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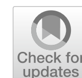

Technical Considerations of Pharmacokinetic Assays for LNP-mRNA Drug Products by RT-qPCR

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Abstract

Lipid nanoparticle-messenger RNA (LNP-mRNA) drug products are a growing class of drug modalities. The unique composition of these drug products requires multiple measurements to account for the different components of these drug modalities. Pharmacokinetic (PK) measurements include measurement of the encapsulated mRNA and components of the LNP in circulation to understand the effectiveness of the therapeutic mRNA. The PK measurements can utilize many different platforms including PCR. Current regulatory guidance documents for bioanalytical method validation are specific to ligand binding and chromatographic assay methods and difficult to interpret for use with molecular workflows. The purpose of this paper is to provide information on considerations for validation of regulated reverse transcription quantitative PCR (RT-qPCR) assays that are used to support the pharmacokinetic analysis of LNP-mRNA drug products.

Keywords LNP · MRNA · PK · RT-dPCR · RT-qPCR

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Introduction

RNA-based drug products are an emerging drug modality with the ability to treat a variety of diseases (1–4). There has been a rapid increase in the development and utilization of RNA-based therapeutic modalities especially after the global success from the recent COVID-19 mRNA vaccines (5). Therapeutic RNA (RNA) structures vary, and could include a cap, 5′UTR (untranslated region), CDS (coding sequence), 3′UTR, and a poly(A) tail. The fidelity of start and stop codon recognition involves the sequence context and translation factor (6). The construct of the mRNA drug product is critical to ensure reliability of translation and stability. There are many different types of RNA species that are currently known and studied including conventional mRNA, self-amplifying RNA, and circular RNA to mention a few examples.

Delivery of mRNA drug product is challenging based on its inherent physical and chemical instability (7), potential to elicit unwanted immune responses, and challenges to deliver to target cells and tissues (8). Because of the structure and charge, mRNAs are susceptible to degradation which causes inefficient delivery to cells (9). Lipid nanoparticles (LNPs) and lipoplexes are key delivery vehicles for mRNA-based drug products. LNPs are typically made up of ionizable lipids, phospholipids, cholesterol, polyethylene glycol (PEG) lipids and a helper lipid-like DSPC (distearoylphosphatidylcholine) (10, 11). These lipid components work synergistically to protect the mRNA, enhance cellular uptake, and ensure the efficient expression of the therapeutic protein. The construct of the LNP can be tailored to administration routes as well as target cells and expression of mRNA-derived proteins (e.g., intravenous, subcutaneous, intramuscular, secreted *versus* intracellular) (12, 13).

The unique modality of mRNA delivery systems (e.g., LNP-based) (14) requires pharmacometrics analysis that includes multiple measurements to account for the unique pharmacokinetic (PK) attributes of these drug products (15). For example, PK of LNP-mRNA drug products require measurement of the encapsulated mRNA and components of the LNP (e.g., ionizable lipid and the PEG lipids) in circulation (e.g., serum, plasma, CSF, urine) (16). Each component plays a crucial role in the delivery and effectiveness of the RNA drug product. In developing pharmacokinetic/pharmacodynamic (PK/PD) models, mRNA PK is linked to dynamics of expressed protein and other biomarkers that help build dose-exposure–response relationships (15). The focus of the paper is on a single component of mRNA drug product PK, which is the mRNA quantitation in circulation.

There are many other bioanalytical techniques (e.g., branched DNA (bDNA)), that utilize hybridization and chemiluminescent reactions for probe detection that can be used to quantify mRNAs in circulation (17). bDNA assays are commonly used to quantify mRNAs; however, this technique is out of scope of discussion in this paper. Herein, the authors will focus on reverse transcription quantitative PCR (RT-qPCR), especially in the absence of prescriptive guidance for bioanalytical method validation for PK analysis using quantitative PCR techniques. Current regulatory guidance documents for bioanalytical method validation (18) are specific to ligand binding and chromatographic assay methods and difficult to interpret for use with molecular workflows. The purpose of this paper is to provide information on assay design and technical considerations for the validation and sample analysis of RT-qPCR regulated assays that are used to support the pharmacokinetic analysis of mRNA-based drug products (e.g., LNP-mRNA) (Fig. 1). Although the focus of the paper is on LNP-mRNAs, assays to detect and quantify naked mRNA and guide RNA (gRNAs) or similar RNA structures in liquid matrices, like serum, plasma or other biological matrices, share similar considerations.

Assay Design Considerations

The general assay design strategies described in the white paper on qPCR and dPCR assays in support of cell and gene therapies (19) also apply to mRNA drug products, therefore only the unique assay design strategies and technical considerations for mRNA drugs will be elaborated here.

Primer/Probe Design and Multiplexing

As mentioned in the introduction, RNA drug products can consist of one or more RNA strands inside an LNP core. It is critical to understand the structure of the RNA(s) before designing an assay for accurate quantitation.

In addition, if multiple RNA species are present (e.g., multiple mRNA packed into an LNP, CRISPR LNPs with both mRNA and gRNA), it should be carefully investigated if assays for all RNA species are required or if selecting one representative RNA species is sufficient to produce reliable PK of the RNA drug product. Multiplexing in RT-qPCR and RT-dPCR (reverse transcription digital PCR) allows simultaneous detection of multiple targets in a single reaction, minimizing the use of reagents, sample volumes, and reaction times, which are particularly valuable for high-throughput assays with limited sample availability. Considerations for designing multiplexed qPCR assays have been extensively described and can be followed for multiplexing assays for mRNA drug products (19).

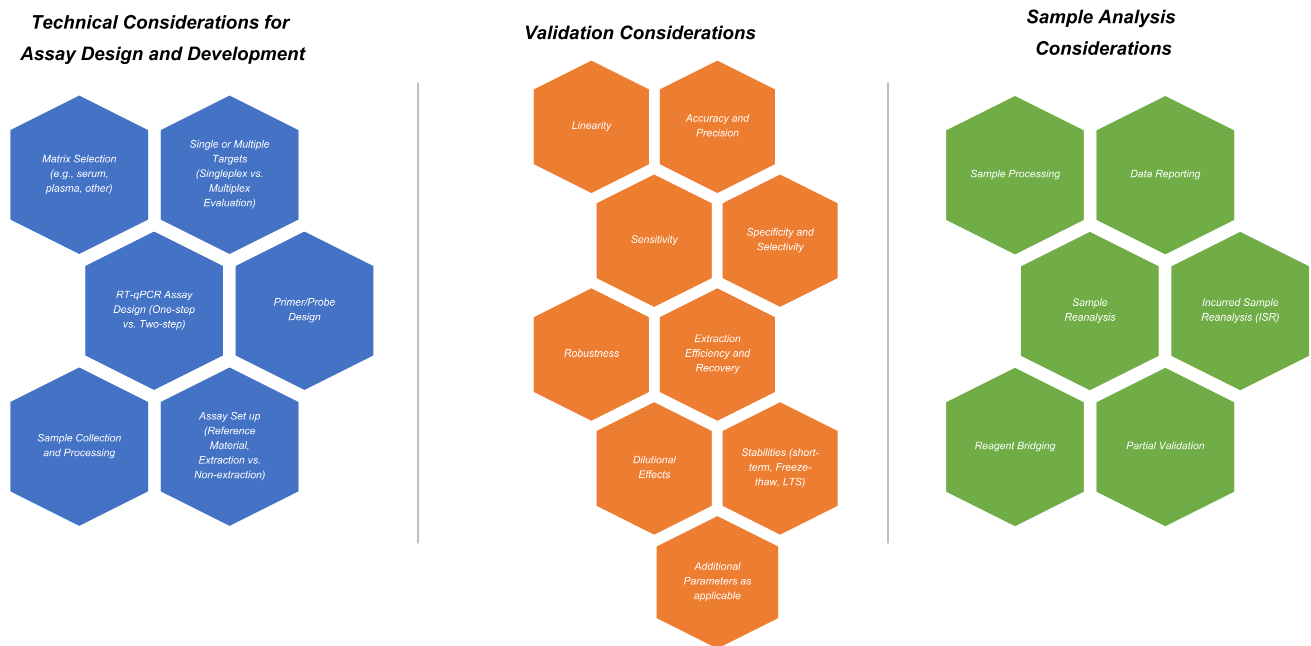

Fig. 1 Assay Design, Validation, and Sample Analysis Considerations for Pharmacokinetic Assays of LNP-mRNA Drug products by RT-qPCR. There are many factors to consider when designing, developing, and validating PK assays for LNP-mRNA drug products

