Impact of cap structures on the performance of *in vitro*–transcribed mRNAs

Exploring strategies for optimal capping to maximize mRNA potency

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Introduction

Capping is a crucial modification of *in vitro*–transcribed mRNAs, enhancing their stability, facilitating translation initiation, and enabling self/non–self-discrimination in therapeutic applications. At its most basic level, capping involves the addition of a 7-methylguanosine (^{m7}G) to the 5' end of the mRNA, generating a Cap-0 structure which is often recognized as non-self in higher eukaryotes. The first transcribed base can also be methylated at the 2' ribose position to generate the more common Cap-1 structure found on endogneous mRNAs in higher eukaryotes.

mRNA capping can occur post-transcriptionally through the action of specific enzymes (enzymatic capping) or co-transcriptionally, as exemplified by TriLink's CleanCap® technology. Enzymatic capping takes place after *in vitro* transcription (IVT) and typically uses Vaccinia Capping Enzyme (VCE) to add ^{m7}G to the 5' end followed by a separate 2'-O-methyltransferase enzyme to modify the first transcribed base and convert Cap 0 to Cap 1. It often requires purification after IVT, increasing processing time and potential yield loss.

Co-transcriptional capping, in contrast, is a one-step process that adds the ^{m7}G cap to the nascent mRNA transcript as it is being synthesized by RNA polymerase. Early versions of co-transctriptional capping such as anti-reverse cap analog (ARCA) utilize a dinucleotide cap with reduced capping efficiencies around 70% and generate a Cap 0, which can result in reduced potency and unwanted innate immunogenicity. This has been improved with CleanCap technology in which a trinucleotide cap analog such as ^{m7}GpppA$_m$pG is added to initiate and cap mRNAs during synthesis. Compared to ARCA, CleanCap technology revolutionizes mRNA synthesis by creating a Cap-1 structure in a single-step reaction with ≥95% efficiency while improving mRNA translation in both *in vitro* and *in vivo* settings[1,2].

In this tech note, we evaluate the quality attributes and protein expression of mRNAs synthesized using CleanCap® AG (original analog), CleanCap® AG 3' OMe (upgraded analog), and CleanCap® M6 (latest analog). Additionally, we compare the efficacy of CleanCap M6 with the enzymatic method. Our findings provide insights into the therapeutic potential of mRNAs capped with CleanCap M6.

Evaluation of mRNA quality attributes with enzymatic capping and CleanCap technology

Enzymatic capping has traditionally been used to modify synthetic RNA. However, CleanCap technology, introduced in 2017, addresses challenges associated with enzymatic capping, such as extended processing times, potential for low yield, and higher costs[3].

CleanCap technology enables co-transcriptional capping while allowing modifications to the cap's chemical structure for enhanced functionality. For instance, 3'-O-methylation of the ^{m7}G ribose facilitates proper cap incorporation[4], while N6-methylated adenosine can increase protein expression at least in part by inhibiting decapping[5]. Following the development of CleanCap AG, we subsequently introduced CleanCap AG 3' OMe and, more recently, CleanCap M6 (**Figure 1**). In our latest peer-reviewed publication[6], we have shown that CleanCap M6 increases mRNA stability, at least in part by impairing Dcp2-mediated decapping.

