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eBook

Manufacturing mRNA for research and beyond

Explore the mRNA universe with Cytiva

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Contents

When it comes to mRNA, many manufacturing investments are being made while still early in the process development workflow. So essentially, we are translating methods from the milliliter scale to the liter scale. A lot of the decisions made on mRNA process will have a great impact on equipment setup, batch cost, and throughput.

Katarina Stenklo
Leader, Enterprise Solutions, Cytiva

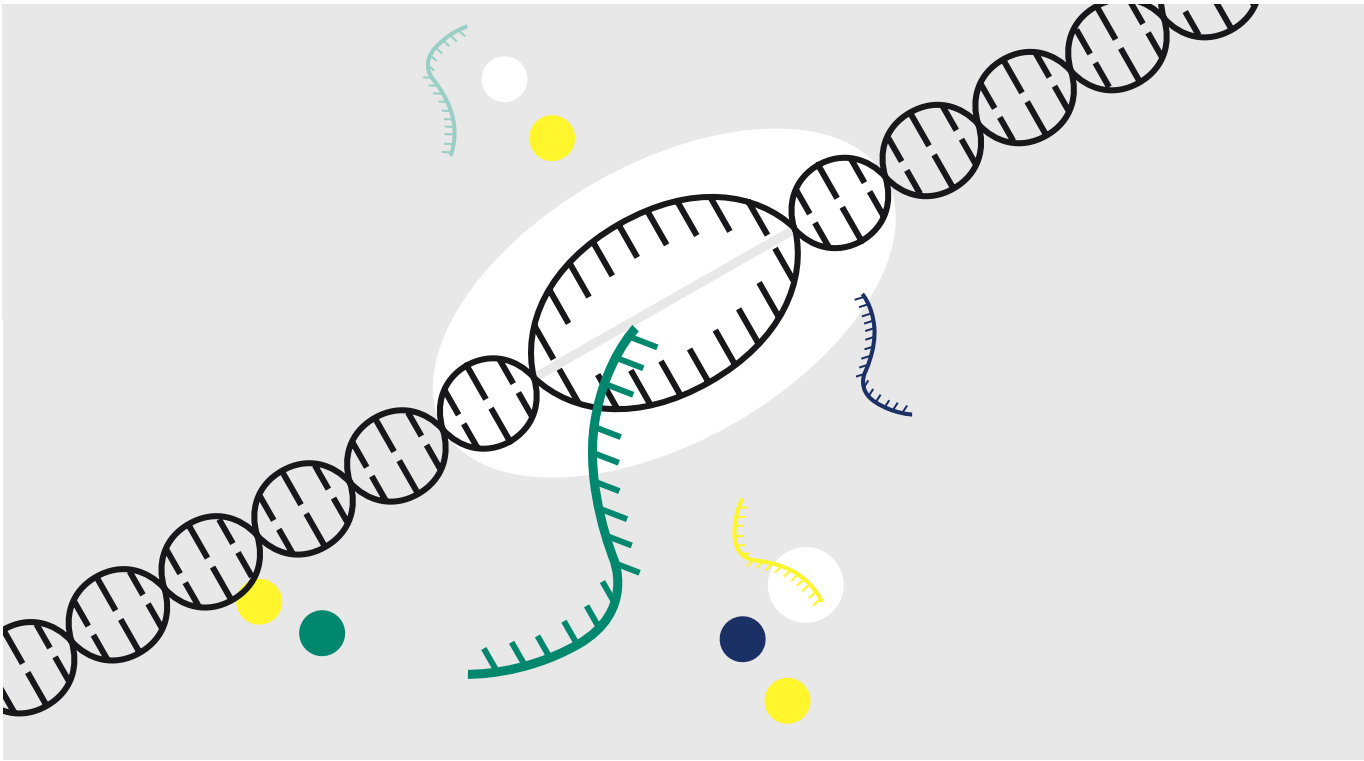
Through peer-to peer interactions, we continue to encourage the spirit of collaboration to accelerate next-generation therapeutics. In this series, our scientists share their insights into mRNA synthesis for research purposes and advice on how to scale.

Introduction to mRNA manufacturing

Over the past two years, the emergence and worldwide spread of the SARS-CoV-2 virus, the cause of coronavirus disease 2019 (COVID-19), has changed the course of scientific research around messenger RNA (mRNA). In fact, the development of mRNA vaccines is undoubtably one of the most significant scientific breakthroughs that has contributed to bringing the world out of the pandemic.

On its own, "naked" mRNA degrades rapidly after injection, a fact that has led scientists to pursue the successful and currently widely used approach of encapsulating mRNA into lipid nanoparticles (LNPs). LNPs act to protect and deliver mRNA to target cells and support cell uptake by endocytosis. Once in the cytoplasm, mRNA is released from the LNP and is translated into a protein. This has led to intensified research within the mRNA–LNP field, and mRNA-based therapeutics are now viewed as key solutions for tackling some of the most difficult and pressing clinical challenges such as cancer, genetic disorders, and infectious diseases.

In this eBook, our scientists share their insights into mRNA synthesis for research purposes. And they share their advice on how to scale. Through peer-to-peer interactions, we continue to encourage the spirit of collaboration developed at a time when new modalities are being used as therapies for the first time.



Part 1: Solutions for mRNA manufacturing

mRNA–LNP therapeutics have tremendous potential to improve or even outclass current therapies with their ability to be adapted to seasonal variants. Setting up an mRNA research laboratory seems deceivingly simple. Compared to developing a conventional vaccine, which can take a decade or more, it took a relatively short period of time to go from sequencing the SARS-CoV-2 genetic code to distributing a safe and effective injectable vaccine. However, there is more to it than meets the eye, with many hurdles and challenges presented along the way to developing mRNA–LNP therapeutics.

This eBook aims to guide you in the practical aspects of setting up an mRNA research laboratory, addressing methods, equipment, reagents, and workflow. Even if your current aim is not process development with the goal of scaling up, it is nevertheless beneficial to consider the feasibility and ease of scaling up. This will in turn save you precious time in the future. Please note that not all methods described here are scalable.

Structural features of mRNA

mRNA is about 10 times larger than the actual protein that it encodes for, and it can range from 500 to 6000 bases of nucleotide subunits. Transcription is an intranuclear (inside the nucleus) process in which DNA serves as a template from which mRNA is transcribed by an enzyme called RNA polymerase. This enzyme binds to the promoter region of DNA and initiates transcription by forming a complex. Nucleoside triphosphates (NTPs) that are complementary to the DNA are incorporated in the 5′-to-3′ direction through the formation of phosphodiester bonds.

In almost all eukaryotes, modifications are made at both ends of the nascent mRNA, a 5′ cap and a 3′ polyadenylated (polyA) tail. The 5′ cap is multifunctional and is involved in mRNA maturation, nucleocytoplasmic transport, and protection from 5′ exonuclease digestion. The polyA tail, a chain of adenine nucleotides at the 3′ end, increases stability of the molecule and appears to play a role in mRNA quality control.

