CASE STUDY

GenScript
Scripting Possibilities

Enhancing mRNA therapeutics with 2'OEt Cap1 AG

A modified cap1 analog designed to improve translational output, slow decapping kinetics, and support mRNA stability *in vivo*.

Challenge

Synthetic mRNA delivered via LNPs has proven successful in vaccines. However, expanding this platform to other therapies-such as protein replacement or *in vivo* CAR-T-faces challenges, including the need for high, frequent dosing to achieve therapeutic efficacy. Even with a natural 5′ cap structure, synthetic mRNA encounters bottlenecks in performance.

Two key limitations hinder current cap analogs: potency and durability. Unlike vaccines, mRNA therapeutics require ~10-fold higher protein expression for efficacy, necessitating novel designs with improved translational efficiency.

Additionally, sustained protein expression is critical. Natural Cap1 mRNA is susceptible to decapping enzymes, leading to rapid degradation. This shortens mRNA half-life *in vivo* and limits cumulative protein output over time.

Solution

We sought to determine whether a modified cap structure could:

- Increase protein expression *in vitro* and *in vivo* across multiple payloads
- Slow decapping kinetics to extend mRNA half-life and improve *in vivo* stability
- Integrate seamlessly into existing IVT protocols without process changes
- Perform consistently across diverse mRNA constructs and payload types

This IVT kit enables preparing an mRNA with a modified cap1 structure, m7G(5′)pppA(2′-O-ethyl)pG, 2'OEt Cap1 AG for short. 2′-O-methyl group at the N1 ribose, found in standard cap1 analogs, is replaced with a bulkier 2′-O-ethyl group. This modification slows decapping kinetics, contributing to extended mRNA half-life and improved stability *in vivo*.

Introduction

2'OEt Cap1 AG addresses two fundamental limitations of conventional cap1 analogs: incomplete translational output and susceptibility to cellular decapping. By substituting a bulkier ethyl group at the 2′-O position of the N1 ribose, this modified cap analog slows the kinetics of decapping enzymes while preserving efficient ribosome engagement. The result is a cap structure that enhances both peak protein expression and expression duration across diverse model systems.

GenScript evaluated 2'OEt Cap1 AG across a range of cell types, payloads, and delivery formats, comparing performance directly against standard cap1 constructs under identical conditions.

Case Studies:

1. mRNA with 2'OEt Cap1 AG modification enables stronger and longer mRNA expression than mRNA with natural Cap1 *in vivo.*

In first experiment, we prepared Fluc with four different cap molecules, named Cap1, 2'OEt Cap1 AG, 3'OMe Cap1, and M6A Cap1 analogues (Figure 1) by co-transcriptional capping method [1] with N1-methyl-pseudoU modification. We then encapsulated the Fluc mRNA with different caps into standard 4-component SM102-LNP [2], and then IV injected to 4-6 week old Balb-C mice at the dose of 0.3 mg/kg. The expression of Fluc mRNA were detected at different time points by IVIS method following IP Intra-peritoneal injection of luciferin. The result showed that Fluc mRNAs capped with 2'OEt Cap1 AG showed higher protein expression than mRNAs capped with standard cap1 analog, or 3'OMe Cap1, M6A Cap1.

