-HPLC) [D]
	Product related impurities - percentage of fragment mRNA	Reversed-phase HPLC (RP-HPLC) [D]
	Process related impurities - residual DNA template	quantitative PCR (qPCR)
	Process related impurities - quantitation of free/non-incorporated nucleosides	Reverse-phase liquid chromatography mass spectroscopy (RP–LC-MS/MS) [D]
	Process related impurities - residual NTP and capping agent	Anion exchange high-performance liquid chromatography (AEX-HPLC) [D]
	Process related impurities - residual T7 RNA polymerase content	Enzyme-linked immunosorbent assay (ELISA)
Potency	Expression of target protein	Cell-based assay
Safety	Endotoxin	USP <85>
	Bioburden	USP <61>, <62>, <1115>
Other	Appearance	USP <790>
	Residual solvents	USP <467>
	pH	USP <791>

D Donated methods

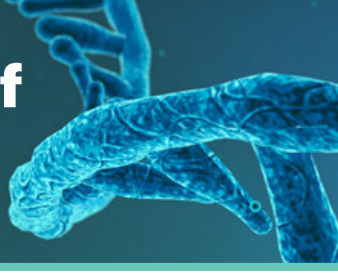


Table 3: Characterization and release testing for mRNA Drug Product

Quality	Attribute	Method
Identity	RNA identification	Sanger sequencing
		Reverse Transcriptase – PCR (RT-PCR)
	Identity of lipids	Reversed-phase high-performance liquid chromatography with charged aerosol detector (RP-HPLC-CAD)
Content	RNA concentration/RNA encapsulation efficiency	Fluorescence-based assay
	Lipid content	Reversed-phase high-performance liquid chromatography with charged aerosol detector (RP-HPLC-CAD)
Integrity	LNP size and polydispersity	Dynamic light scattering (DLS)
	RNA size and integrity	Capillary gel electrophoresis (CGE) [D]
Purity	Product related impurities - aggregate quantitation	Size exclusion-high-performance liquid chromatography (SEC-HPLC) [D]
	Product related impurities - percentage of fragment mRNA	Ion pair reversed-phase high-performance liquid chromatography (IP-RP-HPLC) [D]
Potency	Expression of target protein	Cell-based assay
Safety	Endotoxin	USP <85>
	Sterility	USP <71>
Other	Appearance	USP <790>
	Residual solvents	USP <467>
	Osmolality	USP <785>
	Subvisible particles	USP <787>
	Extractable volume	USP <1>, <698>
	Container closure integrity	USP <1207>
	pH	USP <791>

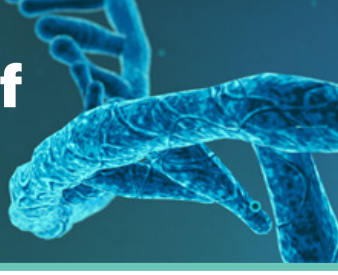
D Donated methods

[Note: all temperatures are written in " ° ", denoting Celsius.]

Identity

Identity of Encoded RNA Sequence by HTS

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 dideoxynucleotide triphosphates (ddNTPs). The ddNTPs act as reversable terminators that prohibit any further attachment of nucleotides at the 3′ end in a given sequencing cycle. 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 HTS is the enrichment of mRNA for the subsequent library construct. Fragmentation and library preparation should be done directly on the RNA.

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 ethylenediaminetetraacetic acid (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 (A260/280 ratio of RNA should be around 2:1). 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 three times 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. First the total RNA is briefly denatured, followed by hybridization of polyadenylated 3′ ends to oligo(dT) beads. All other non-polyadenylated transcripts such as rRNA, tRNA, and degraded RNA are 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 >99.8% 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 appropriate rotation, time, and temperature (at e.g. maximum speed (10,000 x g) for 25 min at 4°). Carefully remove supernatant and wash the pellet 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.

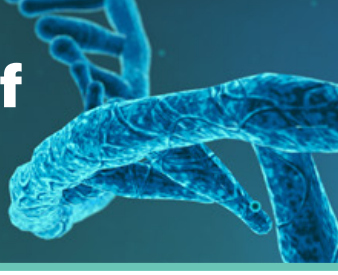
Synthesis of first strand cDNA: RNA fragments are reverse transcribed to cDNA because the DNA is more stable and allows for amplification using DNA polymerases. mRNA can be transcribed from the coding strand (has the same sequence as mRNA) or template strand (used for transcription). This process will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

First strand buffer (5X): Mix 250 mM of tris(hydroxymethyl)aminomethane hydrochloride (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 (≤ 1 µg of total RNA or 50 – 100 ng of poly(A)+ RNA) of mRNA.[3] Incubate the sample in a PCR thermal cycler at 65° for 5 min and then place on ice immediately. Set the thermal cycler to 25°. For each 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 a 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 (~200 U) 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 of 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 purifies the products of the ligation reaction to select a size for enrichment with a gel, beads, or other appropriate methods. The following gel method described is only an example.

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. 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 cleaves 3′ overhangs into blunt ends which can occur due to attachment of non-templated nucleotides.

Preheat a heat block at 20°. 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 onto the next step.

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

Preheat a heat block at 37°. 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.

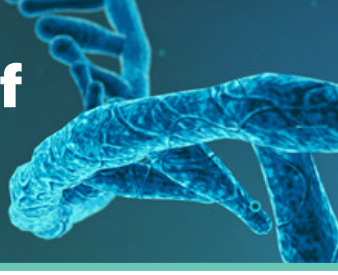
In a 1.5 mL RNase–free tube, add 23 µL of eluted DNA, 25 µL of 2X Rapid T4 DNA Ligase Buffer, 1 µL of PE Adapter Oligo Mix,[10] and 1 µL of T4 DNA Ligase to a total volume of 50 µL. Incubate the sample at room temperature for 15 min, then purify the sample using a PCR purification kit following the instructions 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 of DNA ladder (the use of ladder that covers a range of bp from 100 bp to 2000 bp), 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 (≤400 mg) 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. There are variety of ways to validate the library including using qPCR, ddPCR, Bioanalyzer, or Micro Chip. Next, check the size and purity of the sample. This can also be analyzed by a native PAGE.

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 determine the sequence. There are three steps to HTS 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. Appropriate system suitability controls should be included within the assay to ensure a valid test; assay validity criteria must be established in a case-by-case basis, but could be based on parameters such as number of reads required, Q30, controls, % of reads before and after trimming, % of unmapped reads, etc.

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 ddNTPs of the nucleotide bases. The method is performed by generating double stranded cDNA from mRNA and then sequencing by sanger sequencing. The detailed description provided is an example of a potentially suitable analytical procedure.

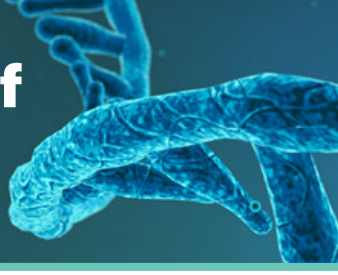
[NOTE- The protocol below is written for sequencing with Applied Biosystems BigDye Direct chemistry and BigDye XTerminator clean-up. It provides one-tube simplicity for Sanger sequencing and post-sequencing processing. For other sequencing chemistries, please see the documentation supplied with the kit.]

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 cDNA Synthesis master mix (containing a gene specific primer or random hexamers and oligo-dT primers),[17] 1 – 15 µL of sample (100 ng – 2.5 µg of mRNA recommended), 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 4. Samples can be held at 4° for up to 8 h or freeze at –20° for longer storage. However, individual reverse transcriptase may differ in terms of optimal working temperatures. Set the temperature conditions as per the specific RT instructions.

Table 4. 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 and reverse primers that focus on specific regions of the target gene.

[NOTE- BDD sequencing requires M13 sequence tag additions to the primers. Add M13 forward (5′-TGTAAAACGACGGCCAGT-3′) and reverse (5′-CAGGAAACAGCTATGACC-3′) sequences to the 5′-end of primers specific for your gene(s)].

Resuspend dried and desalted primers to final concentration of 100 µM with 1xTE buffer solution. Next, make a working stock of PCR amplification primers. 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 primers 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 triplicate, 5 µL of PCR BigDye Direct PCR Master Mix,[18] 1 µL of cDNA sample (20-40 ng of cDNA), and water to final volume of 10 µL. Setup duplicate reactions, since one will be used for forward and another for reverse direction sequencing. Make sure to include a separate positive control sample and a separate negative control sample (no-template). Seal the plate, vortex the mixture briefly and centrifuge for 5–10s at 1,000 x *g*. Put samples in the thermal cycler and run the program detailed in **Table 5**.

Table 5. Thermal Cycler Conditions (Amplification)

	Steps				
	Polymerase Activation	Cycle (40 cycles)			Hold
		Denaturation	Annealing	Extension	
Temperature (°)	95	96	62	68	4
Time (min)	10 min	3 s	25 s	30 s	Indefinitely

Cycle sequencing: Remove the seal from the plate and add 2 µL BigDye Sequencing Master Mix, 1 µL of forward or reverse primer to each of the wells.[19]

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

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 6**.

Table 6. Thermal Cycler Conditions (Cycle Sequencing)

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

Sequencing clean-up: There are methods available for removal of unincorporated nucleotides and salts. The following protocol uses BigDye Xterminator[20] Sequencing clean-up. This kit works directly on the reactions in the wells of the Cycle Sequencing plate, without the transfer of the reaction to new tubes or other manipulations.