In addition to these modifications, a mature mRNA also consists of a 5′ untranslated region (UTR), a coding sequence, and a 3′ UTR (Fig 1). UTRs are known to play a role in the post-transcriptional regulation of gene expression and are a part of the "gene" or DNA template design in the *in vitro* transcription (IVT) process. During mRNA manufacturing, capping of the transcribed mRNA can be done in different ways, for example in the IVT chamber directly (co-capping) or as an additional enzymatic step downstream of IVT. The polyA tail is usually included in the DNA template.

Translation happens outside the nucleus, where the mRNA serves as a template to construct a protein from amino acids. The 5′ cap tags the mRNA as self-RNA to the innate immune system and initiates ribosomal binding. Together with the 5′ cap, the polyA tail promotes translation to protein. UTRs modulate translation efficiency, and the coding sequence contains the gene of interest. The polyA tail promotes mRNA stability (3′ exonuclease protection is one such mechanism) and participates in terminating transcription.

This eBook describes several analytical assays, including polymerase chain reaction (PCR), chromatography, and filtration methods. For a more comprehensive list, please refer to a table compiled by Schoenmaker *et al* (1).

Even a single change (e.g., strand break) in the mRNA strand can halt translation, making it vital to monitor the integrity of the molecule during the process.

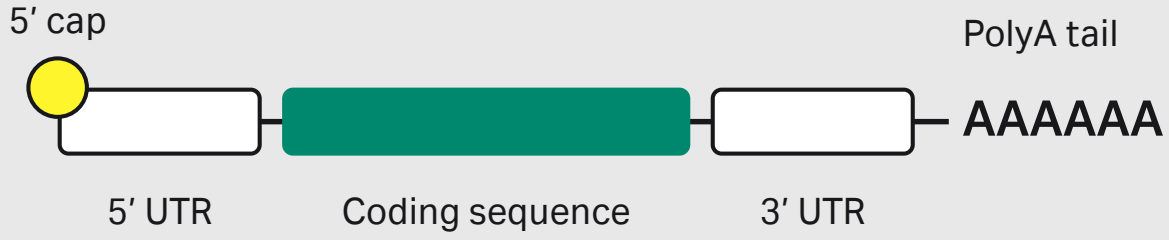


Fig 1. The structure of an mRNA molecule.

RNase-free considerations

Ribonucleases (RNases) are ubiquitous and present the biggest risk when working with RNA due to their ability to rapidly degrade RNA. Hence, it is critical to emphasize the importance of maintaining an environment free from RNases. Besides being potentially present in reagents, tips, tubes, bottles, and surfaces, RNases are also found in bodily fluids (e.g., skin oil and perspiration). You can minimize RNase contamination by:

- Using exclusively RNase-free reagents, solutions, and equipment.
- Wearing protection such as a face mask, gloves, and lab coat.
- Changing gloves often.
- Decontaminating surfaces and pipettes diligently.
- Dedicating equipment and material and labeling them “RNase-free” or “for RNA work only”.
- Using certified RNase-free consumables such as pipette tips with barriers and tubes.
- Creating a separate mRNA lab or an area with equipment and material specifically dedicated for RNA work.

RNases are extremely resistant to most chemical and biological conditions. For instance, RNase A (Thermo Fisher Scientific) tolerates both boiling and autoclaving. So far, there is no RNase elimination method that is compliant with biopharmaceutical production. That means prevention and monitoring are the best ways to control RNase contamination.

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In addition, it has been suggested that autoclaving or baking glassware for at least two hours at 240°C can destroy RNase activity. Unfortunately, these enzymes are very robust and will still retain partial RNase activity. Although diethyl pyrocarbonate (DEPC) has been used to inactivate RNases, leftover traces or byproducts may inhibit subsequent enzymatic reactions. Furthermore, DEPC is incompatible with Tris-based buffers such as IVT buffer, which contains Tris-HCl.

RNases cannot be easily inactivated by ethanol, ethylenediaminetetraacetic acid (EDTA), or other metal chelators. Highly concentrated sodium hydroxide (NaOH) is effective against RNases, but soaking is time-consuming.

For solutions (e.g., buffers) prepared in house, it is highly recommended to test with an RNase activity test kit such as an RNaseAlert kit sold by **Integrated DNA Technologies (IDT)**. The result can be read visually for qualitative assessment of contamination or quantified using fluorometry. IDT also offers an anti-RNase reagent, Nuclease Decontamination Solution, that can easily be applied on and around your workspace to irreversibly inactivate RNases and reduce contamination risk. Moreover, it can be applied to plastic surfaces, which are difficult to sterilize.

Part 2: Creating the DNA template

The creation of an encapsulated mRNA–LNP begins with synthesizing the DNA template. If plasmid DNA (pDNA) is used as the template, additional steps are necessary, such as purification after host cell lysis and linearization of the circular pDNA. The DNA template then undergoes purification prior to IVT, during which mRNA is formed.

Capping of the 5′ end of the mRNA takes place either during or after IVT, and the addition of the polyA tail is usually included in the DNA template. Impurities from the IVT reaction are removed and the purified mRNA can be sterile-filtered before being encapsulated into a lipid-based nanoparticle. Finally, the mRNA–LNP is polished and sterile-filtered (Fig 2).

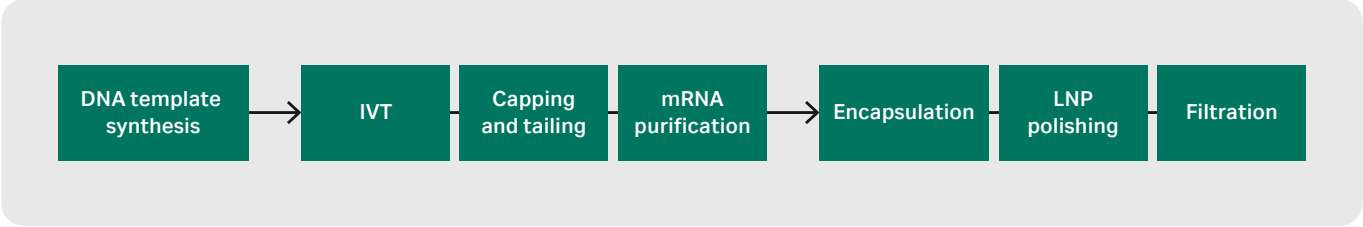


Fig 2. The process of creating an encapsulated mRNA–LNP.

