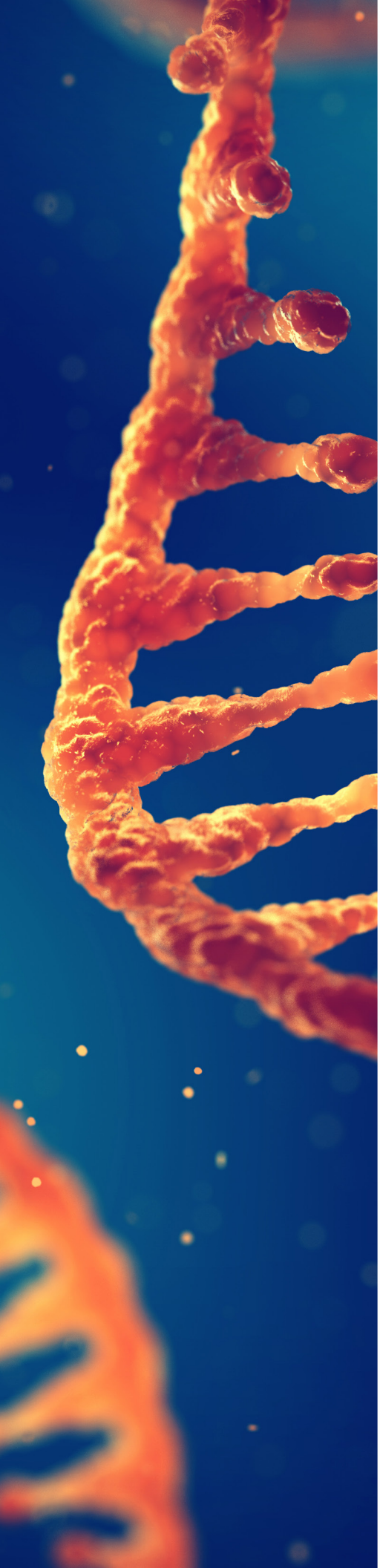
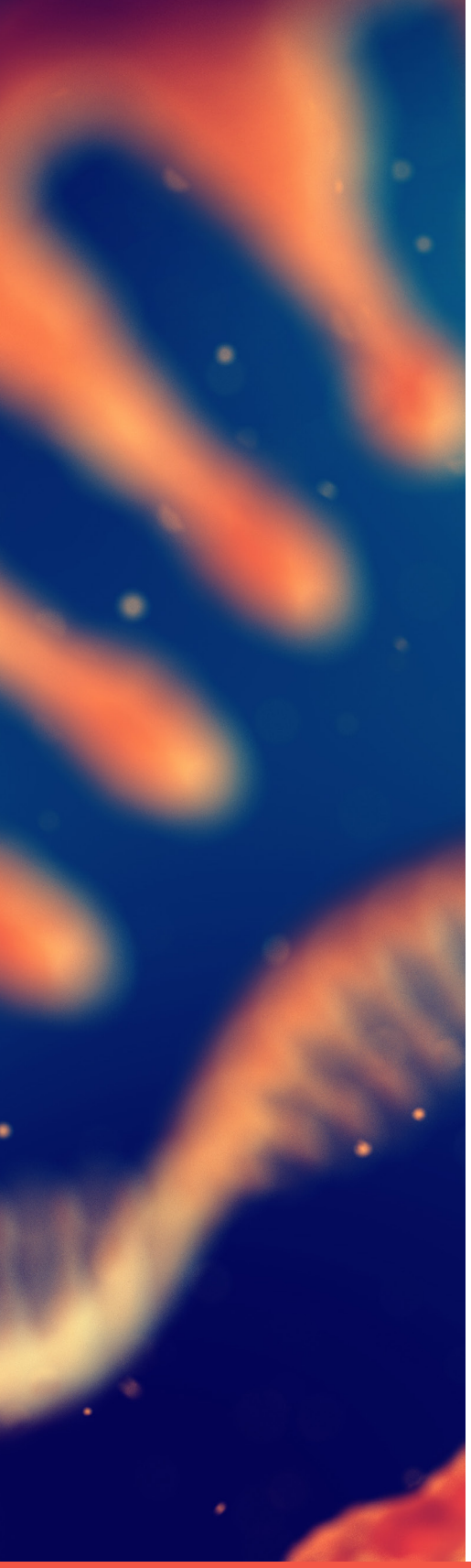


from template *to* transcript

overcoming challenges in mRNA manufacturing

NEW ENGLAND Biolabs®



From template *to* transcript

Introduction

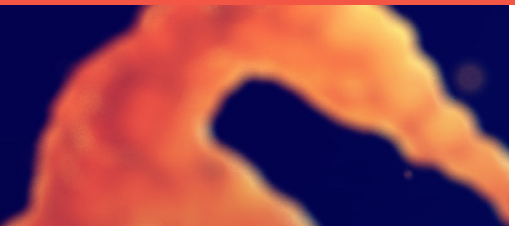
Messenger RNA (mRNA) technologies are now at the forefront of innovative medicines and are being explored for a diverse range of applications, including prophylactic and therapeutic vaccines for infectious disease and oncology, biotherapeutic protein expression, gene therapy, *in vivo* gene editing and cell therapy applications. Before the emergence of SARS-CoV-2 in December 2019 and the declaration of the coronavirus infectious disease 2019 (COVID-19) pandemic by the World Health Organization (WHO), the potential use of mRNA vaccines for pandemic response had been well described [1], but this was the first opportunity for broad deployment of the platform in a real pandemic setting. We witnessed the rapid acceleration of RNA vaccines relative to conventional vaccine technologies, a shift toward cell-free production systems and licensure of the first mRNA-based products under emergency use authorization. This progress has fueled precipitous and accelerated research and discovery efforts across all the diverse therapeutic applications.