The RNA species in the mRNA-LNP are often modified to increase RNA stability (20), caution should be taken during the assay development stage to ensure the modifications on the target RNA species will not impact the accurate quantification. Primers or probes with LNA (locked nucleic acids) are sometimes used to achieve tighter binding when constrained to using a shorter oligo or to using a lower anneal/extend temperature and can be most beneficial for RNA oligos (21). Primer and probe design decisions must involve context of use and required specificity. Other factors can prove important (e.g., high amplification efficiency and secondary structure predictions). The assay design preference could be development stage dependent. For example, during early development the assay could be "backbone"-specific so all constructs during development can be monitored, whereas, when the lead construct is available a drug specific assay can be designed.

One-step vs Two-step Assays

There are two primary ways that RT-qPCR can be carried out, one-step and two-step RT-qPCR (22). In the one-step format, reverse transcription (RT) takes place in the same tube as the qPCR, whereas in the two-step format, the RT and qPCR steps take place in separate reaction tubes. Both have advantages and limitations, and careful consideration should take place in method development for which workflow to be used. One-step assays are more common, especially for liquid matrix samples. Avoiding an extra step allows for less sample handling, and less potential errors in sample analysis. In addition, gene-specific primers are used in the one-step RT-qPCR, assuring enough reverse primers to detect the target RNA at highest levels for a PK study. The random hexamer or oligo dT primers typically used in a two-step RT-qPCR assay may require optimization to quantify target RNA at its highest levels. When sample volume or quantity is limited, a multiplex assay for different targets might be required, thus a two-step assay might be beneficial. Commercial one-step RT-qPCR kits such as Thermo Fisher TaqPath or TaqMan series or others are widely available. Master mixes with buffers specific for difficult templates (GC buffer) are also available to overcome secondary structures when present. Assay design with a one-step or two-step assay should be driven by method development and context of use (COU).

An example of a one-step RT-qPCR assay includes the following components. Sample and final volumes can differ and should be optimized in method development for each assay Table I:

An example of the standard thermal cycling conditions as established based on the results of method development is shown below. The manufacturers' package inserts should be consulted during method development Table II.

Sample Collection and Processing Considerations

Proper clinical sample collection, processing and storage conditions are essential to preserve mRNA integrity.

Table I Example of One-Step RT-qPCR Reaction Set-up

Reagent	Amount
Reference Standard mRNA or LNP-mRNA*	0–10^8 copies[1] or 0 – X ng
Forward Primer	100–900 nM
Reverse Primer	100–900 nM
Probe	100–250 nM
2—4 × Master Mix	1x
Matrix*	10 µL of Matrix RNA or 0.5 µL of matrix
Nuclease-free water	To a final volume of 20 µL

**Reference standard (as defined by the method) mRNA or LNP-mRNA (material containing the target sequence) and matrix are replaced by samples in sample wells; X, ULOQ for an RT-qPCR assay*

[1]*copies and ng equation; molecular weight determination of RNA*

Table II Example of Standard Thermal Cycling Steps and Conditions for RT-qPCR

Cycle	Temperature and Time*	# of Cycles
Reverse Transcription	varies	Typically 1
RT Inactivation/Activation of TaqMan Polymerase	varies	Typically 1
DNA Denaturation	varies	Typically 40
Anneal and extension	varies	

**Times and temperature conditions are dependent on manufacturer reagents and optimization in method development*

Different mRNA-based drug products can consist of different lipid components, with various structures, which can impact the stability of the drug product during collection and storage. The choice of biological matrix is dependent on several factors including, but not limited to, the route of administration, biodistribution (e.g., plasma/serum, CSF, urine). Furthermore, for assessing mRNA in blood circulation, the choice between serum or plasma collection could be based on the convenience of having multiple assessments from the same sample collection tube (e.g., mRNA PK, lipid PK, immunogenicity, soluble biomarkers). The stability of LNP drug products at 4 °C has been observed under lyophilized conditions (23–25), however it is essential to establish the stability of the mRNA component in the collected matrix of choice. While certain LNPs may be stable upon storage in collection tubes, the mRNA component may be unstable when stored without extra additives (26). To overcome instability, several commercially available tubes from different vendors contain proprietary additives that preserve mRNA integrity for long periods of time (e.g., PAXgene ccfDNA, Streck RNA Complete BCT, lysis buffer). One drawback of these specialized tubes is the Limited choices of volume draws, which can be up to 10 mL, leading to an increase in patient burden. An alternative option could be to add lysis buffer or an RNase inhibitor immediately after sample collection; however, this could be operationally challenging. Also, it is important to assess if stabilizers in the samples could inhibit PCR, especially if PCR analysis is to be performed directly after minimum required dilution (MRD). If there is a possibility of inhibition, an extraction process is recommended, as long as it does not compromise the required sensitivity. A common alternative to preserve integrity prior to PCR analysis is to flash freeze the study samples (e.g., in liquid nitrogen or dry ice).

Reference Material

As required for any PK assay, a suitable certificate of analysis (COA) should be provided to document characterization of the reference material used in the assay (27). Basic information such as name of material, lot/batch, source storage conditions, expiration/retest, purity, and concentration should be included. It is especially critical to include information on the molecular weight and nucleotides of the reference material to ensure accuracy for appropriate recovery calculations. It could also be valuable to understand the source of molecular weight (e.g., method used in manufacturing and characterization process) to allow for consistent measurements throughout the use of the material. In general, the reference material is an RNA standard that contains the target sequence (e.g., test article, analyte, drug product, LNP-mRNA, mRNA or synthetic nucleic acid fragment containing the amplicon of interest). Considerations for which material to use should take into account the availability of sufficient volume of the material during method development, validation and sample analysis. The material used should also be consistent in the assay (e.g., the calibrators and QCs should be made from the same reference material).