Centrifuge the reaction plate for 1 min at 1,000 x *g*. Each well requires 45 µL of BigDye Xterminator SAM solution and 10 µL Xterminator solution. Combine enough of these solutions sufficient for each reaction plus a 20% extra volume.

Add 55 µL of this mixture to each of the cycle sequencing wells. Make sure to vortex the XTerminator mixture after every 8-12 additions to re-homogenize the mixture.

Collection of data: Load the plate into the capillary electrophoresis instrument. Select or create an appropriate run module according to capillary length, number of capillaries, and polymer type on the instrument. Make sure BDX running options are chosen. 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: There are multiple ways to analyze data. One way is to use sequence analysis software to generate a report documenting the resulting sequence and QC metrics of the run. Verify the sequence is correct using alignment algorithms such as BLAST. If needed, software is available that will call low frequency somatic variants at a detection level as low as 10% frequency (Applied Biosystems Minor Variant Finder).

Identity by Non-Quantitative RT-PCR

Non-quantitative reverse transcription PCR (RT-PCR) and digital PCR can be used to identify mRNA. Alternatively, a general overview of the qRT-PCR is provided below and is performed in two steps: reverse transcription (first strand of cDNA synthesis), and PCR amplification. The purpose is solely the identification of the mRNA and not quantification of the mRNA drug substance. Note that in general, RT-PCR provides less information by comparison to the sequencing approaches as a maximum of 100 nt can be verified by PCR, in contrast to the full sequence provided by HTS and Sanger methods. However, RT-PCR can be selected as a suitable method for mRNA identity testing under certain circumstances. Thus, the justification of selection of an identity method for DP and DS testing should take all relevant aspects from the product itself, manufacturing process and analytical methods into consideration.

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 7. First-Strand cDNA Solution

Component	Volume
Gene specific primer, random hexamers, oligo-dT primer (2 pmol)	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 8. Reverse Transcription Reaction Solution

Component	Volume
cDNA mixture from above	12 µL
First strand buffer (5X)	4 µL
RNase-free water	Final volume to 19 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—There are different types/suppliers of reverse transcriptase with different condition requirements therefore use as per manufacturer's instructions]

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 9** prepare a 50 µL reaction solution.

Table 9. RT-PCR Reaction Solutions

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)	1 µL
cDNA from first strand reaction	2 µL
ddH_20	Final volume to 50 µL

Gently mix the reaction and place it in the thermal cycler using the following program. This program is an example based on a specific enzyme/process and operators should follow conditions based on the reagents and amplicon they are working with. Follow procedures as per manufacturer's guidance.

Table 10. Thermal Cycler Conditions

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

Content

Content by qPCR

Quantitative PCR (qPCR) can be performed to quantify mRNA using either the fluorescent DNA probes or DNA probe. Following method uses TaqMan as fluorescent dye and a 1/20 volume of the cDNA preparation as template and SuperScript III Platinum kit.[21]

[NOTE- There are currently four different fluorescent DNA probes for RT-qPCR; SYBR Green, TaqMan, Molecular Beacons and Scorpions. Follow manufacturer's instructions for each. Adjustment may be required for the use of other kits or other real time PCR instruments.]

mRNA content by qPCR: Thaw all components including primer/probe mix. Additionally, primer/probe mix can also be purchased. 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.

Master Mix: Prepare the following mixed solution.

Table 11. Master Mix

Component	Volume
Reaction mix 2X	12.5 µL
$MgSO_4$	0.4 µL
Forward primer (10 µM)	1 µL
Reverse primer (10 µM)	1 µL
Probe (10 µM)	0.5 µL
mRNA (1-500 ng)	X µL
Superscript IIIRT/Platinum Taq Mix	1 µL
RNase-free water	Final volume to 25 µL

Heat the mixture at 65° for 5 min, and then quickly cool on ice for 2 min. Centrifuge for 5–10s at 1,000 x *g*.

Assay controls: Each RT-qPCR should include controls in addition to the unknown sample.

Negative control: A known negative template control that is sterile with nuclease-free water. This is used as a control for PCR reagent function and cross-contamination.

Positive control: Used as control for PCR reagent function including primer and probe integrity.

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. Add 20 µL of master mix into each well of a optical plate chilled on ice. Make sure to include at least 2 wells of negative control and 2 wells of positive control.

Run the plate on thermocycler using the following cycling conditions.

Table 12. Thermal Cycler Conditions

Cycling Step	Temperature (°)	Time	Cycles
Enzyme activation	95	10 min	None
Denaturation	95	15 sec	40
Annealing/extension	60	30-60 sec	40

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

Content by digital PCR

Digital PCR can be used to confirm the identity of mRNA without a standard curve. mRNA drug substance is reverse transcribed to cDNA and amplified, followed by running the samples on a digital or droplet digital PCR System.[22]

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 13. First-Strand cDNA Solution

Component	Volume
Gene specific primer (2 pmol)	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 14. Reverse Transcription Reaction Solution

Component	Volume
cDNA mixture from above	12 µL
First strand buffer (5X)	4 µL
RNase-free water	Final volume to 19 µL

NOTE—There are several types/suppliers of reverse transcriptase with different condition requirements therefore use as per manufacturer's instructions]

Gently vortex the mixture for few seconds. If gene specific 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.[23] Mix thoroughly by vortexing each tube for 30s at maximum speed to ensure homogeneity. Centrifuge briefly to collect contents at the bottom of the tube. Prepare the following reaction mixture on ice.

Table 15. dPCR Reaction Mixture

Component	Volume per Reaction (µL)	Final Concentration
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.

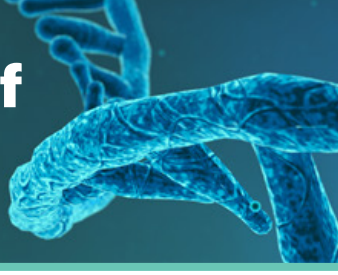
Droplet generation: Load 20 µL of each reaction mixture from above into a sample well of a DG8 cartridge.[24] Add 70 µL of Droplet Generator Oil to the bottom row of the cartridge designed for "oil". Fit rubber DG80 Gasket onto the Cartridge and place it on the Droplet Generator. This process should 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 16. Thermal Cycler Conditions

Cycling Step	Temperature (°)	Time	Cycles
Reverse transcription	42-63	60 min	1
Enzyme activation	95	10 sec	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.

Content 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.

[NOTE: The A260/A280 ratio is dependent on both pH and ionic strength.]

[NOTE: The use of an alternative methods (such as a fluorescence-based assay) for RNA content is also possible at the DS stage.]

Sample solution: Dilute RNA matrix of the mRNA sample to prepare dilutions within the linear range of the method and to obtain a solution with a maximum absorbance at 260 nm.

Perform a background correction by making readings from a blank at 320 nm, 260 nm, and 280 nm. The absorbance at respective wavelengths are acquired with calibrated spectrometer referencing with appropriate blanks.

[NOTE— Acquire a full UV spectrum to detect offsets caused by blank/sample mismatch or additional error sources (particle scattering caused by dust or undissolved particulates, contaminations). For a simple correction of light scattering contribution, OD values at 260 nm and 280 nm can be subtracted by OD value at 320 nm of the blank solution (buffer solution only). For a best correction, second derivative method on most UV spectrum can be used.]

Analysis: The molar extinction coefficient is determined by measuring the absorbance values of an RNA standard (in buffered solution) dilution series in the sample matrix. The slope of the obtained standard curve can be used to determine the molar extinction coefficient of RNA in the specific matrix.

Determine the absorbance of the *Sample solution* using Beer-Lambert Law equation. For highly diluted RNA samples, cuvettes with optical path lengths other than 1 cm might be preferable.

Beer-Lambert Law equation

$$A = \varepsilon b C$$

A = absorbance
ε = molar absorption coefficient
b = path length, 1 cm
C = concentration

[NOTE— The mass extinction coefficient of single-stranded RNA is: $0.025(\mu g/ml)^{-1}\ cm^{-1}$ (absorbance max at 260 nm)].

Integrity

RNA Integrity

A high-resolution analytical method that can measure the integrity of RNA molecules by length is crucial for quality assurance, understanding potency, and for optimization of manufacturing processes. The Fragment Analyzer system is a parallel capillary electrophoresis instrument that can analyze 12, 48 or 96 samples at once of the linearized plasmid DNA purity and IVT RNAs. Capillary gel electrophoresis 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. Data acquisition and analysis can be performed using the instrument's software to determines the size and concentration of the RNA fragments present in the sample.

RNA Integrity by Fragment Analyzer

RNA ladder: Use a suitable RNA ladder.[25]

RNA diluent marker solution: Use a suitable RNA diluent marker solution.[26]

Intercalating dye solution: Use a suitable intercalating dye solution.[27]

Separation gel: Use a suitable separation gel.[28]

Blank solution: Use a suitable blank solution.[29]

Capillary conditioning solution (5X): Use a suitable 5X capillary conditioning solution.[30]

Capillary conditions solution (1X): Mix 1-part *Capillary conditions solution* with 4 parts WFI

Inlet Buffer (5X): Use a suitable Inlet Buffer

Inlet Buffer: Prepare the *Inlet Buffer (5X)* by mixing 1-part Inlet buffer with 4 parts WFI

RNA separation gel: Prepare by mixing *Intercalating Dye Solution (1/10,000 dilution)* with the *RNA Separation gel* to create a sufficient volume depending on the number of samples to be analyzed.