However, this recent excitement and innovation was supported by over 50 years of basic research efforts in labs around the world. In the early 1960s, there were several publications describing the isolation of mRNA and its theoretical role in gene regulation [2, 3, 4]. The complexity of what took place is much more in keeping with what we know about science — a series of different groups attack a problem, using slightly different techniques, seeing the problem from different angles, before eventually a breakthrough makes clear what was previously problematic [4]. The scientific principles of mRNA production using enzymatic *in vitro* transcription (IVT) are based on research from these early pioneering years, and in 1969 Lingrel and Lockard worked together to publish methods for *in vitro* translation of mRNA [5]. It was not until 1984 that we published methods enabling the production of milligram quantities of mRNA [6], which began to fuel excitement for therapeutic applications despite the reservations around the instability of mRNA. For the therapeutic opportunities to be unlocked, delivery systems needed to be developed. In 1987, we witnessed the development of lipoplex delivery with cationic lipids [7], which then led to the development of lipid nanoparticles with ionizable cationic lipids for the delivery of short interfering RNA (siRNA [8]), and then their use for the delivery of mRNA vaccines in 2012 [9].

This very recent deployment of mRNA as a therapeutic modality is supported by the modern tools of molecular and synthetic biology that have been available as basic research tools since the late 1970s. These disciplines have been taught at academic institutions for a similar period and have helped to fuel the mRNA revolution that we are witnessing today.

Andy Geall, Ph.D.
Co-founder and
Chief Development Officer
at Replicate Bioscience, Inc.

With 50 years of experience in enzyme research and reagent manufacturing, NEB has developed a broad portfolio of reagents and tools for the manufacture and analysis of mRNA for use in therapeutic development. This eBook will discuss the challenges encountered throughout mRNA production, exploring how NEB products and services can be utilized for successful synthesis, analysis and supply.



Learn more about products for your mRNA-related workflows at NEBrna.com

Introduction to mRNA synthesis

In vitro transcription

In vitro transcription (IVT) is the core technology that enables mRNA manufacturing. Transcription is a cellular process that produces RNA molecules from DNA. IVT takes advantage of a simplified form of the cellular process to produce RNA from a DNA template under artificial conditions.

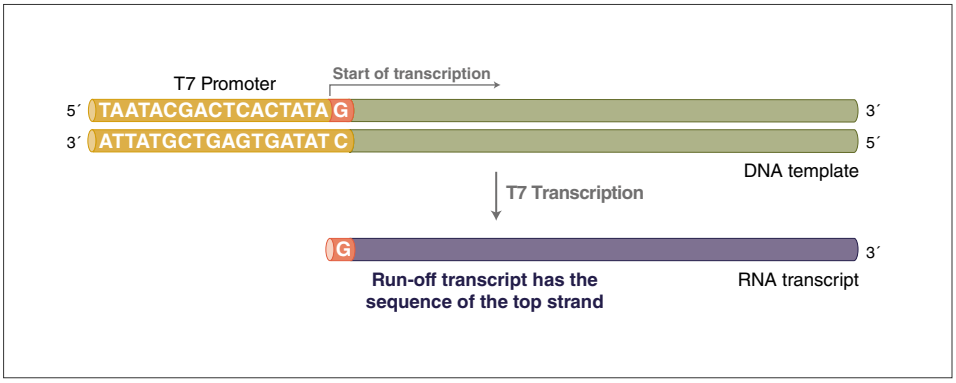
IVT is normally driven by a DNA-dependent RNA polymerase (RNAP) that transcribes DNA into RNA through an RNA polymerization activity. T7 RNA Polymerase (NEB #M0251), the most commonly used polymerase in IVT, recognizes a specific promoter sequence located upstream of the transcription start site where transcription of the synthetic RNA (a transcript) begins (Figure 1) [10].

Ribonucleotides are the building blocks of RNA. However, the delivery of synthetic mRNA using unmodified ribonucleotides, elicits an undesirable immune response in the cell. In 2005, the research team led by Katalin Karikó and Drew Weissman showed that replacing some ribonucleotides in synthetic RNA with their modified forms (e.g., pseudouridines) effectively suppressed innate immune responses and increased translation efficiency. This discovery has led to the development of safe mRNA vaccines and therapeutics. Karikó and Weissman shared the Nobel Prize in Physiology or Medicine in 2023 for the discovery [11, 12].

Messenger RNAs (mRNAs) are linear molecules that require modifications at their 5´- and 3´- ends to promote translation and further evade immune responses in the cell. These modifications, namely, 7-methylguanosine 5´ cap structures and 3´ polyadenosine tails, respectively, can be added to *in vitro* transcripts during transcription, or in subsequent steps.

IVT and subsequent capping and tailing can be readily scaled from benchtop reactions to industrial scale for therapeutic manufacturing.

Figure 1. T7 Transcription



A transcription template contains a T7 promoter sequence followed by the sequence of interest. The T7 promoter is required for transcription to occur. The sequence of the transcript is the same as the top strand of the DNA template and initiates from G residues encoded in the optimal T7 RNA Polymerase promoter.

mRNA capping & tailing

An array of 5´ cap structures exist in nature. While lower eukaryotes (such as yeast) carry a Cap-0 structure, mammalian cells feature a Cap-1 structure that contributes to efficient protein translation and evasion of the innate cellular immune response, among many other functions [13]. Incorporation of the Cap-1 structure into synthetic mRNA can be achieved by two strategies (Figure 2).

Co-transcriptional capping:

5´ caps can be incorporated into synthetic mRNA during transcription using cap analogs. Modified versions of Cap-0 or Cap-1 are incorporated into the synthetic mRNA as the initiating nucleotide. First-generation dinucleotide cap analogs, such as m7G(5´)ppp(5´)G, generate functional mRNA but compromise yield and cap efficiency. This class of cap analogs can be incorporated at the 5´ end of the synthetic RNA in either orientation, therefore further reducing the fraction of functional synthetic mRNA to 50% of the capped RNA molecules. The second generation of cap analogs, known as Anti-Reverse Cap Analog (ARCA) (NEB #S1411) results in transcripts with a correctly oriented Cap-0 structure. However, ARCA and the early cap analogs compete with guanosine triphosphate (GTP) for transcription initiation, which leads to incomplete cap installation and reduces RNA yield. The incorporated ARCA can then be converted to a Cap-1 structure by using a mRNA Cap 2´-O-Methyltransferase (NEB #M0366). Uncapped transcripts are immunostimulatory and require additional treatment for removal from the RNA preparation [14]. The latest generation of cap analogs, the trinucleotide cap analogs, such as CleanCap® Reagent AG, can generate high yields of >95% Cap-1 mRNA in a single reaction.

