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Contract Testing Services

Application Note

Establishing Analytical Methods for mRNA-based Therapies

From plasmid DNA (pDNA) and mRNA delivered as vaccines, to different forms of RNA used to silence or knock down gene expression, nucleic acid-based modalities represent a diverse set of options for addressing unmet medical needs. With proven efficacy as vaccines for SARS-CoV-2, mRNA has demonstrated its versatility and fast-to-market potential. Given the complexity of this emerging modality, rigorous characterization and testing are required to ensure safe and effective mRNA drug products.

mRNA modalities can be manufactured by using a pDNA template generated via an *E. coli* master cell bank as a starting point or via *in vitro* transcription (**Figure 1**). Because mRNA cannot be delivered directly to patients, the drug substance must be formulated into the final drug product for targeted delivery. In many cases, this is accomplished by encapsulating the mRNA into a lipid nanoparticle (LNP).

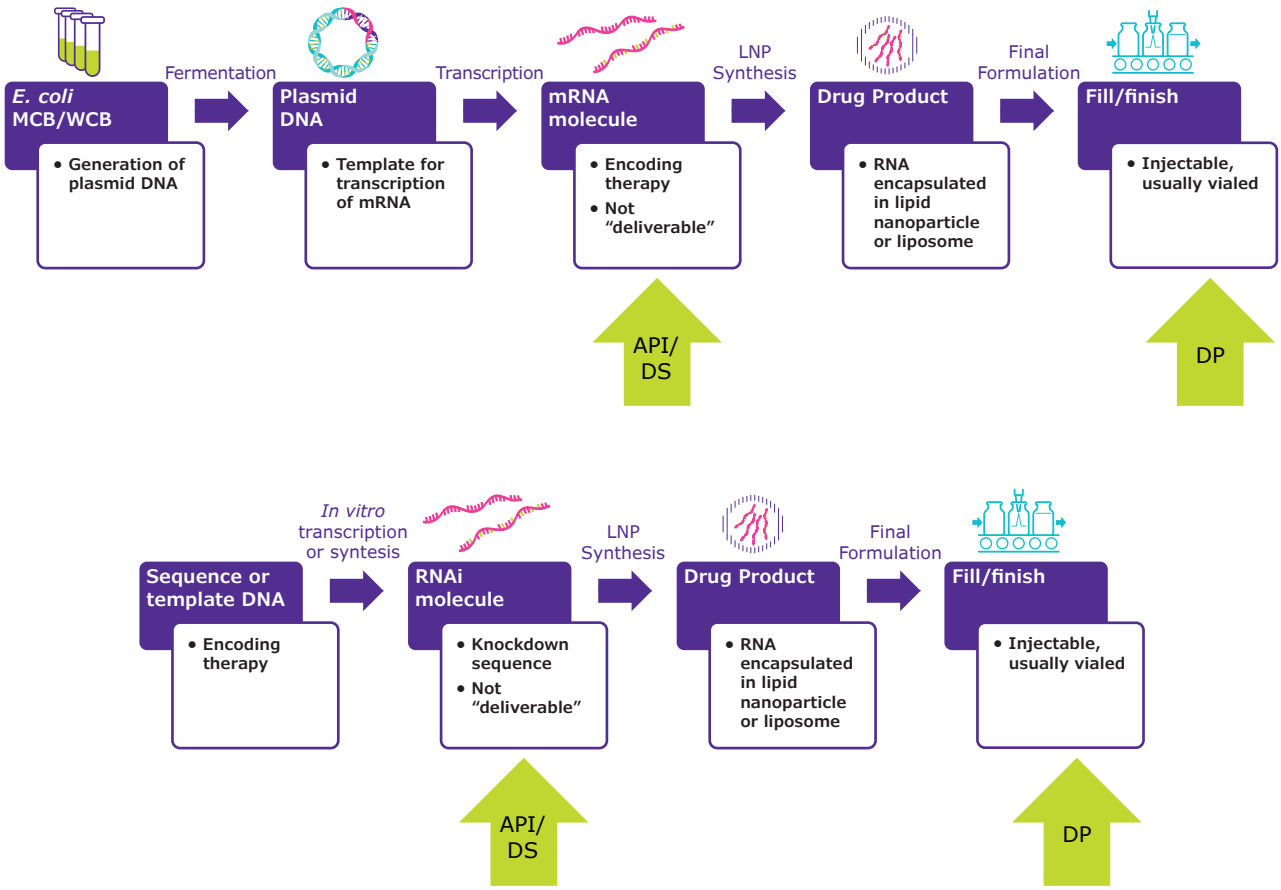

Figure 1. Production of mRNA from an *E. coli* working cell bank (A) and *in vitro* transcription (B).