Figure 1. Structure of Cap1, 3'OMe Cap1, 2'OEt Cap1 AG, and M6A Cap1 analogues

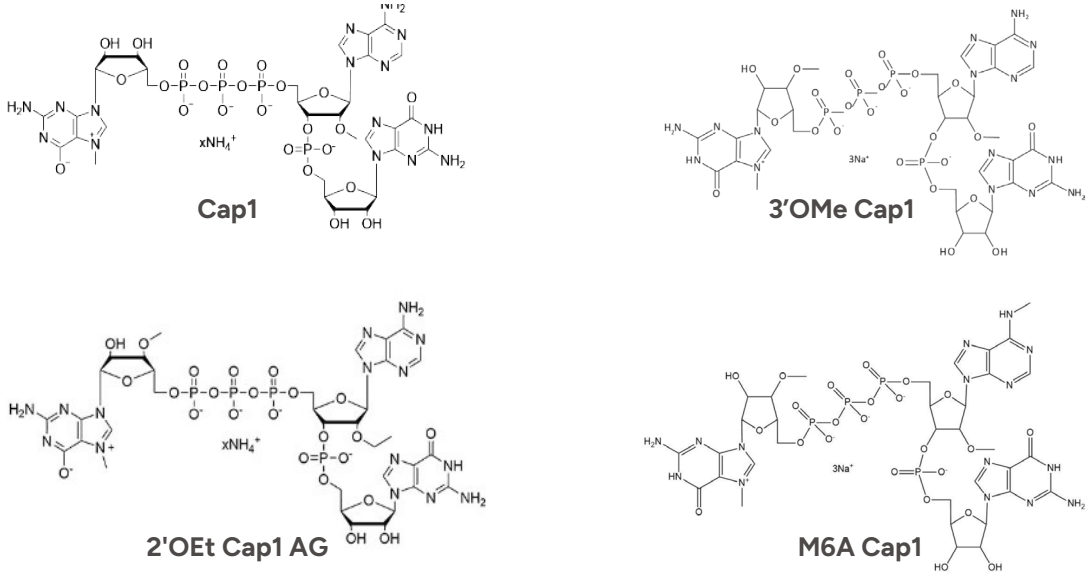
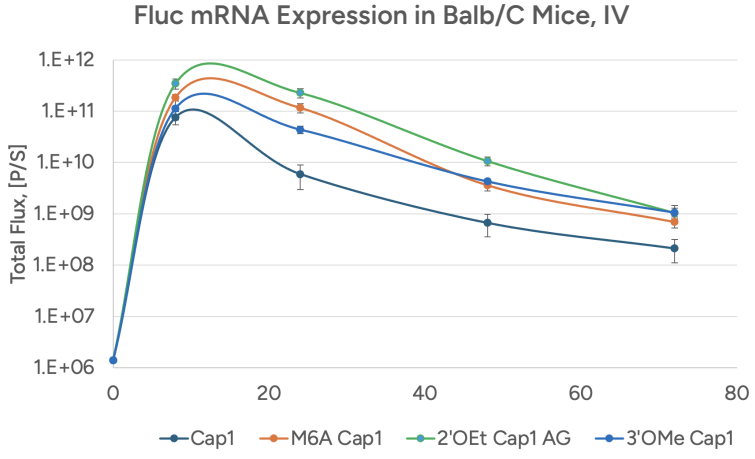


Figure 2. Fluc mRNA with Cap1, 3'OMe Cap1, 2'OEt Cap1 AG, and M6A Cap1 expression in mice after IV injection.

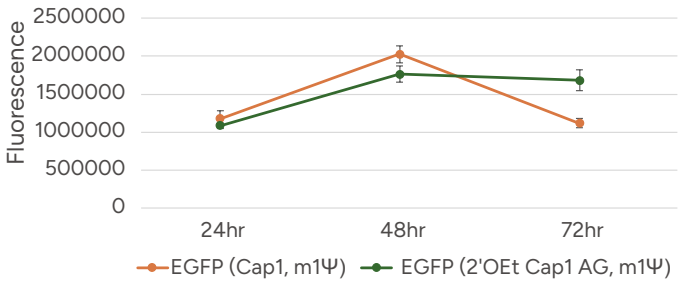


2. mRNA with 2'OEt Cap1 AG modification enables stronger and longer mRNA expression than mRNA with natural Cap1 *in vitro*.

We further explored if 2'OEt Cap1 AG mRNA can have higher protein expression in other difference genes of interest. We prepared EGFP mRNA [3], Fluc mRNA [4] and espCas9 mRNA[5] with both 2'OEt Cap1 AG and natural Cap1 analogues by IVT method, and tested their expression in A549 Cells at different time points. 2'OEt Cap1 AG shows higher and more durable expression across different payloads in EGFP mRNA, Fluc mRNA and espCas9 mRNA(Figure 3A-C).