Calibration Curve

RT-qPCR analysis of PK samples uses a standard curve for absolute quantification of target RNA. During method validation, the standard curve assesses linear dynamic range, sensitivity, linearity, amplification efficiency and (when run in replicate) repeatability. The calibration curve can be prepared by using either LNP-mRNA (drug product) or naked mRNA as the standard reference material measured in nanograms, picograms, or single-stranded copies. The linear dynamic range can span many orders of magnitude but is often limited by RNA extraction. The standard curve range should align with context of use and specific drug program needs, thus targeting expected concentrations of the study samples. There are several approaches to set-up of the standard curve for these assays, of which a few will be discussed here. These approaches can take many forms and can utilize

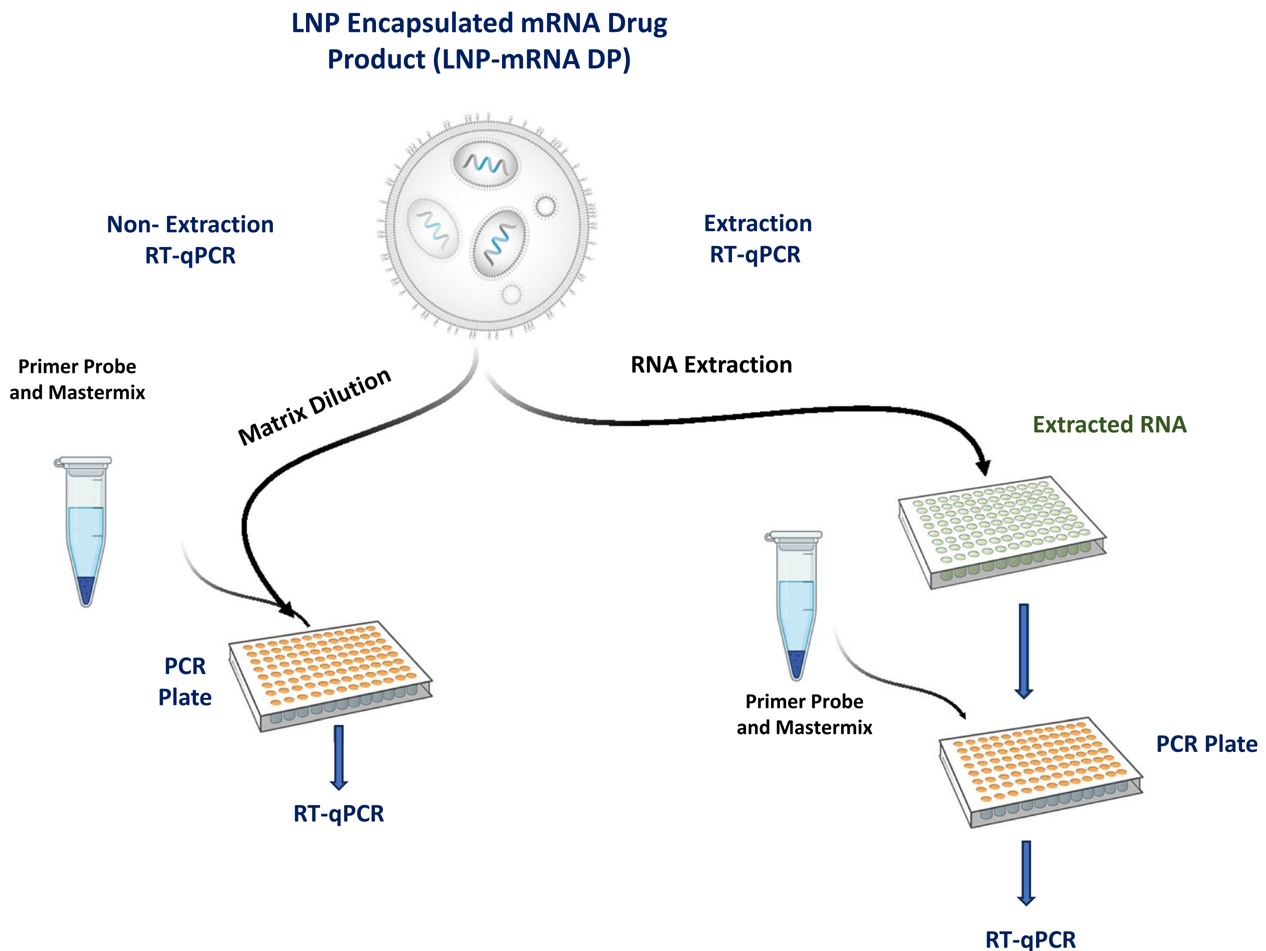

Fig. 2 LNP-mRNA PK assay schematic for non-extraction vs extraction RT-qPCR methods. mRNA quantitation from LNP-mRNA encapsulated drug products can be measured by two approaches. In the non-extraction method, which requires no extraction of encapsulated mRNA, the matrix requires dilution (MRD) to avoid interference, then is mixed with primers/probe, mastermix, and plated on the PCR plate for RT-qPCR quantification. The extraction method requires extraction of encapsulated RNA from drug product, then the extracted RNA along with primer/probe and mastermix is plated on the PCR plate for RT-qPCR quantification

extraction or non-extraction techniques illustrated in Fig. 2. All approaches have been used in different laboratories and are sufficient to support these studies with no clear advantage of one method over another. Other methods not listed could also be used if they are scientifically sound.

Calibration Curve: Non-extraction

In the non-extraction approach, RT-qPCR analysis of matrix is performed without extraction (28–30) using an appropriate RNA standard containing the target sequence. For example, if an LNP-mRNA drug product as reference material is used, calibrators can be prepared by spiking into a naive matrix (e.g., host serum or plasma) at a high concentration. Subsequent calibrators can then be generated through serial dilutions within the same matrix or prepared using a different dilution scheme. Then each standard level in matrix is further diluted with a diluent to the MRD determined during method development. The process of heating to release mRNA from the LNP-mRNA complex can be carried out either at this stage or during the RT-qPCR step. Alternatively, if a standard curve is created using naked mRNA as the reference material, RT-qPCR is performed on the naked mRNA standards in a representative matrix. This is done in the presence of lysate from the matrix, prepared using the same procedure as the sample processing. If naked mRNA standards are used, it is recommended to show a co-linearity between LNP-mRNA drug product, if the drug product is available. In extraction-free RT-qPCR, critical parameters and reagents such as inhibitor-tolerant polymerases, polymerase enhancers, and direct RT-qPCR conditions can be optimized to improve assay conditions (31, 32).

Calibration Curve: Extraction

For the extraction method, two example approaches will be described here. In the first approach, naked mRNA is spiked into extracted RNA from matrix. The RNA extracted from matrix usually exhibits less inhibition on RT-qPCR than the matrix samples. Consequently, more RNA sample can be tested in each RT-qPCR, as extraction concentrates the nucleic acids and can improve assay sensitivity. However, the naked mRNA standards do not account for the recovery of the target RNA from the matrix. With this approach, it is important to evaluate extraction efficiency of the method through implementing extraction QCs or through other assessments in validation to characterize the recovery of the extraction process that samples undergo.

In the second approach, the LNP-mRNA drug product is used as the reference material and spiked into matrix and diluted in naïve host matrix to prepare the standards and QCs. The volume of the sample to be used for extraction should be optimized during assay development (e.g., if a pre-dilution is required). Achieving high and consistent extraction efficiency can be challenging with this method and should be optimized by testing different extraction kits and platforms during method development. Extraction efficiency can be estimated by spiking a control RNA with known concentration or copy number into LNP-mRNA matrix during the extraction process and calculated after RT-qPCR analysis for recovery of the control RNA. However, to minimize the complexity of the introduction of control RNA, if all the standards, QCs and samples are processed together in an extraction plate and undergo the same conditions, a separate estimation of extraction efficiency is irrelevant because the standard curve and QCs have been extracted in the same way as the samples. Adding a carrier RNA improves the yield of RNA from a low LNP-mRNA sample and can be added to all the standards and QCs at the time of preparation.

Optimization of the extraction method can include evaluating material input, extraction kits and components, carrier RNA, buffer selection, DNase treatment, heat treatment, automation, and other parameters as needed (33).

Quality Controls

To validate the analytical methods used for calibration curves and study samples, Quality Control (QC) samples are prepared and analyzed together. Further discussion on extraction and processing of study samples is discussed in the sample processing section below. It is recommended to include QC samples in each analytical run, with QC samples and calibration standards prepared separately. Typically, QC samples are prepared at three concentrations (low, medium, and high) using a relevant matrix spiked with the reference material.