Post-transcriptional capping:

In nature, RNA capping enzymes are responsible for the installation of the cap structures to the nascent pre-mRNA. RNA capping enzymes from vaccinia virus, Vaccinia Capping Enzyme (VCE), and faustovirus, Faustovirus Capping Enzyme (FCE), have been used to install the Cap-0 structure of synthetic RNA *in vitro* [14, 15]. The Cap-1 structure can be generated by including mRNA Cap 2´-O-Methyltransferase (NEB #M0366) in the VCE or FCE reaction.

Poly(A) tailing:

Similar to the 5´ cap, the poly(A) tail contributes to efficient protein translation and the stability of the RNA molecules [16]. Poly(A) tailing can also be performed co-transcriptionally and post-transcriptionally:

- Template encoded: By incorporating a poly(T) sequence into the DNA template, a poly(A) tail can be added to the 3´-end of mRNA during IVT.
- Post-transcription: By using Poly(A) Polymerase (NEB #M0276) and ATP as a donor, polyadenylation at the 3´-end of synthetic mRNA is performed after IVT is complete.

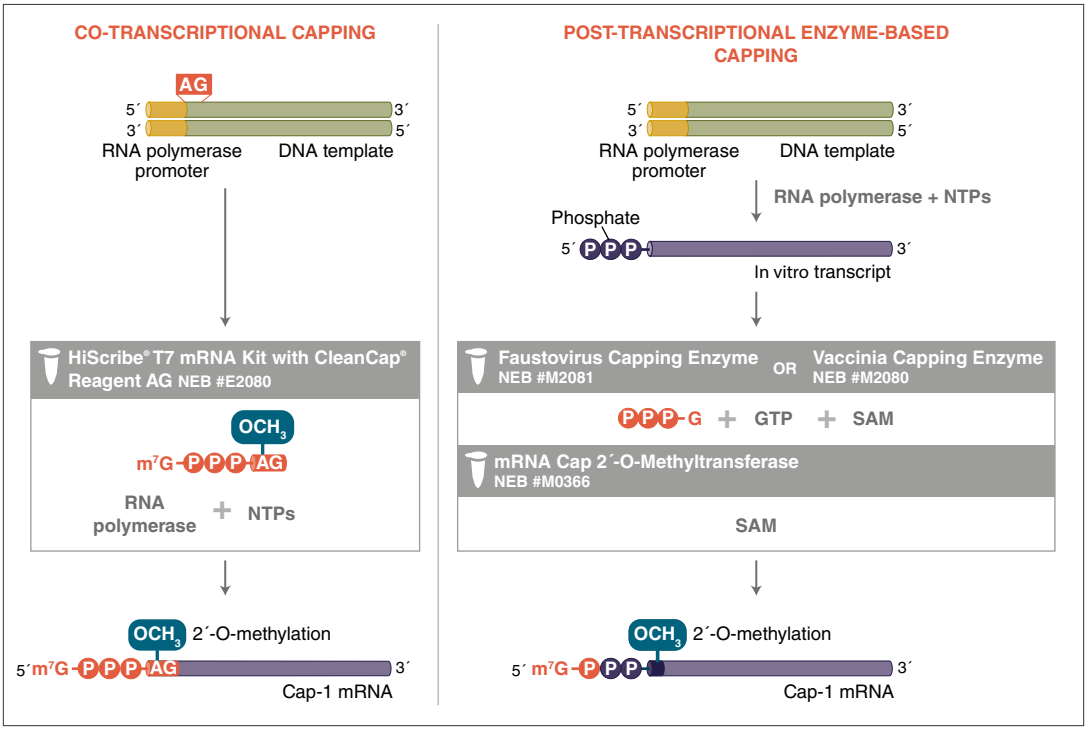
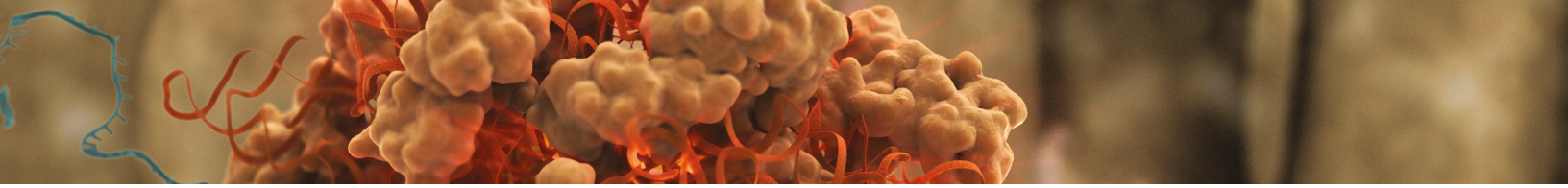


Figure 2.
Generating Cap-1 mRNA

Enzyme-based capping, shown on the right panel, is performed by treatment of synthesized RNA with either Faustovirus Capping Enzyme (FCE) (NEB #M2081) or Vaccinia Capping Enzyme (VCE) (NEB #M2080) GTP, and SAM resulting in a Cap-0 structure. Cap-1 is achieved by 2´O methylation of the first transcribed nucleotide in a subsequent or concurrent reaction with mRNA Cap 2´-O-Methyltransferase (NEB #M0366) and SAM. Co-transcriptional capping, shown on the left panel, can be achieved with trinucleotide cap analogs, e.g., CleanCap Reagent AG, in a single, simple reaction.