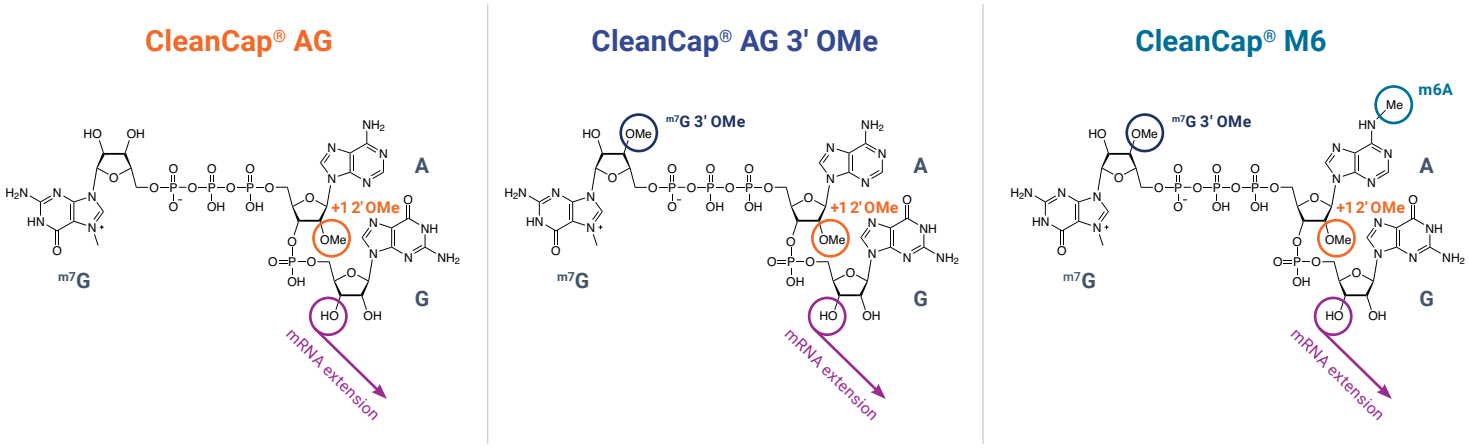

Figure 1. Structures of CleanCap cap analogs for mRNA. CleanCap AG has a 2'-O-methyl modification on the +1A which confers the Cap-1 structure. CleanCap AG 3' OMe additionally features a 3'-O-methyl modification on the ^{m7}G. CleanCap M6 includes an m6A modification on the +1A of CleanCap AG 3' OMe.

Before investigating their functionality, we first compared the quality attributes of mRNAs capped using different analogs. We examined full-length integrity, capping efficiency, and double-stranded RNA (dsRNA) levels, and found comparable mRNA quality across all three cap analogs (**Table 1**).

Following their standard protocols, the enzymatic and CleanCap capping typically yield approximately 5 mg/mL of crude mRNA. However, with CleanCap analog's optimized protocols — such as our proprietary **CleanScript® IVT method** using CleanCap AG or CleanCap AG 3' OMe[7], or the **pulse-feed protocol** with CleanCap M6[8] — we can achieve mRNA yields as high as 10 mg/mL.

Table 1. Comparing mRNA quality attributes across different capping methods. Firefly luciferase (FLuc) mRNAs were synthesized by IVT, capped using different analogs, and incorporated with N1-methylpseudouridines in place of uridines. Their mRNA integrity was analyzed by IP-RP-HPLC, capping efficiencies were determined by LC-MS, and dsRNA levels were assessed by J2 immunoblot.

Capping method (Cap 1)	Full-length integrity	Capping efficiency	dsRNA (J2 dotblot)
Enzymatic	92.8%	99%	<10 ng/µg
CleanCap AG	93.3%	97%	<5 ng/µg
CleanCap AG 3'OMe	94%	96%	<5 ng/µg
CleanCap M6	97%	97%	<5 ng/µg

Assessment of protein expression from mRNAs with three CleanCap analogs

To investigate the influence of 5' cap structures on protein expression, we compared mWasabi mRNAs with CleanCap AG 3' OMe or CleanCap M6 *in vitro*. We selected CleanCap AG 3' OMe for the comparison with CleanCap M6 since we had previously shown CleanCap AG 3' OMe performed better than ARCA and CleanCap AG[2]. We transfected the mRNAs into HeLa and HEK293T cell lines, measured fluorescence at 24 hours post-transfection, and found the M6 modification promoted higher protein expression in both cell lines (**Figure 2**).