It is recommended to add negative controls, such as no template controls (NTCs). Although not a requirement, sentinel controls can also be used to ensure absence of contamination. NTCs are used to monitor for general reagent contamination and are included in all assay runs. Sentinel controls are employed to check for airborne contamination during the RT-qPCR setup. Sentinel controls are prepared in a similar composition to NTCs (e.g., water or buffer) however these preparations are left open to ambient air during assay set up. The negative controls should meet acceptance criteria of undetermined or below the assay LLOQ.

During validation or sample analysis, process controls can be used to monitor the extraction or PCR process. For example, an exogenous RNA oligo or MS2 virus (e.g., an RNA bacteriophage, or other commercially available material) can be spiked prior to the extraction step. The recovery of the material then can be assessed by RT-qPCR to monitor the extraction process. To monitor potential PCR inhibition, exogenous controls (e.g., Xeno control (Thermofisher), SPUD control (Sigma)) can be included in a duplex assay format. The Ct values of these exogenous controls are monitored to ensure that the samples with results below quantifiable values for the target mRNA are truly negative.

It can be useful to monitor DNA contamination in your sample by including a"No Reverse Transcriptase"(No RT) control in the RT-qPCR assay. This control helps differentiate between mRNA and DNA signals, ensuring that the assay specifically quantifies mRNA in the drug product. The inclusion of a No RT control is critical during at least method development and can be included in validation if deemed necessary. This will ensure that the primers and probes used in the assay are specific and no amplifying parts of DNA contaminants are present.

Several methods can be used to incorporate a No RT control including:

- Master Mix without RT: use of a master mix without reverse transcriptase. This requires a master mix containing equivalent components to the one used in the RT-qPCR assay to ensure comparable conditions (e.g., equivalent salt concentrations, Taq polymerase) – just without the RT.
- Heat Inactivation of RT: heat inactivating the reverse transcriptase in the master mix used for RT-qPCR. It should be noted that this approach may not eliminate RT activity, which could limit its effectiveness.
- Ribosomal DNA (rDNA)-based primers which uses primers targeting ribosomal DNA that can also detect genomic DNA (gDNA) contamination due to the rDNA's highly conserved sequences and abundance in the genome (34, 35).

It might be important to assess the potential presence of gDNA in samples, as it can impact the accuracy and reliability of the assay. Evaluating gDNA levels ensures that any interference or bias is identified and appropriately addressed during assay development. There are many different methods to evaluate the presence of gDNA contamination, including comparison of the cycle threshold (Ct) value for the No RT control compared to the Ct value of a sample of known target or use of other commercial techniques such as ValidPrime (36). The technical design considerations for assay design are summarized in Table III.

Assay Validation Considerations

Linearity

RT-qPCR efficiency is evaluated to assess reverse transcription and amplification performance, providing insights into matrix interference, sample dilution linearity, and the use of surrogate positive controls. RT efficiency measures how accurately the RT enzyme converts RNA into complementary cDNA (cDNA). While 100% efficiency would mean total transcription of RNA to cDNA, achieving this is challenging due to factors like RNA quality, enzyme efficiency, target RNA structure, and reaction conditions.

The efficiency of quantitative PCR (qPCR) measures how effectively the PCR polymerase duplicates the template DNA during each cycle of the PCR process. Ideally, PCR efficiency should be close to 100%, it typically ranges due to various factors, including primer design, reaction conditions, template quality and folding, and inhibitors. Biological Matrices May inhibit efficiency, and efficiencies below 90% may be acceptable depending on assay performance and reproducibility (37).

Linearity refers to the relationship between the input RNA amount and the output signal (Cq or Ct value) in RT-qPCR, ideally showing a proportional decrease in Cq or Ct value with increasing RNA on a logarithmic scale. Recommendations for the assessment of linearity are extensively described (19). The linear dynamic range is often limited by preanalytical steps like extraction rather than the RT-qPCR itself. (38). Degree of linearity as indicated by the correlation coefficient (R^2) of the standard curve, with R^2 values of ≥ 0.980 is generally deemed acceptable (19). Representative results illustrating linearity as well are presented in Fig. 3.

Accuracy and Precision

Accuracy and Precision (A&P), a measure of random and systemic error of the assay is typically determined during initial method development and confirmed in assay validation. Accuracy (A) represents the closeness of the measured/observed results to the ‘true’ value and is expressed as a ratio of the absolute error (observed value—true value) to the true value, expressed as a percentage. Precision (P) measures the closeness among the multiple independent measurements and is generally expressed as %CV (linear scale) among the independent measurements. A&P is determined by analyzing a set of quality controls (QCs) prepared independently from the standard curve, each at different concentrations spanning the range of the assay, both within (intra) and between (inter) runs.

QCs are intended to mimic study samples and typically are generated from a spike of a known quantity of reference material into relevant biological matrix (e.g., RNA from relevant biofluids, serum/plasma, lysate). Typically, a standard curve spanning six to eight log concentrations of the analyte along with at least duplicate sets of fresh or frozen QCs and spanning at least five concentrations of the analyte, including upper limit of quantification (ULOQ), high quality control (HQC), mid quality control (MQC), low quality control (LQC) and lower limit of quantification (LLOQ) is used for determination of assay A&P. Each concentration level of the QCs is assayed at least in triplicate. A&P runs during validation are assessed over at least 6 passing runs spanned across ≥ 2 days by at least 2 analysts (19).

General acceptance criteria for precision and accuracy of regulated qPCR assays are referenced in previous publications (19) and are applicable for RT-PCR. In general, intra- and inter-assay precision ≤ 30% CV for QCs and ≤ 50% CV for LOQs in interpolated (qPCR) or absolute copies (dPCR). Intra- and inter-assay accuracy of −50 to 100% RE interpolated copies or ≤ 30% RE for QCs and ≤ 50% LOQs for absolute copies. While generally most PCR assays can achieve the recommended criteria, the assay performance demonstrated in method development and expected needs for the study program should guide the establishment of fit-for-purpose assay acceptance criteria to apply in validation. Since extraction is a significant contributor run to run for inaccuracy and imprecision, it is also important to understand the impact of extraction on the overall precision and accuracy of the method.

Representative example of presentation of Accuracy and Precision results are presented in Table IV.

Sensitivity

As mentioned in the previous paper (19) that extensively describes how to determine assay sensitivity for regulated qPCR assays, regulatory guidance on the target sensitivity is sparse and can vary based on the intended use of the assay and the specific drug program. Oftentimes, the FDA mention of the target sensitivity for preclinical biodistribution assays stated in the Long Term Follow Up After Administration

Table III Technical Considerations and Assay Design for LNP-mRNA PK Assays by RT-qPCR

