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RNA

Defining the required critical quality attributes (CQAs) and phase requirements for mRNA/ LNP product development and manufacture

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About BioPhorum

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Since its inception in 2004, BioPhorum has become the open and trusted environment where senior leaders of the biopharmaceutical industry come together to openly share and discuss the emerging trends and challenges facing their industry.

Growing from an end-user group in 2008, BioPhorum's membership now comprises top leaders and subject matter experts from global biopharmaceutical manufacturers and suppliers, working in both long-established and new Phorums. They articulate the industry's technology roadmap, define the supply partner practices of the future, and develop and adopt best practices in drug substance, fill finish, process development and manufacturing IT.

In each of these Phorums, BioPhorum facilitators bring leaders together to create future visions, mobilize teams of experts on the opportunities, create partnerships that enable change and provide the quickest route to implementation, so that the industry shares, learns and builds the best solutions together.

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1.0

Introduction and scope

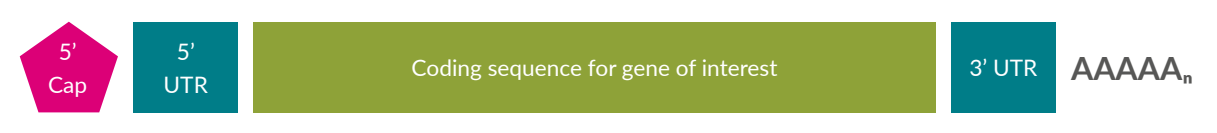
There is a long history of research and development into RNA-based therapeutics and vaccines due to their potential to treat or prevent disease through influencing protein expression. By supplying missing or dysfunctional proteins, or through activation or silencing of gene expression, RNA therapeutics provide treatment possibilities for diseases that cannot be addressed by conventional targeting of proteins and protein pathways. Small RNA molecules such as anti-sense oligonucleotides (ASOs) and small interfering RNA (siRNA) were the first to market as these formats had fewer challenges in terms of delivery to target cells and the potential for immune activation via toll-like receptors (TLRs).

Development activity is now rapidly expanding in the field of mRNA molecules that can drive expression of therapeutic proteins or antigens, spurred on by recent advances in the delivery systems needed to transfer mRNA into cells, which is inherently susceptible to both enzymatic and chemical degradation. This has translated into the success of the two recently approved SARS-CoV-2 vaccines, Comirnaty® and Spikevax®. However, this is still an immature field for which there are limited global regulatory guidelines available to date and little precedent in terms of marketed products and regulatory approvals to help guide developers of this emerging modality through the challenges of chemistry manufacturing and controls (CMC) expectations around quality control.

In vitro transcription (IVT) is the current method for manufacturing mRNA in which RNA is synthesized using a DNA template and nucleoside triphosphates in the presence of RNA polymerase. The purified mRNA resulting from the IVT process is considered the drug substance (DS). The mRNA DS is then combined with lipids formulated into lipid nanoparticles (LNPs) to produce drug product (DP), where DS and DP are the focus for this paper. As mentioned above, mRNA is inherently susceptible to degradation *in vivo*; to that end, lipid nanoparticles have proven to be promising vehicles for RNA delivery. The IVT manufacturing process offers advantages for RNA in comparison to other types of products, for example, proteins where a cellular system is applied. This results in greater process control and reduced biosafety risks for RNA products.

The schematic structural features of an mRNA molecule are outlined in Figure 1. The 5' cap and polyadenosine (poly(A)) tail are critical for stability of the molecule and can impact the translation of the molecule, as can the 5' and 3' untranslated regions (UTRs). The UTRs flank the coding sequence of the gene to be expressed, which is optimized to ensure high levels of protein translation and to avoid secondary structures that could be immunogenic and result in undesirable immune responses. Modified nucleosides are commonly used (e.g. pseudouridine) to reduce the possibility of stimulation of innate immune responses via TLRs.

Figure 1: Schematic diagram showing key structural features of mRNA molecule; the length of the poly(A) tail is variable (n)



LNPs act as the protective capsule for the mRNA, essential since mRNA is extremely sensitive to degradation by nucleases, and they facilitate cellular uptake to enable delivery of the mRNA to the target cell's cytoplasm for translation. The current common mRNA/LNP formulation contains four lipid types: (1) ionizable cationic lipid, (2) phospholipids (helper lipids), (3) cholesterol and (4) PEG-lipid[1]. Lipid formulations may vary from product to product and other formulations or number of lipid components are possible. It is important to not only monitor the quality of the mRNA active substance, but also the quality of the components, and the structure and function of the LNP.