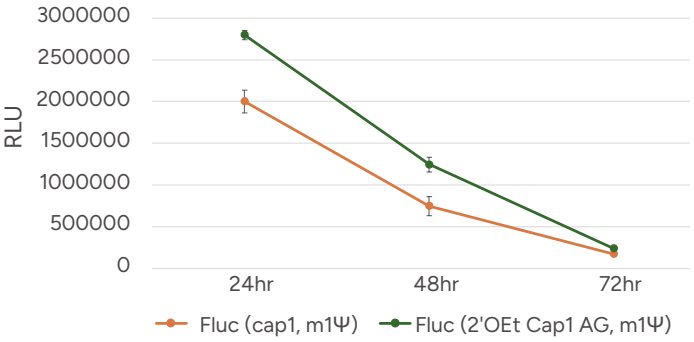
Across *in vitro* cell models, mRNAs incorporating 2'OEt Cap1 AG demonstrated consistently higher protein expression compared to standard cap1 controls across different payloads. mRNA peak fluorescence values were elevated, reflecting enhanced translational efficiency.

Figure 3A. EGFP mRNA expression in A549 cells



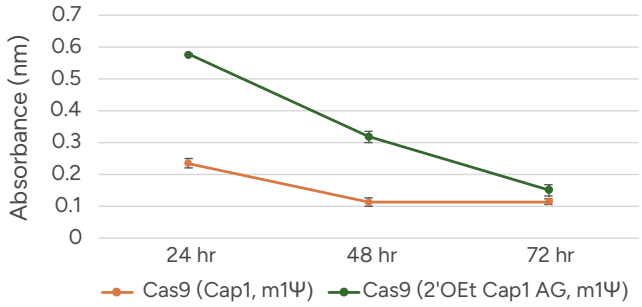
EGFP mRNA [1] were transfected into A549 Cells at 100 ng per well in 96 well plate with lipofectamine messenger-max, and their expression were detected after 24, 48, 72 hr by SpectraMax ID3 plate reader for its fluorescence with excitation at 485 nm, emission at 535 nm

Figure 3B. Fluc expression in A549 cells



Fluc mRNA [2] were transfected into A549 Cells at 100 ng per well in 96 well plate with lipofectamine messengermax, and their expression were detected after 24, 48, 72 hr by SpectraMax ID3 plate reader with Firefly luciferase reporter assay reagent (Promega E4030)

Figure 3C. Cas9 expression in A549 cells



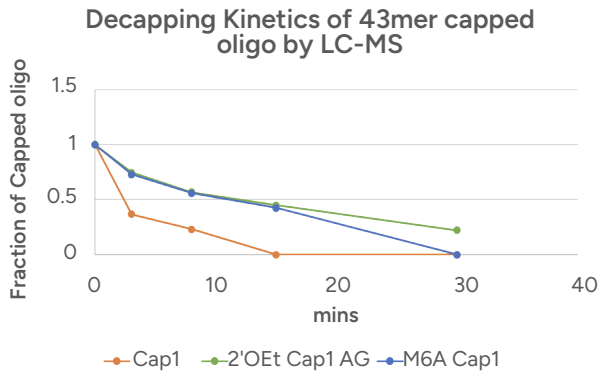
espCas9 mRNA [3] were transfected into A549 Cells at 1 ug per well in 24 well plate with lipofectamine messengermax, and their expression were detected after 24, 48, 72 hr with FastScan Cas9 *(S. pyogenes)* ELISA Kit (Cell signaling, 29666C).

3. Decapping Kinetics and mRNA Stability

The 2'OEt Cap1 AG substitution confers measurable resistance to cellular decapping enzymes. This translates to extended mRNA half-life in cell-based systems, with expression persisting at elevated levels relative to standard cap1 mRNA at later timepoints.

