



Enabling mRNA Therapeutic Development Through Enhanced IVT Capping Efficiency

**Codex® HiCap RNA Polymerase
advances therapeutic development**

Enabling mRNA Therapeutic Development Through Enhanced IVT Capping Efficiency

Codex® HiCap RNA Polymerase advances therapeutic development

Codex® HiCap RNA Polymerase is a uniquely engineered RNA polymerase that enables researchers to produce synthetic capped mRNA at the high yield and purity that today's mRNA-based vaccines and therapeutics demand. It generates fewer undesirable double-stranded RNA (dsRNA) byproducts than wild-type T7 (WT T7) RNA polymerase and incorporates commercially available cap analogs more efficiently by co-transcriptional capping, producing *in vitro* transcription (IVT) products with unparalleled efficiency.

This paper explores how Codex HiCap RNA Polymerase can help deliver safer and more potent therapeutic mRNAs while creating efficiencies to reduce the costs of your clinical development.

New Focus on mRNA Therapeutics

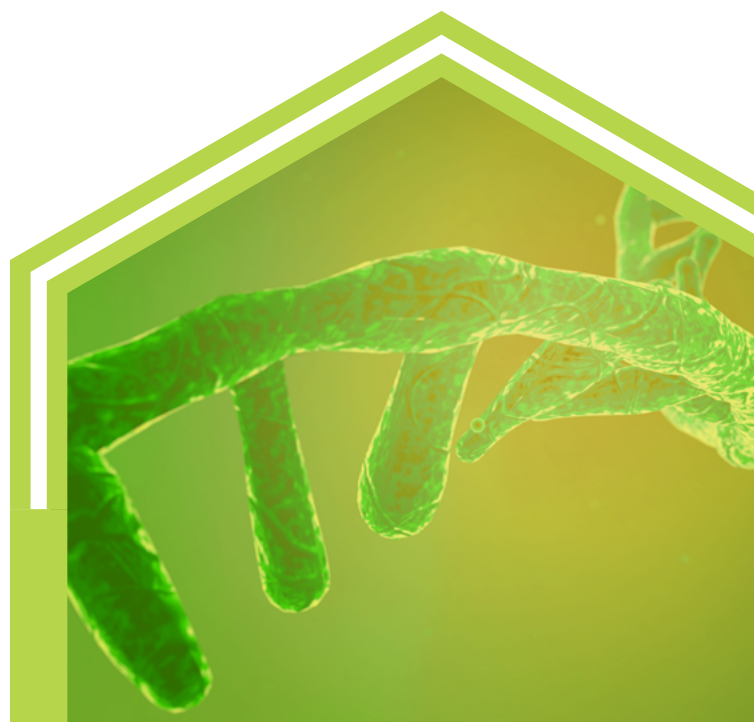
The central dogma of molecular biology, which dictates that genetic information flows from DNA to RNA to protein, has been instrumental in informing academic and translational biological research. In the arena of therapeutics, the upstream DNA and downstream protein ends of the process have received the most attention. More recently, the intermediate (i.e., RNA) between a source genetic sequence and the resulting protein has been the subject of intense therapeutic interest. It's in this area that Codex HiCap RNA Polymerase excels.

The COVID-19 pandemic highlighted the advantages of mRNA-based approaches, which include short development cycles, straightforward manufacturing, and high potential for platform-based development. These advantages position mRNA-based therapies as an attractive avenue to reach previously difficult or undruggable targets. As a result, the clinical pipeline has become rich with mRNA-based

approaches for prophylactic vaccines, cancer immunotherapy, and other indications.¹⁻³ From a product development perspective, contemporary research continues to provide invaluable insight into the anatomy of a functional and potent mRNA-based therapeutic.