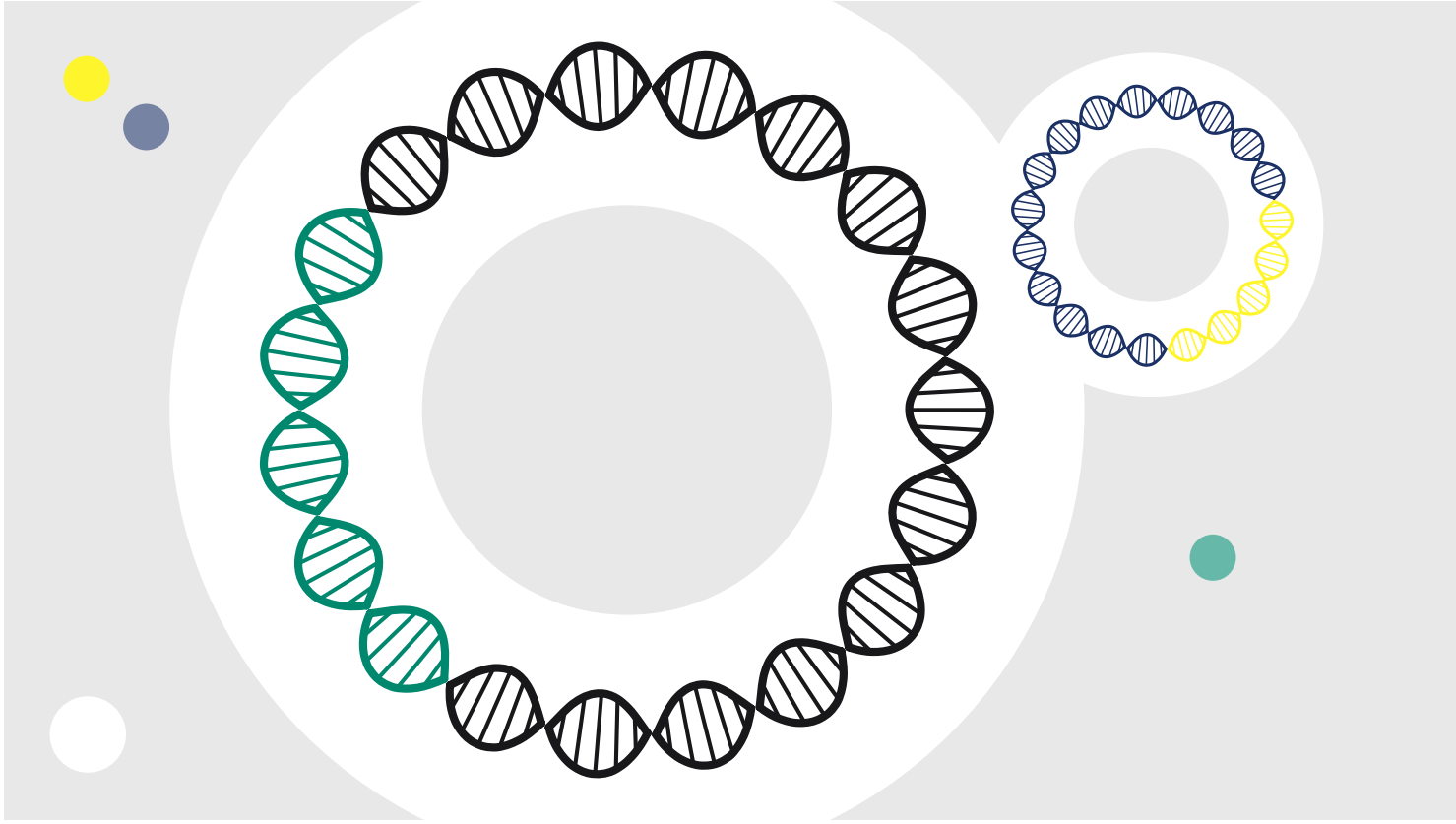
DNA template synthesis

DNA template containing the sequence to be transcribed with an upstream RNA polymerase promoter site serves as starting material for IVT. The template can be derived in several ways, such as from pDNA or by a PCR product. The choice depends on the conditions of the plasmid and intended application, and there are benefits to each option. The pDNA route generates many copies of the same template by hijacking the bacteria's machinery. If the DNA template is derived from plasmids, the pDNA should contain the gene of interest, the RNA polymerase promoter site and untranslated regions (UTRs), a unique restriction enzyme cut site for linearization downstream of the 3′ UTR or polyA tail, origin of replication, polyA tail, and a gene for antibiotic resistance for use as a selectable marker. If elements such as a polyA tail and/or RNA polymerase promoter sequence are missing in the plasmid, a PCR product may be preferred since it is possible to design such sequences in the primers.

The endotoxin-free PCR method offers greater flexibility, creating different templates in a shorter amount of time since there are no incubation and linearization requirements. On the other hand, it is a highly viable solution if only small amounts are needed, since it is more expensive to perform at scale.

pDNA is first transformed into competent cells such as *Escherichia coli* (*E. coli*) bacteria via thermal, chemical, or electrical means. These cells are later cultured in a suitable media, for example, Luria broth (LB), plated onto an LB agar plate containing an appropriate antibiotic, and incubated overnight. The bacterial cells that survive due to the incorporation of pDNA that contain an antibiotic resistance gene form colonies that are subsequently selected to be amplified/expanded overnight in shake flasks with media. The next day the cells are lysed under alkaline conditions to extract pDNA, which is then purified to remove bacterial host cell contaminants. Both cell lysis and pDNA purification are ideally performed using a plasmid purification kit.

Finally, purified pDNA, which is circular, must be linearized by restriction enzymes that cut downstream of the desired 3′ end. Blunt or protruding 5′ ends are preferred for proper transcription by RNA polymerases. To obtain the desired length, pDNA must be completely digested. If complete digestion is unachievable, agarose gel electrophoresis followed by purification via a gel purification kit is strongly recommended to obtain homogenous DNA templates, though some pDNA loss is expected. As an alternative, a different restriction enzyme may be used. Note that these kits are meant for research use and are not scalable. Therefore, it is useful to plan and consider chromatography for larger laboratory-scale purification and concentration analysis.



Generating a DNA template using PCR

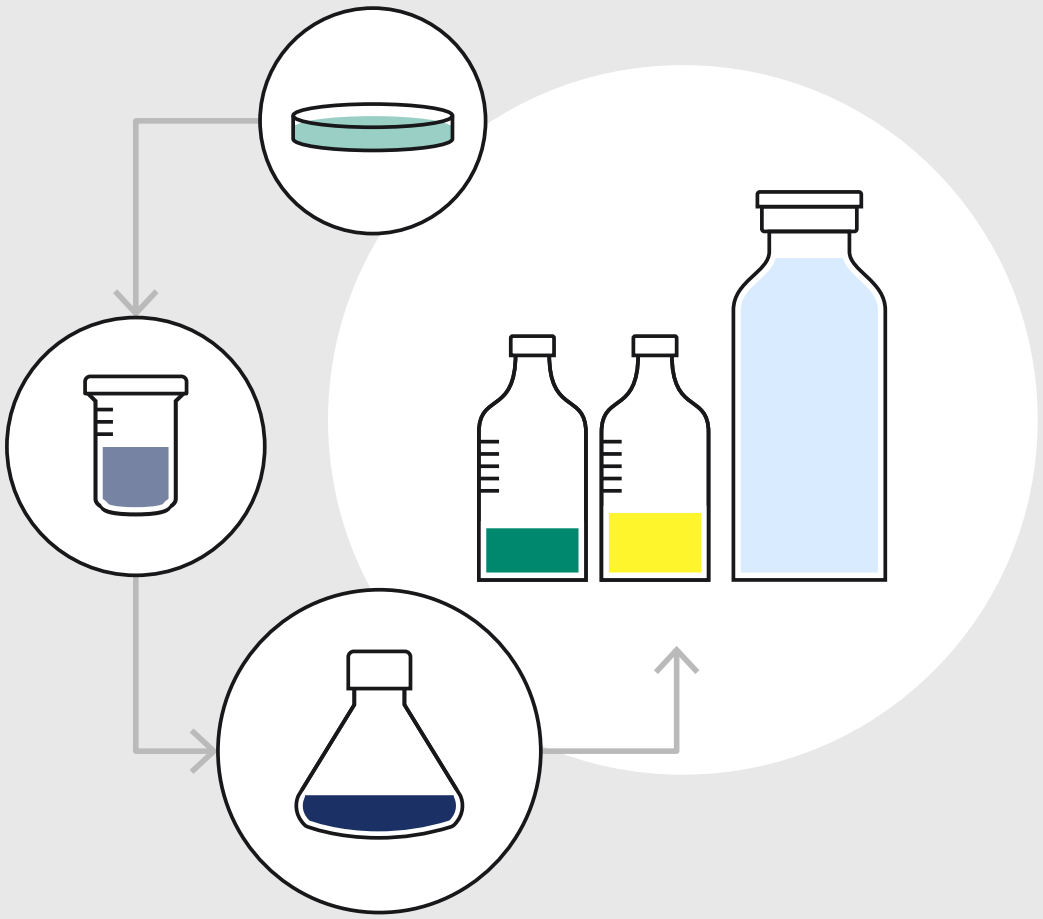
PCR amplifies the DNA sequence of interest by using a reaction mixture consisting of the DNA sequence, primers, DNA polymerase, deoxyribonucleotides (dNTPs), and PCR buffer, and then using thermal cycling to help the replication process. After the reaction, the PCR product is readily purified using a PCR purification kit. PCR allows the conversion of any DNA fragment to a transcription template by affixing an RNA polymerase promoter (e.g., T7) to the forward primer.