As with all biotherapeutics, a comprehensive set of analytical tests are required to ensure the quality and safety of these nucleic acid assets. The RNA drug substance and the formulated drug product should be subjected to stringent testing prior to lot release.

To ensure our customers can develop safe and efficacious mRNA therapies, we provide a comprehensive package of assays for mRNA starting materials, drug substances, and drug products. Our assay packages and phase-appropriate assay development and validation capabilities provide the product and process knowledge you need to develop a quality target product profile (QTPP) and identify the critical quality attributes (CQAs) to unlock the disruptive potential of mRNA vaccines and therapies. Our assays provide an extensive, quantitative understanding of your mRNA products to de-risk clinical development and regulatory approvals. These assays are backed by best-in-class biologics release testing services, leading bioinformatics platforms, and robust regulatory and technical expertise.

This whitepaper provides a detailed description of assays for sequence identification and LNP compositional, two critical quality attributes of mRNA-LNP products. These assays are part of our extensive portfolio of mRNA drug substance and drug product testing services (**Table 1**). Many assays are necessary during the production and formulation of mRNA as a vaccine or therapeutic.

Table 1. Many assays are necessary during the production and formulation of mRNA as a vaccine or therapeutic.

	Identity	Quantity/Potency	Purity	Residuals
starting Materials	• pDNA sequence (NGS)	• UV spectrophotometry	• Homogeneity of plasmid (CE-LIF)	• Residual *E. coli* Protein (BCA or ELISA) • Residual *E. coli* DNA (qPCR) • Residual *E. coli* RNA (qPCR)
Drug substance	• mRNAsequence (NGS)	• mRNA content (A260 or fluorometric assay) • Cell-based expression and/or potency assays	• mRNA purity (CGE) • Capping efficiency (LC or LC-MS) • Poly(A) tail length (LC-MS) • Contaminating dsRNA (ELISA)	• Residual template pDNA (qPCR) • Residual enzymes and proteins
Drug product	• mRNA identity following de-formulation (NGS) • Lipid ID (HPLC-CAD)	• *In vitro* or cell-based potency assays • Lipid content (HPLC-CAD) • RNA content (fluorometric assay)	• Encapsulation efficiency (fluorometric assay) • LNP size/polydispersity (DLS) • LNP surface charge (zetapotential)	• Residual solvents (GC)
Compendial attributes for starting materials, drug substance, and drug product				

Sequence Identification and Variant Detection

Confirmation of sequence identity is a regulatory expectation and ensures that the drug product includes the expected sequence. In the context of mRNA-based vaccines and therapeutics, having the intended sequence is essential as it is subsequently expressed once administered. The presence of an incorrect sequence could have unintended, and in some cases, serious consequences, which is why an identity test is required.

For most viruses and viral vectors, the recommendation is that each is fully sequenced with a complete annotation of the entire vector. It is also recommended to evaluate and detail the significance of differences between the expected sequence and the experimentally derived sequences. This testing should be performed at strategic points in the manufacturing process to ensure that quality materials go into the process, checks are performed during the process (for example, testing bulk harvest), and quality products come out of the process (**Figure 2**).