Unprecedented growth in the mRNA space is exciting but causing some growing pains for the field. The rapid expansion of this technology has forced regulatory agencies to intensify scrutiny of these novel therapies. Even though two mRNA vaccines are currently approved, there is still significant uncertainty as to how to classify this novel therapeutic modality.⁴

Ultimately, the classification of mRNA therapeutics will better define their regulatory path to approval.⁴ Consensus among experts is that even though the industry as a whole is still grappling with the classification of mRNAs, increased scrutiny from regulators will be centered on manufacturing methods, with a keen focus on mRNA purity.⁴



To further complicate the nonlinear growth, global supply chains have witnessed a surge in demand for GMP-grade raw materials to support this mRNA boom. While many suppliers attempted to jump onto this bandwagon, only well-prepared and experienced suppliers were set up to be successful in both the short and long term. These supply chain challenges, in combination with periods of inflation, have placed strain on biotech companies, including those in the mRNA space.⁵ As a result, a keen focus on manufacturing process efficiency, through more conservative usage of input materials, has emerged to reduce overall cost of goods in mRNA production.^{5,6}

We are in a golden age of advancement for mRNA therapeutics. A collective industry focus on overcoming the key challenges of enhanced regulatory scrutiny, strained supply chains, and a deeper understanding of the immunogenicity of mRNA is required to facilitate the realization of their vast potential. Optimizing the mRNA manufacturing process is a primary avenue to enhance transcript yields, improve input reagent efficiencies, reduce costs, and eliminate harmful byproducts to produce a safe and effective therapeutic mRNA.

The Importance of the 5' Cap

The typical structure of a traditional mRNA consists of the desired protein encoded in the open reading frame (ORF), flanked on either end by the 5' and 3' untranslated regions (UTRs).⁷ The 3' end contains the polyadenylated (poly(A)) tail and the 5' end contains a cap structure, both of which are important for stability and translation of the mRNA.

Therapeutic mRNAs are developed and manufactured using *in vitro* transcription from DNA templates via RNA polymerase.^{8–10} Although the IVT process is somewhat straightforward from a chemistry perspective, the process and specific attributes of the target transcript must be optimized to avoid unwanted byproducts. Two such undesirable byproducts are truncated RNA and double-stranded RNAs (dsRNAs), both of which can cause undesirable immunogenic effects in the host.

Truncated RNAs are short transcripts that arise from aborted transcription.¹¹ Double-stranded RNAs can arise from a few different mechanisms, resulting in the annealing of complementary sequences and subsequent continued transcription.^{11,12} Both of these IVT byproducts should be minimized as these impurities can compromise both the safety and efficacy of

the mRNA therapeutic by eliciting unwanted immunogenic responses.^{12–14} Codex HiCap RNA Polymerase is proven to limit dsRNA.

Another major byproduct is improperly capped mRNA species. When it comes to improper capping of the mRNA, the 5' cap, a standard component of eukaryotic mRNA, is of central importance. The 5' cap renders the transcript active in cellular functions and allows the host immune system to recognize the mRNA as “self.”¹⁵ Specifically, the cap stabilizes the mRNA transcript¹⁶, mediates splicing¹⁷, is involved in the nuclear export of the transcript¹⁸, initiates translation of the transcript^{19,20}, and mediates mRNA decay rates.²¹ Multiple studies have demonstrated that targeted modifications of the mRNA cap can enhance the translation capability and stability of the transcript as well as significantly reduce innate immune receptor activation.^{22,23}

Implications of mRNA Capping

In vivo, capping is the first modification made to the nascent RNA during transcription and occurs in the nucleus when the transcript is 25–30 nt long.²⁴ Capping of synthetic mRNA produced in an IVT process is accomplished either through enzymatic post-transcriptional capping or using a co-transcriptional cap analog.¹⁵ In post-transcriptional capping, the uncapped IVT product is subjected to a separate enzymatic reaction to add the 5' cap to the full-length transcript. The most widely used version of this capping strategy requires the use of recombinant Vaccinia capping enzyme, a two-subunit enzyme. Although somewhat effective, post-translational capping is not considered ideal for capping synthetic mRNAs as it adds a separate required step downstream of *in vitro* transcription, and the post-transcriptional capping reaction is relatively inefficient for many transcripts, especially in the context of longer transcripts or those with a 3' end complementarity.^{25–27}

Co-transcriptional capping is ideal because it eliminates the additional reaction step, as the 5' cap is added to the transcript during the IVT process. This approach, while simpler from a manufacturing perspective, does possess some inherent complexities that need to be considered. Not all mRNA obtained from the IVT reaction is capped, therefore capping efficiency, or the percentage of mRNA transcript that is successfully capped during transcription, is crucial.