Early identification of CQAs reduces the risk of delays to product development and ensures alignment of product quality testing and control with regulatory agency expectations. The purpose of this article is to provide an industry resource of (potential) critical quality attributes ((p)CQAs) for products from the early stages of drug development to commercial manufacturing, enabling quick identification of relevant CQAs. When additional product and process knowledge is available, these (p)CQAs may be adjusted to non-critical quality attributes (nCQAs), pCQAs or CQAs. Hereafter in this article, pCQAs will be mentioned as CQAs. It should be noted that various product quality attributes will be critical based on regulatory guidance.

BioPhorum participants have sought to review existing knowledge[2, 3, 4, 5] of mRNA DS and LNP delivery systems to identify CQAs that are controlled at various stages of the product manufacturing process, characterization or release and stability testing. A control strategy for each of the CQAs must be defined by the manufacturing process, release, stability and/or characterization testing.

2.0

mRNA/LNP CQAs and methods

This section gives an overview and summary of the pCQAs of the mRNA/LNP DP and a comprehensive list of the CQAs for mRNA product manufacture, including the importance of testing at different stages of DP development (Section 2.6).

2.1 Purity and product-related impurities

The purity of the mRNA may impact its translational efficiency and stability. mRNA impurities typically originate from the IVT synthesis process as well as the degradation products that occur during manufacturing, material handling or storage of materials.

Common process-related impurities from IVT synthesis may include residual reacting components, residual enzyme, residual DNA templates and incorrect or variable sequences (such as short fragments or extended sequences) generated by the self-priming process. These impurities may impact mRNA efficiency and can potentially introduce unwanted immunological responses.

The 5' cap and 3' poly(A) tail are introduced either co-transcriptionally or in a post-transcriptional reaction. The 5' cap structure is responsible for mRNA binding to the ribosomal initiation factors. The 3' poly(A) tail is involved in binding to the ribosomal machinery. Both the 5' cap and 3' poly(A) tail are important to mRNA *in vivo* stability. Therefore, the percentage of capped and polyadenylated mRNA, as well as the poly(A) tail length and distribution, will have a direct impact on the translational efficiency and DS *in vivo* stability.

Additionally, mRNA is typically encapsulated in delivery vehicles such as LNPs, which facilitate mRNA entering the cell cytoplasm and protect them from nuclease degradation. Impurities from the LNP encapsulation process, as well as lipid raw material impurities and the degradation products of lipids, also need to be considered for potential product safety and efficacy concerns.

While impurities introduced by the manufacturing process are unlikely to increase over time, degradation of the active substance and the DP may occur upon exposure to various conditions during handling or storage, such as heat, light exposure or shear stress. Although there is no evidence indicating instability of the cap or poly(A) tail of the mRNA, characterization of the mRNA stability profile, integrity, 5' cap and poly(A) tail is recommended to allow for informed decision-making regarding the quality control strategy.

mRNA purity and impurities associated with safety, efficacy and immunogenicity should be measured at release and stability time points, and determined using a quality risk-based approach.

2.2 Safety tests

For pharmaceutical products, it is critical to control bioburden and bacterial endotoxins. Bioburden tests the microbiological contamination level in a preparation. This is an important safety test that is monitored for all pharmaceutical products to ensure the product meets established microbiological quality standards. The presence of endotoxins in medicinal and pharmaceutical products might be detrimental to human health once administered. Hence, these endotoxins are to be controlled in the parenteral preparations.

Sterility testing is performed to ensure sterile products do not contain viable microorganisms prior to release. Container closure integrity testing can be performed as an alternative to sterility testing to demonstrate the potential contamination over the course of the product shelf life. These may include tests such as seal force, microbiological, dye penetration tests, etc.

2.3 Strength, identity and potency

Strength, identity and potency affect efficacy and are typically considered obligatory CQAs. For identity, it is important to confirm the correct sequence of the mRNA to ensure the production of the desired protein. The lipid composition/identity and content play critical roles in delivering the mRNA into the cell and impact the efficacy of the product; this attribute should be characterized and typically included as part of the release and stability testing.

2.4 Product quality and characteristics

Size, polydispersity index, surface charge and encapsulation efficiency are CQAs for DP quality. Size and charge of a delivery system can affect transitivity and distribution. Polydispersity index is a measure of quality with respect to size distribution. Additionally, zeta potential can also indicate the stability of the formulation. Encapsulation of the DS in the delivery vehicle is a key process parameter that directly impacts product quality. The encapsulation efficiency is a measure of the ability of the process to effectively drive the encapsulation of the DS inside of the particle.