Matrix Selection, Collection, and Processing	Assay Design	Reference Material	Assay Method (Non-extraction *versus* Extraction)
Matrix Type Biofluid of interest (e.g., serum, plasma, CSF, urine) May be chosen based on the convenience of sample matrix used for other assays or other biological considerations	**Primer/Probe Design** Can be specific target or "backbone" target depending on phase Can use LNAs to achieve tighter binding or using a lower anneal/extend temperature	RNA standard that contains the target sequence (e.g., test article, analyte, drug product, LNP-mRNA, mRNA or synthetic nucleic acid fragment containing the amplicon of interest) Consider if source material is available in sufficient quantities for assay	Various methods available for RT-qPCR. Consider method and calibration curve set up when understanding how to validate the assay Standard curve spanning six to eight log concentrations of the analyte. The dynamic range should target the expected concentrations (C_{max} and C_{trough}) from study samples
Sample Collection Assess collection tube type, storage temperature, shipping conditions, etc ***Considerations*** Can evaluate RNAse Stabilizers in collection tubes or flash freezing after processing	**Multiplexing** If multiple targets, can evaluate the effect of multiplexing or the need for multiple single assays If multiple RNA species are present (e.g., multiple mRNA packed into an LNP, CRISPR LNPs with both mRNA and gRNA), it should be carefully investigated if assays for all RNA species are required or if selecting one representative RNA species is sufficient to produce reliable PK of the RNA drug product	Should be consistent in assay (used for both calibrators and QCs) Suitable characterization with Certificate of Analysis (COA) containing information on molecular weight measurements	***Non-extraction Method*** Reference material is spiked into matrix to prepare calibrators/QCs then further diluted to MRD. Can also include heat treatment in processing step to release mRNA if using LNP-mRNA reference material
Handling and Processing ***Considerations*** Stability assessments for Freeze/Thaw, storage at ambient, refrigerated or frozen, Mixing (e.g., gentle *versus* vortexing)	**RTqPCR Reaction** ***One-step*** allows for consolidated sample handling and less potential errors. Requires maximizing primers to ensure detection of low target RNA ***Two-step*** can be advantageous for low expected sample volumes or quantities	**Co-Linearity** Consider assessing co-linearity if material is available to demonstrate mRNA and LNP-mRNA comparability in the assay	***Extraction Method*** Reference material spiked into extracted RNA from matrix to prepare calibrators/QCs Another method would include spiking reference material into matrix to prepare calibrators/QCs and extracted along with samples

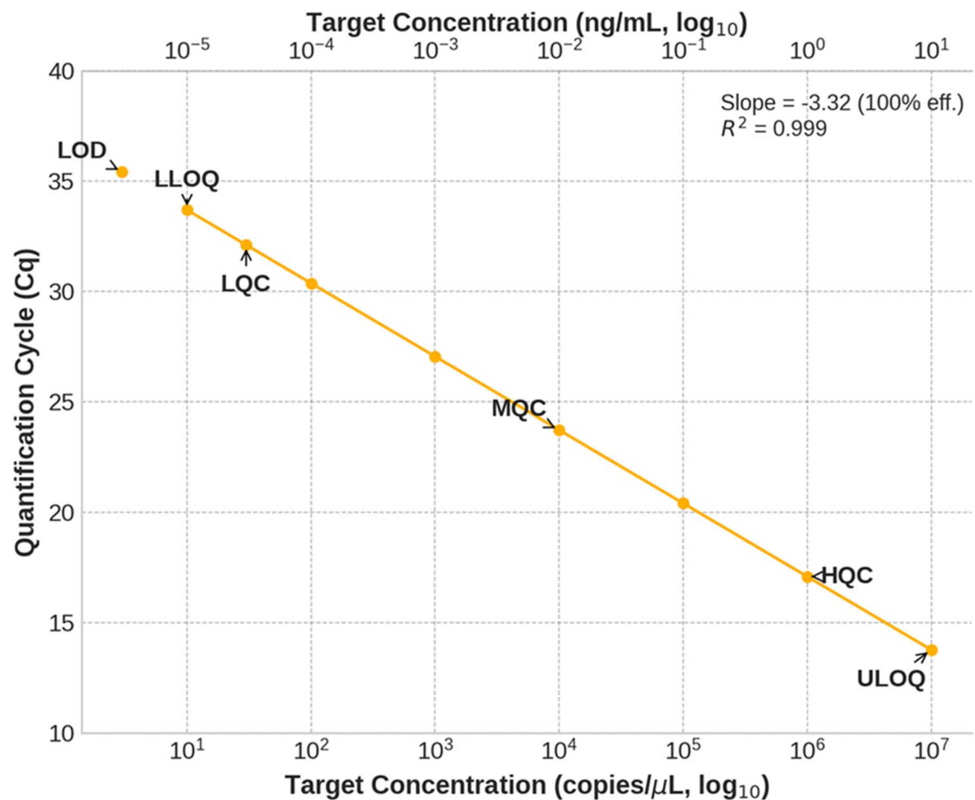

Fig. 3 Representative Calibration Curve Data. Representative calibration curve (in ng/mL and copies/uL of mRNA reference standard) demonstrating acceptable linearity and amplification efficiency as well as QC level placement along quantitation range of the method

of Human Gene Therapy Products (39) is referenced for all qPCR applications. The statement itself contains some ambiguity around defining the LLOQ *versus* the LOD and in many instances is not scientifically feasible with matrices that contain little to no nucleic acid. In this context, for the purpose of PK assessment of LNP-mRNA in circulation, the assay dynamic range and sensitivity should target the expected concentration (C_{max} and C_{trough}) from study samples as with any PK bioanalytical assay (15).

Experimental design for determining assay sensitivity in regulated PCR assays has already been described (19). Although sensitivity can be determined in RT-qPCR assays defining the assay limit of blank (LOB), limit of detection (LOD), and limit of quantification (LOQ), in this context for PK measurement of LNP-mRNA drugs, the lower limit of quantification (LLOQ) is what designates the assay sensitivity limit. The LOD is typically irrelevant and not determined or needed in these assays given that sample analysis results that fall below the LLOQ are determined as BLQ (below the limit of quantification).

The assessment of LLOQ is typically performed in the precision and accuracy runs (≥ 6 runs, ≥ 2 analysts, ≥ 2 days). Acceptance criteria are listed in the A&P section with intra- and inter-assay precision of ≤ 50% CV and %RE.

Specificity and Selectivity

Specificity is designed to evaluate the primer–probe sets ability to accurately detect the target analyte in the presence and absence of total RNA or other interfering substances (e.g., cross reactivity with other mRNAs cargos that are present in a multi-mRNA drug product) in the biological matrices. Specificity is crucial because it ensures that the results obtained are due to the presence of the target analyte and not due to other non-target substances that could lead to false-positive data. Selectivity, on the other hand, refers to the potential of the analytical method to measure the target analyte in the presence of endogenous matrix components such as metabolites or decomposition products within representative individual matrix samples (e.g., hemolyzed and lipemic samples). Selectivity is essential to ensure that the method provides reliable and accurate results for real-world samples where various interfering substances are often present.

Specificity of the primers and probe is evaluated during the designing of these sequences against a nucleotide database using BLAST and alignment tools to assess in silico binding of any non-specific targets. This is performed as a first screening in selecting specific primers and probe against

