Unleashing the potential of mRNA therapeutics with high-quality bioreagents and optimized *in vitro* transcription

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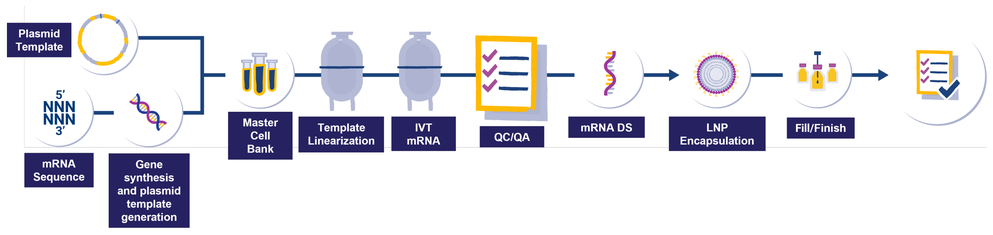
Abstract

In mRNA manufacturing, two key challenges lie in the realms of analytical testing across the workflow, including raw material quality and drug product purity testing, posing obstacles to manufacturing mRNA at a larger scale and cost-effectively. The work described in this poster explores potential solutions for advancing mRNA manufacturing. More robust tools and streamlined processes are needed to ensure the timely production of mRNA for clinical trials and potential commercial launches. Additionally, meeting regulatory standards adds another layer of complexity to the manufacturing process. Using a cGMP grade DTT (dithiothreitol) reagent, which is a reducing agent protecting proteins against oxidation, is crucial in in vitro transcription buffer, as it protects enzymes such as T7 RNA polymerase, inorganic pyrophosphatase, and RNAse inhibitors, therefore facilitating, high-yielding reactions and high-quality mRNA transcripts.

Introduction

The utilisation of mRNA therapeutics poses challenges in terms of mRNA degradation, stability and delivery of lipid nanoparticles (LNPs), and immunogenicity upon delivery. The operation of manufacturing mRNA therapeutics (Figure 1) is complex in and of itself because it is difficult to achieve high-quality and highly pure mRNA with scalable manufacturing processes.

Figure 1. Basic mRNA manufacturing process

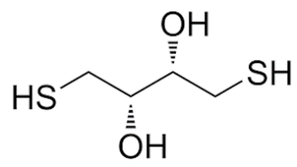


Methods

Utilizing GMP-grade raw materials

The most straightforward method to streamline manufacturability is to utilise an *in vitro* transcription process that yields the greatest amount of material at the highest quality and then preserves that quality throughout the downstream processing. For example, BIOVECTRA's process relies on GMP-grade raw materials for batch-to-batch consistency, even for early-phase clinical trials. This ultimately creates a consistent process and drug product. Using GMP-grade raw materials also eliminates the risk of needing to switch materials later in the development process, thus reducing the risk of program delays and regulatory concerns.

One such GMP-grade material is dithiothreitol (DTT), a reducing agent used to prolong the life of enzymes in a reaction tube by limiting the natural oxidation that would inactivate them. This high-quality material, manufactured by BIOVECTRA and distributed by Thermo Fisher Scientific, is crucial in in vitro transcription buffers, as it protects enzymes such as T7 RNA polymerase, inorganic pyrophosphatase, and RNAse inhibitors. It facilitates high-yielding reactions and high-quality mRNA transcripts.



Methodology for the HPLC and ELISA

100 ng of supercoiled plasmid is injected onto the column to obtain the % supercoiled result and the amount of multimers present. The diluent is typically water or 10 mM TE, pH 8.0, but moderate amounts of other salts are well tolerated and do not affect the separation. Linearization is performed using commercially available enzymes and 100 ng of the linearized crude or purified product is loaded onto the column and the linearization efficiency determined using the amount of supercoiled plasmid remaining.