RNA ladder solution: Thaw ladder on ice. Heat-denature the ladder at 70° for 2 min, immediately cool to 4° and keep on ice. Use 2 µL of the 96 ng/µL ladder for every run.

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 suitably validated concentration range (e.g. 1 ng/µL to 100 ng/µL). If the concentration of the sample is above this range, dilute with RNase-free water. Prepare each sample in duplicate.

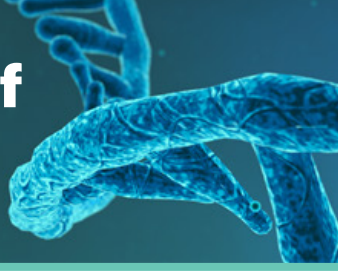
Sample plate preparation: Using a fresh RNase-free 96-well sample plate, pipette 22 µL of the *RNA diluent marker (15 nt)* to each well in a row that is to contain sample or *RNA ladder solution*. Pipette 2 µL of each denatured RNA sample into the assigned well on the plate containing 22 µL of *RNA diluent marker solution (15 nt)*. The *RNA ladder solution* must be run in parallel with the samples for each experiment to ensure accurate quantification and sizing. Pipette 2 µL of denatured *RNA ladder solution* into the 22 µL of *RNA diluent marker solution (15 nt)* in the designated ladder well. Mix the contents of each well by pipetting up and down 10x or by using a plate shaker set to 3000 rpm for 2 minutes. Fill any unused wells within the row of the sample plate with 24 µL of Blank solution. 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.

Instrument set up: Pipet 1 mL of fresh Inlet buffer into a deep well plate and place plate into drawer 'B' on the instrument. Pipet 200 µL of 0.25x TE Rinse buffer into each well of a 96 well plate and place in drawer 'M'. Load prepared gel and prepared conditioning solution into the instrument.

Separation procedure and analysis: Load prepared sample plate into the instruments and enter sample names. Select Add to Queue and select the corresponding method based on the appropriate capillary length of the installed capillary array. Select OK to add the method to the queue. Click the 'GO' button to start the separation. For IVT RNA separations, it is recommended to increase the separation time by 5 minutes to ensure that the entire sample separates out. This can be done when the method is loaded and clicking on Edit Method, then increasing the time to 45 minutes. After the separation is completed, the samples can be automatically analyzed, and reports generated with the instrument data analysis software.

RNA Integrity by Capillary Gel Electrophoresis

A high-resolution analytical method that can measure the integrity of RNA molecules by length is crucial for quality assurance, and for optimization of manufacturing processes. The following two methods for capillary gel electrophoresis (CGE) with Light Induced Fluorescence (LIF) detection is used to evaluate the total RNA integrity. Option A is suitable for separation of RNA fragments varying from 200 to 6,500 bases and option B from 50 to 9,000 bases.



Separation Buffer

Option A: Protocol: 1X TBE buffer: 89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3.

Option B: RNA 9000 Purity & Integrity Kit[31]: Buffer not required.

Separation Gel

Option A: 1% Polyvinylpyrrolidone (PVP) at 1.3 MDa in 1X TBE buffer with 4 M Urea and 50,000x dilution or 0.002% SYBR™ Green II Stain[31]. Suitable for separation of RNA fragments varying from 200 to 6,500 bases.

Option B: RNA 9000 Purity & Integrity Kit[31]: Validated and ready-to-use separation gel. SYBR™ Green II Stain included and diluted at optimized concentration for mixing with the separation gel for single stranded RNA integrity and purity analysis. Suitable for separation of RNA fragments varying from 50 to 9,000 bases.

RNA ladder and marker

Option A: Dilute RNA ladder in nuclease-free water to 25 µg/mL.[32] If necessary, spike the ladder with a 1.2 Kb RNA marker to assess the separation method. Denature the solution for 5 minutes at 65° and place the sample mixture on ice or in an ice water-bath to cool the sample down for a minimum of 5 minutes.

Option B: RNA 9000 Purity & Integrity Kit: Add 2 µL of the ssRNA ladder to 48 µL of nuclease-free water or Sample Loading Solution. Heat the sample at 70° for 10 minutes, and immediately after the heating step, put the mixture on ice or in an ice water-bath to cool the sample down for a minimum of 2 minutes.

[NOTE: RNA ladders should be used as guides for size estimation and/or indicators for analytical variability. The user should consider RNA chemical modifications, the purine: pyrimidine ratio, and GC-content that may affect the expected migration time of analytes in comparison to the RNA ladder or markers.]

Sample preparation

To release the RNA from the mRNA-LNP particle for capillary electrophoresis (CE) analysis without a purification step, mix 10 µL of the mRNA-LNP sample(s) with 20 µL of a 0.3% Triton X-100 solution and incubate at room temperature for 20 minutes. Next, heat the sample(s) at 70° for 5 minutes, and immediately after the heating step cool down the sample(s) by placing on ice or in an ice-water bath for a minimum of 5 minutes. Lastly, add 60 µL of CE-grade water to the sample(s) and analyze by CE. This procedure can also be used for the analysis of free or in vitro transcribed RNA. An RNA detection range from 50 ng/mL to 50 µg/mL has been reported using the PA 800 Plus Pharmaceutical Analysis System or BioPhase 8800 System.

[NOTE - Occasionally, higher molecular weight (HMW) RNA products may be detected by capillary electrophoresis. The nature of these HMW products is of great interest in the field. Reports have shown that breaking up the mRNA-LNP or dissolving the released RNA material from the mRNA-LNP in the presence of water-based formamide solution (>80%) can dissociate these HMW products. The user should use caution interpreting these HMW RNA products.]

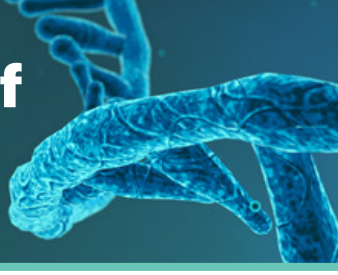
Cartridge

PA 800 Plus Pharmaceutical Analysis System: EZ cartridge pre-assembled with bare fused-silica capillary (50 µm I.D., 30 cm total length, 20 cm effective length).

BioPhase 8800 System: Pre-assembled BioPhase bare fused-silica capillary cartridge (8 capillaries, 30 cm total length).

Capillary gel electrophoresis

Option A: Suitable for RNA fragments ranging from 200 to 6,500 bases. Perform capillary electrophoresis by reverse polarity 200 V/cm electrical field (6 kV) at 25° within 18 minutes of running time. Sample introduction into the inlet of the capillary can be achieved electrokinetically at 5kV for 3 seconds. The sample tray temperature can be set at 10° with the LIF detector configured to 488 nm laser with an emission filter of 520 nm. A calibration curve using the RNA ladder described above can be generated to estimate the size of an unknown sample peaks. The user can add samples as needed to obtain the desired number of replicate measurements for statistical analyses.



Option B: Suitable for RNA fragments ranging from 50 to 9,000 bases. The recommended capillary electrophoresis settings for the PA 800 Plus Pharmaceutical Analysis System or the BioPhase 8800 System include a 6 kV reverse polarity with a capillary temperature of 30° with a ramp time of 2.0 minutes and a duration of 22 minutes. Automated sample introduction into the inlet of the capillary can be achieved first by a water-plug step using 0.5 psi for 5 seconds followed by pressure injection at 1.0 psi for 5 seconds. The sample tray can be set at 10° and the LIF detector configured to 488 nm laser with an emission filter of 520 nm. This separation method can analyze up to 8 samples or replicates in a single run using the BioPhase 8800 System. For detailed instrument settings refer to RNA 9000 Purity & Integrity Kit application guide.

Data analysis
32 Karat software is compatible with the PA 800 Plus Pharmaceutical Analysis System. Samples run with the BioPhase 8800 System can be analyzed with BioPhase software. 32 Karat data files are compatible with the BioPhase software. Automated data processing can determine main product percent purity composition based on corrected peak area %.

RNA Integrity by Agarose Gel Electrophoresis

MOPS buffer (10X): Dissolve 41.86 g of 200 mM MOPS (free acid), 6.80 g of 50 mM sodium acetate, 3.72 g of 10 mM EDTA-2$H_2$0 and 3.80 g of 10 mM EGTA (free acid) in 850 ml of RNase-free water. Adjust pH to 7.0 ± 0.2 with 10 M NaOH and adjust volume to 1000 mL with RNase-free water. Filter the solution through 0.2 µm pore size filter.

Running buffer (1X): Dilute *10X MOPS buffer* with deionized water, 1:50.

Loading dye: Add *10X MOPS buffer*, 0.5 M 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 enough of the loading buffer 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.]

mRNA sample preparation: Add the freshly prepared 2X loading buffer 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. 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 (for a 1% gel) in 72mL of deionized water until dissolved. Cool agarose to 60°. Place in fume hood. Add 10 mL of *10X MOPS buffer* and 5.5 mL of prewarmed 37% 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 5 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 known RNA standards.

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

DNAzyme-mediated mRNA capping efficiency assay by LC-MS
A DNAzyme is a synthetic oligonucleotide capable of catalytically cleaving RNA to which it hybridizes. Applying DNAzyme to cleave the specified 5′ end of the in-vitro transcribed mRNA allows for subsequent analysis of capped and uncapped 5′-RNA fragments by LC-MS.