Table IV Representative Accuracy and Precision Results for an LNP-mRNA Assay

		Replicate Concentration (ng/mL)			Intra-Assay Statistics					Inter-Assay Statistics			
QC Level	Run ID	Replicate 1	Replicate 2	Replicate 3	Mean (ng/mL)	SD	%CV	%RE	Pooled Intra-Assay Precision (%CV)	N	Mean Bias (% Relative Error)	Inter-Assay Precision (%CV)	% Total Error (|Mean Accuracy|+ Mean Precision)
LLOQ (2.00 ng/mL)	1	2.32	1.74	1.91	1.99	0.30	15.0	−1.0					
	2	2.05	1.98	2.06	2.03	0.04	2.0	2.0					
	3	1.88	1.95	2.12	1.98	0.12	6.0	−1.0	7.3	18	0.9	13.6	14.5
	4	1.93	1.94	1.79	1.89	0.08	4.0	−6.0					
	5	1.81	1.71	1.73	1.75	0.05	3.0	−13.0					
	6	2.60	2.41	2.41	2.47	0.11	5.0	24.0					
LQC (5.00 ng/mL)	1	5.11	5.11	4.62	4.95	0.28	6.0	−1.0					
	2	5.13	4.60	5.17	4.97	0.32	6.0	−1.0					
	3	4.23	5.10	5.33	4.89	0.58	12.0	−2.0	6.6	18	−1.4	11.4	12.9
	4	4.57	4.22	4.41	4.4	0.18	4.0	−12.0					
	5	4.80	4.35	4.45	4.53	0.24	5.0	−9.0					
	6	6.10	5.65	5.76	5.84	0.23	5.0	17.0					
MQC (50.0 ng/mL)	1	48.8	52.5	51.2	50.8	1.88	4.0	2.0					
	2	50.9	53.8	53.6	52.8	1.62	3.0	6.0					
	3	43.8	51.1	50.7	48.5	4.10	8.0	−3.0	4.5	18	−1.2	8.2	9.4
	4	47.9	44.5	44.3	45.6	2.02	4.0	−9.0					
	5	43.6	44.6	46.6	44.9	1.53	3.0	−10.0					
	6	54.7	52.6	53.9	53.7	1.06	2.0	7.0					
HQC (300 ng/mL)	1	293	298	315	302	11.5	4.0	1.0					
	2	316	328	340	328	12.0	4.0	9.0					
	3	289	332	317	313	21.8	7.0	4.0	4.4	18	2.4	7.9	10.4
	4	289	274	277	280	7.94	3.0	−7.0					
	5	293	277	295	288	9.87	3.0	−4.0					
	6	346	325	328	333	11.4	4.0	11.0					
ULOQ (400 ng/mL)	1	396	419	432	416	18.2	5.0	4.0					
	2	421	408	453	427	23.2	6.0	7.0					
	3	391	417	468	425	39.2	10.0	6.0	5.2	18	2.8	7.2	10.0
	4	388	384	377	383	5.57	1.0	−4.0					
	5	375	378	390	381	7.94	2.0	−5.0					
	6	447	425	430	434	11.5	3.0	9.0					

the intended target sequences. However, empirical assessment of specificity should be performed experimentally in method development or validation. This can simply be demonstrated with NTCs or a negative control containing a relevant nucleic acid background matrix such as RNA extracted from naïve matrix. Specificity is demonstrated with interpolated or absolute results that are ≤ LLOQ.

If assessing specificity against potential interfering mRNAs that could be present in the Matrix, then 100% of specificity samples spiked with the non-relevant mRNA/target should demonstrate measured results below the LLOQ suggesting no cross reactivity with the potential interfering target.

Expanding the specificity parameter to include individual evaluations allows for an additional assessment of selectivity. This means that the method's performance is further validated by assessing individual samples from the biological Matrix of interest, ensuring that it can accurately measure the analyte in the presence of various interfering and endogenous substances. By including individual Matrix samples in such evaluations, robustness of the method is further confirmed, providing greater confidence in its suitability to deliver accurate and reliable results in diverse and complex biological samples. Where possible, at least 10 individual samples should be evaluated unspiked as well as spiked with reference material at a low concentration (e.g., LQC). Other concentrations can also be assessed (e.g., HQC or MQC). The RNAs are quantified and analyzed for target specific amplification and Matrix interference. All the unspiked individual samples must demonstrate undetermined Ct values or values below the LLOQ. At least 80% (8 of 10) of the spiked individual samples should meet the acceptance criteria consistent with accuracy and precision criteria.

Robustness

Depending on the scope of the assay validation, a formal robustness assessment may not be included. Robustness is usually not performed during method development, though data between days could be compared if there are not significant changes to assay method. The robustness of an assay can be evaluated through batch testing, wherein changes can be introduced between runs. Common variables to change include analyst, instrument, day, and master mix. Additionally, variations could stem from differences in sample preparation. A robustness assessment will lessen the concern that the assay is not reliable, especially when new critical reagent lots need to be bridged into the assay down the road.

Extraction Efficiency/Recovery

To assess the extraction efficiency, an extraction efficiency evaluation should be performed for each matrix but at minimum in primary matrix and surrogate matrix (or buffer e.g., phosphate buffered saline), if multiple similar biofluid matrices are being evaluated. This can be assessed during method development with one or more runs over the concentration range after the sample extraction procedure has been optimized. High, mid, and low spike extraction samples or varying spike levels (6–8) over a concentration range depending on prospective downstream sample analysis plan can be prepared and flash frozen or stored as per the sample analysis plan and dosing prediction used with drug formulation (drug substance or drug product). The recovery may be established as a part of method validation for each matrix in question over multiple runs (as the availability of matrix allows) and the acceptance criteria should be based on the assay's context of use.

For method development, the bioanalytical method will need to be optimized to be specific and robust, to quantify the recovery of RNA from the LNP formulation. This may involve optimizing the extraction method, selecting appropriate sample preparation techniques, and choosing an analytical platform such as qPCR or dPCR depending on the type of matrices that will be evaluated. It may also be valuable to determine MRD of samples to evaluate the recovery of the undiluted spiked samples and at the MRD.

To assess the recovery of the target RNA from the spiked LNP samples, matrix spiked with known amounts of drug product (LNP-mRNA) can be prepared and extracted followed by RT-qPCR or RT-dPCR. The measured recovery concentration to the expected concentration can be evaluated to calculate the recovery percentage or extraction efficiency to evaluate the accuracy and reliability of the recovery measurement. Recovery should ideally be performed in an unspiked matrix or surrogate matrix, or PBS should be included for each matrix tested. Extraction efficiency should be conducted in validation as described previously(19) with an additional investigation at the expected Cmax of the LNP-mRNA to ensure the extraction method is suitable for extraction of both high and low levels of LNP-mRNA in the matrix. Extraction efficiency should be characterized and reported in assay validation. Acceptable extraction efficiency values should be driven by the context of use and consistency in performance during method development. If the method does not require an RNA extraction step, then no extraction efficiency evaluation is needed. Alternatively, if the samples follow a dilution/heat treatment method, an evaluation can be conducted to ensure the efficiency of the LNP releasing the target payload mRNA.

Dilutional Effects

Dilution effects assess the sample dilution procedure to ensure that it doesn't affect the measured concentration of

the analyte. The goal is to show that a sample with an analyte concentration above the validated range can be diluted into a measurable range with accuracy and precision. Dilutional effects can be assessed using several approaches. One method is by spiking the matrix with the test material at concentrations above the upper limit of quantitation (e.g., 10X ULOQ) and diluting this sample with blank matrix. Accuracy (recovery) and precision should be within the set criteria across dilutions. Another approach is to extract the analyte spiked samples and dilute the extracted material. Accuracy (recovery) and precision should be within the set criteria across dilutions. The approach to this assessment must be identical with how samples will be tested in study.

Stabilities

Diverse and numerous agents in the environment and in biology lead to the destruction of RNA and thus preserving stability of RNA during sample processing and handling is important. PK analysis often involves batch testing in order to avoid inter-assay imprecision contributions. Even with this approach, stability assessments are needed in order to avoid data interpretation that inadvertently confuses instability for biologically relevant differences in RNA concentration. When possible, the drug product should be spiked into matrix to assess stability. Drug products that have protection (e.g., LNP) around the RNA target can be more stable. Special considerations are needed for when using naked mRNA as the assay reference material. Due to the inherent instability of mRNAs, study samples are typically immediately frozen. Therefore, the baseline QC is first frozen overnight and used as a comparator to subsequent stability timepoints. The baseline should be clearly defined based on the condition of the sample the laboratory will receive.