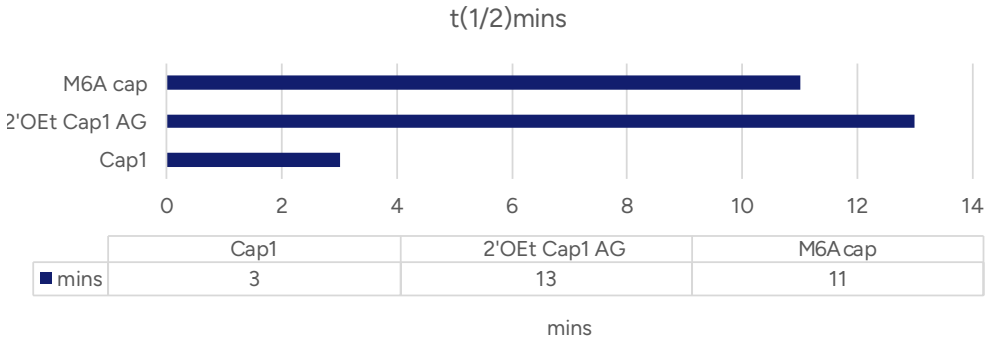
To understand why 2'OEt Cap1 AG mRNA shows higher *in vitro* and *in vivo* expression and durability than natural Cap1 mRNA, we studied the decapping kinetics of differently capped oligos. A 43-mer oligo was chemically capped with natural Cap1, 2'OEt Cap1 AG, or M6A Cap1, and its decapping kinetics were measured against the yDcpS scavenger decapping enzyme (NEB) by LC-MS. Following NEB's protocol for yDcpS, 50 µL aliquots of the decapping reaction mixture were taken at 1, 3, 8, 15, and 30 minutes and 1 hour, then heat-inactivated at 70 °C for 15 minutes. The oligos were purified using magnetic beads and analyzed by LC-MS to determine the fraction of intact capped oligo at each time point (Figure 4A and B). The natural Cap1 oligo had the shortest half-life (~3 min), the M6A Cap1 oligo ~11 min, and the 2'OEt Cap1 AG oligo the longest at ~13 min.

Figure 4A. *In-vitro* decapping assay for different caps



mRNA decapping susceptibility was assessed using NEB Human mRNA Decapping Enzyme (NEB #M0608) [4], with reactions performed in 1X mRNA Decapping Enzyme Reaction Buffer in a 20 µl total reaction volume at 37°C for 1 hour. Decapping efficiency was quantified by LC-MS using 43 nt chemically capped oligonucleotides, with 2'OEt Cap1 AG constructs compared directly against standard cap1 controls under identical conditions.

Figure 4B. Half- Life of different caps



Cap half-life was determined by one phase decay analysis of decapping kinetics over time. The standard cap1 analog showed a half-life of 3 minutes, while M6A extended this to 11 minutes. 2'OEt Cap1 AG demonstrated the greatest resistance to decapping with a half-life of 13 minutes, consistent with the bulkier ethyl group sterically hindering decapping enzyme access to the 5′ cap.

Conclusion

2'OEt Cap1 AG delivers meaningful improvements in both peak protein output and expression duration compared to standard cap1 analogs, without requiring changes to upstream IVT or downstream purification workflows. 2'OEt Cap1 AG integrates seamlessly into existing co-transcriptional IVT protocols as a drop-in replacement, requiring no changes to reaction conditions, nucleotide ratios, or purification steps. Performance is consistent across diverse payloads and construct designs, making it suitable for a broad range of mRNA therapeutic and vaccine applications. These gains translate directly into:

- Reduced mRNA dose requirements to achieve equivalent therapeutic protein levels
- Extended functional windows for cell-based assays and in vivo studies
- Improved confidence in construct comparison and candidate selection
- A drop-in upgrade path compatible with existing manufacturing processes

Future Direction

These results position 2'OEt Cap1 AG as a compelling enhancement to existing mRNA therapeutic platforms. Ongoing evaluation will explore performance across additional therapeutic payloads, cell types, and *in vivo* delivery formats. As mRNA applications expand in complexity and scale, the ability to improve translational output and mRNA durability through targeted cap modification offers a practical and broadly applicable strategy for next-generation mRNA design.

References:

1. GenScript IVT Kit manual.PDF
2. RP-A00025, GenLNP-S01-Fluc mRNA (m1Ψ) manual.PDF
3. RP-A00009, eGFP mRNA (Cap1, m1Ψ) manual. PDF
4. RP-A00023, Fluc mRNA (Cap1, m1Ψ) manual. PDF
5. RP-A00018, espCas9 mRNA (Cap1, m1Ψ) manual. PDF
6. Grudzien-Nogalska E, et al. New Insights into Decapping Enzymes and Selective mRNA Decay. Wiley Interdiscip Rev RNA. 2017;8(1):e1379.