Technical Note

In vivo expression of exogenous mRNA synthesized with co-transcriptional capping

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INTRODUCTION

The mRNA platform for biotechnology and drug development spans vast applications due to its effectiveness, flexibility, and speed.[1-4] However, *in vivo* activity of exogenously delivered mRNA depends on several factors, including delivery mechanism, minimal innate immune response activation, and translation of the message into a functional protein.

mRNA translational efficiency depends in part on the 5' cap structure of the mRNA. Obtaining a proper cap structure can present technical challenges during *in vitro* synthesis. Therefore, chemically synthesized co-transcriptional cap analogs, such as TriLink's CleanCap® products, advance the manufacturability and accessibility of mRNA products.

In this technical note, we compare the *in vivo* expression of luciferase mRNA synthesized *in vitro* with co-transcriptional capping via cap analogs resulting in three unique cap structures: ARCA (Cap0,) CleanCap Reagent AG (Cap1) and CleanCap Reagent AG (3' OMe) (Cap1.) Lipid nanoparticle encapsulated mRNA delivered to mice demonstrate higher expression with the CleanCap Cap1 trinucleotide caps compared with the ARCA dinucleotide Cap0 capped mRNA. Improved expression and manufacturing advantages make CleanCap Cap1 structures the ideal mRNA cap for in vivo use.

WHY USE CLEANCAP FOR IN VIVO mRNA EXPRESSION?

The 5' cap serves several important biological functions, including translation initiation, mRNA stability, and self-recognition.[5-8] Therapeutically administered mRNAs that closely mimic host mRNAs best avoid unwanted immune responses and achieve the highest expression. Historically, enzymatic reactions to cap synthetic RNA led to long synthesis times, risk of low product quality, and high cost. Alternatively, co-transcriptional capping with a cap analog allows for a streamlined mRNA synthesis but first-generation synthesis methods generate a Cap0 structure while eukaryotic mRNAs have a Cap1 structure.

The need for an updated process gave rise to novel cap analogs, such as the CleanCap trinucleotide, which generates a Cap1 structure at greater than 90% capping efficiency without sequence and low yield limitations.[9,10] The CleanCap trinucleotide approach vastly improves synthetic mRNA manufacturing capabilities.

With high synthesis yields, excellent mRNA integrity, and low dsRNA contaminants, the CleanCap process produces an mRNA poised for the best possible *in vivo* expression.

In this technical note, we demonstrate the efficacy of *in vivo* mRNA expression of co-transcriptional synthesis approaches by directly comparing mRNAs synthesized via ARCA or CleanCap methods.

MATERIALS AND METHODS

mRNA Synthesis and Analysis

Firefly luciferase mRNA was transcribed with CleanCap Reagent AG, CleanCap Reagent AG (3' OMe), or ARCA. Crude mRNA yields and mRNA integrity were assessed by analytical IP-RP-HPLC against a standard curve (UltiMate 3000 HPLC, Thermo Fisher).

LNP Formulation

mRNA samples were encapsulated into GenVoy-ILM lipid nanoparticles using the NxGen microfluidic platform (Precision NanoSystems, Vancouver, BC). Total mRNA content and encapsulation efficiency (Quant-iT RiboGreen RNA Assay, Thermo Fisher) and mRNA integrity (Agilent 2100 BioAnalyzer, Agilent) were quantified before injection.

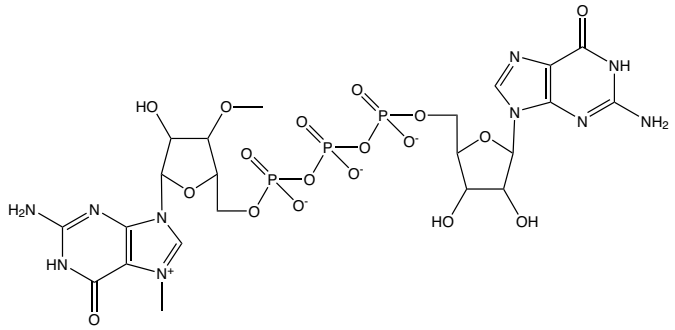
In vivo Delivery

Female CD-1 mice (8–10 weeks old) were randomized into a group based on body weight (Charles River Laboratories, Discovery Research Services, North Carolina). LNP:mRNA test articles were diluted in PBS to deliver 1 mg/kg in a single bolus by tail-vein injection. Luciferase activity was measured by whole-body bioluminescence imaging on the IVIS Spectrum CT system (Perkin Elmer, Greenville, SC) at seven time points post mRNA injection with D-luciferin intraperitoneal injection 10 minutes prior to imaging sessions (150 mg/kg total).