PolyA-tailed reverse primers can be used during PCR to generate transcription templates with polyA tails, obviating the need for an additional polyadenylation step after transcription. These reverse primers are particularly useful for screening different polyA tail lengths, especially for short mRNAs. For long mRNAs, post-transcriptional polyadenylation is a better option since the reverse primers can be very long and may be more costly. Although phenol-chloroform extraction or ethanol precipitation are purification methods traditionally used in the laboratory, incomplete removal of organic solvents will cripple subsequent enzymatic steps. For example, these solvents can denature enzymes, leading to failed IVT reactions or low mRNA yield. Scalability is also an issue.

To determine DNA quantity and purity, a spectrophotometer is commonly used to measure UV absorption (A_{260}/A_{280}). Agarose gel electrophoresis, sequencing using primers, or a bioanalyzer can be used to check the integrity of the DNA template.

Challenges

- DNA template construction is a labor-intensive and time-consuming process.
- Linearization (pDNA only) and purification of the DNA template can result in variable purity and quality.
- DNA template quality affects transcription yield and mRNA integrity. The higher the purity, the greater the transcription yield.
- Handling of organic solvents (phenol and chloroform) and their presence (if not fully removed) denature linearization restriction enzymes and IVT enzymes.
- It is difficult to maintain a nuclease-free environment and acquire high quality nuclease-free reagents (enzymes, nucleotides, and primers) for PCR and sequencing.
- There is a need for simplified, lab-friendly preparative chromatography steps and resins for larger-scale purification.



Strategies and product offerings

- **Aldevron** offers high quality standard and linearized pDNA, ranging from microgram to multi-gram scales, complete with Certificate of Analysis.

- illustra™ plasmidPrep kits from **Cytiva** simplify pDNA extraction and purification.
  - The Mini Spin Kit can yield 6–15 µg of purified pDNA from 1.5–3 mL *E. coli* cultures for molecular cloning (A_{260}/A_{280} > 1.8) in less than 10 min without the use of organic solvents.
  - For larger culture volumes and transfection quality (A_{260}/A_{280} = 1.8–2.0) pDNA, the Midi Flow Kit can yield 20–250 µg from 25–150 mL culture medium.

- The **Cytiva** illustra™ GFX PCR DNA and Gel Band Purification Kit is ideal for DNA volumes from PCR (up to 100 µL) or from gel slices (up to 900 mg), purifying DNA ranging from 50 bp up to 10 kbp in size.

- If handling large numbers of samples in solution and DNA 100 bp–10 kbp in length, the illustra™ GFX 96 PCR Purification Kit from **Cytiva** allows purification of up to 96 PCR products simultaneously in 15 min.

- **Cytiva** and **Pall Corporation** offer solutions for purification for gene therapy and vaccine applications.
  - Anion exchange chromatography: Mustang® Q membrane absorbers from **Pall Corporation** are used in this pDNA capture step with process intensification and overall recovery in mind. These membrane absorbers are multilayered, compact, and possess high dynamic capacity. Within the Mustang® Q family, the Mustang® Q XT Acrodisc® chromatography units are fit for laboratory scale. The membrane volume of < 1 mL reduces the amount of sample required for evaluation, and the female Luer lock inlet and outlet simplifies connection to typical low-pressure chromatography systems.
  - Hydrophobic interaction chromatography: Capto™ PlasmidSelect resins from **Cytiva** are involved in this isoform separation step. These resins allow high-flow separation of supercoiled, covalently-closed, circular forms of pDNA from open circular forms and are available prepacked in HiTrap™ and HiScreen™ columns from **Cytiva**. In addition, these resins can be used to determine pDNA concentration and analyze the ratio of supercoiled to open circular pDNA.

- **Cytiva** offers high quality nuclease-free PCR and sequencing reagents.
  - Taq and Thermo Sequenase™ DNA polymerases.
  - dNTPs and dideoxyribonucleotides (ddNTPs).

- In addition to RNaseAlert kit and Nuclease Decontamination Solution, **IDT** also provides nuclease-free water and custom primers.

Part 3: mRNA synthesis with IVT

In vitro transcription (IVT) is the synthesis of RNA molecules, allowing a researcher to tailor synthesis and introduce modifications to produce a transcript. It's worthwhile to spend some time optimizing mRNA template design, IVT, and other process parameters to simplify downstream processing and ensure the appropriate balance of yield and secondary structure formation.

During IVT, DNA template, RNA polymerase, nucleoside triphosphates (NTPs), RNase inhibitor, pyrophosphatase, and IVT buffer are combined to produce mRNA. RNase inhibitor prevents degradation of newly formed mRNA. As NTPs are incorporated into the nascent mRNA, pyrophosphates bind to free magnesium ions and are released, inhibiting RNA polymerization. Pyrophosphatases must be added to catalyze the hydrolysis of pyrophosphates into inactive single orthophosphate ions that don't bind magnesium ions, which increases the yield of mRNA. T7, T3, or SP6 prokaryotic phage RNA polymerases are widely used.

Depending on the purification step downstream, it may or may not be necessary to remove the DNA template by adding DNase I after IVT. This step is required, for example, when using a purification method that doesn't separate mRNA from DNA. It's also necessary when there's a concern that DNA fragments may hybridize to mRNA.

In vitro transcription, or IVT, is an enzymatic reaction. In our lab, we've found that IVT is very sensitive to the quality and concentrations of the ingoing components, such as the plasmid template.

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There are two methods to add a 5′ cap (Fig 3) *in vitro*: either via a multi-step enzymatic reaction or via co-transcription. The aim is to produce a methylated first nucleotide at the ribose 2′-O position (Cap 1), which has superior *in vivo* properties compared to a 7-methylguanylate cap structure (Cap 0) at the 5′ end. This is true because the Cap 0 structure can still be recognized as non-self, inducing interferon response and hence a more rapid degradation.