Since low capping efficiency can produce mRNA that has significantly reduced function and can be a potent immunostimulant to the host, multiple process inputs have been investigated to alleviate the issue. High molar excess of the cap analog has been shown to bias the capping reaction toward cap initiation as the cap analog competes with nucleotides as the initiator component.^{15,28} Although improvements in capping efficiency upwards of 70% have been reported, this improvement comes at the expense of both significant increases in process cost due to the cost of the additional cap analog needed, and overall reduction of mRNA product yields. Using high concentrations required for this competition-based strategy is even more cost prohibitive when using advantageous but more expensive cap analogs, such as CleanCap® Reagent AG.

Another potential solution to increase the content of capped mRNA is to remove uncapped species through digestion with a 5'-polyphosphatase-dependent exonuclease.¹⁵ While possible, this approach of using additional enzymatic reactions negates the initial benefit of co-transcriptional capping being a simpler and more cost-effective process.

An additional consideration for co-transcriptional capping includes inverted elongation, where elongation occurs in the reverse direction at the 3'-OH of m7G.¹⁵ These reverse transcripts will fail to be translated and thus can significantly impact potency of the mRNA product.²⁹ The potential for reverse elongation can be eliminated by utilizing anti-reverse cap analogs such as ARCA which

contain modifications that inhibit elongation in the wrong direction.¹⁵

High capping efficiency is crucial for a potent and safe therapeutic mRNA, given the importance of the 5' cap for mRNA transcript functionality and minimizing unwanted immunogenic response. Co-transcriptional capping, from a process simplicity and cost perspective, is the most attractive approach to IVT capping as it eliminates additional post-transcriptional and downstream unit operations. In the end, co-transcriptional 5' capping is ideal for IVT-produced therapeutic mRNA.

Demonstration of Improved IVT Capping Efficiency

As a novel and strategic approach to increasing efficiency of the 5' capping in IVT production of mRNA products, an engineered RNA polymerase was developed to increase the efficiency of the capping reaction. This novel RNA polymerase, Codex® HiCap RNA Polymerase (previously referred to as T7-68 during development work and early publications), was engineered through directed evolution to selectively incorporate di- and tri-nucleotide cap analogs while retaining high mRNA yield.³⁰ These development efforts produced an RNA polymerase with exceptional capping efficiency that enables significant cost savings in the IVT process.

IVT reactions were conducted to express firefly luciferase mRNA and to highlight the enhanced capping efficiency of the Codex HiCap RNA Polymerase. This model, given the size of this