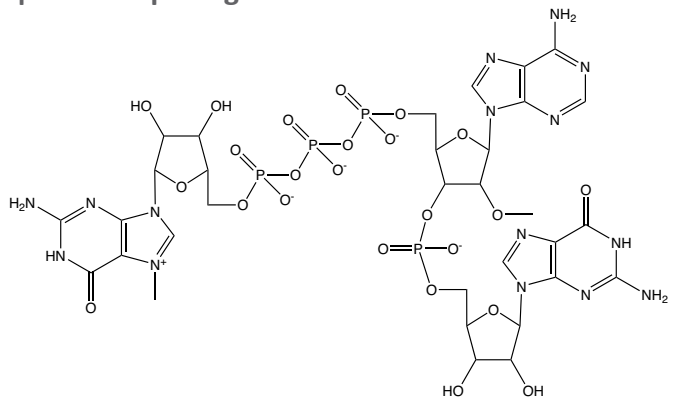
RESULTS & DISCUSSION

Here, we compare the manufacturability and *in vivo* expression levels from Firefly luciferase (Fluc) mRNA synthesized by two capping strategies: co-transcriptional ARCA dinucleotide GG Cap0 and co-transcriptional trinucleotides CleanCap Reagent AG and CleanCap Reagent AG (3’ OMe) (Figure 1). This study reflects the advantages of the CleanCap co-transcriptional trinucleotide cap analogs for in vivo mRNA applications

A | ARCA



B | CleanCap Reagents AG



C | CleanCap Reagents AG (3’ OMe)

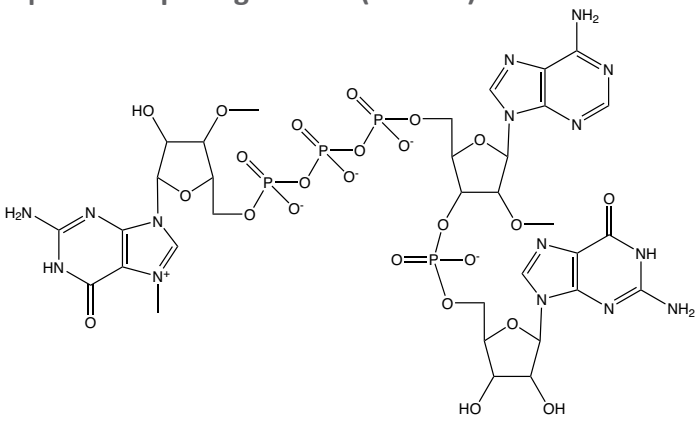


FIGURE 1. Cap Structures. A) Anti-reverse cap analog (ARCA), a Cap0 dinucleotide. B) CleanCap Reagent AG, a Cap1 trinucleotide. C) CleanCap Reagent AG (3’ OMe), a Cap1 trinucleotide.

In vitro mRNA Synthesis and Capping Manufacturability

All mRNA samples were assessed for yield, capping efficiency, quality, and dsRNA content.

TABLE 1. QC assessments of *in vitro* mRNA synthesis with different cap analogs

Cap Analog Name	Cap type	Relative Yield	Capping Efficiency	Relative mRNA Integrity	dsRNA content
ARCA	Cap0	Lowest	Lowest	Lowest	Highest
CleanCap Reagent AG	Cap1	High	High	High	Lower
CleanCap Reagent AG (3' OMe)	Cap1	High	High	High	Lower

In Vivo mRNA Expression

mRNA samples were encapsulated into lipid nanoparticles (LNPs) and evaluated for similar particle size, polydispersity, zeta potential, and encapsulation efficiency before use. LNPs were delivered to CD-1 female mice as a single 1 mg/kg teil-vein bolus. No deaths or extreme body weight loss occurred during the study.

Whole body bioluminescent imaging at 3, 6, 9, 12, 24, 48, and 96 hours post-injection assayed for luciferase mRNA expression (**Figure 2, 3**). Quantified luciferase expression levels (integrated photons/second) across the entire 96 h period (**Figure 4**) reflect the visible trends in whole-body imaging (**Figure 2 and 3**).