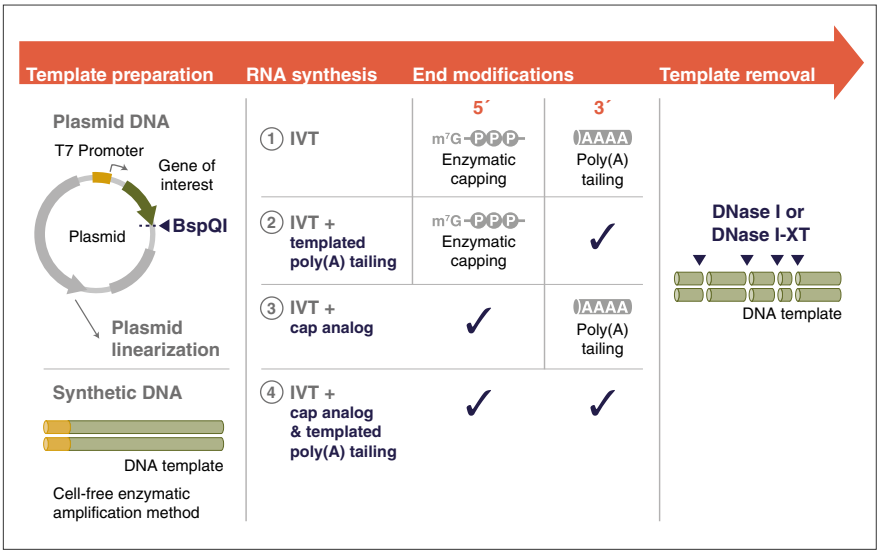
Overcoming challenges and streamlining IVT mRNA synthesis

Considerations for high-yield, high-quality mRNA synthesis

While *in vitro* synthesis of mRNA can be done using defined enzymes and reagents, the development of a robust, high-yield process for the manufacture of high-quality mRNA requires deliberate design and engineering (Figure 3). These are some areas that require attention.

- **Template design, generation and purification:** The design and quality of the template can directly impact the quality, yield, level of 5´ cap and 3´ poly(A) tail incorporation, as well as translation efficiency of the mRNA molecules. Therefore, thoughtful design (including the template construct, restriction enzyme choice and purification methods) and preparation of the DNA template are essential for high-quality mRNA synthesis.
- **Reaction conditions and format for IVT and 5´ capping:** Although the underlying mechanism is unclear, it is generally accepted that the RNA sequence of the synthetic mRNA has a major impact on the outcome of IVT and the effectiveness of 5´ capping. Therefore, optimization of reaction conditions (such as enzyme concentration, nucleoside triphosphate (NTP) concentration, reaction time and format) should be considered when high-yield, high-quality mRNA synthesis is desired. The use of commercial kits such as the HiScribe® T7 High Yield RNA Synthesis Kit (NEB# E2040) and the HiScribe T7 mRNA Kit with CleanCap Reagent AG (NEB# E2080) requires little need for optimization if a small amount of synthetic mRNA is needed for exploratory research.
- **Analytics:** Analytics are important not only at the mRNA product validation stage but also crucial for evaluating method development for IVT, capping and tailing. Areas such as cap identity, cap incorporation rate, RNA integrity, sequence identity and tail length are important quality control (QC) attributes.

Figure 3. RNA Synthesis workflow options



Recommended products for high-yield, high-accuracy DNA Construction

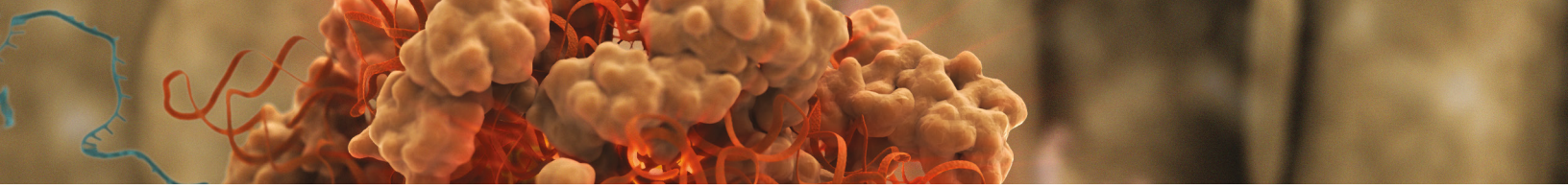
- **NEBuilder® HiFi DNA Assembly Master Mix (NEB #E2621):** Enables seamless, error-free assembly of DNA fragments using a high-fidelity DNA polymerase.
- **NEBridge® Ligase Master Mix (NEB #M1100):** Combines high-fidelity Golden Gate Assembly with Type IIS restriction enzymes for scarless assembly and swapping of DNA sequences.

Tools for template design, construction and synthesis

Template design is important for IVT as it can impact transcription efficiency and mRNA quality and yield (Figure 4).

Building on years of research on DNA ligases and DNA assembly, NEB has created tools to simplify the template design and construction processes. Free online tools such as NEBridge Ligase Fidelity Tool and NEBuilder Assembly Tool enable fast construction of complex assemblies as well as, for example, rapid swapping of regulatory sequences and the genes the synthetic mRNA encodes [17, 18].

Once the construct is prepared, plasmid DNA can be linearized using a restriction enzyme and used as the template in IVT. It is important to optimize the restriction enzyme reaction conditions to avoid insufficient linearization and potential star activity, which can lead to poor yields and transcription failure.