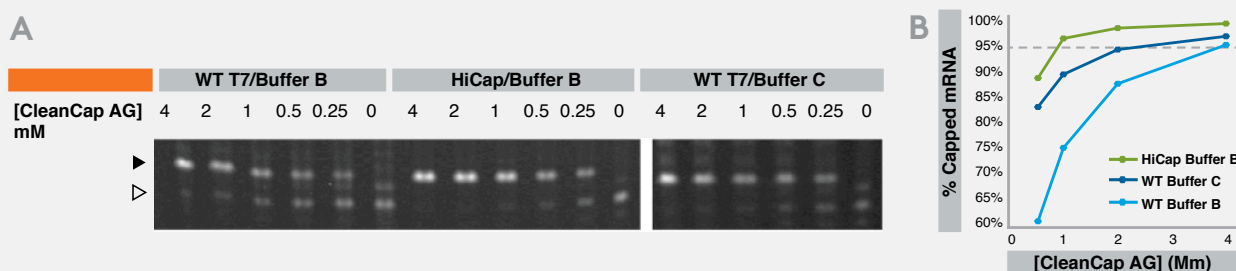


Figure 1. Capping efficiency comparison for CleanCap AG. Trinucleotide analog capping efficiency in different buffers. **(A)** Codex HiCap RNA Polymerase or WT T7 polymerases were used in IVT reactions with 0–4 mM CleanCap AG. IVT products were analyzed by polyacrylamide gel electrophoresis showing the capped species (black arrow) were more prevalent in the Codex HiCap RNA Polymerase-produced mRNAs compared to those produced using WT T7, which demonstrated more uncapped mRNAs (white arrow). **(B)** Densitometry to quantify the extent of mRNA capping showed that Codex HiCap RNA Polymerase was able to produce capping efficiencies of greater than 95% with CleanCap AG concentrations ranging from 1–4 mM, whereas WT T7 only reached more than 95% capping efficiency when using the highest cap analog concentration used in the study.

1.8kb construct, is representative of therapeutic mRNA size and complexity. The capping efficiency of Codex HiCap RNA Polymerase was compared to the popular wild-type T7 (WT T7) polymerase, the most utilized RNA polymerase for IVT, using the trinucleotide analog cap CleanCap Reagent AG.³¹ This cap generally improves capping efficiency over dinucleotide analogs due to the selective incorporation by RNA polymerases compared to nucleotides. CleanCap AG reactions also do not require nucleotide starvation to achieve high capping efficiencies. For these experiments GTP was held constant at 5 mM and IVT reactions were performed across a range of 0–4 mM CleanCap AG. Resulting products were analyzed for capping efficiency via polyacrylamide gel electrophoresis (PAGE) (Figure 1A). It was observed in different buffers that Codex HiCap RNA Polymerase performed consistently well while WT T7 exhibited reduced capping efficiencies, consistently below 95%. In the optimized Buffer B, Codex HiCap RNA Polymerase achieved a 97% capping efficiency, which was equivalent to WT T7 only when using 4 times excess concentration of the cap (Figure 1B).³⁰

Similar studies were performed using dinucleotide caps as well. Titrations of the anti-reverse cap ARCA (Figure 2A) and the symmetrical sCap analogs (Figure 2B) ranging from 0–5 mM, with GTP ratios totaling 6 mM.

This titration range ensures the cap analog becomes limiting, and thus evaluates the true capping efficiency during the IVT reaction. Again, the Codex HiCap RNA Polymerase was compared to the popular WT T7 polymerase. Similar to the CleanCap AG studies, resulting IVT products were analyzed for capping efficiency using PAGE. IVT products for both cap types generated with the Codex HiCap RNA Polymerase exhibited a significant increase in capped mRNA species, especially prevalent in lower cap concentration ranges when compared to the WT T7 polymerase (Figure 2A and 2B).³⁰

To quantify these results, densitometry across the entire dilution series was performed (Figure 2C). Capping efficiencies of Codex HiCap RNA Polymerase-transcribed IVT products in the presence of a 5 mM cap realized >95% efficacy for both cap analogs (Figure 2C). These proved to be significantly better capping efficiencies when compared to IVT products generated with WT T7 polymerase (~81%) for both dinucleotide caps.³⁰ The overall mRNA yield was assessed across this cap/GTP dilution range using a fluorescent intercalating dye assay to determine whether the increased capping efficiency demonstrated by Codex HiCap RNA Polymerase was at the expense of total transcript yield.³⁰ No loss in overall productivity was found with the HiCap polymerase using either ARCA or sCap (Figure 2D).