In co-transcriptional capping, a cap analog is added directly into the same IVT reaction mixture along with NTPs. This analog is incorporated as the first nucleotide of the transcript, resulting in Cap1 mRNAs. A large proportion of mRNAs are initially capped. But the final reaction contains a mix of capped and uncapped variants because guanosine triphosphates (GTPs) are depleted.

In contrast, enzymatic capping is performed separately after IVT using guanylyltransferase, followed by 2′-O-methyltransferase. The vaccinia virus capping enzyme executes the three steps needed for the addition of Cap 0 to the 5′ end. These steps are mediated by enzymes RNA triphosphatase, guanylyltransferase, and guanine methyltransferase.

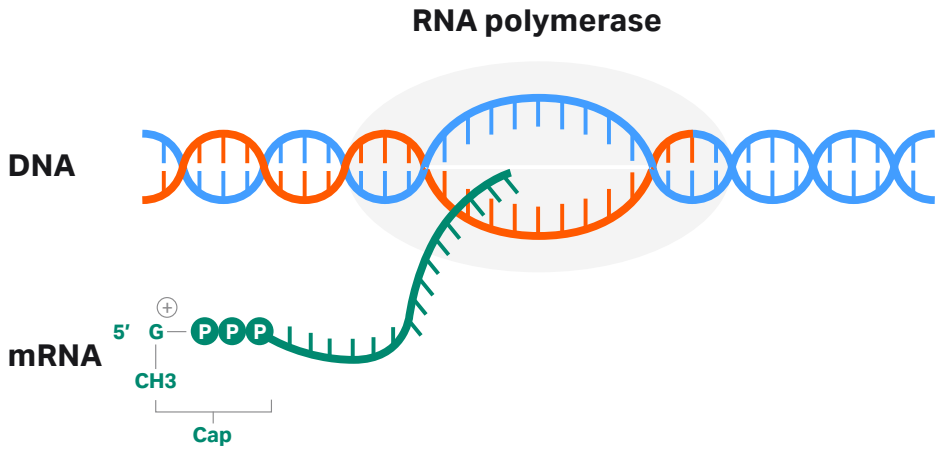

Fig 3. The process of adding a 5′ cap to an mRNA molecule.

The steps lead to the conversion of the 5′ triphosphate of nascent RNA to the cap 0 structure. First, a phosphate is removed from the 5′ triphosphate to generate 5′ diphosphate mRNA. Then, a guanosine monophosphate (GMP) group is transferred from GTP to the 5′ diphosphate. Finally, a methyl group is added to the N7 amine of the guanine cap to form the cap 0 structure. Cap 0 mRNAs are subsequently converted to Cap 1 mRNAs by 2′-O-methyltransferase by methylating the 2′-OH of the first nucleotide adjacent to the cap structure. The methyl groups are donated by S-adenosylmethionine.

Although the co-transcriptional method appears to be simpler with fewer steps and enzymes, the yields of capped mRNA are usually lower than the more cost-efficient, reliable, and effective enzymatic method. Another drawback of the co-transcriptional method is that a manufacturing license may be required. On the other hand, enzymatic capping can require more time because mRNA needs to be purified before and after capping in some cases, which may also cause some loss of mRNA.

If the polyA tail is not designed in the pDNA or PCR template, a polyA polymerase can be used to catalyze the addition of adenosine monophosphate (AMP) from adenosine triphosphate (ATP) to the 3′ ends. The length of the polyA tail can range from 40 to 250 residues long. The optimal polyA tail length may vary by application. This method may generate a heterogeneous population of polyA-tailed mRNAs.

At this point, unpurified (crude) mRNA can be analyzed in several ways. Agarose gel electrophoresis can check for any potential degradation of mRNA, while concentration can be determined by fluorescence-based, RNA-specific assays.

We've found that there isn't a universal generic protocol that works for every IVT reaction—each new mRNA sequence requires optimization. The same is true for reagents from different vendors. Optimizing the reaction so frequently may seem tedious, but we've found it's worth doing because it saves you time later.

Susanna Lindman, PhD
Principal R&D Scientist, Cytiva

Challenges

- Need for high quality of enzymes, reagents, and filters.
- Must choose the capping strategy, bearing in mind costs, time, and reliability.

Strategies and product offerings

- Use high quality IVT enzymes from **Aldevron**, which can be custom made to meet your specific requirements from mg-to-g scale.
  - Transcription: T7 RNA polymerase, pyrophosphatase, RNase inhibitor.
  - Template removal: DNase I.
  - Multi-step capping: guanylyltransferase (3 enzymatic activities) and 2'-O-methyltransferase.
  - Tailing: polyA polymerase.
- Use NTPs from **Cytiva**, which are tested for RNase activity and are extensively purified (≥ 98%). They are available either as individual products or in a convenient set containing ATP, cytidine triphosphate (CTP), GTP, and uridine triphosphate (UTP) in separate tubes.

Part 4: mRNA purification post-IVT

Impurities from IVT—including DNA template, enzymes, NTPs, salts, double-stranded (ds) RNA, and truncated mRNA—can induce undesired immune responses and negatively impact translation efficiency. For these reasons, the mRNA must be purified. There are several options to purify mRNA: lithium chloride (LiCl) precipitation, oligo deoxythymidine (dT) magnetic particles, and chromatography. When designing a purification process, consider scalability and suitability for a manufacturing environment. Read on to discover the pros and cons of each option.

DNA removal by DNase I digestion followed by LiCl precipitation is a common laboratory-scale purification method for mRNA. LiCl precipitation uses elevated concentrations of lithium cations to selectively precipitate mRNA, leaving behind impurities. The precipitated mRNA is pelleted, and the supernatant is discarded. Although mRNA is specifically separated from DNA, DNase I treatment is still recommended for complete DNA removal. EDTA can be used to inactivate DNase I after digestion. A major disadvantage is that undesired aberrant mRNAs such as dsRNA and truncated mRNA are still present.

Separation via oligo(dT) magnetic particles is based on specific hybridization to the polyA tails. The process is simple (Fig 4). When the particles are added to the sample, the polyA tails hybridize to the oligo(dT) ligands on the surface of particles. When an external magnetic field is applied, the beads with the bound mRNA are attracted and immobilized to the outer edge of the tube. The contaminants are removed during the washing steps. Next, the addition of an elution buffer releases mRNAs from the immobilized beads as a purified sample. Finally, the magnetic field is removed, and the beads are released, ready for re-use.