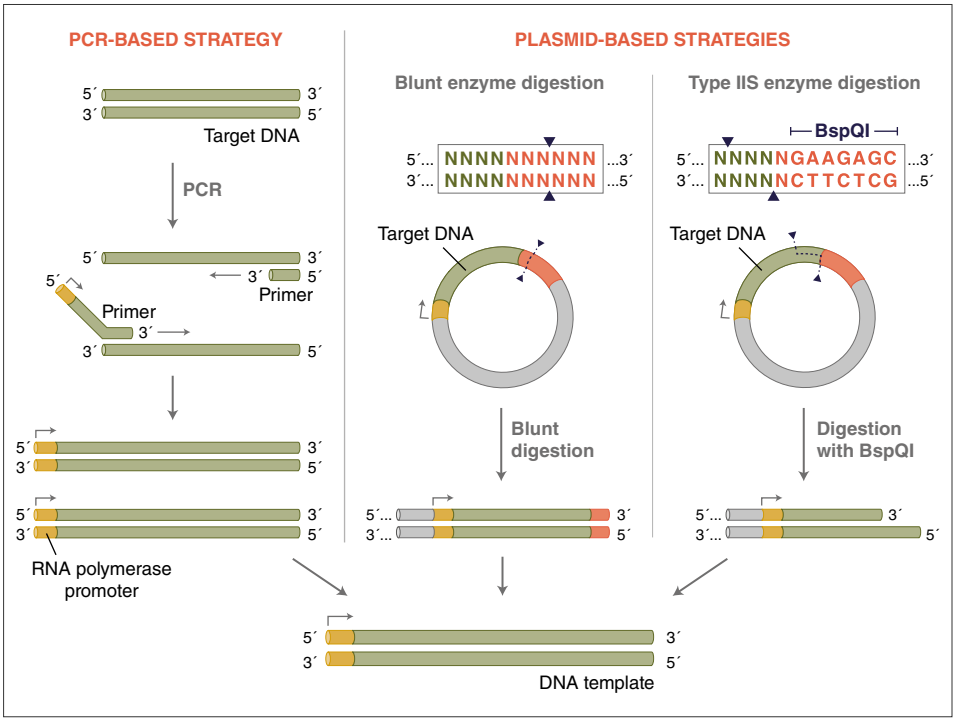
Alternatively, plasmid DNA can be used as a template for DNA amplification to generate synthetic IVT templates. In this case, it is important to choose a high-fidelity DNA polymerase and optimize reaction conditions to minimize the introduction of errors during PCR. Errors in the DNA template can lead to mutations in the final protein product and significantly impact the functionality of the synthetic mRNA.

Whichever method is used, conditions should be optimized to generate accurate, high-quality IVT templates. The following products can be used for the generation of fully linearized DNA templates:

- **Plasmid linearization:** Type IIS restriction enzymes, e.g., BspQI (NEB #R0712), BsaI-HF®v2 (NEB #R3733), generate double-strand breaks away from their recognition sequence. This property has been used to generate IVT templates with a scarless 3´ runoff site - no additional nucleotides derived from the restriction site are added to the 3´ end of the synthetic RNA. This facilitates the generation of synthetic mRNA with a custom 3´ end (such as poly(A) tail).
- **PCR amplification:** With fidelity ~280X of *Taq* DNA Polymerase, Q5® High-Fidelity DNA Polymerase (NEB #M0491) is a fast and high-fidelity DNA Polymerase developed by NEB. Q5 DNA Polymerase is available in standalone, master mix and kit formats. The hot start formulation, Q5® Hot Start High-Fidelity DNA Polymerase (NEB #M0493), allows reactions to be set up at room temperature, further reducing non-specific amplification.
- **Isothermal amplification:** Template DNA can also be produced through isothermal amplification. The strand displacement activity and high fidelity of phi29 DNA Polymerase allow for accurate amplification of double-stranded DNA at a single, low temperature. While products tend to be long and branched, there are various approaches to process the DNA for the creation of clean, discrete template molecules. To ensure resulting DNA is free of unwanted structures and products, T5 Exonuclease (NEB #M0663) can be used to remove any non-template DNA. The performance of these methods is regularly enhanced, for example, by the engineered phi29-XT Polymerase (NEB #E1603) that can reduce workflow times and increase DNA product yield significantly.

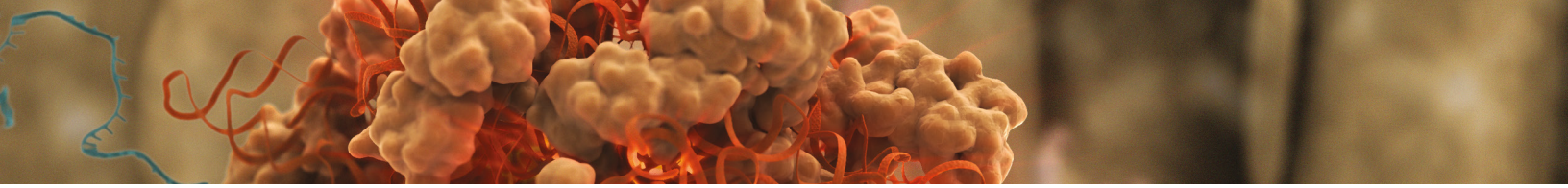
After template linearization or amplification, the template DNA should be purified to remove the enzymes and reaction reagents. Spin column purification using kits such as Monarch® PCR and DNA Cleanup Kit (NEB #T1030) is often the method of choice for quick and easy purification of high-quality template DNA for IVT.

Figure 4. Transcription Template Generation



(A) PCR can be used to amplify target DNA prior to transcription. A polymerase promoter can be introduced via the upstream primer. Choose a high-fidelity polymerase to ensure that your template is error-free.

(B) When using plasmid DNA as a template, linearize with an enzyme that produces blunt or 5´-overhanging ends. Using a type IIS restriction enzyme (e.g., BspQI) allows RNA synthesis with no additional 3´-nucleotide sequence from the restriction site.



Achieving high quality and high yield in IVT

With the consideration of template design, template generation and IVT conditions discussed above, NEB has developed a large selection of IVT kits that combine high yield with flexibility on the choice of RNAP (T7 vs. SP6), fine-tuning reagent concentrations or inclusion of modified nucleotides, or co-transcriptional capping using CleanCap AG or ARCA with optional post-transcriptional tailing. The HiScribe T7 Quick High Yield RNA Synthesis Kit (NEB #E2050) consolidates IVT reagents into master mixes for ultimate convenience.

The HiScribe® RNA synthesis kits seamlessly interface with the Monarch® RNA Cleanup Kit (NEB #T2030, T2040, T2050). Users can achieve rapid purification of RNA from IVT reactions with high recovery rates (70-100%) and the flexibility to generate high-concentration RNA preparations (~2 µg/µl for 10 µg or >2 µg/µl for 500 µg scale purification).

Achieving high capping and tailing efficiency

5´ capping and 3´ poly(A) tail incorporation are critical quality attributes (CQAs) of synthetic mRNA manufacturing because of their impact on mRNA stability, functionality and reduced immunogenicity. NEB offers capping and tailing options that are highly efficient and amenable to upscaling.