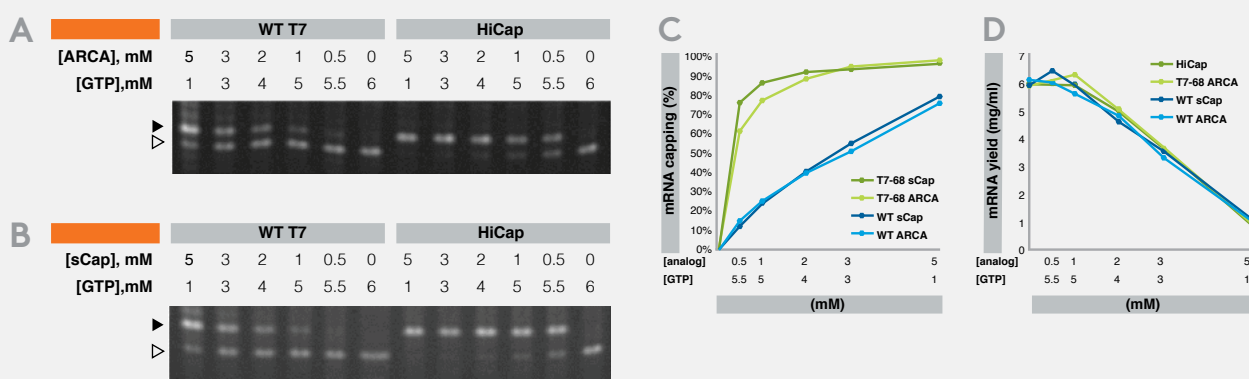


Figure 2. Capping efficiency comparison for sCap and ARCA. Stepwise reduction in cap analog concentrations of ARCA (**A**) and sCap (**B**) determined a stark difference between capped (black arrow) and uncapped mRNA content (white arrow) between HiCap RNA Polymerase and WT T7 polymerase as determined by polyacrylamide PAGE gel electrophoresis. (**C**) Quantification via densitometry over the entire dilution series demonstrated that at cap concentrations of 1mM, HiCap RNA Polymerase produced approximately 3-fold higher capped mRNA relative to WT T7 polymerase. It was determined that this enhanced capping efficiency was not at the expense of overall mRNA yield.

Enhance Safety and Potency

Codex HiCap RNA Polymerase demonstrated superior capping efficiencies of synthetic mRNAs with therapeutic relevance in the context of transcript size and cap types when compared to the popular WT T7 polymerase. These benefits were observed with three different cap analogs (CleanCap AG, ARCA, and sCap). Performing a series of IVT reactions in the presence of stepwise reduced cap concentrations, it was found that the concentration of cap analog was reduced at least 4-fold while still achieving equal, if not better, capping efficiencies.³⁰ Codex HiCap RNA Polymerase exhibited significant advantages over the WT T7 polymerase, thus offering a significant reduction in cap inclusion levels and associated costs of this expensive reagent. And Codex HiCap RNA Polymerase's enhanced capping efficiency didn't compromise the overall mRNA yield.

When the per-base error rate of the Codex HiCap RNA Polymerase was compared to that of the WT T7, an equivalent transcriptional fidelity was demonstrated. Furthermore, the Codex HiCap RNA Polymerase exhibited full compatibility with modified uridine analogs – deimmunizing feature widely recognized as critical for therapeutic mRNAs.³⁰

Codex HiCap RNA Polymerase produced significantly fewer dsRNA byproducts, known to be potent *in vivo* immunogens.¹² Crude Codex HiCap RNA Polymerase-transcribed mRNA exhibited dsRNA byproducts equivalent to that of High Performance Liquid Chromatography (HPLC)-purified WT T7 transcribed mRNAs and a significantly reduced amount compared to crude WT T7 transcribed preps.³⁰ This significant reduction in dsRNA contamination translated to a less stringent immune response in a cell-based assay and an 8-fold higher translation rate.

Overall, the Codex HiCap RNA Polymerase offers better flexibility for IVT process optimization and delivers a more cost-effective route for manufacturing mRNA therapeutics. Significant cost savings are realized through reducing the need for excess concentration of costly cap analogs as well as removing the need for polishing chromatography steps to remove known immunogenic IVT byproducts. Specifically, Codex HiCap RNA Polymerase enabled up to 62% reduction in co-capping reagent demand and the resulting crude mRNA transcript contained less dsRNA than HPLC-purified WT T7 transcribed material.³⁰

Ultimately, Codex HiCap RNA Polymerase has the potential to help mRNA developers deliver safer and more potent therapeutic mRNAs that translate to more efficient clinical development and delivery of these novel therapeutics to patients who need them most.