[NOTE – DNAzyme must be optimized for each RNA sequence, otherwise it will not bind to the sample.]

Annealing Buffer: 15 mM NaCl, 5 mM Tris-HCl, pH 7.5, 0.1 mM EDTA

Annealing Reaction: 100 pmol-454 pmol of mRNA and DNAzyme (2-fold excess as a minimum) are mixed in annealing buffer, heated for 3 min at 95°C and slowly cooled to 37°C.

Cleavage reaction: One-tenth of the volume of 10x cleavage buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 200 mM $MgCl_2$) is added and the mixture is incubated for 3 hours at 37°C.

Phosphatase treatment: The post DNAzyme reaction mixture is treated with a mixture of T4 polynucleotide kinase and CIP by adding one-tenths of the volume of T4 PNK T4 buffer, 10 units of each enzyme per 1 pmol of mRNA and incubating mixture for 1 hour at 37°C.

Quenching the reaction: Add appropriate volume of 0.5 M EDTA to phosphatase-treated reaction mixture.

Sample workup: The reaction mixture is concentrated and desalted using 3K MWCO ultracentrifugation filters. The resulting concentrated sample is diluted with Mobile phase A to target 2 pmol/µL RNA with at least a 1:1 ratio of filter solution and Mobile phase A and used for LCMS analysis.

Alternatively, RNA is isolated from the reaction mixture using phenol-chloroform extraction and NaOAc-Ethanol precipitation. The resulting RNA pellet is re-suspended in Mobile phase A and is used for LCMS analysis.

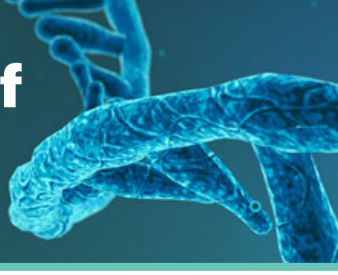
Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)
[NOTE - Alternative settings can be applied, if justified and validated for intended use.]

Mode: LC-MS
Solution A: 1% Hexafluoroisopropanol (HFIP), 0.1% Diisopropylethylamine (DIPEA), 2 µM EDTA in water
Solution B: 0.075% HFIP, 0.0375% DIEA, 2 µM EDTA in 65/35 v/v ACN/Water
Column: ACQUITY UPLC BEH C18 Column, 130Å, 1.7 µm, 2.1 mm X 100 mm reverse phase
Flow rate: 400 µL/min
Detector: UV 260nm and ESI-MS
Column Temperature: 70°
Autosampler compartment temperature: 10°
Injection volume: 50-100 µL (100 pmoles)

Table 17. LC Gradient Table

Time (min)	Solution A (%)	Solution B (%)
0.0	98	2
3.0	98	2
3.1	94	6
22.1	85	15
33.1	80	20
33.2	0	100
35.2	0	100
35.3	98	2
42.0	98	2



ESI parameters: Gas temperature 263°, gas flow 13 L/min, nebulizer pressure 40 psi, sheath gas temperature 425°, sheath gas flow 12 L/min, capillary voltage 3500 V, nozzle voltage 0 V

Analysis

Measure the peak areas of the respective peaks.

Purity

mRNA 5′- CAP by RP–LC-MS/MS

This method allows accurate detection and quantitative assessment of ribose-methylated and ribose-unmethylated cap structures, which can be separated and quantified using LC-MS/MS. To this end, it is necessary to digest the mRNA using Nuclease P1 to produce cap dinucleotides.

Solution A: 30 mM of ammonium acetate buffer. The pH is adjusted to pH 7.5 by addition of ammonium hydroxide

Solution B: Acetonitrile

[NOTE- Use only LC-MS grade chemicals.]

Mobile phase: See the gradient below.

Table 18. RP-HPLC Gradient Table

Time (min)	Solution A (%)	Solution B (%)
0	100	0
1	100	0
7	70	30
9	50	50
10.5	50	50
12.5	100	0
20	100	0

Sample preparation: Treat up to 20 µg mRNA with 0.6 U nuclease P1 in 20 mM ammonium acetate (pH 5.3) and 0.1 mM zinc chloride. Incubate the reaction at 50°C for 1 h. Nuclease P1 hydrolyze RNA to nucleoside monophosphates. Since phosphodiester bonds of cap structures are spared out, it also results cap dinucleotides (e.g., m7GpppN). Optionally, the resulting nucleoside monophosphates can be hydrolyzed to nucleoside level using Alkaline Phosphatase.

Chromatographic system

[NOTE: Alternative method settings can be applied, if justified and validated for intended use.]

Column: Poroshell 120EC-C18 3x150 mm 2.7 µm
Column temperature: 25°
Flow rate: 0.5 mL/min

Mass parameters

Mass spectrometer: Triple quadrupole (QQQ) equipped with an electrospray ion source (ESI).
Mode: Positive ion mode, MRM (multiple reaction monitoring).
ESI parameters: Gas temperature 300°C, gas flow 7 L/min, nebulizer pressure 40 psi, sheath gas temperature 350°C, sheath gas flow 12 L/min, capillary voltage 3500 V, nozzle voltage 0 V.
QQQ parameters: Depending on which cap dinucleotides must be detected. It is recommended to optimize the instrument parameters (fragmentor voltage, collision energy, and cell accelerator voltages) for each mass spectrometer to achieve optimal sensitivity.

Table 19. Examples for QQQ Parameters

Compound Name	Precursor Ion (m/z)	Product Ion (m/z)	Fragmentor (V)	Collision Energy (V)	Cell Accelerator Voltage (V)
m^7GpppAm	801	136	135	68	7
m^7GpppA	787	136	135	60	7
m^7GpppGm	817	166	135	68	7
m^7GpppG	803	248	135	20	7

Analysis

Measure the peak areas of the dinucleotide containing the ribose methylation and of the dinucleotide not containing the ribose methylation.

Calculate the percentage of ribose-methylated cap-dinucleotides:

$$\text{Result} = [\ A_{rm} / (A_{rm} + A_u)] * 100$$

A_{rm} = area of the ribose-methylated cap dinucleotide peak

A_u = area of the ribose-unmethylated cap dinucleotide peak

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 thus a critical quality attribute for mRNA-based vaccines. Capped and uncapped mRNA fragments can be separated and quantitated using ion pair reverse-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.

Solution A: 100 mM of triethylammonium acetate buffer, pH 7.0
Solution B: Solution A with 25% (v/v) acetonitrile
Mobile phase: See the gradient table below.

Table 20. 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 concentration should be 120% of the mRNA concentration to ensure complete hybridization of the mRNA. The RNA cleavage probe is product specific and should be chosen to produce a 5′-cap fragment of sufficient size (~25 nt) 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. 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*.)

[NOTE: Alternative method settings can be applied, if justified and validated for intended use.]

Mode: LC
Detector: UV 260 nm
Column: Acquity Premier Oligonucleotide C18 column, 130 Å,1.7µm,2.1 x 100 mm
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 peaks 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

Percent Poly(A) Tail Length by IP-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 mRNA vaccine. mRNA molecules with and without a poly(A) tail (tailless) can be separated and quantitated using ion pair reverse-phase high performance liquid chromatography (IP-RP-HPLC). It may be necessary to perform site-specific cleavage of the mRNA molecule using RNAse T1 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]

Buffer A: 100 mM of triethylammonium acetate buffer, pH 7.0
Buffer B: *Solution A* containing 25% acetonitrile.
Mobile phase: See the gradient table below.

Table 21. RP-HPLC Gradient

Time (min)	Buffer A (%)	Buffer B (%)
0	57	43
0.5	50	50
3.5	46	54
4	46	54
4.5	57	43
5	57	43

Chromatographic system

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

[NOTE: Alternative settings can be applied, if justified and validated for intended use.]

Mode: LC
Detector: UV 260 nm
Column: RNASep, 7.8 x 50 mm; packing non-porous, PS/DVB resin matrix
Column temperature: 61°
Flow rate: 0.9 mL/min
Injection volume: 10 µL

Analysis

Samples: *Sample solution*

Measure the areas of the peaks. There could be multiple peaks due to different lengths of poly(A) tail and different tailless fragments.

Calculate the percentage of poly(A) mRNA:

$$\text{Result} = [A_b / (A_b + A_e)] \times 100$$

A_b = area of the poly(A) mRNA peak
A_e = area of the tailless mRNA peak

Inaccurate results can occur due to over estimation of poly(A) tail percentages. If the enzyme fails to digest properly, it can result in underestimation for tailless fragments or overestimation for poly(A) mRNA. Second, peak areas are proportional to the lengths of digestion products: longer poly(A) tail have stronger signal than shorter tailless mRNA. This could lead to over-estimation percentage of poly(A) mRNA.

Poly(A)-tail Length and Polydispersity Analysis in mRNA by LC-MS

Liquid chromatography mass spectroscopy (LC-MS) method can be used to analyze poly(A) tail length ≤ 130 nucleotides (nt).

The method involves initial digestion of the mRNA with a combination of nucleotide-specific endonucleases that leave the long poly(A) fragments intact while reducing the rest of the mRNA to mononucleotides and small oligonucleotide fragments. The resulting digest is analyzed using IP-RP-HPLC-ESI-MS method. The data is processed using deconvolution software to determine composition, lengths, and polydispersity of the poly(A) species.

mRNA digestion procedure: Add 50-100 pmole mRNA in digestion buffer and incubated at 90° for 10 min. Add appropriate amounts of RNAse A and RNAse T1 to the mRNA solution and incubate the reaction at 37° C for 1 -3 h. Analyze the reaction mixture by LCMS.

