The Evolution of
Cap Analogs

ARETERNA
A Synthgene Company

INTRODUCTION

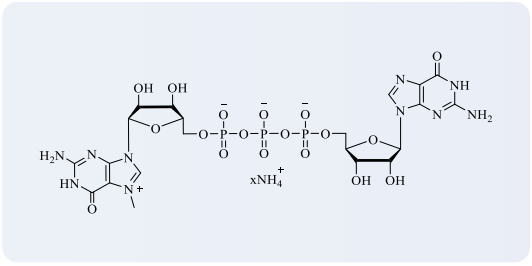
In the intricate world of mRNA synthesis, the cap structure plays a pivotal role in regulating gene expression. Scientists have made significant strides in the development of various cap analogs to enhance mRNA stability, translational efficiency, and therapeutic potential. We'll delve into the different types of cap analogs–m7G cap, ARCA, CleanCap®, and more–exploring their basic principles, advantages, disadvantages, and the factors that guide the choice of one cap over another.

UNDERSTANDING THE BASICS

The mRNA cap is a modified guanosine nucleotide added to the 5' end during transcription. This modification serves as a critical determinant for mRNA stability and efficient translation. The classic cap structure, m7G cap, features a methylated guanosine linked to the mRNA via a 5'-5' triphosphate bridge.

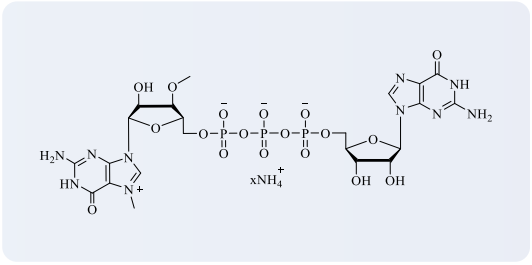
m7GpppG is the first generation of cap analog.

The RNA or DNA chain is extended from 5'-end to 3'-end by the monophosphate bridge. If you say CpG, we know the C and G are on the same strand rather than C-G pairing. If you think of 5'-end as head and 3'-end as tail, an RNA chain would be something like 5'-GpGpApG-pNpNp…3' from head to tail. But the m7GpppG is connected by two heads (5'-5') hence has two 3'-end tails, either tail can be extended by the RNA Polymerase in the In-Vitro Transcription (IVT) reaction. If the next nucleotide is added in the wrong direction, i.e. on the m7G instead of the G, the IVT product will have no functioning cap because it lacks the m7 modification. That is the problem.



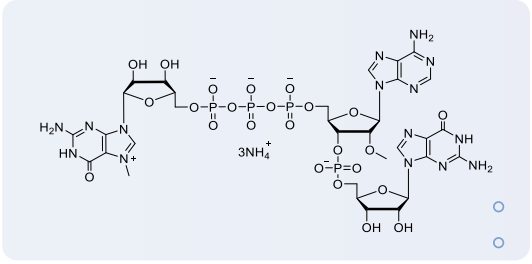
ARCA (Anti-Reverse Cap Analog) is the solution.

By adding a methyl group to the 3'-end of m7G, it prevents the mRNA chain from extending in the wrong direction. With ARCA, more functional mRNAs can be made from one IVT reaction. But ARCA has its own problems, 1) ARCA can only generate Cap0 structure, not Cap1; and 2) GTP competes with ARCA for the first position on the mRNA chain.



The emergence of trinucleotide cap analogs

Cap1 means the first nucleotide of mRNA has 2'-O-methyl modification. This modification is found in all higher eukaryotic mRNAs and marks mRNA as "self". Attempt was made to modify ARCA with 2'-O-methyl group, however, once the 2'-poisiton was occupied by a methyl group, the chain extension on the 3'-position would be hindered. The solution to Cap1 is trinucleotide cap, for example m7GpppAmG as shown on the right.



Areterna-LLC
sales@areterna.com
15810 Gaither Drive, Suite 230
Gaithersburg, MD 20877

V1.0 202312

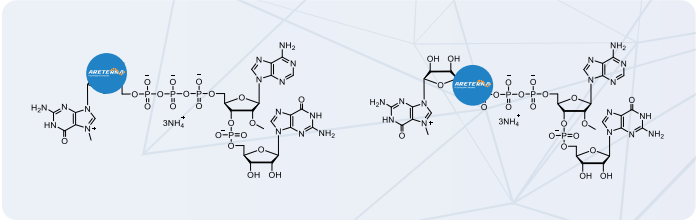
By adding an un-methylated G after the 2'-O-methyl-A, the mRNA chain can be extended from the terminal G as its 2'-OH allows the addition of the next nucleotide. This new cap, with AG initiating mRNA transcription, will not compete with GTP for chain initiation, so capping efficiency and IVT yield can be improved over ARCA.