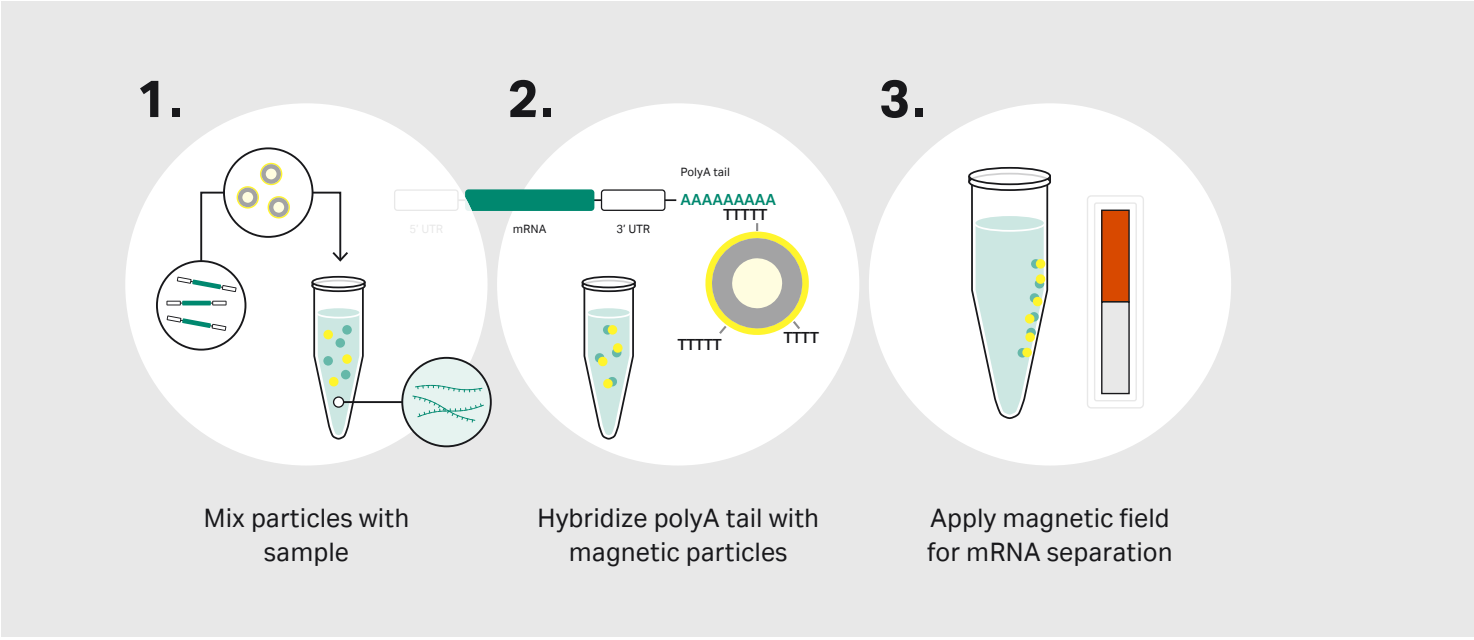

Fig 4. mRNA capture with oligo(dT) magnetic particles.

With this method, there's no need for centrifugation or vacuum, eliminating stress or shear force. In addition, the process is amenable to automation in 24-, 96-, and 384-well plates. Like LiCl precipitation, DNase I treatment is optional but recommended. Untailed mRNAs are removed, but other forms of aberrant mRNAs with polyA tails remain. Neither LiCl precipitation nor oligo(dT) magnetic particle separation is scalable.

Chromatography, a purification process routinely used in the pharmaceutical industry, is gradually replacing traditional precipitation methods. This is true because chromatography offers higher reproducibility, recovery, and purity as well as better scalability. Another advantage is that chromatography can effectively remove DNA template, aberrant RNA, residuals, and impurities. A variety of techniques are available, including oligo(dT) affinity, anion exchange, hydrophobic interaction, and multimodal chromatography.

Oligo(dT) affinity chromatography captures mRNA by binding to the polyA tails via oligo(dT)-tagged beads. DNA, NTPs, enzymes, buffer components, and any other impurities without polyA tails are washed away. The downside is that aberrant RNAs aren't removed, which is why this step is usually followed by another chromatography step. Anion exchange chromatography (AEX) is an attractive option for polishing because it provides good separation of single-stranded mRNA from immunogenic DNA, RNA–DNA hybrids, dsRNA, and other aberrant RNAs.

AEX is based on the reversible interaction between the negatively charged sugar-phosphate backbone of mRNA and the positively charged chromatography resin. An alternative is hydrophobic interaction chromatography, which is based on the reversible reaction between mRNA and the hydrophobic ligand of the resin. Core bead chromatography, a form of multimodal (mixed-mode) chromatography, is another effective technique to remove low molecular weight impurities. It combines the power of size exclusion and binding separation such that small impurities are trapped inside the beads and the large mRNA product flows through.

One of the biggest challenges is maintaining the stability of the mRNA while maximizing yield and purity. Keeping the reaction and all equipment in RNase-free conditions is a must. But don't assume it's RNase if you're having problems—the type of buffer and salt concentration, as well as time and temperature, also affect the stability of the mRNA during purification.

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After purification, the mRNA can be quantified with either fluorescence-based, RNA-specific assays or a spectrophotometer. An absorbance ratio (A_{260}/A_{280}) less than 2.0 may indicate the presence of residual DNA or protein, and the mRNA product may not be suitable for downstream applications unless further purified. High performance liquid chromatography (HPLC), enzyme-linked immunosorbent assay (ELISA), or dot blotting can be used to check for unwanted truncated or dsRNA. It's important to pre-filter mRNA prior to an HPLC run to remove particulates that can damage columns.

The stock concentration for encapsulation is 1 mg/mL. The concentration of mRNA may need to be adjusted after purification by diluting in a suitable aqueous buffer. To reduce the risk of mRNA degradation, work quickly and wear gloves. If there's time between the steps, consider keeping the mRNA cold.

Prior to encapsulation, perform a sterile filtration step to remove potential microbial contaminants.

Challenges

- Choosing the optimal combination of methods to remove impurities, especially aberrant mRNA.
- Choosing the analysis method for aberrant RNA.
- Need for high-quality 0.2 µm filters.

Strategies and product offerings

- Choose mRNA capture, polishing, and analysis products from **Cytiva**:
  - Capture mRNA using Sera-Mag™ Oligo(dT) magnetic particles.
  - Perform microscale purification by converting ÄKTA pure™ 25 chromatography system using a Micro kit.
  - Simplify the workflow with HPLC-certified regenerated cellulose (RC) filters, as they're compatible with many solvents to minimize extractables.
  - Perform dot blotting with Nytran™ SuPerCharge (SPC) nylon membrane and Cy™3/Cy™5 secondary antibodies.
- 0.2 µm pre-sterilized Acrodisc® syringe filters from **Pall Corporation** equipped with Supor® or Fluorodyne® membranes are good for filtering fluids up to 150 mL with a holdup volume as low as 60 µL and are available in 13-, 25- and 32-mm sizes. Fluorodyne® filters incorporate a high-capacity asymmetric polyethersulfone (PES) pre-filter layer over a polyvinylidene fluoride membrane layer, while Supor® filters have a PES membrane with broad pH compatibility. Sterilization by gamma irradiation prevents the contamination risk that can result from ethylene oxide sterilization.

Part 5: LNP formulation to encapsulate mRNA

Microfluidic mixing is one of the most effective means to formulate lipid nanoparticles (LNPs) because of its scalability and reproducibility. An aqueous buffer containing mRNAs and an organic lipid mixture are rapidly mixed in a controlled and non-turbulent manner. This step facilitates self-assembly into stable nanostructures such that mRNAs are encapsulated in the interior core through interactions with ionizable lipids, cholesterol, helper lipids, and stabilizers. This protective structure allows the mRNA to evade degradation by endosomes and increases stability in physiological fluid. It's important to consider how the combination of cationic or ionizable lipids and the lipid composition (i.e., the ratio of helper lipids and surfactants) impact mRNA encapsulation.