Optimizing 5´ capping

As previously discussed, 5´ capping can be achieved co-transcriptionally or post-transcriptionally. Co-transcriptional capping using ARCA (NEB #S1411) can generate translatable transcripts (Cap-0) in less than two hours. The HiScribe T7 ARCA mRNA Kit (NEB #E2065) has been optimized for co-transcriptional capping using ARCA. However, the use of ARCA generally results in lower yields and lower capping efficiency than more recently developed trinucleotide cap analogs. Trinucleotide cap analogs such as CleanCap reagent AG from TriLink® can be used to produce a Cap-1 structure in a single reaction. With the appropriate initiating nucleotide in the IVT template, the Hiscribe T7 mRNA Kit with CleanCap Reagent AG (NEB #E2080) can generate >95% Cap-1 transcripts with reaction yields comparable to using the Hiscribe T7 High Yield RNA Synthesis Kit (NEB #E2040) or (NEB #E2050). The simple reaction setup makes HiScribe T7 mRNA Kit with CleanCap Reagent AG the method of choice for generating a large number of transcripts for screening purposes.

Post-transcriptional capping using VCE (NEB #M2080) in conjunction with mRNA Cap 2´-O-Methyltransferase (NEB #M0366) offers a more cost-effective means to generate high-level Cap-1 incorporation. More recently, NEB has discovered and characterized FCE (NEB #M2081). FCE exhibits improved capping activity even on difficult substrates and a broad temperature range. The latter permits capping at low temperatures, desirable for maintaining the integrity of long RNAs such as self-amplifying RNAs, or high temperatures, where capping of structured 5´ ends may be improved. With reaction condition optimization, enzymatic capping can routinely achieve >95% Cap-1 incorporation.

Understanding and reducing double-stranded RNA (dsRNA) formation

dsRNA is a major by-product of IVT. Numerous reports have shown that dsRNA is highly immunostimulatory, an undesirable property for therapeutic mRNA. Although downstream purification can be devised to reduce dsRNA content to undetectable levels, understanding the mechanism of formation and addressing these mechanisms in IVT can help reduce the dsRNA load for downstream processing. NEB scientists have helped elucidate possible mechanisms of dsDNA formation during IVT [19]:

- **3´-extension by-products:** The 3´ end of the run-off products anneal to complementary sequences (cis or trans, DNA template or RNA product) to form extended duplexes.
- **Antisense by-products:** T7 RNAP re-initiates and transcribes from the non-template strand of the dsDNA template, forming extended antisense RNA molecules that form a duplex with the run-off transcript.

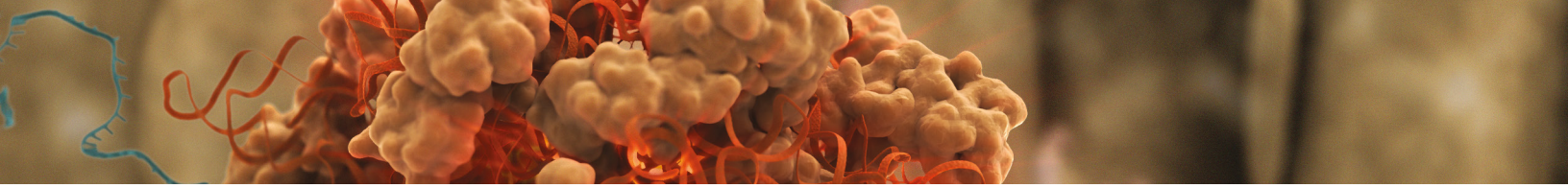
Elevating IVT reaction temperature has been shown to reduce dsRNA formation. When used at elevated temperatures, the wild-type T7 RNAP and Hi-T7 RNAP (NEB #M0658), a thermostable RNAP developed by NEB scientists, can reduce the formation of dsRNA byproducts [19].

Incorporation of the poly(A) sequence into the DNA template can significantly reduce the formation of dsRNA via 3´-extension [20].

Recommended products for high-quality, high-yield IVT

HiScribe *in vitro* RNA synthesis kits available from NEB:

- HiScribe T7 High Yield mRNA Synthesis Kit (NEB #E2040)
- HiScribe T7 Quick High Yield RNA Synthesis Kit (NEB #E2050)
- HiScribe T7 mRNA Kit with CleanCap® Reagent AG (NEB #E2080)
- HiScribe T7 ARCA mRNA Kit (NEB #E2065) (with tailing) (NEB #E2060)
- HiScribe SP6 RNA Synthesis Kit (NEB #E2070)



Post-IVT treatment

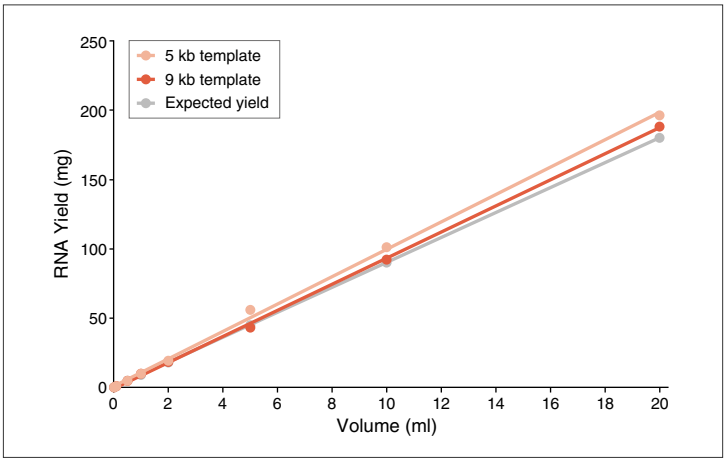
Removing the template DNA after IVT can greatly simplify downstream processing. Enzymatic digestion of template DNA is the method of choice due to its simple implementation. NEB supplies DNase I (RNase-free) (NEB #M0303) and DNase I-XT (NEB #M0570) which are highly effective in digesting template DNA after IVT. While DNase I (RNase-free) is inhibited by salt concentrations >50 mM, DNase I-XT, which has been engineered by NEB scientists for high salt tolerance, exhibits optimal activity between 50-100 mM salt and retains 65% and ~40% activity in 200 and 300 mM salt, respectively. This increased salt tolerance enables DNase I digestion without buffer adjustment, further simplifying the workflow. After DNase I digestion, the IVT reactions can be subjected to downstream processing to remove IVT reagents, enzymes and DNase I from the synthetic mRNA. NEB's Monarch RNA Cleanup Kits (NEB #T2030, T2040, T2050) are designed for the purification of 10–500 µg of IVT RNA in a simple and quick spin-column format.