2.5 Other obligatory CQAs

Appearance, pH and sub-visible particles are product quality tests that are monitored over the product shelf life and the specifications are set to maintain critical product quality. Additional release tests including osmolality, moisture, reconstitution time (for lyophilized product) and other tests (as described in Table 1) are evaluated as per compendial testing requirements.

2.6 CQAs for mRNA product development and manufacture

Table 1 gives a comprehensive list of the CQAs for mRNA/LNP manufacture including the importance of testing at different stages of DP development. However, the application of some or all of the CQAs is to be assessed by the respective manufacturer in conjunction with the relevant regulator/authority. Furthermore, extensive studies demonstrating control of a specific CQA (e.g. by means of the manufacturing process) could remove the requirement for measuring it at release and/or stability.

Table 1: Summary of CQAs for mRNA product development and manufacture

Attribute quality	CQA*	Justification of importance	Stage tested	Stage of manufacture (DS/DP)	Is CQA unique for RNA products?	Regulatory precedence?	Release and stability assay methods	General comments
Purity	Percentage capped mRNA	Evaluates the 5' capping efficiency/ percentage of mRNA species that are capped. Critical for mRNA translational efficacy and for RNA stability	Stability/Release/ Characterization	DS	Yes	Yes	LC-MS IPRP-LC	
	Poly(A) tail length and distribution	Crucial for RNA stability and protects RNA from degradation. Has a function in translation initiation and influences translational efficiency	Stability/Release/ Characterization	DS	Yes	Yes	LC-MS IPRP-LC	
	RNA integrity	Impacts efficacy of mRNA, identifying how much of the RNA is desired product. Impurities can contribute to immunological response	Stability/Release/ Characterization	DS/DP	Yes	Yes	IPRP-LC Gel/capillary electrophoresis	
	Percentage poly(A) mRNA	Impacts stability and influences translation efficiency	Stability/Release/ Characterization	DS	Yes	Yes	IPRP-LC qPCR/dPCR	**For Stability**—Product dependent for poly(A) tail length or percent poly(A) mRNA at stability stage
Process-related impurities	Residual nucleoside 5'-triphosphate (NTP)	Ensures removal of reacting components	Characterization	DS	Yes	Yes	LC-MS AEX-LC	Can be tested at release until validated, if validated then does not need to be tested at release
	Residual enzyme	Residual enzymes potentially cause immunogenic activity, reduced efficacy of the final product and toxicity in patients	Characterization	DS	No	Yes	ELISA	Can be tested at release until validated, if validated then does not need to be tested at release
	Residual DNA templates	Demonstrates effective purification during manufacture, can potentially cause immunogenic activity. The presence of residual DNA may lead to oncogenicity, infectivity and immunomodulatory risks	Release	DS	No	Yes	qPCR	
	Residual solvents	Residual solvents can have toxicity and carcinogenicity effects and need to be limited according to ICH guidelines to ensure product quality and safety[6]	Characterization	DP	No	Yes	GC-FID	Can be tested at release until validated, if validated then does not need to be tested at release
	Lipid-related DP impurities	Lipid-related DP impurities can impact efficacy and safety if present	Characterization/ Release	DP	No	Yes	LC-MS LC-CAD LC-ELSD	**For Release**—Does not necessarily need to be a specific method as the integrity method may be able to detect impurities. If lipid adducts are present then will require testing on release and stability
	dsRNA	Can impact safety and efficacy and translation of protein antigen	Release	DS	No	Yes	Antibody-based—ELISA-HTRF-immunoblot Cell-based reporter assays—TLRs, IFN	

*Note that the CQAs contained in the table may be considered as (potential) critical quality attributes ((p)CQAs), nCQA (non-critical quality attributes) or CQA (critical quality attributes) depending upon individual product, process knowledge and regulatory/legislative requirements.

Table 1: Summary of CQAs for mRNA product development and manufacture (continued)