pDNA linearization

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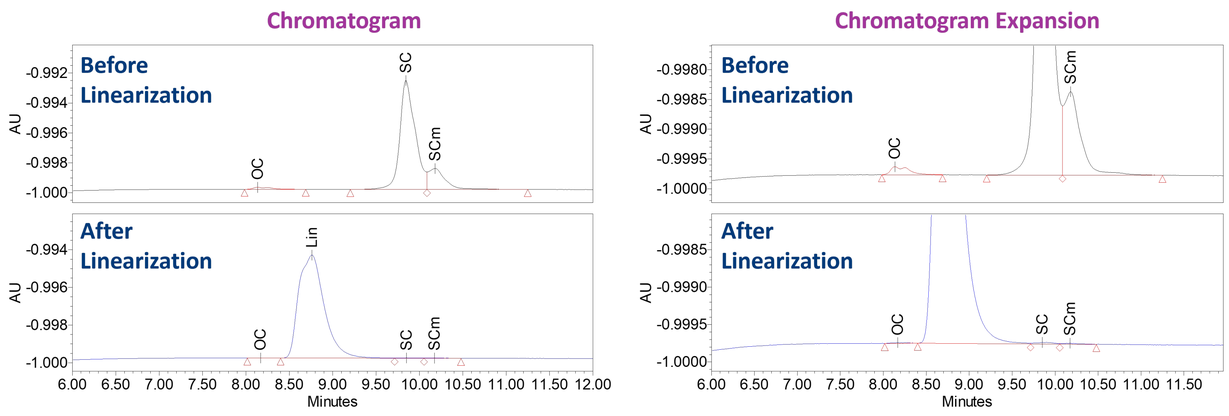
Figure 2. Plasmid topology and linearization efficiency can be assess using HPLC. On the left are full size chromatograms and the right are zoomed in chromatograms to better visualize the baseline. The HPLC method demonstrates that we can resolve the different isoforms of the plasmid DNA impurities. "OC" is open circle. "SC" is supercoiled. "SCm" is supercoiled multimers. "Lin" is linearized pDNA. It is important to be able to detect the different isoforms and impurities that are present in your linearized plasmid DNA. Fully linearized pDNA is the optimal starting material for a high-quality in vitro transcription (IVT) reaction