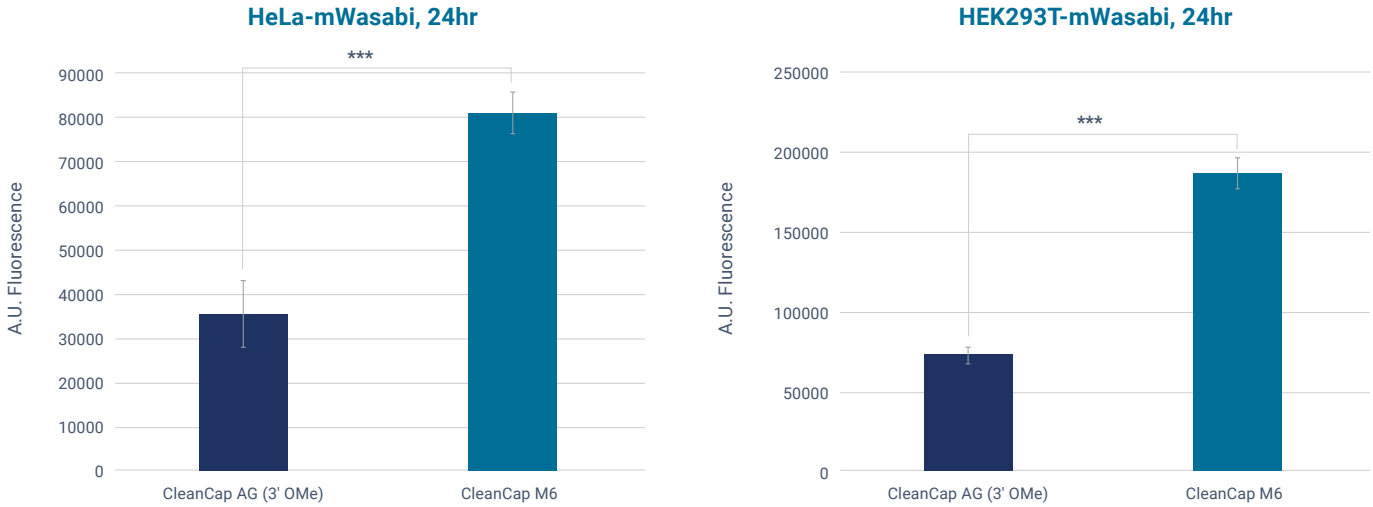

Figure 2. Protein expression in cultured cells. mWasabi mRNA was capped with either CleanCap AG 3' OMe or CleanCap M6, transfected into HeLa and HEK293T cells (n = 5 technical replicates per group), and measured for fluorescence at 24 hrs post transfection. (*** $p < 0.001$, t-tailed *t*-test. Error bars are standard error of mean.)

Next, to examine protein expression *in vivo*, we encapsulated N1-methylpseudouridine–modified FLuc mRNAs that were capped with CleanCap AG, CleanCap AG 3' OMe, or CleanCap M6 into lipid nanoparticles (LNPs) and administered them through tail vein injection to female CD-1 mice (n = 5 per group). We then monitored luciferase activity for 48 hours using whole-body bioluminescence imaging at various time points. Consistent with the *in vitro* data, CleanCap M6–capped mRNAs exhibited significantly higher luminescence over time compared to those capped with CleanCap AG and CleanCap AG 3' OMe (**Figure 3**).

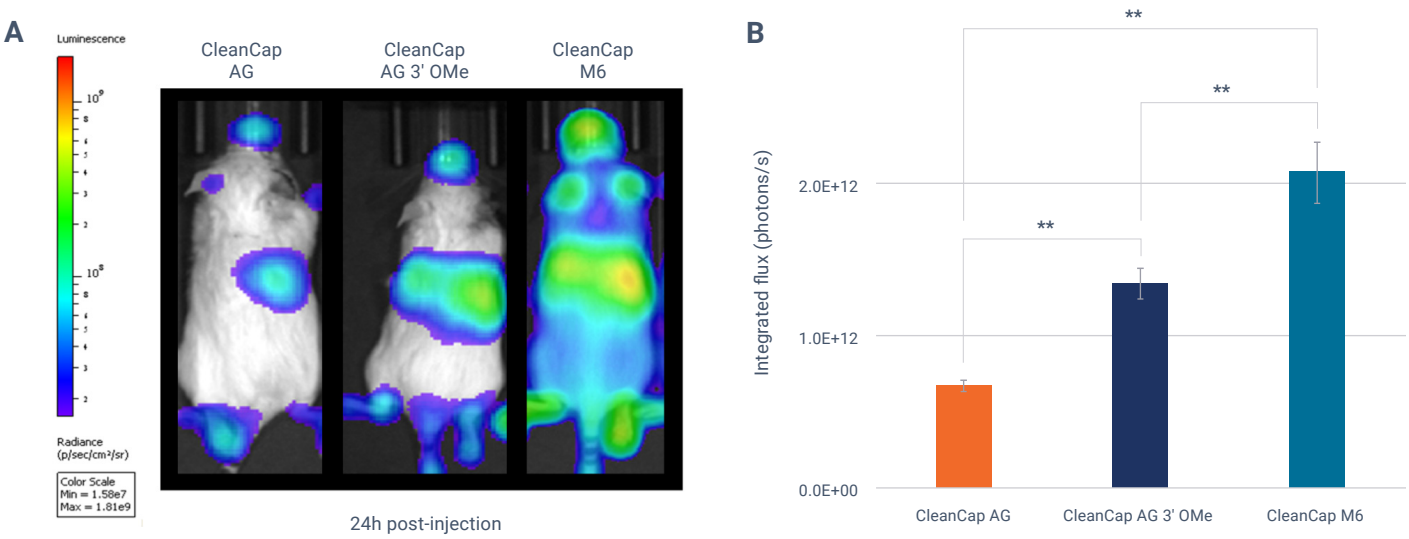

Figure 3. *In vivo* protein expression from mRNA with three CleanCap analogs. (A) N1-methylpseudouridine–modified, uridine-depleted FLuc mRNA was transcribed with the indicated cap analogs. 1 mg/kg dose of mRNA:LNP was delivered to mice by tail vein injection. Representative mouse per cohort by whole-body luciferase activity at 24 hrs post delivery is shown. **(B)** Measurement of the total luciferase activity from six timepoints (3-48 hrs post mRNA:LNP delivery) using area-under-the-curve calculations is shown. (*** $p < 0.01$, one-way ANOVA; error bars are standard error of mean.)