Chromatographic system

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

[NOTE: Alternative settings can be applied, if justified and validated for intended use.]

Mode: LC-MS
Solution A: 1% Hexafluoroisopropanol (HFIP) and 0.2% TEA in water
Solution B: 50/50 Acetonitrile/methanol
Column: ACQUITY UPLC BEH C18 Column, 130Å, 1.7 µm, 2.1 mm X 100 mm reverse phase
Flow rate: 250 µL/min
Detector: UV 260 nm and ESI-MS
Column Temperature: 70°
Autosampler compartment temperature: 10°
Injection volume: 100 µL

Table 22. RP-HPLC Gradient Table

Time (min)	Solution A (%)	Solution B (%)
0	99	1
1.0	99	1
16.0	86	14
16.2	0	100
17.2	0	100
17.5	99	1
23.0	99	1

ESI parameters: Gas temperature 250°, gas flow 13 L/min, nebulizer pressure 40 psi, sheath gas temperature 275°, sheath gas flow 12 L/min, capillary voltage 3500 V, nozzle voltage 0 V

Analysis: Analyze the poly(A) peak by MS to determine composition, lengths, and polydispersity of the poly(A) species

Measure the Poly A tail distribution of mRNA using the following equation:

Weighted Average of the Poly A tail length = SUM(Rounded A count * Intensity)/ SUM(Intensity)

[NOTE - To determine the Rounded A count - open the Deconvolution peak and copy and paste the values in excel. Highlight the masses with rel% between 20 - 100, then divide all the highlighted masses by 329 and round to the nearest integer to estimate the number of A's.]

mRNA Purity by IP-RP-HPLC

Ion-pair reverse-phase high performance liquid chromatography (IP-RP-HPLC) can be utilized for the determination of the integrity of the transcribed mRNA.

Chromatographic system

(See *Chromatography* <621>, *System Suitability*.)
[NOTE - Alternative settings can be applied, if justified and validated for intended use.]

Mode: LC
Solution A: 100 mM Triethylammonium acetate (TEAA), 1 mM EDTA in water, pH 7.3
Solution B: 100 mM TEAA, 1 mM EDTA, 25% Acetonitrile in water, pH 7.3
Column: RNASep, 7.8 x 50 mm; packing non-porous, PS/DVB resin matrix
Flow rate: see gradient table below.
Detector: UV 260 nm, collect 3D data from 200-400 nm
Column Temperature: 65°
Autosampler compartment temperature: 10°
Maximum column backpressure: 4000 psi
Injection volume: 5 µL
Sample preparation: Dilute mRNA samples to 0.2 mg/mL of water.
Blank preparation: Prepare blank vials with 100-200 µL of water into HPLC vials. Run blank samples bracketing each set of samples.

Table 23. RP-HPLC Gradient Table

Time (min)	Solution A (%)	Solution B (%)
0	0.3	30
1	0.3	30
1.1	0.3	40
2	0.3	40
16	0.3	60
16.5	1	30
19	1	30
19.6	0.3	30
20	0.3	30

Analysis

Measure the percent areas of the respective peaks.

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 release assay.]

TBS-T buffer: Prepare a solution of 25 mM Tris-HCl, 150mM NaCl, 0.05% of tween-20, pH7.4.

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

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

dsRNA Antibody Solution: Dilute the reconstituted antibody 1:5000 in *Incubation Buffer*.[33]

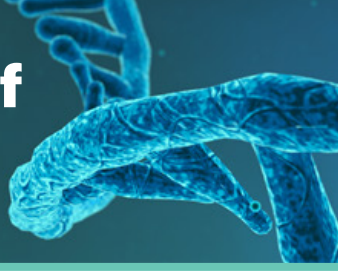
[NOTE- Dilute the reconstituted antibody as per your antibodies manufacturer's instructions. This recommendation is specific to this antibody only.]

Detection Antibody Solution: Dilute the reconstituted HRP-conjugated donkey anti-mouse IgG 1:5000 in *Incubation Buffer*.[34]

Detection Reagent: Chemiluminescent (ECL) substrate for low femtogram protein level detection

Procedure and Analysis: Blot 200ng of the mRNA test sample and a dsRNA reference sample at the limit of detection onto a positively charged Nylon membrane (0.45µM) 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 the membrane with *Detection Reagent*. Capture images with an appropriate digital imaging system.

[NOTE- Buffer/matrix interference must be factored into the final format since signal intensity could be higher depending on the buffer used.]



dsRNA by ELISA

ELISA can be used to measure a specific antigen in a biological sample. It can be used to quantitate the amount of dsRNA. This example uses K1 IgG2a monoclonal antibody.

[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 release assay.]

Sample diluent buffer: Prepare the solution by combining equal volume of 0.1 M NaCl in 1X TE solution, and RNA storage solution.

To determine the concentration of dsRNA, generate a standard curve using antigens of a known concentration then calculate the concentration of dsRNA using the optical density (OD).

ELISA can be performed by capturing anti-dsRNA monoclonal antibody, by diluting K1 IgG2a to a concentration of 300ng/mL in blocking buffer. Add 100 µL of this mixture to a protein A coated 96-well microplate. Incubate the plate overnight at 5°. Wash plate with 1X PBS containing 0.05% Tween-20. Dilute samples in *sample diluent buffer*. Add 200 µL of this mixture to the plate and incubate for 2 hrs at RT with shaking at 500 rpm. Wash plate with 1X PBS containing 0.05% Tween-20. After washing add 50 µL of neat K2 IgM hybridoma supernatant to the plate and incubate for 1 hr at RT with shaking at 500 rpm. Wash plate with 1X PBS containing 0.05% Tween-20. Dilute HRP-conjugated goat anti-mouse IgM, chain specific polyclonal antibody to 1:16,000-fold in blocking buffer. Add 100 µL of this to the plate and incubate for 1 hr at RT with shaking at 500 rpm. Wash plate with 1X PBS containing 0.05% Tween-20. Add 100 µL 1-Step Ultra TMB-ELISA substrate Solution to the plate following the instruction provided with the substrate. Incubate as instructed.

Analysis

Read the absorbance values at 450 nm on a plate reader. The concentration of dsRNA in the test sample can be determined from the standard curve prepared from a 142-bp dsRNA standard and the response of the standard concentrations fit to a 4-parameter logistic equation.

mRNA Aggregation by SEC-HPLC

Size Exclusion Chromatography (SEC-HPLC) can be utilized for its quick and reliable method in many applications such as purification or aggregate quantification. This SEC method allows a quick and reliable to quantify the percentage of aggregates of mRNA sample. Further characterization of each SEC peak can be performed using RP-HPLC as an orthogonal method (see below).

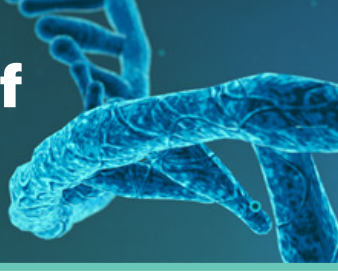
Chromatographic system

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

Mode: LC
Mobile phase: 150 mM Phosphate buffer, pH 7.0
Column: SRT SEC-1000, 5 µ, 1000Å, 7.8x300 mm
Flow rate: 1.0 mL/min
Detector: UV260 nm
Column Temperature: 25°
Sample: mRNA sample (single-stranded, 1000 nucleotides, 300-600 kDa) (0.5 mg/mL)
Injection volume: 5 µL

Analysis

Measure the peak areas of the respective peaks (main peak, high molecular weight, and low molecular weight).



mRNA Percent of Fragments by RP-HPLC

Ion-pair reverse-phase high performance liquid chromatography (IP RP HPLC) can be utilized for the determination of the integrity of IVT-transcribed mRNA.

Chromatographic system

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

[NOTE: Alternative settings can be applied, if justified and validated for intended use.]

Mode: LC
Solution A: 100 mM triethylammonium acetate (TEAA), pH 7.0
Solution B: 100 mM TEAA/ 25% Acetonitrile, pH 7.0
Column: Proteomix RP-1000, 5 µm, 1000Å, 2.1x100 mm
Flow rate: 0.3 mL/min
Detector: UV 260 nm
Column Temperature: 50°
Sample: mRNA sample (single-stranded, 1000 nucleotides, 300-600 kDa) (0.5 mg/mL)
Injection volume: 10 µL

Table 24. RP-HPLC Gradient Table

Time (min)	Solution A (%)	Solution B (%)
0	90	10
1	90	10
5	40	60
20	30	70

Analysis

Measure the peak areas of the respective peaks

Residual DNA Template (qPCR)

(See *Residual DNA Testing* <509>)

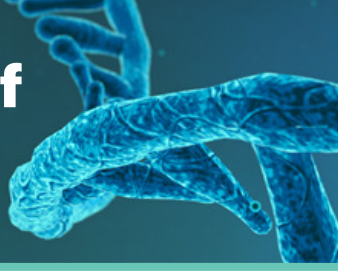
The following method is suitable for measurement of residual host cell DNA in mRNA vaccines drug substance. Extraction may not be required for drug substances; therefore, a quantitative polymerase chain reaction (qPCR)-based method can be directly used for the measurement of residual host cell DNA template. 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. Use method suitable for nucleic acid extraction that is appropriate for mRNA to be examined. 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-HCl and 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.

