CSOs in CDMOs giving mRNA Manufacturing Considerations *Standard and Practical Process to Fast-Track mRNA Drug Product Manufacturing*

The central dogma of life has DNA on one side, protein on the other, and RNA sitting right in the centre. RNA, specifically mRNA, is the intermediary step in this dogma and is being used as the essential cargo in many therapies. Expertise in manufacturing high-quality DNA and enzymes used in the *in vitro* transcription (IVT), capping, and the tailing process is fundamental to mRNA production for several therapeutic modalities. Based on our 25 years of experience as a contract development and manufacturing organisation (CDMO), we present considerations and practical solutions at each step of production to streamline the workflow with the appropriate quality system to deliver mRNA therapeutically.

THE STANDARD WORKFLOW OF AN IVT REACTION

Plasmid Vector Design Serving as a Linear Template

Using a consciously designed plasmid backbone that has optimized untranslated regions (UTRs) and poly(A) tail with a well-placed restriction site for linearisation is critical in the first step of mRNA manufacturing as they may affect the downstream processes. The 5' and 3' UTRs regulate the transcription of the gene of interest, affect translation efficiency, and provide molecule stability. This is where experience in the design process provides the opportunity for optimisation. For example, customers who decide to encode a poly(A) tail within their linear DNA template design also must contend with the limitations of *E. coli* in propagating structural DNA elements like the poly(A) tail. Once the template is manufactured, linearisation with a blunt end restriction site is recommended as a best practice (Step 1 in Figure 1). Before proceeding into the IVT reaction (Step 2 in Figure 1), the digestion should be fully optimised to a 100% rate. Failure to achieve complete digestion prior to IVT can produce impurities with subsequent steps of the reactions. These considerations in plasmid vector design should be accounted for when choosing a manufacturing partner to get the optimal mRNA synthesis.

Cap it – By Either Enzymatic or Co-transcriptional Methods

Both enzymatic and co-transcriptional capping is accepted by industry standards for the manufacturing of mRNA. With co-transcriptional capping, the big advantage is that it is a one-pot reaction. However, enzymatic capping has been observed to be more efficient than co-transcriptional since it is the natural capping method using wild-type enzymes (Step 3 in Figure 1).

When establishing manufacturing standards, capping efficiency can be determined by state-of-the-art analytical methods. The challenge is that conditions must be optimised for every RNA construct. Best practices include using liquid chromatography followed by mass spectrometry to evaluate the final product. Therefore, when selecting your manufacturing partners, ask what methods will be utilised in determining capping efficiency. It should be clear how the capping efficiency is being measured, and the process should be tailored to the specific individual construct(s).

Finish with Tailing

The poly(A) tailing of RNA can be done in two ways. One is encoding the sequence into the plasmid. The other way of doing it is enzymatically after the RNA transcription (Step 4 in Figure 1). When the poly(A) tail is encoded within the DNA template, the risk is that bacteria do not see long poly(A) tails as natural, often leading to the tail's truncation. Typically, anything that has more than 95 to 100 bases is very difficult to retain in an *E. coli* strain when propagating a plasmid. Therefore, the ideal length is somewhere around 90. Also, selecting a dedicated bacterial stain engineered to handle constructs with these elements provide high yield, and fidelity should be considered. Therefore, working with a CDMO that understands the importance of the cell strains and certain conditions that can retain long poly(A)s is necessary.