Contact Aldevron to advance your program with Codex® HiCap RNA Polymerase.

1. Fang, E. *et al.* Advances in COVID-19 mRNA vaccine development. *Signal Transduction and Targeted Therapy* vol. 7 Preprint at <https://doi.org/10.1038/s41392-022-00950-y> (2022).
2. Wang, Y. *et al.* mRNA vaccine: a potential therapeutic strategy. *Molecular Cancer* vol. 20 Preprint at <https://doi.org/10.1186/s12943-021-01311-z> (2021).
3. Miao, L., Zhang, Y. & Huang, L. mRNA vaccine for cancer immunotherapy. *Molecular Cancer* vol. 20 Preprint at <https://doi.org/10.1186/s12943-021-01335-5> (2021).
4. Kate Goodwin. Medicine's Hot New Modality, mRNA, Faces Unclear Regulatory Landscape. <https://www.biospace.com/article/medicine-s-hot-new-modality-faces-unclear-regulatory-landscape/>.
5. Matt Chessum. Biotech sector remains under pressure despite previous COVID gains. <https://www.spglobal.com/market-intelligence/en/news-insights/research/biotech-sector-remains-under-pressure-despite-previous-covid-gains>.
6. Jingtao Zhang. mRNA Development and Manufacturing: CMC Challenges and Solutions. <https://cellculturedish.com/mrna-development-and-manufacturing-cmc-challenges-and-solutions/>
7. Gote, V. *et al.* A Comprehensive Review of mRNA Vaccines. *International Journal of Molecular Sciences* vol. 24 Preprint at <https://doi.org/10.3390/ijms24032700> (2023).
8. Whitley, J. *et al.* Development of mRNA manufacturing for vaccines and therapeutics: mRNA platform requirements and development of a scalable production process to support early phase clinical trials. *Translational Research* vol. 242 38–55 Preprint at <https://doi.org/10.1016/j.trsl.2021.11.009> (2022).
9. Damase, T. R. *et al.* The Limitless Future of RNA Therapeutics. *Frontiers in Bioengineering and Biotechnology* vol. 9 Preprint at <https://doi.org/10.3389/fbioe.2021.628137> (2021).
10. Karikó, K., Muramatsu, H., Ludwig, J. & Weissman, D. Generating the optimal mRNA for therapy: HPLC purification eliminates immune activation and improves translation of nucleoside-modified, protein-encoding mRNA. *Nucleic Acids Res* **39**, (2011).
11. Milligan, J. F., Groebe, D. R., Witherell, G. W. & Uhlenbeck, O. C. *Nucleic Acids Research Oligoribonucleotide Synthesis Using T7 RNA Polymerase and Synthetic DNA Templates*. vol. 15.
12. Mu, X., Greenwald, E., Ahmad, S. & Hur, S. An origin of the immunogenicity of *in vitro* transcribed RNA. *Nucleic Acids Res* **46**, 5239–5249 (2018).
13. Triana-Alonso, F. J., Dabrowski, M., Wadzack, J. & Nierhaus, K. H. Self-coded 3'-extension of run-off transcripts produces aberrant products during *in vitro* transcription with T7 RNA polymerase. *Journal of Biological Chemistry* **270**, 6298–6307 (1995).
14. Gholamalipour, Y., Karunanayake Mudiyansele, A. & Martin, C. T. NAR breakthrough article 3 end additions by T7 RNA polymerase are RNA self-templated, distributive and diverse in character—RNA-Seq analyses. *Nucleic Acids Res* **46**, 9253–9263 (2018).
15. Muttach, F., Muthmann, N. & Rentmeister, A. Synthetic mRNA capping. *Beilstein Journal of Organic Chemistry* **13**, 2819–2832 (2017).
16. Furuichi Yasuhiro, La Fiandra Alba & Shatkin Aaron. 5'-Terminal structure and mRNA stability. *Nature* **266**, 235–239 (1977).
17. Edery, I. & Sonenberg, N. *Cap-Dependent RNA Splicing in a HeLa Nuclear Extract (Cap Analogues)*. *Biochemistry* vol. 82 (1985).