Long Term Stability (LTS)

Both −20°C and −80°C storage conditions should be evaluated for long-term stability, as not all sites have −80°C storage capabilities, which facilitates easier site screening, especially in developing countries. If mRNAs are unstable at −20°C, conduct a minimum period investigation and transfer samples to −80°C storage at regular intervals. All samples must meet established stability criteria. Frozen stability can be validated for approximately 1 week and 1 month or other study relevant timepoints, with results included in the validation report. Stability can also be assessed at longer intervals (e.g., 3, 6, and 12 months or the length of estimated time that samples are stored for the study, using appropriate levels of QCs (e.g., HQC, LQC).

Other Stabilities

Other stability assessments, such as benchtop, freeze–thaw, and whole blood stability, can also be included in assay validation based on how samples are stored and processed in study. Recommendations include, if applicable:

- Dilutional QC (projected Cmax or 10 × ULOQ) included where there is concern regarding stability of samples with high mRNA concentrations in storage.
- Whole blood stability to determine processing impact on serum or plasma from whole blood. This can be performed by spiking samples and testing at incremental times (e.g., 1 h, 2 h, 4 h).
- Assessment of benchtop stability at room temperature (RT) and 4°C.
- Assessment of freeze–thaw cycles to support sample freeze/thaw in-study.
- Extracted RNA frozen stability to demonstrate stability if samples need to be retested from extracted RNA.

Generally, samples are considered stable when the stability sample results are within 70–130% of the baseline samples with ≤ 30% CV between replicates. If the method includes extraction, then extraction efficiency variability should be taken into consideration when setting appropriate acceptance criteria.

Considerations for RT-dPCR

Digital PCR (dPCR) is an absolute quantification method that does not rely on the use of standard curve and is less impacted by matrix effects compared to qPCR. When reverse transcription dPCR (RT-dPCR) is used as a bioanalytical method in LNP-mRNA drug programs, it is the complementary DNA (cDNA) copy number in the RNA extract that is being quantified as a surrogate of the mRNA copy number in the original biological sample.

For programs in which low drug levels are anticipated, digital PCR should be considered as it is more precise and accurate at low copy numbers compared to RT-qPCR. Both technologies may reach the ultimate sensitivity of detecting a single target molecule if present. Assay sensitivity is then limited by the total amount of sample that is analyzed. The dPCR platforms offer a convenient option to combine experimental data from multiple runs increasing the total amount of mRNA loaded (or reaction volume) resulting in a higher sensitivity compared to an individual reaction, which can be an option for multiplexing to support LNPs with multiple mRNA cargos.

Overall, RT-dPCR methods have a narrower dynamic range (3–4 logs) compared to RT-qPCR (7–8 logs) which can necessitate a high dilution factor during sample analysis.

Table V Validation Considerations for LNP-mRNA PK Assays by RT-qPCR

Quality Controls	Accuracy and Precision	Sensitivity	Specificity/ Selectivity	Extraction Efficiency	Dilutional Effects	Stability
General Considerations and Experimental Design						
QCs prepared independently with the same reference material as calibrators Include negative controls (e.g., NTCs, can include sentinel controls, and No-RT controls, if applicable)	QCs in duplicate sets spanning at least five concentrations of the analyte (ULOQ, HQC, MQC, LQC, LLOQ) Each concentration level of the QCs is assayed at least in triplicate A&P runs during validation are assessed over at least 6 passing runs spanned across ≥ 2 days by at least 2 analysts	The assay sensitivity should target the expected concentration (C_{trough}) from study samples The LLOQ is what designates sensitivity. The LOD is typically not determined or needed as sample results that fall below the LLOQ are determined as BLQ	Specificity can be demonstrated with NTCs or a negative control containing a relevant nucleic acid background matrix such as RNA extracted from naïve matrix For selectivity, at least 10 individual samples should be evaluated unspiked as well as spiked with reference material at a low concentration (e.g., LQC) and evaluated in the assay	Biofluid samples spiked with known amounts of drug at minimum of one QC level and at Cmax prepared and diluted/heat treated, per the method, followed by RT-qPCR An unspiked replicate should be included for each matrix tested	Can be assessed using different approaches as described below with the non-extraction or extraction method	Stabilities should be assessed based on expected conditions of sample processing and storage and performed if applicable to the specific study Can be demonstrated with QCs subjected to varying conditions Can also include a dilutional QC (projected Cmax or 10 × ULOQ) if there is concern regarding the stability of samples with high mRNA concentrations in storage
Non-Extraction Method Considerations						
If samples are to be extracted as compared to the calibration curve preparation, consider including an extraction control or evaluate extraction efficiency in assay validation			Contrived samples are subsequently diluted, heat-treated (if applicable), and analyzed for target specific amplification and matrix interference	If the method does not require an RNA extraction step, then no extraction efficiency evaluation is needed	Matrix is spiked with reference material at a concentration > ULOQ. The sample is then diluted with blank matrix to a level within the curve, followed by RT-qPCR to evaluate A&P of dilutions	
Extraction Method Considerations						

Table V (continued)

Quality Controls	Accuracy and Precision	Sensitivity	Specificity/ Selectivity	Extraction Efficiency	Dilutional Effects	Stability
If spiking naked mRNA with extracted RNA from matrix, consider evaluating an extraction control or demonstrate extraction efficiency in assay validation to understand the percent recovery of samples An extraction QC is not needed in methods where calibrators/QCs are extracted along with samples			Total RNAs are subsequently extracted and analyzed for target specific amplification and matrix interference	If the samples follow a dilution/heat treatment method, an evaluation can be conducted to ensure the efficiency of the LNP to release the mRNA	Extract total RNA from samples spiked with high analyte concentration (> ULOQ), dilute the extracted material, followed by RT-qPCR to evaluate A&P of the dilution(s)	If the method includes extraction, then extraction efficiency variability should be taken into consideration when setting appropriate acceptance criteria If the extraction method includes calibrator and QC extraction along with samples, stability acceptance can be aligned with general stability acceptance (e.g. 70–130% of the baseline samples with ≤ 30% CV between replicates or recovery of stability samples is within acceptance. For example %RE ± 30.0%)
Suggested Acceptance Criteria						
The negative controls should meet acceptance criteria of undetermined or below the assay LLOQ	The assay performance demonstrated in method development and expected needs for the study program should guide the required acceptance criteria in validation In general, intra- and inter-assay precision ≤ 30% CV for QCs and ≤ 50% CV for LOQs in interpolated (qPCR) or absolute copies (dPCR) Intra- and inter-assay accuracy of −50 to 100% RE interpolated copies or ≤ 30% RE for QCs and ≤ 50% LOQs for absolute copies	The assessment of LLOQ is typically performed in the precision and accuracy runs (≥ 6 runs, ≥ 2 analysts, ≥ 2 days) Acceptance criteria are listed in the A&P section with intra- and inter-assay precision of ≤ 50% CV and %RE	Specificity is demonstrated with interpolated or absolute results that are ≤ LLOQ If assessing specificity against potential interfering mRNAs that could be present in the Matrix, then 100% of specificity samples spiked with the non-relevant mRNA/target should demonstrate measured results below the LLOQ suggesting no cross reactivity with the potential interfering target	Extraction efficiency is reported and generally acceptable with a spike recovery between > 20% and < 120% or based on the assay performance	Accuracy (recovery) and precision should be within the set A&P criteria across dilutions	Generally, samples are considered stable when the stability sample results are within 70–130% of the baseline samples with ≤ 30% CV between replicates

It is therefore important to assess dilutional linearity and to possibly include a dilution QC in method validation where appropriate.

Considerations for method validation are summarized in Table V.