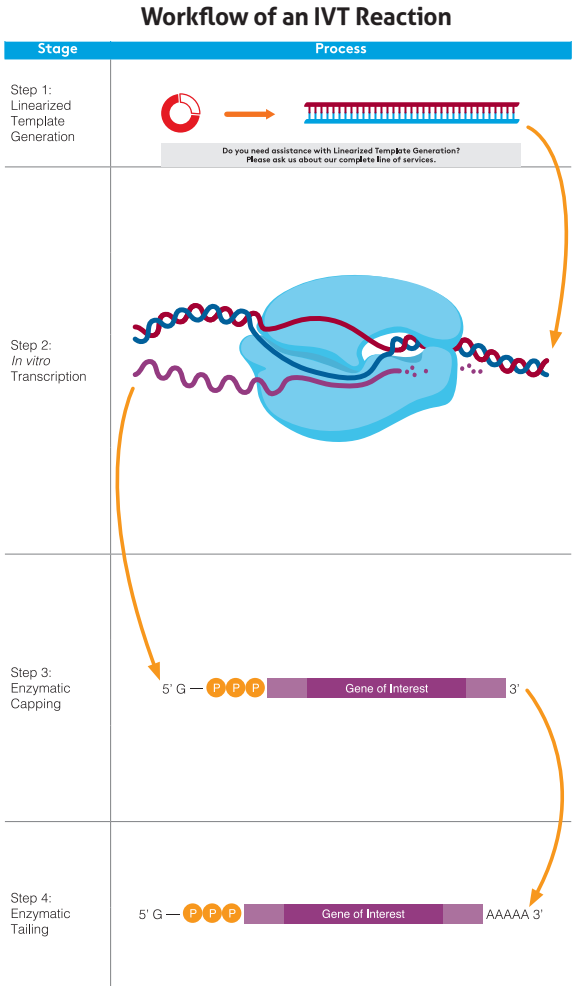

Figure 1. The mRNA synthesis process starts with the linearisation of the plasmid DNA template in the first step. Then this linearised template can be used in step 2 for the in vitro transcription reaction that generates the mRNA molecule. In the final steps, the mRNA is capped in Step 3 and tailed in Step 4 by enzymatic reactions to finalize the drug substance.

CONSIDERATIONS FOR MRNA SAFETY AND QUALITY

Getting Rid of the Impurities

Initially, part of the impurities originates from *E. coli*, including specific genomic DNA, RNases, and endotoxins as a by-product of plasmid manufacturing. Therefore, it is highly recommended to perform quality control (QC) and assurance to ensure the aforementioned impurities are not present before going into the IVT process used to make RNA. After template linearisation, IVT, and capping, the final mRNA product still contains impurities that include the linearised DNA, so it is necessary to detect and purify them at this step. On the other hand, no impurities are generated in the IVT reaction, including no endotoxin, given that it is a cell-free process. So, it is very important to make sure that these are being measured at the appropriate stage and that there is no carryover to the next step.

Once IVT enzymes generate the mRNA, it must be isolated and purified from the reaction mixture using multiple purification steps to achieve clinical purity standards. Typically, double-stranded RNA (dsRNA) is a major impurity generated because the polymerase can run over, forming additional sequences and folding back on itself. The best way to remove double-stranded RNA is by reverse-phase chromatography. However, a challenge with reverse-phase chromatography is that it uses flammable solvents like acetonitrile. Not all RNA manufacturers have fully equipped facilities with the safety and capacity to handle the solvents in this process. Therefore, it is highly recommended to have a clear understanding of a manufacturer's full suite of capabilities to prevent surprises later that risk delaying or eliminating your program for production.

Highly experienced manufacturers are positioned well to deal with impurities and remove them through various forms of purification. These steps are critical, especially for mRNA therapeutics and vaccine programs, because these impurities can trigger an undesired innate immune response within patients. Therefore, when considering mRNA manufacturing, it is always worthwhile to find a CDMO that offers a complete production workflow, analytical testing, and a quality release system all under one roof.

Optimise the Analytics Methods

The basic analytics must measure both the process impurities of the drug substance and the impurities within the final drug product for appropriate quality release. As mentioned earlier, for the process impurities, testing is necessary for the stages of RNA synthesis, from any residual DNA to any of the small molecules, such as nucleotides and the other reaction components. It is extremely critical to have a strong analytical panel for testing the drug substance through downstream purification, as this will be the material moving forward to produce the final drug product.

For drug substance, it is critical to evaluate the performance of the mRNA. Therefore, part of industry-standard analytical assays in the release panel includes identifying the sequence of the RNA, RNA integrity, potency, capping efficiencies, and poly(A) tail length. This can be challenging for most because it needs to be developed for every construct. The complexity also lies in the development of the proper analytical assay based on the mRNA molecule to evaluate the potency and integrity of the RNA. Therefore, relying on a manufacturing partner that can accommodate the construct specificity for analytics is extremely beneficial.

Another very important consideration is selecting a capping method with the highest efficiency. Insufficient capping can not only introduce uncapped RNA, as mentioned above, but it can also generate dsRNA impurities within the product. Both the dsRNA and uncapped RNA can trigger unintended immunogenicity. Therefore, it is extremely necessary to identify the purity of the mRNA molecule developed for your therapeutic application.

In the case of the drug product, the analytical panel and release depend mostly on the formulations and the technology of the delivery system. The essential analytics to confirm the RNA sequence, purity, and potency of the RNA are similar between drug substance and drug product. Still, in the case of final product release, it is also important to look at the concentration and encapsulation efficiency. Furthermore, the industry is moving away from random mixing and using state-of-the-art technology in microfluidics to help control drug formulation and mitigate these impurities.