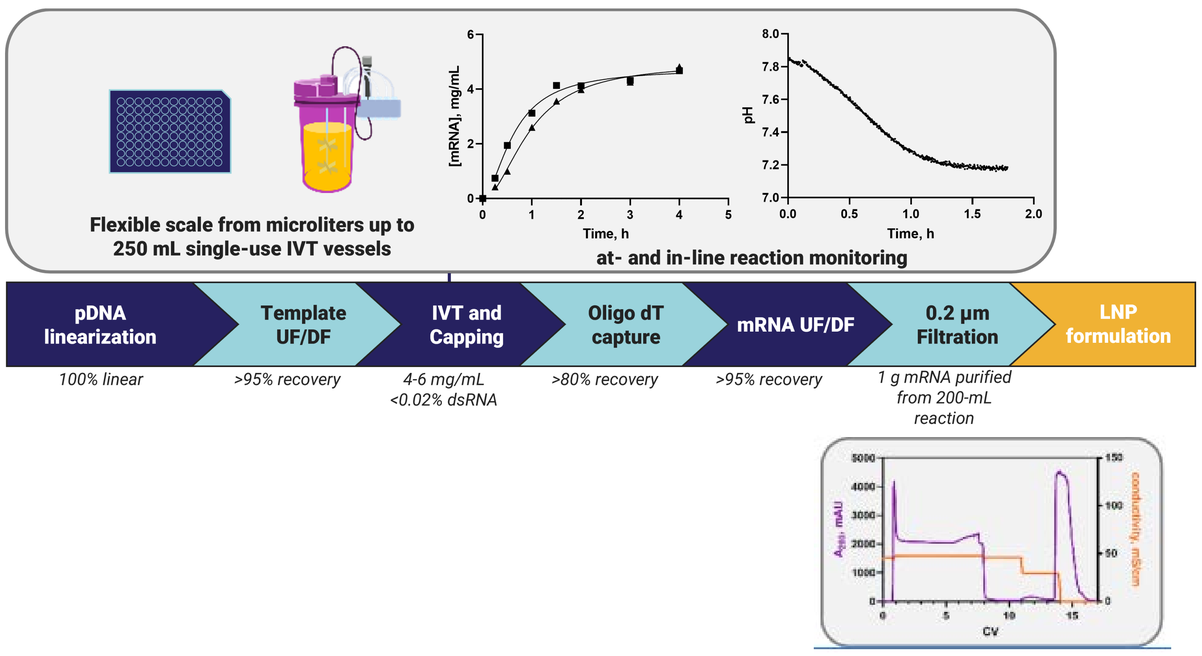


IVT and purification

After linearization is ensured, BIOVECTRA's *in vitro* transcription process (Figure 3) can be followed to achieve high-yield, low double-stranded RNA, and high purity full-length species with or without co-transcriptional capping.

- Proficiency using N1-methylpseudoUTP, trinucleotide cap-1 analogues, wild-type and mutant T7 RNA polymerases.
- Minimal process exceeds safety specifications, but optional DNase I and polishing steps available.
- Efficient fed-batch process in development.

Figure 3. BIOVECTRA's *in vitro* transcription process



Comprehensive analytical assays

Throughout the mRNA manufacturing process, comprehensive and GMP-compliant analytical assays are required for method development, method familiarisation, qualification, and validation. A commercially available kit from Novus Biologics (Cat# NBP3-11368 was used.Thermo Fisher plates (PI1504) were used for coating with Novus coating antibody, detection antibodies, and secondary antibodies from the K1 kit (NBP3-11368). Experiments were ran in triplicates.

Table 1. Analytical assays supporting all phases of clinical development

Quality Attribute	Product Attribute		
	Plasmid DNA	mRNA Drug Substance	LNP Drug Product
Content	DNA concentration	RNA concentration	Lipid Content Encapsulation Efficiency mRNA Quantitation Nucleic Acid Content
Purity/Integrity	Purity (Total plasmid) by agarose gel electrophoresis Purity (Supercoiled DNA) by capillary electrophoresis Residual protein by SDS-PAGE Host cell protein by ELISA Residual host cell DNA by qPCR Residual host cell RNA by HPLC Residual kanamycin	Poly A Tail Length/Homogeneity Capping Efficiency Purity by agarose gel electrophoresis Fragment Analyzer HPLC Residual protein dsRNA by ELISA Residual DNA by qPCR DNAse Contamination RNAse Contamination	Polydispersity/Size mRNA Integrity
Safety	Endotoxin Bioburden Sterility	Endotoxin Bioburden Sterility	Endotoxin Bioburden Sterility
Identity/Potency	Identity by restriction digest Sanger Sequencing	Sanger Sequencing *In Vitro* Potency/Functional Translation	Lipid Components
Other	Appearance pH	Appearance pH Osmolality Residual Solvents	Appearance pH Osmolality Residual Solvents Lipid Related Impurities

dsRNA quantification by ELISA

Double stranded RNA or DSRNA is a major impurity of therapeutic mRNA produced by *in vitro* transcription. Impurity levels of this transcriptional byproduct need to be tightly monitored in mRNA production lots to ensure efficient mRNA expression and to reduce the risk of undesired high immunogenicity.

The most used assay is dot blot, as it is a fast and easy method for the detection of double stranded RNA both in crude and feature transcription mixes, as well as purified mRNA. However, a dot blot assay can be difficult to quantify.

Rather than using a dot blot, BIOVECTRA has developed an ELISA method, which is more robust and allows for better quantitation of the extremely immunogenic impurity transcripts made with RN. Optimised *in vitro* conditions results in double stranded RNAs of less than .02%.

Figure 4. Example of dsRNA impurities quantification in an IVT reaction. This sample was purposely designed to yield high dsRNA impurities (approximately 3,000 ng/mL).