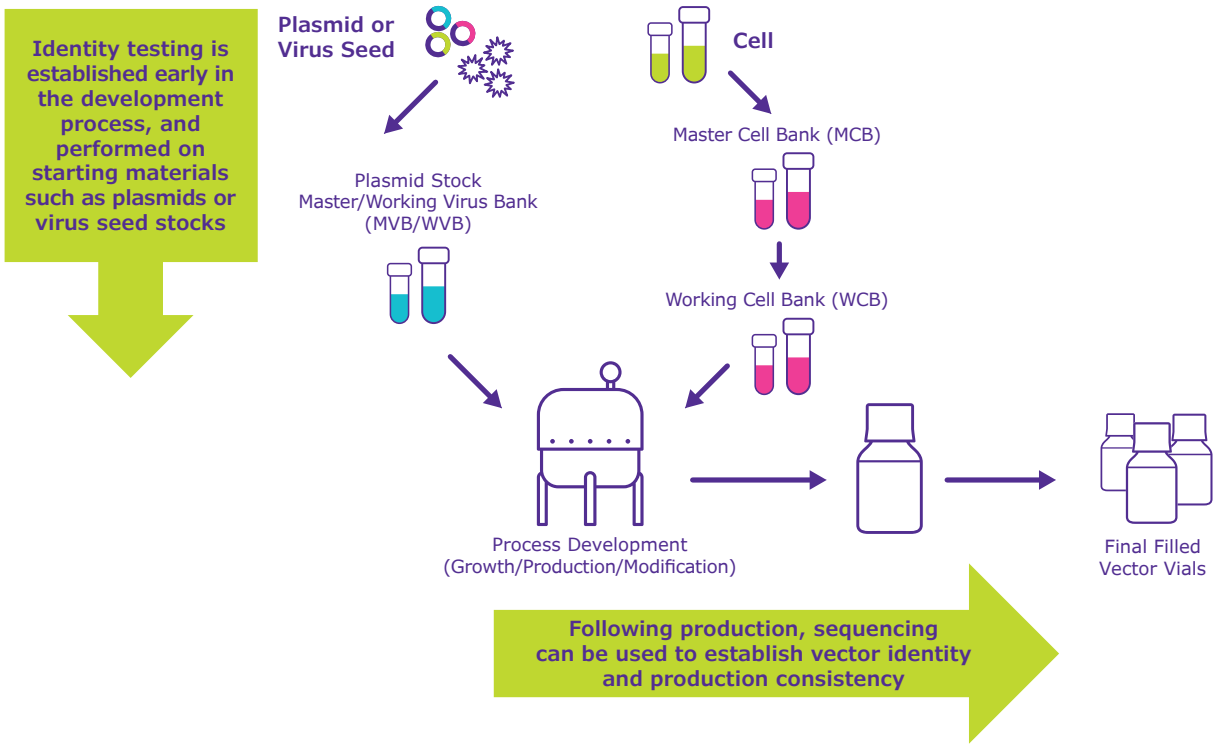

Figure 2. Identity testing should be performed across the manufacturing workflow.

Historically, identity testing has been performed using a variety of molecular-based methods such as qPCR and restriction enzyme analysis. A major shortcoming of these approaches is that they do not confirm that the entire sequence is correct, only that a portion of the sequence is generating the correct profile—for example, amplification or specific fragments or patterns on a gel. As such, subtle changes may not be detected.

As an alternative, Sanger sequencing can be used to confirm the consensus sequence, generally from a PCR amplified product. Detection of low-level variants, however, is impractical using this technology. Detection of individual base pair changes is not possible unless the variant is at least 20% of the population, and even at that level, there is some subjectivity to the call based on background. Detection of small insertions and deletions is near impossible with Sanger sequencing because of phase shifting which is not easily discernable.

In contrast to Sanger sequencing, next-generation sequencing (NGS) is particularly well suited for genomic characterization, including nucleotide variant or sub-population detection. For this type of analysis, the sequence data set is aligned against one or more defined reference sequences. In instances where sufficient depth of coverage is achieved across the genome or region of interest (ROI), sequence variant detection is possible, enabling the detection of sub-populations within a test sample. NGS uses sequence-by-synthesis, a different type of technology to perform individual molecule sequencing leading to much greater depth of coverage.

Whereas Sanger sequencing is typically assessed in replicate reactions (4X being industry standard), NGS assesses each molecule individually. With this granularity of sequence assessment, millions of reads with numbers typically greater than 10,000X coverage per base are used to determine the sequence. This allows for a much higher level of variant detection, down to 1% or less in a population and enables identification of small insertions and deletions with pin-point accuracy, which is challenging for Sanger sequencing due to limitations of the technology. NGS can therefore confirm identity and flag small variations which can have an impact on the product.