Analytics can be put in two buckets. The first is analytical innovation/method development, and the second is method validation. Analytical innovation requires a highly skilled technical expert to provide the appropriate data. It is very rare to have this expertise in the industry. For method validation, working with an experienced partner that understands the assay readout is critical to manage the risk and move your mRNA program toward success. In the table below, the specific considerations and the potential negative outcome should be evaluated for each of the production steps to prevent any delays or failures in mRNA drug product manufacturing. Therefore, discussing the specification in detail with a manufacturing partner and determining their in-house capabilities based on your specific needs and final application is critical.

Analytics		
Steps	**Considerations**	**Risks**
Template Optimisation	• Design of the UTR • poly(A) tail stabilisation	• Inefficient IVT reaction • Diminished mRNA expression • Immunogenicity • Loss of poly(A) tail in a bacterial production cell line
Polymerases	• Endotoxin • Residual plasmid DNA interference	• Lower mRNA synthesis • Impurities of the final product
Capping or poly(A) tailing	• Optimal synthesis methods • Method development of liquid chromatography and mass spectrometry	• Immunogenicity by uncapped mRNA • Timeline delays
Delivery Methods	• LNP Formulation • Method assay of encapsulation efficiency	• Timeline delays • Ineffective mixing • Impurities of product • mRNA efficacy loss • Inefficient Drug Product delivery

THE NEW FRONTIER FOR DELIVERY AND THERAPEUTICS

mRNA Delivery

In the last few years, new therapeutic developers have utilised a range of synthetic materials, including lipid nanoparticles

(LNPs) and polymers, for clinical application to facilitate the delivery of mRNA to target cells. Traditionally, viral vectors such as an adeno-associated virus (AAV) had been used but were often overkill, as it delivers the cargo to the nucleus. In the case of mRNA, the target is delivered to the cytoplasm to utilise the cellular translational framework to produce the target antigen. In the case of LNP and polymers, the delivery mechanism meets the demand to deliver the mRNA to the cytoplasm efficiently.

There is increased interest in using LNP for mRNA delivery, mainly due to the multiple formulations currently available for targeting specificity. The recent years of vaccine development has leveraged the field to the next set of discoveries utilising mRNA-LNP as a drug product for therapeutics. Leading the charge in this new era of drug development has been a collaboration between academics and the biotech industry to test different formulations of LNPs for optimal RNA delivery to specific target sites.

mRNA Therapeutics

mRNA-LNP has opened a new perspective for different vaccine and therapeutic applications. The impact of mRNA COVID vaccines is not behind us, as it paves the way for other mRNA-based vaccines targeting pathogens like influenza and respiratory syncytial virus (RSV). Furthermore, the field has been pushing the possibility of a multivalent vaccine immunising against COVID, influenza, and/or RSV. That is an exciting prospect and serves to highlight the potential of mRNA technologies to impact global healthcare.

An alternative approach to LNP targeting is to modulate and control tissue-specific expression, thereby enabling expression only in appropriate cells or tissues. Therefore, the industry has begun exploring frontiers for mRNA cancer vaccines and therapies that would likely be part of a patient's overall treatment plan and be combined with traditional chemotherapeutics. Furthermore, rare diseases are a consideration and can be a possibility in the near future.

The possibilities are endless based on the way RNA is designed, despite the basic elements remaining the same. How it is optimised for therapeutic programs will always be different, given the need for analytical tests required for each of the individual constructs. This is only the beginning, mRNA is well known and has had a jump start in different therapies. However, research has expanded the sequence design to include self-amplifying RNA (saRNA) and circular RNA (circRNA). As told in their names, saRNA can replicate using molecular machinery and is not as dependent on the cell, whereas circRNA is a closed-loop structure that is more stable, but each of these three RNA sequences has its advantages and disadvantages. The only limitation is the time for the field to determine how to maximise each RNA sequence and for which of the many diseases and disorders the industry has just begun to address.

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Venkata Indurthi, Ph.D., has been a member of the Aldevron team since he received his doctorate in pharmaceutical sciences from North Dakota State University, Fargo, ND, in 2016. He has held a variety of positions in increasing responsibility and focus, including Senior Scientist in product and process design, Director of RNA Operations, Director and then Vice President of Research and Development before being named Chief Scientific Officer in 2022. Indurthi received his Bachelor of Science in Biotechnology from SRM University, Chennai, India, in 2010. He has been recognized with several awards and honors from a variety of organizations, served on or lead many panels, and has authored or participated in numerous published articles.