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 C_t.

Calculate the slope and the intercept.

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

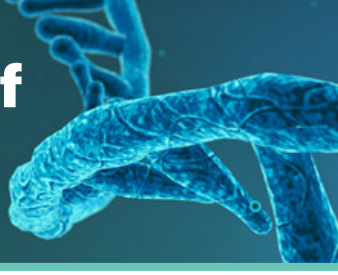
$$\text{Result} = 10^{(C_t - b/m)}$$

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.



Quantitation of Free/Non-Incorporated Nucleosides in mRNA by RP-LC-MS/MS

This method allows for detection and quantitation of most modified nucleosides in synthetic mRNA. This includes not only modifications that were intentionally introduced via their triphosphates during in vitro transcription, but also impurities introduced via commercial NTP charges of synthetic or biological origin, as well as impurities such as oxidized nucleosides. The method also detects m7G or ribose-methylated modifications released from cap structures. Using LC-MS/MS, it is possible to analyze mRNA at nucleoside level to detect and quantify dozens of different modified nucleosides in a single run. The method can easily be adapted for quality control of NTP charges used for in vitro transcription.

Solution A: 5 mM of ammonium acetate buffer. The pH is adjusted to pH 5.3 by addition of acetic acid

Solution B: Acetonitrile It is obligatory to use only LC-MS grade chemicals.

Mobile phase: See the gradient below.

Table 25. RP-HPLC Gradient Table

Time (min)	Solution A (%)	Solution B (%)
0	100	0
10	92	8
20	60	40
23	100	0
30	100	0

Sample preparation

Up to 10 µg RNA are digested to nucleoside level using 0.6 U nuclease P1, 0.2 U snake venom phosphodiesterase, 0.2 U bovine intestine phosphatase and 10 U benzonase in 5 mM Tris (pH 8) and 1 mM magnesium chloride for 2 h at 37°. Optionally, it is possible to add deaminase inhibitors like pentostatin (A-deaminase inhibitor, 200 ng) and tetrahydrouridine (C-deaminase inhibitor, 500 ng) to avoid nucleoside degradation.

[NOTE: Alternative settings can be applied, if justified and validated for intended use.]

Chromatographic system

Column: Synergi Fusion 4 µm particle size, 80 A pore size, 250 x 2.0 mm; Phenomenex
Column temperature: 35°
Flow rate: 0.35 mL/min
Detector: UV 254 nm

Mass parameters

Mass spectrometer: Triple quadrupole (QQQ) equipped with an electrospray ion source (ESI)

Mode: Positive ion mode, dMRM (dynamic multiple reaction monitoring)

ESI parameters: Gas temperature 300°, gas flow 7 L/min, nebulizer pressure 60 psi, sheath gas temperature 400°, sheath gas flow 12 L/min, capillary voltage 3000 V, nozzle voltage 0 V

QQQ parameters: Depending on which modified nucleoside must be detected. It is recommended to optimize the instrument parameters (fragmentor, collision energy, cell accelerator voltage) for each mass spectrometer to achieve optimal sensitivity.

Table 26. Examples for QQQ Parameters

Compound Name	Precursor Ion (m/z)	Product Ion (m/z)	Retention Time (min)	Delta Ret Time (min)	Fragmentor (V)	Collision Energy (V)	Cell Accelerator Voltage (V)
m^7G	298	166	6.5	4	80	13	4
Am	282	136	14.8	3	105	17	4
m^5C	258	126	7.6	3	75	13	4
8oxoG	300	168	9.5	3	100	13	4

Analysis – Relative quantification

Measure the peak areas of the respective modified nucleoside by MS/MS.

The amount of modified nucleoside is normalized to the amount of adenosine, to account for differences in the injected RNA amount. For this purpose, extract the peak areas of adenosine from the UV chromatogram recorded at 254 nm.

Quantification is performed in a relative manner by adding isotope-labeled standards to each sample.

$$\text{relative abundance} = \frac{A_{(\text{MS mod})} * n_{(\text{ISTD})}}{A_{(\text{MS ISTD})} * A_{(\text{UV adenosine})}}$$

$A_{(\text{MS mod})}$ = area of the MS peak of the respective modification

$n_{(\text{ISTD})}$ = amount of internal standard in each sample

$A_{(\text{MS ISTD})}$ = area of the MS peak of the internal standard

$A_{(\text{UV adenosine})}$ = area of the UV peak of adenosine

Alternatively, another main nucleoside (C, U or G) can be chosen for normalization. For example, in the case of enzymatic polyadenylation which leads to unknown or different amounts of adenosine.

Analysis – Absolute quantification

Measure the peak areas of the respective modified nucleoside by MS/MS.

The amount of modified nucleoside is normalized to the amount of adenosine, to account for differences in the injected RNA amount. For this purpose, extract the peak areas of adenosine from the UV chromatogram recorded at 254 nm.

Absolute quantification is performed by using external calibration solutions. Prepare calibration solutions with concentrations of 0.1, 0.5, 1, 5, 10, 50, 100 and 500 nM for MS/MS detected modifications each containing equal amounts of internal standard. Inject 10 µL of each dilution to achieve a calibration in a range from 1 – 5000 fmol. For adenosine, prepare calibration solutions with concentrations of 0.1, 1, 10 and 100 µmol. Inject 5 µL of each dilution to achieve a calibration in a range from 0.5 – 500 pmol. The response factor corresponds to the slope of the linear fit of the calibration curves (peak areas are plotted against the respective amount).

$$x_{(\text{mod per main nucleoside})} = \frac{A_{(\text{MS mod})} * n_{(\text{ISTD})} * rf_{(\text{UV adenosine})}}{A_{(\text{MS ISTD})} * rf_{\left(\frac{\text{MS mod}}{\text{MS ISTD}}\right)} * A_{(\text{UV adenosine})}}$$

$x_{(\text{mod per main nucleoside})}$ = absolute quantification, amount of modification per main nucleoside)

$A_{(\text{MS mod})}$ = area of the MS peak of the respective modification

$A_{(MS\ ISTD)}$ = area of the MS peak of the internal standard

$n_{(ISTD)}$ = amount of internal standard in each sample

$rf_{(MS\ mod/\ MS\ ISTD)}$ = response factor of the ratio of the respective modification and the internal standard

$A_{(UV\ adenosine)}$ = area of the UV peak of the respective modification

$rf_{(UV\ adenosine)}$ = response factor of the respective modification

For mRNA of defined known sequence:

The amount of modified nucleoside can be normalized to the amount of RNA molecules.

$$x_{(mod\ per\ RNA)} = \frac{A_{(MS\ mod)} * n_{(ISTD)} * rf_{(UV\ adenosine)} * N_{(adenosine)}}{A_{(MS\ ISTD)} * rf_{\left(\frac{MS\ mod}{MS\ ISTD}\right)} * A_{(UV\ adenosine)}}$$

$x_{(mod\ per\ RNA)}$ = absolute quantification, amount of modification per RNA molecule

$N_{(adenosine)}$ = number of the respective modification in the RNA sequence

Alternatively, another main nucleoside (C, U or G) can be chosen for normalization. For example, in the case of enzymatic polyadenylation which leads to unknown amounts of adenosine

Residual T7 RNA Polymerase by ELISA

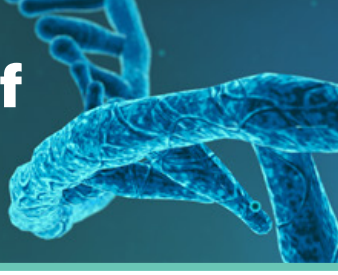
ELISA can be used to assess residual protein impurities specific for the detection of T7 RNA polymerase.

To determine the concentration of T7 RNA polymerase, generate a standard curve using antigens of a known concentration then calculate the concentration of T7 RNA polymerase using the optical density (OD).

Coating plate: Dilute rabbit anti-T7 RNA polymerase polyclonal antibody to 3 µg/mL in 1X PBS. Coat high binding 96-well microplate with 100 µL of this mixture and incubate overnight at 5°. Wash plate with 1X PBS containing 0.05% Tween-20. Block with Blocker Casein in PBS for 1.5 h at RT with shaking at 500 rpm. Wash plate with 1X PBS containing 0.05% Tween-20. Dilute T7 RNA polymerase and test samples in 1X PBS with 0.05% Tween and 0.1% BSA. Add 100 µL of this mixture to the 96-well microplate and incubate for 2 h at RT with shaking at 500 rpm. Wash plate with 1X PBS containing 0.05% Tween-20. Dilute horseradish peroxidase-conjugated rabbit anti-T7 RNA polymerase antibody to 3.3 µg/mL in Blocker Casein in PBS. Add 50 µL of this solution to the plate and incubate for 1 hr at RT with shaking at 500 rpm. Wash plate with 1X PBS containing 0.05% Tween-20. Add 100 µL 1-Step Ultra TMB-ELISA substrate Solution to the plate following the instruction provided with the substrate. Incubate as instructed.

Analysis

Read the absorbance values at 450 nm on a plate reader. The concentration of T7 RNA polymerase in the test sample can be determined from the standard curve prepared from a purified T7 polymerase standard and the response of the standard concentrations fit to a 4-parameter logistic equation.