As a leading service provider, we have pioneered the use of NGS to expand our portfolio of GMP service offerings. Our fully validated NGS identity method consists of three fundamental steps: sample processing, sequencing, and data analysis. While the entire workflow is governed by rigorous quality control (QC) guidance, additional QC steps are performed during sample processing to ensure the highest data quality. In the context of mRNA, this approach enables rapid characterization of the sequence to ensure safe and effective translation. Our suite of NGS services is based on complementary technology platforms that rapidly generate deep sequencing for comprehensive data analysis, backed by biosafety experts who deliver intelligent results tailored to your unique testing needs.

LNP Compositional Analysis

The LNP plays an important role in enabling targeted delivery of the mRNA to specific cell types and tissues, while also protecting the mRNA from degradation. LNPs typically consist of four different lipids formulated in a defined ratio:

- **Ionizable cationic lipids** form a complex with the RNA via electrostatic interactions, overcoming the nucleic acid's negative charge to enable delivery to the target cell.
- **Polyethylene glycol (PEG) lipid**s provide colloidal stability and prevent protein binding to the particle, thereby shielding it from the immune system and achieving longer circulation.
- **Neutral/anionic lipids** provide structural stability and play a role in defining the fusogenicity and biodistribution.
- **Cholesterol** is used to modulate the bilayer density and fluidity and uptake of the LNP.

The efficacy of an mRNA-based vaccine or therapeutic is determined in large part by the lipid composition of the LNP, which varies from product to product. A small change in the relative abundance of a particular lipid could have a significant impact on how that drug is delivered and its efficacy. As such, the identity, quantity, and purity of the LNP lipids are critical quality attributes of mRNA-LNP products, as well as their ratio in the final formulation.

Ultra-high performance liquid chromatography (UHPLC) coupled with charged aerosol detection (CAD) is a powerful technique for analysis of compounds such as lipids that do not contain chromophores and as such, do not absorb UV light. To assess lipid content, the LNP is dissolved in an appropriate sample diluent, resulting in disassembly of the LNP into its constituent lipids. The individual lipids are then separated by reversed phase UHPLC and detected by CAD. Each lipid is quantified relative to a calibration curve obtained from a series of lipid standards. Due to the variations in LNP compositions and the availability of novel, proprietary lipids, it is important to leverage a combination of product-specific method development and ready-to-use approaches.

Figure 3 shows the CAD chromatogram of the constituent lipids generated from an internal study using an mRNA-LNP formulation. Each of the individual lipids were detected and well-resolved, demonstrating the utility of UHPLC-CAD for the simultaneous analysis of LNP components. A series of dilutions were prepared for three of the lipids that were present in the sample: cholesterol, DMG-PEG (a lipid formed by the PEGylation of myristoyl diglyceride), and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC). Data for the proprietary, cationic lipid were not obtained. As shown in **Figure 4**, the response of the assay is linear ($R^2 \geq 0.995$) for each lipid over the range studied, allowing the quantity of the individual lipids to be determined.

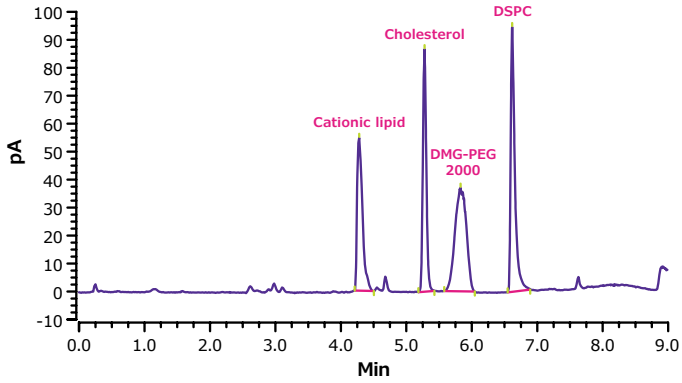

Figure 3. UHPLC-CAD chromatogram of an LNP formulation.