After mRNA is encapsulated, the LNPs are diluted with a suitable aqueous buffer to reduce the organic solvent content. Nanoparticle size and polydispersity index (PDI), which gives the size distribution of nanoparticles, can be determined by using a dynamic light scattering bioanalyzer. A high PDI indicates a broad or multimodal size distribution. Hence, the lower the PDI, the better. Encapsulation efficiency can be measured using fluorescence-based, RNA-specific assays.

Current mRNA–LNP vaccines for COVID-19 must be frozen for long-term storage and may be sensitive to shaking. However, this may change in the future.

Every additional step reduces the total process yield and increases the amount of consumables used and therefore, the total process cost.

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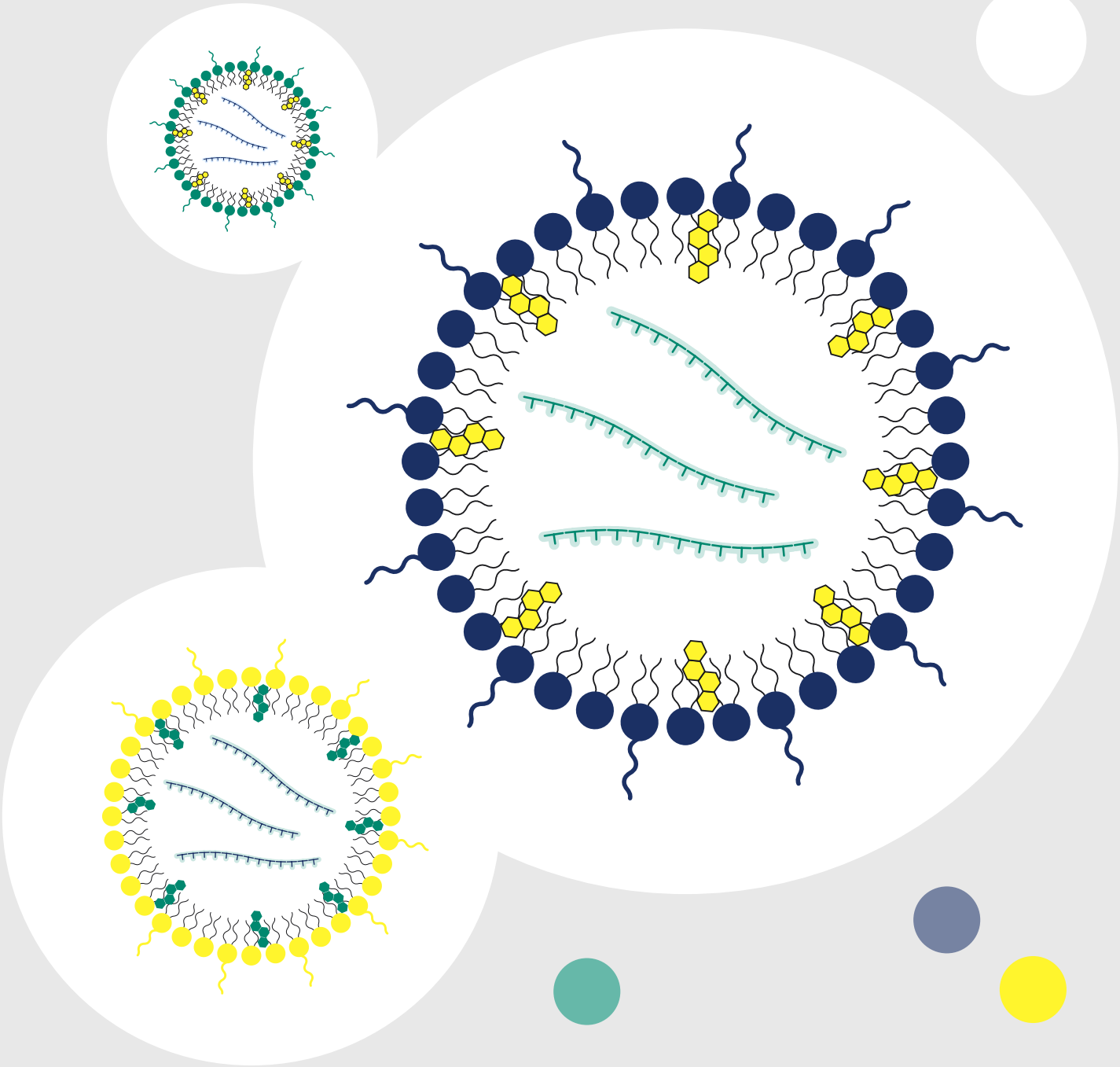


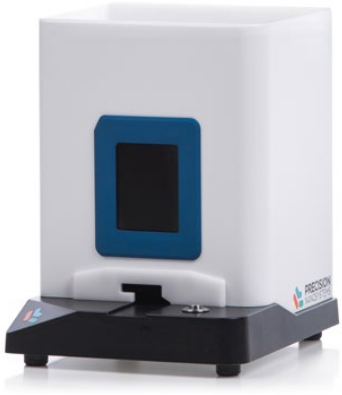
Challenges

- Need for reliable and user-friendly encapsulation systems.
- Need for a ready-to-use lipid mixture that has the right combination of lipids and lipid composition.
- Need for scalable manufacturing processes throughout development, from preclinical to commercial manufacturing.

Strategies and product offerings

Precision NanoSystems offers a range of systems, reagents, lipids, and services based on NxGen™ microfluidic technology, resulting in reproducible and high-quality LNPs.

- NanoAssemblr® Spark™ is the smallest system that formulates between 25–250 µL of LNPs, encapsulating mRNA on the microgram scale (Fig 5). The single-use NxGen™ microfluidic mixer cartridge minimizes cross-contamination, and simple one-button operation reduces user variability. The petite Spark™ system can easily be placed on the benchtop or in a sterile biosafety hood. Since formulation takes barely 10 s per run, LNPs can be made on-demand for immediate use.



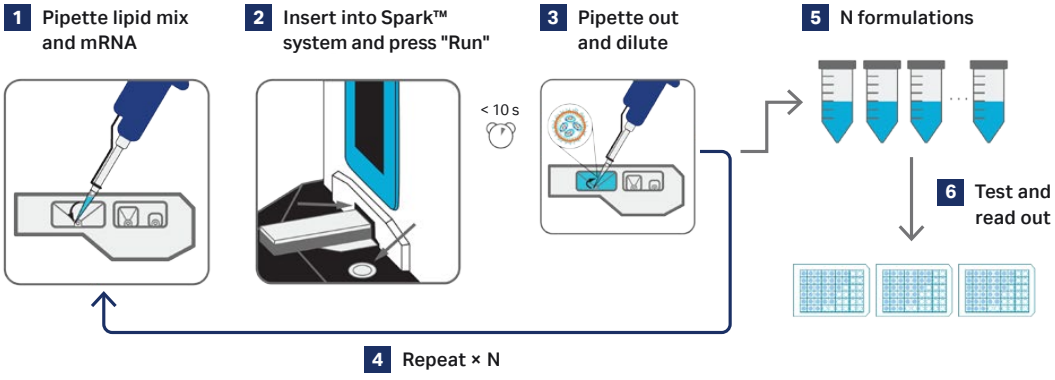


Fig 5. Precision NanoSystems NanoAssemblr® Spark™ system and workflow.