The first report of the m7GpppAmG structure was by Yamaguchi et al. in 1984 (https://pubmed.ncbi.nlm.nih.gov-/6369253/). Then in 2009 Ishikawa et al. published a paper (https://pubmed.ncbi.nlm.nih.gov/19749294/) citing the usage of this cap analog to make luciferase mRNA by IVT.

TriLink first commercialized this m7GpppAmG cap analog under the name of CleanCap® Reagent AG -(N-7113). A variation of this molecule, CleanCap® Reagent AG (3'-O-Me) - (N-7413), was used to manufacture the COVID-19 vaccine by Pfizer/BioNTech (https://www.nature.com/articles/s41586-020-2814-7)

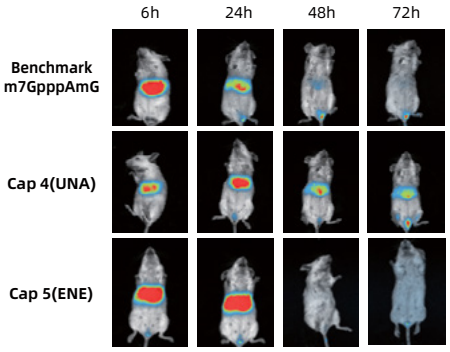
Areterna's novel trinucleotide cap analogs

Areterna's parent company Synthgene was founded in 2018 with a mission to democratize mRNA vaccine. Since cap analogs contribute substantially to the total material cost for synthesizing mRNA, the company has devoted a big portion of its R&D effort to develop new cap analogs, two of which are highlighted here.



Both CAP4 and CAP5 are variations of m7GpppAmG trinucleotide. The innovation is on the m7G cap, with CAP4 having a UNA (unlocked nucleic acid) structure and CAP5 having a double bond between the ribose ring and the first phosphate group.

We made 3 different luciferase mRNA constructs, each with different cap analogs, namely CAP4, CAP5 and the benchmark m7GpppAmG, using the same DNA templates and the same IVT conditions. The mRNAs were then encapsulated with LNPs and injected into mice. Pictures were taken at different time points to assess luciferase protein expression.



The mRNA capped with Benchmark m7GpppAmG showed strong luciferase signal at the 6-hour time point, but the luciferase signal was almost all gone at 48 hours. In contrast, CAP4 mRNA showed weaker expression at 6 hours, but the protein expression impressively persisted beyond 72 hours. CAP5 mRNA gave the highest protein expression initially among the 3 constructs and the strong luciferase signal carried from 6 hours to 24 hours, then died down at 48 hours. Thinking of applications, CAP4 would be great for protein replacement where persistent expression is desirable, and CAP5 would be ideal for gene editing where the effector protein gets made quickly, does the cutting and disappears to avoid off-target effects.

CONCLUSION

The cap analogs have evolved from m7G cap, ARCA to trinucleotides. While the first-generation dinucleotides suffer from the Cap0 limitation and unsatisfactory IVT yield, the trinucleotides, particularly m7GpppAmG variants, offer Cap1 structure, high capping efficiency and improved yield. The new generation CAP4 and CAP5 are fine-tuned for different therapeutic applications. These two cap analogs will be made in GMP grade and offered at competitive price to enable wider adoption of mRNA as vaccines and therapeutics.

References

1. Kazuo Yamaguchi, Iwao Nakagawa, Mitsuo Sekine, Tsujiaki Hata, Kunitada Shimotohno, Michiyo Hiruta, Kin-ichiro Miura, Chemical synthesis of the 5'-terminal part bearing cap structure of messenger RNA of cytoplasmic polyhedrosis virus (CPV): m7G5'pppAmpG and m7G5'pppAmpGpU, *Nucleic Acids Research*, Volume 12, Issue 6, 26 March 1984, Pages 2939–2954, https://doi.org/10.1093/nar/12.6.2939

2. Masahide Ishikawa, Ryuta Murai, Hiroyuki Hagiwara, Tetsuya Hoshino, Kamui Suyama, Preparation of eukaryotic mRNA having differently methylated adenosine at the 5'-terminus and the effect of the methyl group in translation, *Nucleic Acids Symposium Series*, Volume 53, Issue 1, September-October 2009, Pages 129–130, https://doi.org/10.1093/nass/nrp065

3. Sahin, U., Muik, A., Derhovanessian, E. et al. COVID-19 vaccine BNT162b1 elicits human antibody and TH1 T cell responses. *Nature* 586, 594–599 (2020). https://doi.org/10.1038/s41586-020-2814-7

BIORON

Distributed in Europe by

BIORON GmbH
In den Rauhweiden 20
67354 Römerberg
Germany

Phone +49 623 229845-0
Fax +49 623 229845-29
info@bioron.de
www.bioron.de

V1.0 202312