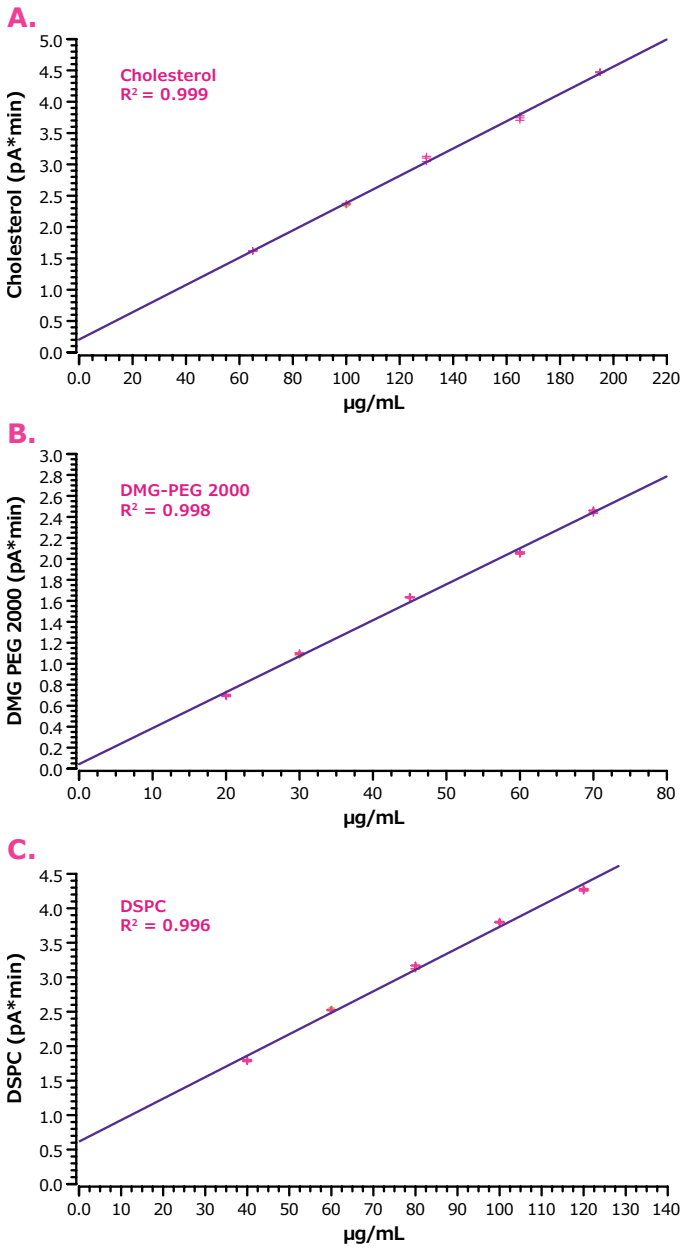

Figure 4. Calibration curves for cholesterol, DMG-PEG 2000, and DSPC.

Conclusion

Nucleic acid modalities and LNP delivery systems are revolutionizing how diseases are treated and prevented. As with other modalities, their success relies on robust and consistent processes to make and purify the drug substance and then formulate it to ensure successful delivery to the intended target. A range of analytical techniques is necessary to ensure product quality and patient safety.

In addition to the methods described above, we offer an extensive package of assays for pDNA, mRNA drug substance, and mRNA drug product to confirm the required critical quality attributes for various stages of development. Our extensive assay packages are backed by analytical development and biosafety experts to deliver results tailored to your unique testing needs. Partner with us throughout your development journey to gain access to best-in-class biologics testing services, a leading bioinformatics platform, and robust regulatory and technical expertise.

Learn more: **SigmaAldrich.com/mRNATesting**

Contact Us

Europe
Todd Campus
West of Scotland Science Park
Glasgow, G20 0XA
Scotland
Tel: +44 (0)141 946 9999
Biosafety@merckgroup.com

North America
14920 Broschart Road
Rockville, MD 20850-3349
USA
Tel: (301) 738-1000
Biosafety@merckgroup.com

Singapore
#2 Science Park Drive
#04-01/12 Ascent Building, Tower A
Singapore 118222
BiosafetyAP@merckgroup.com

China
15-18F, No.3, Building C,
The New Bund World Trade Center
(Phase II)
Lane 227 Dongyu Road, Pudong
New District
Shanghai, China
BiosafetyCN@merckgroup.com

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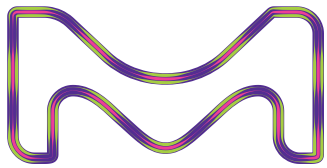

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