Scaling up

Therapeutic exploratory and early-stage studies require vast amounts of RNA. Large-volume and high-yield IVT reactions, in which all components are scaled linearly, can be achieved with a few key considerations. The quality, type (PCR, synthetic dsDNA, linearized plasmid) and source of the dsDNA template are key to a successful reaction and will require advanced planning. Keep in mind that sourcing templates from an outside supplier may be the most efficient avenue to acquire the amount of dsDNA needed.

NEB offers two main options for large-scale IVT, which include individual IVT enzymes or high-yield kits. High-yield, HiScribe kits contain all the components necessary for IVT and have been developed to work with a wide range of template lengths. Optimization of reaction conditions (NTP, enzyme and $MgCl_2$ concentration) is required when working with individual enzymes, and it is recommended to carry out these optimization steps at low volumes before scaling up. It is important to note that the optimal amount of template (by weight) to achieve the desired high yield for every sequence and length of RNA to be transcribed will need to be determined first, as fewer moles are present for longer templates when following guidelines for equal input template weight. There is a direct correlation between template length and reaction time as shorter templates require more RNA polymerase initiation events to attain the same amount of RNA (by weight) as longer templates (Figure 5). Therefore, shorter templates generally require a longer incubation.

Figure 5. RNA extraction using HiScribe



The yield of RNA when extracted at different volumes using 5 kb and 9 kb templates compared with the expected yield. All reaction components are scaled linearly from the protocol recommendation in The HiScribe® T7 High Yield RNA Synthesis Kit (NEB# E2040) using linearized plasmids as templates. Reactions were incubated for 1 hour at 37°C.

A key consideration when scaling is balancing the reaction time where high yield is achieved while limiting the amount of reaction byproducts that can form over time — longer isn't always better.

Once the amount of required template and reaction time is determined at the small-scale level, reactions can be scaled linearly. When working at larger scales, gentle mixing (to avoid bubble formation) and keeping a constant temperature within the reaction vessel will be key for a successful large-scale IVT that scales linearly with volume.

Scaling out

For some therapeutic approaches, such as personalized therapeutics, pharmaceutical manufacturing quality systems and practices must be applied to bespoke medicines manufactured for a single patient. Sourcing requirements may require individual packaging for the raw materials used in a manufacturing run at considerably smaller sizes than used in batch manufacturing. To meet these needs, NEB can work with the customer to provide custom product formats for both research use and good manufacturing practice (GMP)-grade* products.

** GMP-grade is a branding term NEB uses to describe products manufactured or finished at NEB's Rowley facility. The Rowley facility was designed to manufacture products under more rigorous infrastructure and process controls to achieve more stringent product specifications and customer requirements. Products manufactured at NEB's Rowley facility are manufactured in compliance with ISO 9001 and ISO 13485 quality management system standards. However, at this time, NEB does not manufacture or sell products known as active pharmaceutical ingredients (APIs), nor does NEB manufacture its products in compliance with all of the current good manufacturing practice regulations (cGMP).*

Leveraging advanced solutions for mRNA analytics success

Leveraging advanced solutions

Due to the accelerated progress of mRNA-based medicines, the regulatory standards outlining aspects of mRNA quality, such as analytical testing (e.g., identity verification, control of impurities and dosage concentration), are still evolving. To ensure process and product quality, efficacy and safety, mRNA process developers are working to further advance analytics based on the specifics of the quality attributes in question.

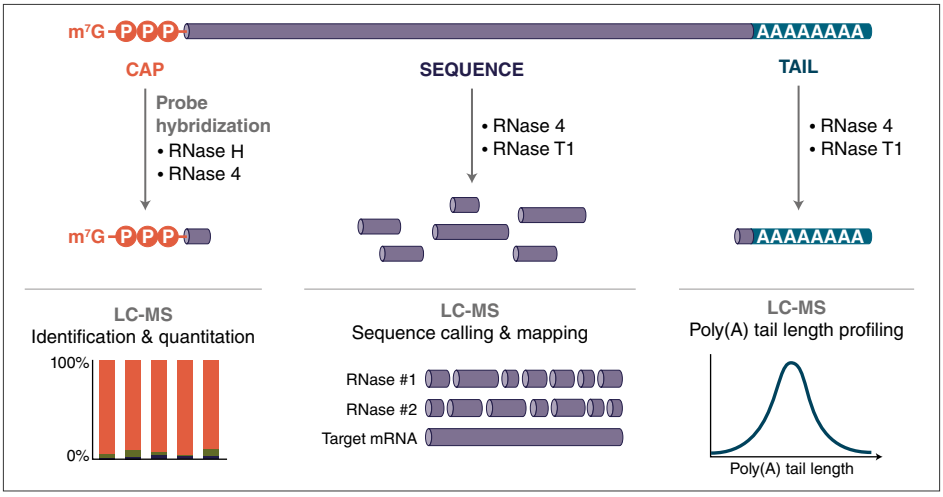
To help establish an analytics framework and standards for the novel mRNA therapeutics, the USP has published specific chapters, using information across industry, government and academia to define standards for mRNA-based products. This encompasses the best practice for methods such as RT-qPCR and RNA sequencing, to identity the mRNA drug substances [21].

Tandem liquid chromatography-mass spectrometry (LC-MS) is one of the few direct detection methods that can provide reproducible and accurate identification, as well as qualitative and quantitative analysis of RNA (Figure 6). LC-MS allows for direct analysis of mRNA without amplification or conversion into DNA [22]. Modifications that can be challenging for sequence-based methods (such as N1-methylpseudouridine 5′ capping and 3′ poly(A) tailing) can be detected and, in some cases, quantified using LC-MS analysis [23].