Residual NTPs and capping reagent in mRNA by AEX-HPLC

Anion exchange high performance liquid chromatography (AEX-HPLC) can be utilized to verify and quantify the presence of residual NTPs and capping reagent in mRNA.

Preparation of Samples and Controls

NTP stock solution: Prepare intermediate solution by diluting mixed NTPs to 1 mM in DNase/RNase free water into a microcentrifuge tube.

Capping stock solution: Prepare intermediate solution by diluting capping agent to 10 mM in DNase/RNase free water into a microcentrifuge tube.

NTP control: Add 8 µL of 1 mM mixed intermediate dilution, 50 µL of 100 mM NaCl and 142 µL of water. Vortex and spin down in a microcentrifuge.

Capping reagent control: Add 3.2 µL of 10 mM mixed intermediate dilution, 50 µL of 100 mM NaCl and 146.8 µL of water. Vortex and spin down in a microcentrifuge.

mRNA sample: Add 150 µL of mRNA and 50 µL of 100 mM NaCl to a microcentrifuge tube. Vortex and spin down in a microcentrifuge.

Blank preparation: Prepare blank vials by mixing 50 µL of 100 mM of NaCl with 150 µL of water.

Filtration: Transfer blank, controls, and mRNA samples into a separate 10kDa ultrafiltration unit. Centrifuge the vials at 13,000 x *g* for at least 20 minutes. Transfer the filtrate into HPLC vials.

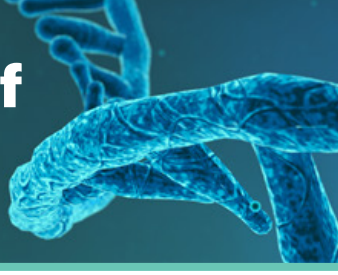
Chromatographic system

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

Mode: LC
Solution A: 25 mM TRIS base
Solution B: 25 mM TRIS base, 1 M lithium chloride
Column: DNAPac PA200 RS, 4 µm, 4.6 x150 mm
Flow rate: 1.0 mL/min
Detector: UV 260 nm
Column Temperature: 25°
Autosampler compartment temperature: 10°
Injection volume: 5 µL

Table 27. RP-HPLC Gradient Table

Time (min)	Solution A (%)	Solution B (%)
0	90	10
0.5	90	10
8.0	74	26
8.5	0	100
9.5	0	100
10.0	90	10
15.0	90	10



Analysis

Measure the peak areas of the respective peaks.

Calculate the mM of NTPs in the mRNA sample using the following equation.

$$\frac{Sample\ Peak\ Area}{Standard\ Peak\ Area} \times Standard\ NTP\ Concentration = Sample\ NTP\ Concentration$$

Biological Activity by Cell-Based Assay

Potency testing intends to determine cellular functionality of the DP (uptake, release, translation of RNA). DP potency can be demonstrated by verifying the cellular translation of the DP-containing mRNA into the encoded protein. There are two ways to detect cellular translation, I) antibody dependent detection of the target protein (FACS, ELISA, WB etc.) or II) antibody independent detection (aptamers, MS etc.). The method described here uses ELISA as the protein detection method, but other methods may also be used.

Functional binding assay of the expressed protein from transfected cells can be used to demonstrate potency of the mRNA-LNP.

[NOTE- This example of cell-based expression uses HepG2 cells. Other cell lines or procedures can be used.]

Sample preparation

Culture HepG2 cells in Minimum Essential Media (EMEM) containing 10% fetal bovine serum. For transfections with mRNA samples, seed cells at 1.0 X 10^6 cells in a 12 well tissue culture plate and incubate at 37˚ and 5% CO_2 in a humidified incubator for 16-24h prior to the transfection. Transfect cells with 4 µg of RNA using the Lipofectamine MessengerMax reagent and Opti-MEM according to manufacturer's instructions. Incubate the transfected cells at 37˚ and 5% CO_2 in a humidified incubator for 24 to 72 hrs. After 24-72 hrs., clarify the supernatant containing the expressed protein by centrifugation at maximum rpm for 1-2 min. Clarified supernatants can be stored at -80˚ if not used immediately.

For transfections with mRNA-LNP samples, seed cells as described above. Transfect cells with a total of 1.25 mg mRNA-LNP in Opti-MEM. Transfection of the LNP can be facilitated by the inclusion of ApoE3. Dilute ApoE3 to 1 mg/mL in the transfection media. Add the transfection media to the LNP and incubate at 37˚ and 5% CO_2 in a humidified incubator for 3 to 4 hrs. Add complete growth media to each well and incubate the plates at 37˚ and 5% CO_2 in a humidified incubator for 24 to 48 hrs., clarify the supernatant containing the expressed protein by centrifugation at maximum rpm for 1-2 min. Clarified supernatants can be stored at -80˚ if not used immediately.

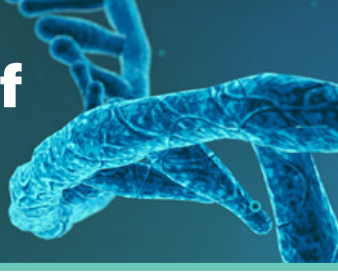
Next, perform ELISA on the expressed protein from transfected cells to demonstrate potency.

Analysis

Read the absorbance values at appropriate wavelength on a plate reader. The concentration of the mRNA sample can be determined from the standard curve prepared and the response of the standard concentrations fit to a 4-parameter logistic equation.

mRNA Drug Product Testing

The mRNA vaccines drug product can be properly analyzed by a list of compendial and non-compendial methods as described in Table 2 above. This section summarizes each test method utilized for drug product release. In addition, some of the analysis may require extraction of the RNA from mRNA-LNP or LNP from mRNA-LNP for further analysis using the methods described in the drug substance section.



Extraction Methods

RNA Extraction From mRNA-LNP

The purpose of RNA extraction is to obtain high quality purified sample for applications described below for some of the drug product analysis. There are several common reagents (e.g., TRIzol, chloroform) and kits (using chemical or beads) for RNA extractions. Follow the instructions provided in the kit. Two examples have been provided below.

Extract mRNA from mRNA-LNP formulation by isopropanol precipitation

[NOTE- Use only glass vials and syringes.]

Extract mRNA from the mRNA-LNP formulation by isopropanol (IPA) precipitation. Perform a 10-fold dilution by adding 100 µL of mRNA-LNP to 900 µL of 60 mM ammonium acetate in 100% isopropanol. Vortex and mix thoroughly. Centrifuge the sample at 14,000 x *g* for 15 minutes at 4°. Discard supernatant then wash the pellet with 1 mL of 100% isopropanol. Vortex the sample and centrifuge again at 14,000 x *g* for 15 minutes at 4°. Wash the mRNA pellet with 70% ethanol before drying the samples in speedvac for 20 min at RT. Dry samples can be stored or resuspended in 100 µL of RNase-free water at room temperature for quantification by UV absorbance.

Extract mRNA from mRNA-LNP formulation using chemical mixture

[NOTE- Use only glass vials and syringes.]

Incubate 100 µL of mRNA-LNP under agitation for 10 min at 50° in 10 µL of 1% Triton X-100. Next, extract the mRNA by adding 900 µL of a mixture of Phenol\Chloroform\Isoamyl alcohol 25:24:1. Next precipitate from the extracted solution by adding 0.1 volume of 3M sodium acetate, pH 5.2 and 2.5 volume of 100% ethanol. Incubate for 12 h at -20°. Centrifuge the sample at 12,000 x *g* for 10 minutes at 4°. Wash the mRNA pellet with 70% ethanol before drying the samples in speedvac for 20 min at RT. Dry samples can be stored or resuspended in 100 µL of RNase-free water at room temperature for quantification by UV absorbance.

Lipid Extraction from mRNA-LNP

The purpose of lipid extraction is to separate lipids from other constituents, such as nucleic acids and to preserve these lipids for further analysis. Polar solvents can be used to separate lipids whereas nonpolar solvents are used to dissolve lipids. There are several common kits and reagents available for lipid extractions. Follow the instructions provided in the kit. Following is a methanol:chloroform extraction method.

Extract of LNP from mRNA-LNP formulation using methanol:chloroform

[NOTE- Use only glass vials and syringes.]

Add 100 µL of mRNA-LNP sample into 200 µL of 100% cold methanol in 2 mL glass vial. Vortex and mix thoroughly for protein precipitation. Add 500 µL of chloroform with glass syringe and vortex. Keep sample on ice for 10 min. Add 200 µL of nucleic acid free water for phase separation. Again, vortex and keep on ice for 10 min. Insert vial into the 15- or 50-mL falcon tubes then centrifuge at 600 rpm for 5 minutes. Carefully remove bottom chloroform layer of about 300 µL using syringe and transfer into a new amber color glass vial with glass syringe. Dry the samples in a speedvac for 20 min at RT or dry under nitrogen gas stream. Store dried sample in -20° until analysis. Sample can be reconstituted IPA:methanol (1:1) for analysis.

Identity and Content Determination of Lipids by RP-UPLC-CAD

Reverse phase ultra-performance liquid chromatography with charged aerosol detection (RP-UPLC-CAD) can be used to determine identity of each individual lipid component and/or the associated degradation products.

[NOTE-Use glass pipets to transfer lipids/LNPs in organic solvents and use glass inserts, vials, and bottle to store lipids and LNP samples.]