Sample Analysis and Post-Validation Considerations

Sample Processing

For sample extractions from matrix, a straightforward approach is to dilute the sample to the MRD followed by heat exposure to release the RNA (40). This heat exposure can be part of or before the RT-qPCR step, if higher temperature is necessary to release the RNA. The heat treatment process (temperature and duration), MRD value, and diluent should be optimized for different conditions, relevant to the target patient population. The MRD diluent should be selected through optimization during method development and can include options such as water, formulation buffer, TE buffer, or PBS.

Alternatively, a bead-based or column-based extraction procedure may be desired (19). For sample extractions, commercial kits may be used. The selection of the kits would be a fit for purpose decision with extraction efficiency optimization conducted in method development and confirmed in validation. If standards and QC samples are prepared independent of extraction, we recommend including an extraction control. This would allow monitoring of the extraction efficiency independent of the assay performance, and the assay standards will not be impacted by extraction efficiency. It is also possible to have both standards and QC samples go through the extraction process; in which case an extraction control is not necessary. If the assay method does not include extraction of standards and QCs, then the sample extraction needs to be evaluated in method development.

Sample Purity & Integrity

Assessment for sample purity (260/280 and 260/230 nm ratio or electrophoresis) and integrity check (electrophoresis or RIN, RNA Integrity Number) would not apply for the typical PK analysis of an mRNA LNP drug in matrix as there is no measurable RNA concentration typically in serum/plasma. Usually, a small amount of DNA contamination in matrix is not a concern unless the targeted mRNA assay sequence is not designed across exon-exon junctions or is a direct match to an endogenous cDNA sequence.

Data Reporting

The PK data reporting for mRNA drug products is different from the viral vector-derived PK reporting, which is usually vector genome copies per volume of biofluid or per mass of total DNA or RNA extracted. The drug product (formulated LNP encapsulated mRNA) received (from manufacturing) for assay development are usually with unit of copies of mRNA per volume (e.g., copies/mL) or mass of mRNA per volume (e.g., ng/mL). It is not difficult to convert the unit from mass to copy number, if the accurate molecular weight is available. It is important that the same unit used for assay development and validation should be used for data reporting and should conform to the requirements and needs of the clinical pharmacologists. For samples with PK quantitation below the quantifiable levels, the data reporting can be BLOQ, below the limit of quantification (41). For RNA drug products where the payload portion will be amplified (e.g., self amplifying RNA) or multiple payloads are packed into the LNP (e.g., multiple payload RNA species or circular RNA that contains multiple payloads), the reporting unit may be copies per volume of biofluid if applicable. During assay development and validation stage, the unit conversions from mass to copy number or from per reaction to per volume of biofluid should be clearly stated to avoid confusion when samples are analyzed, and results are reported. If the standard curve is extracted and prepared in ng/mL, there is no unit conversion required beyond application of the dilution factor and data can be reported in ng/mL.

If the standard curve is run independent of extraction and converted to copies per reaction, it is necessary to perform a unit conversion of the sample data from copies per reaction to copies per mL and then to ng per mL. It is important to ensure consistency in the calculations used throughout the study. The recommended conversion calculations are below:

$$Copies\ per\ mL = \frac{Copies\ per\ reaction}{\mu L\ eluted\ RNA\ per\ reaction} \times Elution\ Volume\ (\mu L) \times \frac{Dilution\ factor}{\mu L\ plasma\ extracted} \times 1000\ \mu L/mL$$

$$ng\ per\ copy = \frac{[Molecular\ Weight\ of\ target]}{6.0221\times 10^{23}} \times 1,000,000,000$$

$$ng\ per\ mL = Copies\ per\ mL\ plasma \times ng\ per\ copy$$

Sample Retesting and Reanalysis

It is important to have a sample retesting procedure outlined in the sample analysis plan in cases that require the sample to be retested for reasons of precision failure or if the sample is ALQ (above the limit of quantification). Sample retesting can be performed by re-extracting sample from matrix or can be retested from the extracted RNA. If retesting is performed on the extracted RNA, stability of frozen RNA extracts can be demonstrated in method validation or supported through historical data and literature (19).

Incurred Sample Reanalysis (ISR)

ISR can be beneficial for confirming the long-term performance of a method with incurred samples and could provide additional confidence in the study results (42). However, the performance of PCR-based methods (including RT-qPCR and RT-dPCR) is unique from other platforms such as LC–MS or ELISA. If the acceptance criteria for ISR is set too narrow or too broadly, it could render the data insignificant or allow for errors in data interpretation. There are also cases where appropriate sample volumes are not available to conduct ISR (e.g., pediatric studies, rare disease studies). In most cases, a scientific review of the data could be sufficient and longitudinal monitoring of assay performance through QC assessment could provide sufficient value to the study. If ISR is considered an added value to the study, then considerations on extraction efficiency, recovery, assay precision, and stability need to be considered before designing ISR experiments and implementing appropriate acceptance criteria.

Reagent Bridging and Partial Validation

Maintenance of bioanalytical assays is an important aspect of any analytical method life cycle which includes post-validation qualification or bridging of reagents. A qualification run ensures that a new lot or preparation of a critical reagent performs per the assay specifications established during the method validation prior to use in-study. A bridging run compares a previous lot or preparation to a newer lot in a side-by-side comparison, typically on a single RT-qPCR run. The number of runs associated with a qualification or bridging event may vary depending upon method complexity. While many of the changes can be covered via a qualification or bridging run, depending on the severity of the change, a partial/cross-validation or new validation may be needed (19).

The scope of qualifying or bridging a new reagent includes reagents, assay execution, procedural changes and associated extraction/analytical instrumentation/platform. Considerations for context of use and severity of the change should be part of any decision-making process on if and how to qualify/bridge, partial/cross-validate, or fully validate the change(s). Examples of changes where qualifying/bridging runs are recommended are changes in production lots of critical reagent, and changes in reagent production or manufacturing processes. A qualification/bridging run is needed to assess changes in how a reagent or reaction is mixed to ensure continued assay performance. For traceability and good documentation practices tracking of critical reagent lot usage is required if lot changes are not qualified/bridged prior to use in sample analysis. For example, a new lot of master mix may not require a bridging or qualifying run, especially if more than one lot was tested in the validation but does require traceability across all runs/experiments. Critical reagents should be identified in method development and can include assay components such as reference material lots/batches, restriction enzymes. Matrix pool lots can also be considered a critical reagent that may require bridging across lots.

More significant changes in reagents, such using a different extraction kit/process, or a different manufacturer's master mix, can require a partial/cross-validation or new validation depending on the extent of the change. Changes such as a change in the sequence of a primer, probe, or target sequence will require a new validation, as would a change in the intended study matrix (e.g., from plasma to whole blood).

Conclusions

RNA drug products are a growing class of drug modalities that have unique compositions that require multiple pharmacokinetic measurements including the RNA cargo. RT-qPCR is a bioanalytical method that can be used to quantify RNAs in circulation. Current regulatory guidance documents for bioanalytical method validation (18) are specific to ligand binding and chromatographic assay methods and difficult to interpret for use with molecular workflows. The purpose of this publication is to provide information on assay design and technical considerations when validating RT-qPCR regulated assays that are used to support the pharmacokinetic analysis of mRNA-based drug products. The considerations herein should help to improve consistency, clarity, and provide sound scientific recommendations for RT-qPCR for regulatory submissions. Although the focus of the paper has been primarily on LNP-mRNA drug products, assays that detect other RNA therapy components (e.g., guide RNA, other RNA modalities) in circulation can share similar technical considerations.

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Data Availability Any data used herein are examples and not used from data repositories. A data availability statement is not applicable.

Declarations

Conflict of interest The authors are employed by and receive compensation from their affiliations that are listed in this manuscript. The authors have no other relevant affiliations or financial involvements with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. No writing assistance was utilized in the production of this manuscript.

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