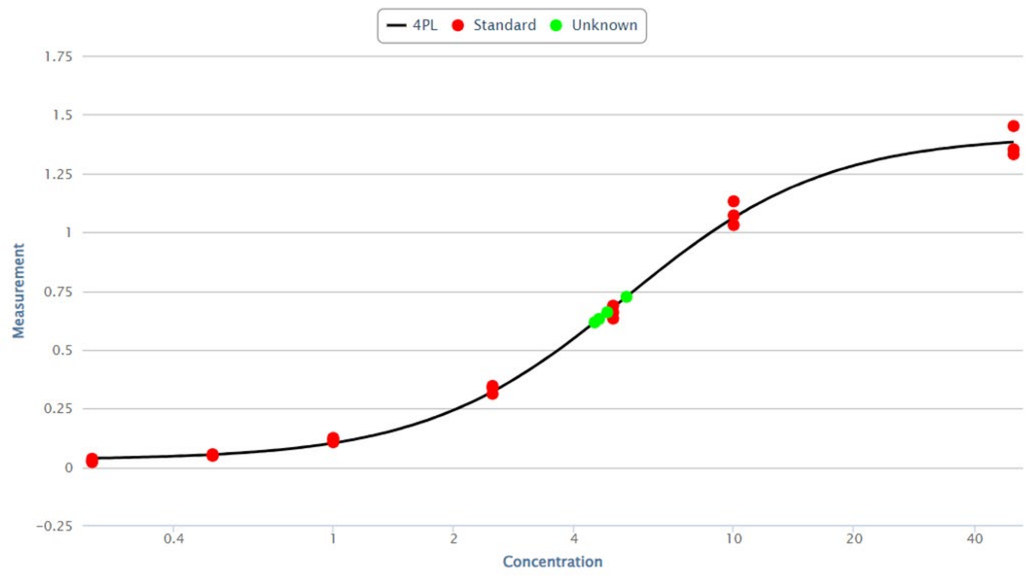


Figure 5. DTT GCMP vs. Non-GMP. Difference in the product release testing.

	DTT - Reagent Grade	DTT - Molecular Biology Grade	DTT - GMP Grade
	Industry-leading quality and reliability (Purity > 99%). Reagent used for maintaining thiol groups in a reduced state during protein processing, isolation, and denaturing. Incorporated into some leading COVID-19 testing kits	Includes additional testing for: Protease, RNase, DNase, ICP metals	Includes additional testing residual solvents
I.R.	✓	✓	✓
Titration ex-SH (iodometric)	✓	✓	✓
T.L.C	✓	✓	✓
UV Assays	✓	✓	✓
Appearance	✓	✓	✓
% Oxidized	✓	✓	✓
Water Insoluble Material Content	✓	✓	✓
Protease		✓	✓
RNase		✓	✓
DNase		✓	✓
ICP-Metal Analysis		✓	✓
Residual Solvents			

Conclusions

Using high-quality raw materials to optimize *in vitro* transcription conditions is one method to address common challenges in the mRNA manufacturing process, such as low yields, low purity, and high double-stranded RNA, to help ensure safe and effective drug products. Low yeilds will increase your product costs. Low purity of your mRNA ttranscripts will result in lower translation of the mRNA in vivo, resulting in lower therapeutic or vaccine efficacy. Double-stranded RNA (dsRNA), a major by-product of the in vitro transcription (IVT) process, will trigger of cellular immune responses. Toll-like receptors 3, 7, & 8 recognize single stranded and double stranded RNA (dsRNA) when trafficking through endosomes. dsRNA is also detected by cytosolic sensors Protein Kinase R (PKR) and MDA5. If high amounts of dsRNA are present, your mRNA product will activate the innate immune system, negatively affect your functional translation, and lower your product's efficacy as well as cause safety concerns.

Trademarks/licensing

BIOVECTRA's GMP-grade DTT, can be sourced through Thermo Fisher Scientific. Request information on BIOVECTRA's bioreagent portfolio at thermofisher.com/biovectra

Thermo Fisher Scientific is an authorised distributor of BIOVECTRA's bioreagent