LNP extraction: Follow Lipid extraction method from above to extract all the components of lipids from mRNA-LNP. Alternatively, mRNA-LNP can be diluted in 100% ethanol. Analyze the supernatant.

Lipid controls: Dilute each one of the purified lipid controls (cationic lipids, auxiliary lipids, cholesterol, and polyethylene glycol (PEG) separately. These will be used to identify and quantify lipid components.

Chromatographic system

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

[NOTE—Alternative method settings can be applied, if justified and validated for intended use.]

Mode: LC
Column: ACE Excel 2 Super C18 column, 2.1 X 150 mm
Solution A: 0.1% trifluoroacetic acid (TFA) in water
Solution B: 60/40/0.1% isopropyl alcohol/tetrahydrofuran/TFA
Column temperature: 60 °
Autosampler temperature: 15°
Flow rate: 0.5 mL/min
Injection volume: 5 µL

CAD settings

CAD evaporative temperature: 35°
Power function: 1.0
Gas resolution mode: Analytical
Data rate: 2 Hz
Filter: 3.6

Mobile phase: See the gradient table below.

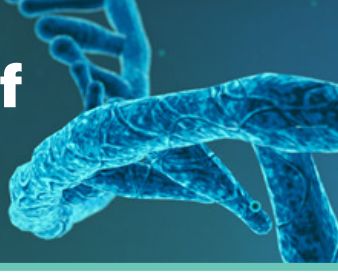
Table 28. RP-UPLC-CAD Gradient Table

Time (min)	Solution A (%)	Solution B (%)
0	95	5
1.5	95	5
5.5	52	48
9.5	52	48
10.5	44	56
22.5	44	56
30.5	4	96
32.5	4	96
35	95	5
40	95	5

Analysis

Sample: Sample solution

Measure the areas of the lipid peaks and calculate the percentage of each.



RNA Encapsulation Efficiency by Ribogreen Assay

Encapsulation efficiency can be measured by a RiboGreen assay. RiboGreen is a fluorescent nucleic acid stain used for RNA quantitation.

[NOTE—All solutions should be prepared in sterile nuclease-free glassware, using nuclease-free pipettes.]

RNA standard curve:

Prepare a 2 µg/mL solution of RNA in TE (10 mM Tris-HCl (pH 8.0) 1.0 mM EDTA) using nuclease-free tube. Dilute the 2 µg/mL RNA solution into disposable cuvettes (10 levels) between 1000 – 10 ng/mL. Add 1.0 mL of 1:200 diluted (in TE buffer) RiboGreen reagent.

Prepare a separate standard curve as described above with the addition of 0.15% of Triton X-100 into the 2 µg/mL RNA solution.

Controls:

Dilute 16S and 23S ribosomal RNA standard 50-fold in TE buffer to make 2 µg/mL working solution.

To determine the concentration of free mRNA, dilute mRNA-LNP sample to 2 µg/mL by adding 1X TE buffer to a final volume of 2000 µL. Add 1000 µL of RiboGreen solution. To release the encapsulated mRNA, add 0.15% of Triton X-100 into the mixture. Transfer the content to 4 mL disposable cuvette and measure the fluorescence response. at 1_{ex} = 480 nm and 1_{em} = 520 nm. Transfer the sample to the cuvette port of the microplate reader.

Analysis

Measure the fluorescence response at 1_{ex} = 480 nm and 1_{em} = 520 m. Determine the concentration of samples from calibration curve that were generated in the presence and absence of detergent and fit to a linear regression model with 1/x weighing.

Particle Size by DLS

Particle size affects biodistribution and cellular uptake and is a critical metric for LNPs. Dynamic light scattering (DLS) can be used for characterization studies and during quality control release testing to determine the average particle size and polydispersity index (PDI) of mRNA-LNP samples.

Sample preparation

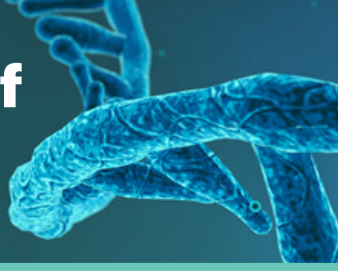
Dilute LNP samples in a suitable diluent (if necessary) at the concentration used at a drug product level or the working concentration during production. The actual dilution of the sample may need to be adjusted depending on the DLS system to enable accurate sizing reads. Perform measurements with the recommended backscatter angle and dispersant refractive index by the instrument manufacturer. Set viscosity parameters to corresponding DP samples and dispersant, respectively.

Analysis

Run DLS measurements for 100s and measure the mean hydrodynamic diameter of each sample.

RNA Size and Integrity by Capillary Gel Electrophoresis

The PA800 plus Pharmaceutical Analysis System from SCIEX System or the multi-capillary BioPhase 8800 System with Light Induced Fluorescence (LIF) detection can be used to evaluate the total RNA integrity. The above method for DS can also be applied to DP post mRNA extraction (see above) or by adding Triton X-100 at 2% (w/v) to the RNA sample mixture to release the encapsulated mRNA.



mRNA Aggregation Quantitation by SEC-HPLC

Size Exclusion Chromatography (SEC-HPLC) can be utilized for its quick and reliable method in many applications such as purification or aggregate quantification.

Chromatographic system

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

[NOTE: Alternative settings can be applied, if justified and validated for intended use.]

Mode: LC
Mobile phase: 100 Mm Tris acetate/ 2.5 mM EDTA pH 8
Column: Zenix SEC-300, 3 µm, 300Å, 4.6x150 mm
Flow rate: 0.25 mL/min
Detector: UV260 nm
Column Temperature: 25°
Sample: mRNA extracted from formulated mRNA-LNP
Injection volume: 5 µL

Analysis

Measure the peak areas of the respective peaks (main peak, high molecular weight, and low molecular weight).

Percentage of mRNA Fragment by IP-RP-HPLC

Ion pair reverse phase high performance liquid chromatography (IP-RP-HPLC) can be utilized to identify impurities formed through mRNA:lipid reactions.

Chromatographic system

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

Mode: LC
Solution A: 50 mM dibutylammonium acetate, 100 mM triethylammonium acetate
Solution B: 50 mM dibutylammonium acetate, 100 mM triethylammonium acetate, 50% acetonitrile
Column: RNASep, 7.8 x 50 mm; packing non-porous, PS/DVB resin matrix
Flow rate: 0.25 mL/min
Detector: UV 260nm
Column Temperature: 25°
Sample: mRNA extracted from formulated mRNA-LNP
Injection mass: 2µg of mRNA

Mobile phase: See the gradient table below.

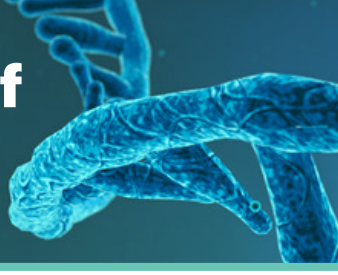
Table 29. IP-RP-HPLC Gradient Table

Time (min)	Solution A (%)	Solution B (%)
0	75	25
1.5	75	25
4.5	50	50
19	44	56
19.5	0	100
22.5	0	100
30.5	75	25
35.0	75	25
35	95	5
40	95	5

Analysis

Sample: *Sample solution*

Measure the areas of each peak and calculate the relative percentage of the late eluting peak of the total chromatographic peak area.



Endnotes

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
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
17. Suitable cDNA synthesis master mix can be obtained from ThermoFisher, Product Code 117565500 or equivalent.
18. BigDye Direct Cycle Sequencing Kit can be obtained from ThermoFisher, Product Code 4458688 or equivalent.
19. BigDye Direct Cycle Sequencing Kit can be obtained from ThermoFisher, Product Code 4458688 or equivalent.
20. BigDye XTerminator Purification Kit can be obtained from ThermoFisher, Product Code 4376486 or equivalent.
21. SuperScript III Platnium One-Step qRT-PCR kit obtained from ThermoFisher, Product Code 11732088 or equivanlent.
22. QX200 or QX100 Droplet Digital PCR System from BioRad Product Code 186 or equivalent.
23. One-Step RT-ddPCR Advanced Kit for Probes from Bio-Rad, Product Code 1864021.
24. DG8 Cartridges for QX200/QX100 Droplet Generator from BioRad Product Code 1864008 or equivalent.
25. Suitable RNA Ladder can be obtained from Agilent, Product Code DNF-386-U015 or equivalent.
26. Suitable RNA Diluent Marker Solution can be obtained from Agilent, Product Code DNF-370-0004.
27. Suitable Intercalating Dye Solution can be obtained from Agilent, Product Code DNF-600-U030.
28. Suitable Separation Gel can be obtained from Agilent, Product Code DNF-265-0240.
29. Suitable Blank Solution can be obtained from Agilent, Product Code DNF-301-0008.
30. Suitable 5X Capillary Conditioning Solution can be obtained from Agilent, Product Code DNF-475-0050.
31. RNA 9000 Purity & Integrity Kit can be obtained from Sciex, Product Code C48231 or equivalent
32. 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.
33. Suitable dsRNA antibody can be obtained from SCICONS English & Scientific Consulting, Product Code 10010500 or equivalent.
34. Suitable Detection Antibody can be obtained from Jackson ImmunoResearch, Product Code 715-035-151 or equivalent.
35. Suitable Detection Reagent can be obtained from Cytiva, Product Code RPN2109 or equivalent.