18. Köhler, A. & Hurt, E. Exporting RNA from the nucleus to the cytoplasm. *Nature Reviews Molecular Cell Biology* vol. 8 761–773 Preprint at <https://doi.org/10.1038/nrm2255> (2007).
19. Sonenberg, N. & Hinnebusch, A. G. Regulation of Translation Initiation in Eukaryotes: Mechanisms and Biological Targets. *Cell* vol. 136 731–745 Preprint at <https://doi.org/10.1016/j.cell.2009.01.042> (2009).
20. Müller-Mcnicoll, M. & Neugebauer, K. M. How cells get the message: Dynamic assembly and function of mRNA-protein complexes. *Nature Reviews Genetics* vol. 14 275–287 Preprint at <https://doi.org/10.1038/nrg3434> (2013).
21. Wang, Z., Jiao, X., Carr-Schmid, A. & Kiledjian, M. *The HDcp2 Protein Is a Mammalian mRNA Decapping Enzyme*. *Source* vol. 99 (2002).
22. Daffis, S. *et al.* 2'-O methylation of the viral mRNA cap evades host restriction by IFIT family members. *Nature* **468**, 452–456 (2010).
23. Ishikawa, M., Murai, R., Hagiwara, H., Hoshino, T. & Suyama, K. Preparation of eukaryotic mRNA having differently methylated adenosine at the 5'-terminus and the effect of the methyl group in translation. *Nucleic Acids Symp Ser (Oxf)* **129–130** (2009) doi:10.1093/nass/nrp065.
24. Ramanathan, A., Robb, G. B. & Chan, S. H. mRNA capping: Biological functions and applications. *Nucleic Acids Research* vol. 44 7511–7526 Preprint at <https://doi.org/10.1093/nar/gkw551> (2016).
25. Shuman, S., Surks, M., Furneaux, H. & Hurwitz, J. Purification and characterization of a GTP-pyrophosphate exchange activity from vaccinia virions. Association of the GTP-pyrophosphate exchange activity with vaccinia mRNA guanylyltransferase. RNA (guanine-7)-methyltransferase complex (capping enzyme). *Journal of Biological Chemistry* **255**, 11588–11598 (1980).
26. Paterson Bruce & Rosenberg Martin. Efficient translation of prokaryotic mRNAs in a eukaryotic cell-free system requires addition of a cap structure. *Nature* **279**, 692–696 (1979).
27. Fuchs, A. L., Neu, A. & Sprangers, R. A general method for rapid and cost-efficient large-scale production of 5' capped RNA. *RNA* **22**, 1454–1466 (2016).
28. Vaidyanathan, S. *et al.* Uridine Depletion and Chemical Modification Increase Cas9 mRNA Activity and Reduce Immunogenicity without HPLC Purification. *Mol Ther Nucleic Acids* **12**, 530–542 (2018).
29. 8548660.
30. Miller, M. *et al.* An Engineered T7 RNA Polymerase for efficient co-transcriptional capping with reduced dsRNA byproducts in mRNA synthesis. doi:10.1101/2022.09.01.506264.
31. Wang, W. *et al.* Bacteriophage T7 transcription system: an enabling tool in synthetic biology. *Biotechnology Advances* vol. 36 2129–2137 Preprint at <https://doi.org/10.1016/j.biotechadv.2018.10.001> (2018).



Contact us to speak with a custom protein expert to explore how we can help you today.

Supplying plasmid DNA, mRNA and proteins from research through clinical development

4055 41st Avenue South, Fargo, ND 58104 USA • +1 (701) 297-9256 • Toll-free (U.S. and Canada): +1 (877) 787-3362

aldevron.com



© 2025 Aldevron LLC. All rights reserved. Aldevron, the stylized logo, and the Aldevron product and service marks mentioned herein are trademarks or registered trademarks of Aldevron LLC in the United States and other countries. All other trademarks are the property of their respective owners.
*Codex® is a trademark of Codexis, Inc. Any other third-party trademarks are the property of their respective owners.