Attribute quality	CQA*	Justification of importance	Stage tested	Stage of manufacture (DS/DP)	Is CQA unique for RNA products?	Regulatory precedence?	Release and stability assay methods	General comments
Safety	Bioburden	Ensures the safety of a manufactured product—effective quality control and accurate test results are essential to minimize risks for consumers	IPC Release	DS	No	Yes	Ph. Eur. 2.6.12 USP <61	
	Sterility	Sterility testing is a GMP microbiology testing requirement used to confirm sterile products do not contain viable microorganisms prior to release and patient administration	Release	DP	No	Yes	Ph. Eur. 2.6.1 USP <71>	
	Endotoxins	Ensures the safety of a manufactured product. Effective quality control and accurate test results are essential to minimize risks for consumers	IPC (DS) Release	DS/DP	No	Yes	Ph. Eur. 2.6.14 USP<85>	
Identity	Lipid ID	Confirms the identity of the appropriate lipids	Release	DP	No	Yes	LC-MS LC-ELSD LC-CAD	
	Sequence identification	Confirms the identity of the sequence to distinguish the product and to ensure no changes to sequence	Release	DS/DP	No	Yes	Sanger sequencing NGS RT-PCR	RT-PCR can be used for DS
Potency	Drug product potency	Set on the minimum dose used to demonstrate efficacy in clinical trials and immunogenicity data, an upper limit to be defined based on available human safety data	Stability/Release	DP	No	Yes	Cell-based assay	Determined on a case-by-case basis. 'Matrix' approaches can be adapted to capture multiple mechanisms of action[7]
	Drug substance potency	DS potency is different to DP potency: at DS level you are looking at correct expression of expected protein and ability to translate, which is not equivalent to potency of the DP	Characterization	DS	No	Yes	On a case-by-case basis	Determined on a case-by-case basis. 'Matrix' approaches can be adapted to capture multiple mechanisms of action[7]
Strength	mRNA content	mRNA content changes could impact on safety and efficacy, also needed for dosing and label claim	Stability/Release	DS/DP	No	Yes	RP-LC-UV-Vis RT-qPCR dPCR RP-LC-Fluorescence detection	
	RNA ratio	Required for confirmation of mRNA content	Release	DP	No	Yes	IPRP-LC dPCR	If more than one RNA species are present

*Note that the CQAs contained in the table may be considered as (potential) critical quality attributes ((p)CQAs), nCQA (non-critical quality attributes) or CQA (critical quality attributes) depending upon individual product, process knowledge and regulatory/legislative requirements.

Table 1: Summary of CQAs for mRNA product development and manufacture (continued)

Attribute quality	CQA*	Justification of importance	Stage tested	Stage of manufacture (DS/DP)	Is CQA unique for RNA products?	Regulatory precedence?	Release and stability assay methods	General comments
Product characterization and quality	Encapsulation efficiency	Encapsulation protects the mRNA from degradation and stabilizes it, it is needed for delivery and efficacy, and is an important measure of quality	Stability/Release	DP	No	Yes	RP-LC-Fluorescence detection	
	Surface charge	Surface charge affects the distribution of nanoparticles and affects performance and process consistency	Characterization	DP	No	Yes	ELS iCIEF TNS binding assay	
	LNP size	Affects distribution and uptake of nanoparticles and impacts safety and efficacy	Stability/Release	DP	No	Yes	DLS (SR-DLS or RT-MALS in/online option) NTA	
	LNP polydispersity	Polydispersity is a measure of the homogeneity and impacts safety and efficacy	Stability/Release	DP	No	Yes	DLS (SR-DLS or RT-MALS in/online option)	
	Lipid content	Lipid content impacts function of the DP	Stability/Release	DP	No	Yes	LC-CAD LC-ELSD	

*Note that the CQAs contained in the table may be considered as (potential) critical quality attributes ((p)CQAs), nCQA (non-critical quality attributes) or CQA (critical quality attributes) depending upon individual product, process knowledge and regulatory/legislative requirements.knowledge and regulatory/legislative requirements.

Table 1: Summary of CQAs for mRNA product development and manufacture (continued)

Attribute quality	CQA*	Justification of importance	Stage tested	Stage of manufacture (DS/DP)	Is CQA unique for RNA products?	Regulatory precedence?	Release and stability assay methods	General comments
Compendial testing	Appearance	A visual indicator of product quality	Stability/Release	DS/DP	No	Yes	Ph. Eur. 2.2.1 Ph. Eur. 2.2.2 Ph. Eur. 2.9.20 USP <790> USP <1>	Methods for different aspects of appearance, e.g. color, visible particles, etc.
	Moisture content	Affects stability of the product	Stability/Release	DS/DP	No	Yes	USP <921> Karl Fischer	Only for lyophilized products
	Osmolality	Ensures tonicity for injections	Release	DP	No	Yes	Ph. Eur. 2.2.35 USP <785>	Can be used as an orthogonal method to verify correct formulation
	Reconstitution time	A measure of lyophilization efficiency and ease of administration	Stability (DP)/ Release	DS/DP	No	Yes	Reconstitution time Product specific	Only for lyophilized products
	Sub-visible particle	Affects product quality and it is an ICH requirement	Stability/Release	DP	No	Yes	Ph. Eur. 2.9.19 USP <788>	
	pH value	Indicates product quality—correct formulation	Stability/Release	DS/DP	No	Yes	Ph. Eur. 2.2.3 USP <791>	
	Content uniformity	Ensuring process consistency within a batch	IPC	DP	No	Yes	Ph. Eur. 2.9.40 USP <905>	Can be validated and tested as an in-process control (IPC) instead of release testing
	Extractable volume	Confirmation of container content for dosing and label claim	Release	DP	No	Yes	Ph. Eur. 2.9.17 USP <697>	For liquid-only products

*Note that the CQAs contained in the table may be considered as (potential) critical quality attributes ((p)CQAs), nCQA (non-critical quality attributes) or CQA (critical quality attributes) depending upon individual product, process knowledge and regulatory/legislative requirements.