For the analysis of global nucleoside content, the Nucleoside Digestion Mix (NEB #M0649) provides a convenient single-step conversion of RNA molecules into nucleosides from which RNA modifications can be accurately identified and quantified.

For RNA sequence verification, kilobase-long RNA molecules are broken down into oligonucleotides amenable to LC-MS analysis. Here, site-specific endoribonucleases can be used individually or in parallel to generate short oligonucleotides for LC-MS analysis [22]:

Figure 6. Overview of LC-MS/MS based synthetic mRNA analysis



To verify mRNA sequence and modification status, the sample is digested using one or more nucleotide-specific endoribonucleases (e.g., RNase 4 and RNase T1) in parallel to generate a series of oligonucleotide cleavage products, and are subjected to LC–MS/MS analysis. After mass matching and sequence assignment, a sequence coverage map is determined by mapping the oligonucleotide to the reference sequence.

To assess 5′ cap incorporation, a predefined fragment near the 5′ end of the transcript is hybridized with a probe and cleaved using RNase H or RNase 4. The probe can contain an affinity tag for isolation of the cleaved product prior to LC–MS/MS. To assess the 3′ tail, a nucleotide-specific endoribonuclease is used to cut and release the poly(A) tail for profiling by LC–MS/MS.

- **RNase T1:** RNase T1 digests single-stranded RNA at G-residues. With a single-base specificity, RNase T1 often generates short cleavage products that are not uniquely mappable, reducing coverage and making oligonucleotide annotation challenging [22].

- **RNase 4:** RNase 4 (NEB #M1284) digests single-stranded RNA at uridine-adenosine or uridine-guanosine dinucleotide sites. Compared to RNase T1, the longer sequence specificity produces oligonucleotides of optimal length distribution for LC-MS analysis. hRNase 4 has been used to map synthetic mRNAs of various lengths, including upwards of 4,000 nucleotides. The use of RNase 4 also allowed for direct assessment of 5′-cap incorporation onto mRNA [22].

To verify the identity of the 5′ cap structure and the extent of cap incorporation, it is necessary to analyze a small defined 5′ fragment of the synthetic mRNA, which is often thousands of nucleotides in length. NEB scientists have reported two related methods that can be used in cap analysis.

- **RNase H:** RNase H recognizes an RNA-DNA hybrid and cleaves the RNA strand. By appropriate design of a nucleic acid probe, RNase H can be directed to cut synthetic RNA in a sequence-specific manner [24]. The resulting 5′ fragments can then be isolated and analyzed by LC-MS/MS. NEB offers RNase H derived from *E. coli* (NEB #M0297) and a thermophilic bacteria (NEB #M0523). Although RNase H has been shown to generate minor cuts at +1 and -1 nt sites, methods have been published to effectively select highly precise cleavage-guiding probes. In addition, RNase H allows for the generation of a 3′ recessive end on the RNA such that the cleavage fragments are amenable for labeling with a fluorescent nucleotide for gel- or capillary-electrophoresis analysis [25].

- **RNase 4:** Taking advantage of the ssRNA and uridine-adenosine/guanosine specificity, NEB scientists devised a method where the target 5′ sequence is protected by a ssDNA probe. When incubated with hRNase 4, all the UA and UG sites, except those in the 5′ region protected by the DNA probe, cleaved, generating precise fragments. The DNA-RNA duplex containing the 5′ region and the cap structure can then be enriched and analyzed by LC-MS/MS [26].

Research-grade versus GMP-grade: Supporting mRNA scale-up

Built for quality by design

When transitioning from bench research and early process development to clinical, and later commercial manufacturing, NEB provides products with additional quality attributes. These GMP-grade materials are manufactured to satisfy your internal sourcing requirements and regulatory requirements, which clearly state manufacturers of cell and gene therapy products (including mRNA products) are required to use the highest grade of raw materials available.

Research-grade and GMP-grade materials are functionally equivalent, allowing for an easy transition between the two. The difference lies with the additional controls over raw material sourcing, batch records, clean room manufacturing environment and release standards for GMP-grade products. With strict bioburden and endotoxin limits, as well as terminal filtration processes in place, these additional steps ensure quality following the strict product specifications that are designed to address potential risks to our customer's patient population. GMP-grade enzymes are manufactured using processes that are completely animal-free and come certified transmissible spongiform encephalopathy (TSE)/ bovine spongiform encephalopathy (BSE)-free.

The documentation package associated with these products supports all stages of clinical development and eventual commercial release. NEB's experienced quality and technical team can provide consultation and support for additional requirements that may arise during the filing process.

The analytical methods used for in-process and release testing are state-of-the-art quantitative assays. The method qualification process provides documentation of their best-in-class accuracy and precision.

NEB's GMP-grade products are manufactured in a state-of-the-art, purpose-built, 43,000-square-foot production facility that opened in 2018 in Rowley, MA. The facility was designed and constructed with an emphasis on robust operation, ease of maintenance and system redundancy.

The facility is ISO 13485 and 9001-certified and the operating and quality systems voluntarily align with the relevant sections of CFR 21 Part 820. In addition to offering on-site quality audits to support your sourcing process, NEB encourages current and prospective customers to visit the facility and speak with our highly trained and experienced staff.

All the aforementioned technical prowess, regulatory experience and production capacity have been pressure tested and validated by our ability to supply reagents supporting the global production of commercially approved COVID-19 vaccines.

> *"New England Biolabs had the deep manufacturing and scientific expertise necessary to dramatically scale enzyme manufacturing at the unprecedented pace required for Moderna to address the COVID-19 global pandemic."*

Jerh Collins, Ph.D.
Chief Technical Officer,
Moderna.

We deliver

With a long and established history in the marketplace, NEB has been a key supplier in the biopharmaceutical industry since its inception in the mid-1970s. With a robust and reliable supply chain network, we can supply customers with what they need, when they need it. We provide custom solutions, flexibility and short lead times to product quantities from bench to commercial-scale.

The combination of 50 years of manufacturing experience, scientific expertise and an extensive global supply network uniquely positions NEB to provide market insights that meet your ever-developing process needs and provide business continuity.

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