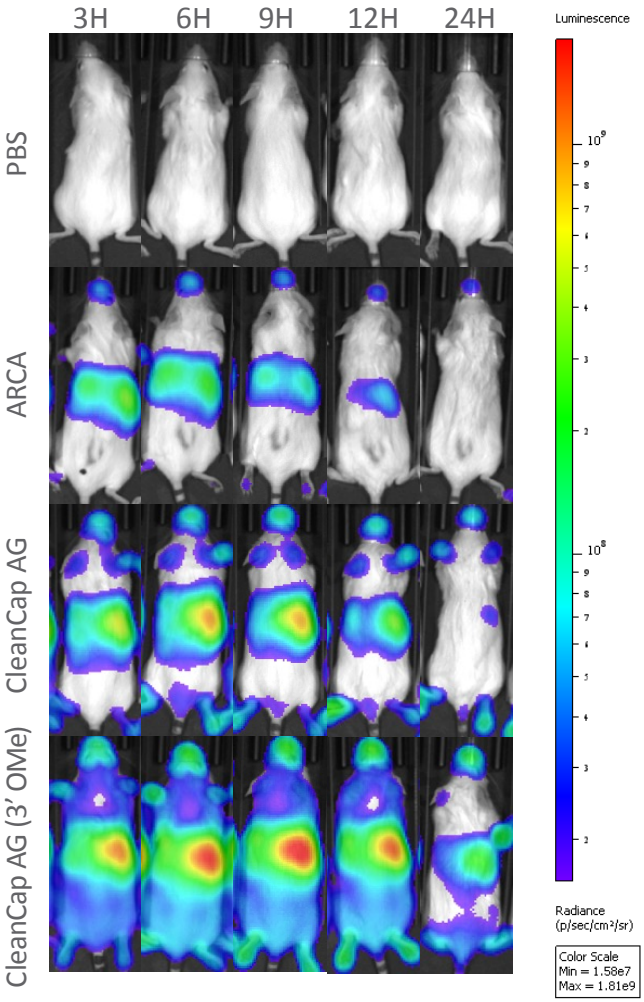


FIGURE 2. ***In vivo*** **mRNA expression across whole mouse body.** One representative animal (n=5 per cohort) was imaged at 3, 6, 9, 12, and 24 hours post mRNA delivery with a luminescence scale. Representative animal chosen as the animal with the flux value closest to the median of all animals at the last time point.

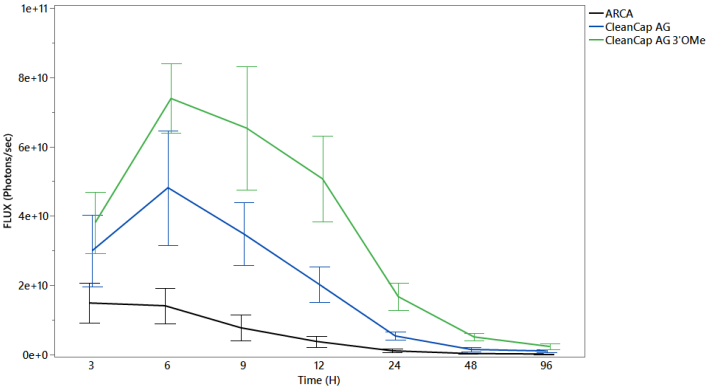


FIGURE 3. Mean luminescence signal (Flux in photons/second) with standard deviation for all mRNA test subjects over all time points.

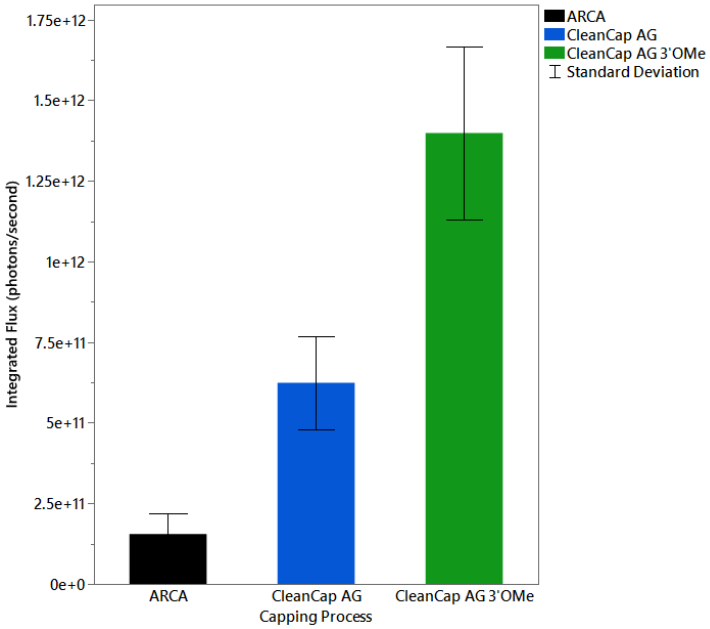


FIGURE 4. Integrated luminescent signal from N=5 animals across all time points displayed as mean with standard deviation. Two-tailed student's t-test was performed for all comparisons. ARCA signal is significantly lower than all other mRNAs (***p<0.0001, *p<0.01). CleanCap Reagent AG (3' OMe) is significantly higher than CleanCap Reagent AG (**p<0.001).

The CleanCap analogs show significantly higher in vivo expression levels than the ARCA mRNA. Expression levels for the CleanCap analogs peaked at 6 h post-delivery, while the ARCA analog expression was highest (at a much lower level) from 3-6 h (**Figure 2 and 3**). Fluc with the CleanCap Reagent AG (3' OMe) performed the best with the quickest expression onset and the highest overall, most systemic, and most prolonged expression of the three mRNAs (**Figure 2–4**).

CONCLUSION

In this Application Note, we demonstrate the advantages of CleanCap analogs for manufacturing and improved *in vivo* expression compared to ARCA. In addition, the data here show that the CleanCap Reagent AG (3' OMe) trinucleotide permits the highest, most widely distributed, and longest sustained *in vivo* expression of FLuc mRNA. This *in vivo* expression pattern is complemented by the simplicity and efficiency of its manufacturing. The CleanCap Reagent AG (3' OMe) is a highly proven[1], first-class capping analog for in vivo applications.

ACKNOWLEDGMENTS

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