- NanoAssemblr® Ignite™ and Ignite+™ (Fig 6) are the larger siblings of the Spark™ system, capable of formulating 1–60 mL. By adjusting key parameters such as flow rate ratio, total flow rate, and dilution, lipid composition can be optimized to obtain the desired LNP size and structure. In addition, flow rates of up to 200 mL/min model clinically relevant process parameters at the bench scale, enabling rapid progression to large-scale NanoAssemblr® systems. Short formulation times (< 1 min/run) and easy setup with a touch-screen interface allow for an efficient systematic approach to finding lead drug candidates. The optional in-line dilution via the choice of appropriate single-use NxGen™ cartridges enhances flexibility and reduces cross contamination.

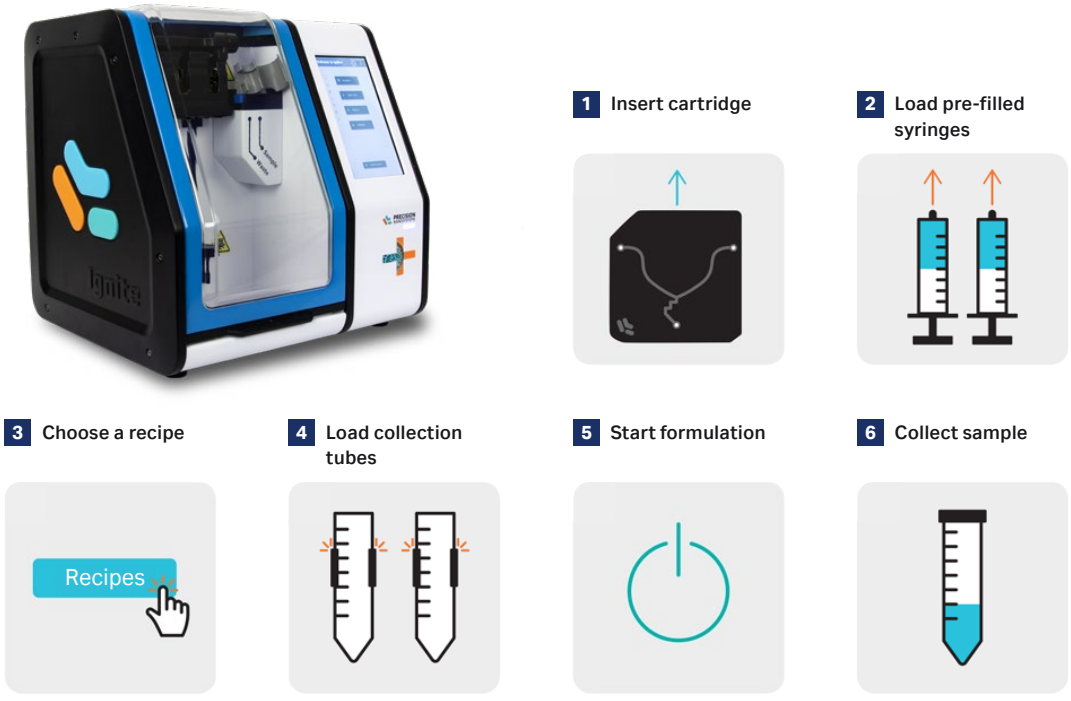


Fig 6. Precision NanoSystems NanoAssemblr® Ignite+™ system and workflow.

- NanoAssemblr® Blaze™ and Blaze+™ are process development systems that formulate 20 mL–10 L of LNPs at flow rates of up to 115 mL/min. These parameters enable efficacy and *in vivo* toxicity studies in secondary animal species as well as investigational new drug (IND)-enabling studies encompassing all manufacturing steps with shorter run times than clinical systems while using the same NxGen™ microfluidic technology to accelerate scale-up. Process development of upstream and downstream activities including tangential flow filtration (TFF) and sterile filtration help to prepare lead RNA–LNP drug candidates for technology transfer to good manufacturing practice (GMP).

- For late stage clinical and commercial RNA–LNP manufacturing, custom NanoAssemblr® instruments are available to support flow rates of up to 6.4 L/min under current good manufacturing practice (cGMP) conditions through multiplexing of NxGen™ mixing modules. This ensures reproducibility, minimizes process optimization, and simplifies technology transfer for single-product, high-dose, or rapid manufacturing of RNA–LNP drug products for pandemic preparedness. These systems meet all safety and controlled space requirements including 21 CFR Part 11 compliance.

- GenVoy-Ionizable Lipid Mix (GenVoy-ILM™) is a pre-optimized Research Use Only lipid mixture designed for encapsulation that can be used at various stages of drug development, from discovery to late preclinical, effectively delivering mRNA to cells with high efficiency and low toxicity. It comprises lipid components at defined ratios and is optimized for use with the NanoAssemblr® systems. This lipid mixture circumvents formulation-related challenges, allowing a quick start to setting up an mRNA–LNP laboratory.

Learn more about Precision NanoSystems' offerings, including LNP delivery formulations, that can be optimized and licensed through clinical development and commercialization. In addition, our Biopharmaceutical Contract Services team brings deep expertise and technical knowledge to accelerate RNA–LNP drug development, including formulation development, analytical development, and QC support.

LNP polishing

This step exchanges the buffer and removes excess lipids and non-encapsulated mRNA to further increase the purity. This fast and relatively inexpensive process can be readily executed in the laboratory by centrifugal ultrafiltration. The primary basis for separation is molecular size. Molecules larger than the membrane pores will be retained, but not bound, at the surface of the membrane and concentrated during the ultrafiltration process. The retention properties of ultrafiltration membranes are expressed as molecular weight cut-off (MWCO), which is based on the ability to retain > 90% of a solute of a known molecular weight (in daltons). Different membranes are available, and it's important to choose the ones with low nonspecific biomolecule binding that can provide > 90% recovery. At larger scales, centrifugal ultrafiltration will be replaced with tangential flow filtration systems.

Challenges

- Need for high-quality centrifugal ultrafiltration devices with a wide range of MWCOs and suitable membrane.

Strategies and product offerings

- Centrifugal ultrafiltration devices from **Pall Corporation** allow polishing of LNPs for volumes of < 50 µL to 60 mL. For individual samples, there are various sizes (Nanosep®, Microsep™, Macrosep®, and Jumbosep™) to choose from. For automation purposes, AcroPrep™ plates come as 24-, 96-, and 384-well filter plates, suitable for volumes of 80 µL–7 mL. Omega™ (modified polyethersulfone) ultrafiltration membranes provide high flow rates and low protein binding and are available in a variety of MWCOs.

Sterile filtration of mRNA–LNPs

This final sterile filtration step removes potential microbial contaminants. The same filters (Acrodisc® syringe filters with Supor® or Fluorodyne® from Pall Corporation) recommended for sterile filtration of mRNA also work well with mRNA–LNPs.

Reference

1. Schoenmaker L, Witzigmann D, Kulkarni JA, et al. mRNA-lipid nanoparticle COVID-19 vaccines: structure and stability. *Int J Pharm.* 2021;601:120586.

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