3.0

Conclusion

Ensuring consistent high-quality mRNA drug product manufacturing under continually compressed timelines remains a significant challenge for mRNA developers. mRNA is still a new modality with increasing knowledge and developing global regulatory guidelines. There is little precedent in terms of marketed products and regulatory approvals to help guide developers through the challenges associated with manufacturing and establishing quality control strategies. This situation could adversely impact control strategies and timelines and, in turn, increase the cost of batch product.

This article supports developers to identify relevant CQAs, the stage at which their testing may occur and the category of control strategy (e.g. release, stability, etc.).
A justification is provided for each CQA category and for each individual attribute.
The justifications provide further information, based on industry experience, on whether these CQAs should be considered for products, as there are many different mRNA products, and each company will have their own in-depth understanding and specific characterization information to inform their quality control strategies.

However, challenges remain in terms of understanding CQAs of the mRNA molecule and LNP delivery systems. For example, sensitivity and specificity of current methods may limit the ability to further understand the *in vitro/in vivo* efficacy or potency and any process-related impurities to ensure desirable product quality. Looking ahead, current methods may be continuously improved or novel analytical tools introduced to gain more insight into the use of these CQAs. Furthermore, characterization of mRNA/LNP and monitoring of consistency of the final product is essential to confirm product quality.

Glossary

Term	Definition
AEX-LC	Anion exchange-liquid chromatography
ASO	Anti-sense oligonucleotide
dPCR	Digital polymerase chain reaction
DLS	Dynamic light scattering
DP	Drug product (liquid nanoparticle)
DS	Drug substance (mRNA)
ELISA	Enzyme-linked immunosorbent assay
ELS	Electrophoretic light scattering
GC-FID	Gas chromatography-flame ionization detection
HTRF	Homogeneous time-resolved fluorescence
iCIEF	Imaged capillary isoelectric focusing
IPC	In-process control
IFN	Interferon
IPRP-LC	Ion pair reversed phase-liquid chromatography
IVT	In vitro transcription
LC-CAD	Liquid chromatography with charged aerosol detection
LC-MS	Liquid chromatography mass spectrometry
LC-ELSD	Liquid chromatography evaporative light scattering detection
LNP	Liquid nanoparticle
mRNA	Messenger ribonucleic acid
NGS	Next-generation sequencing
NTA	Nano particle tracking analysis
Ph. Eur.	European Pharmacopeia
Ph. Eur. 2.2.1	Clarity and degree of opalescence of liquids
Ph. Eur. 2.2.2	Degree of coloration of liquids
Ph. Eur. 2.2.3	Potentiometric determination of pH
Ph. Eur. 2.2.35	Osmolality
Ph. Eur. 2.6.1	Sterility
Ph. Eur. 2.6.12	Microbiological examination of non-sterile products: microbial enumeration tests
Ph. Eur. 2.9.17	Test for extractable volume of parenteral preparations
Ph. Eur. 2.9.19	Particulate contamination: sub-visible particles

Glossary (continued)

Term	Definition
Ph. Eur. 2.9.20	Particulate contamination: visible particles
Ph. Eur. 2.9.40	Uniformity of dosage units
TLR	Toll-like receptors
TNS Binding	2-p-ToluidinylNaphthylene-6-Sulfonate Binding
qPCR	Quantitative polymerase chain reaction
RP-LC	Reversed-phase liquid chromatography
RT-MALS	Real-time multiangle light scattering
RT-qPCR	Real-time quantitative polymerase chain reaction
siRNA	Small interfering RNA
SR-DLS	Spatially resolved dynamic light scattering
USP	United States Pharmacopeia
USP 1	Injections and Implanted Drug Products (Parenterals)—Product Quality Tests
USP 61	Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests
USP 71	Sterility Tests
USP 85	Bacterial Endotoxins Test
USP 697	Container Content for Injections
USP 785	Osmolality and Osmolarity
USP 788	Particulate Matter in Injections
USP 790	Visible Particulates in Injections Test
USP 791	pH Determination
USP 921	Water Determination

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