In vivo comparison of protein expression using CleanCap M6 and enzymatic capping

We investigated the performance of CleanCap M6, our most potent analog, *in vivo* by comparing it to enzymatically capped mRNA. Notably, enzymatic production of the M6 modification is currently not possible due to enzyme limitations, and thus the enzymatically capped mRNA is a canonical Cap-1 cap. Similar to previous experiments,

we injected LNP-encapsulated FLuc mRNAs into mice (n = 9 per group) via tail vein and subsequently measured luciferase activity over 48 hours post-injection. The results indicate higher protein expression with CleanCap M6 (**Figure 4**), suggesting that the M6 modification strongly enhances the potency of mRNA compared to enzymatic capping.

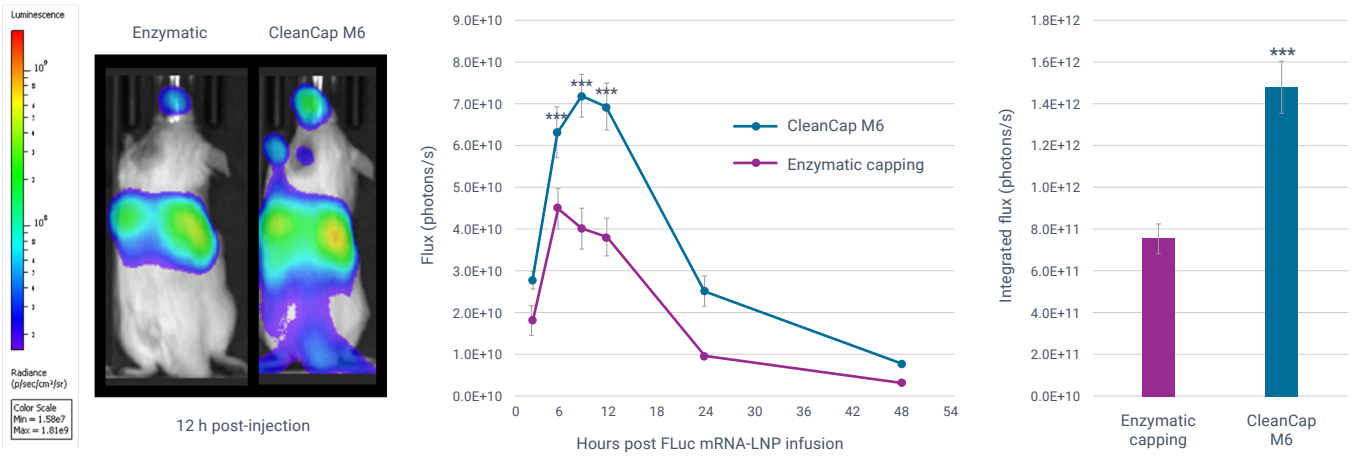

Figure 4. *In vivo* protein expression of CleanCap M6 and enzymatically capped mRNAs. (A) Whole-body luciferase activity of mice after 12 hrs post injection of 1 mg/kg FLuc mRNA:LNP is shown. The only difference between the two group was mRNA capping strategy. **(B)** Time-course measurements of luciferase activity after FLuc mRNA:LNP delivery are plotted. (*** $p < 0.001$, two-tailed *t*-test. Error bars are standard error of mean. n = 9/group) **(C)** Measurement of the total luciferase activity from six timepoints (3-48 hrs post mRNA:LNP delivery) using area-under-the-curve calculations is shown. (*** $p < 0.001$, two-tailed *t*-test. Error bars are standard error of mean. n = 9/group)

Reduced dosing with CleanCap M6 mRNA

To explore potential dose-sparing benefits, we compared CleanCap M6 mRNA to CleanCap AG mRNA in mice. We administered equal doses (1 mg/kg) of both mRNA types and then introduced a significantly lower dose (0.3 mg/kg) of CleanCap M6 mRNA.

We consistently observed increased protein expression with CleanCap M6 when equal doses of both mRNA types were administered at 1 mg/kg (**Figure 5**). Furthermore, our findings revealed that CleanCap M6 mRNA, at a dose of 0.3 mg/kg, achieved comparable *in vivo* expression levels to CleanCap AG mRNA at 1 mg/kg. This suggests the potential for reduced dosing requirements. Reducing the necessary dose while maintaining effectiveness can lead to cost savings and may minimize dose-related side effects.

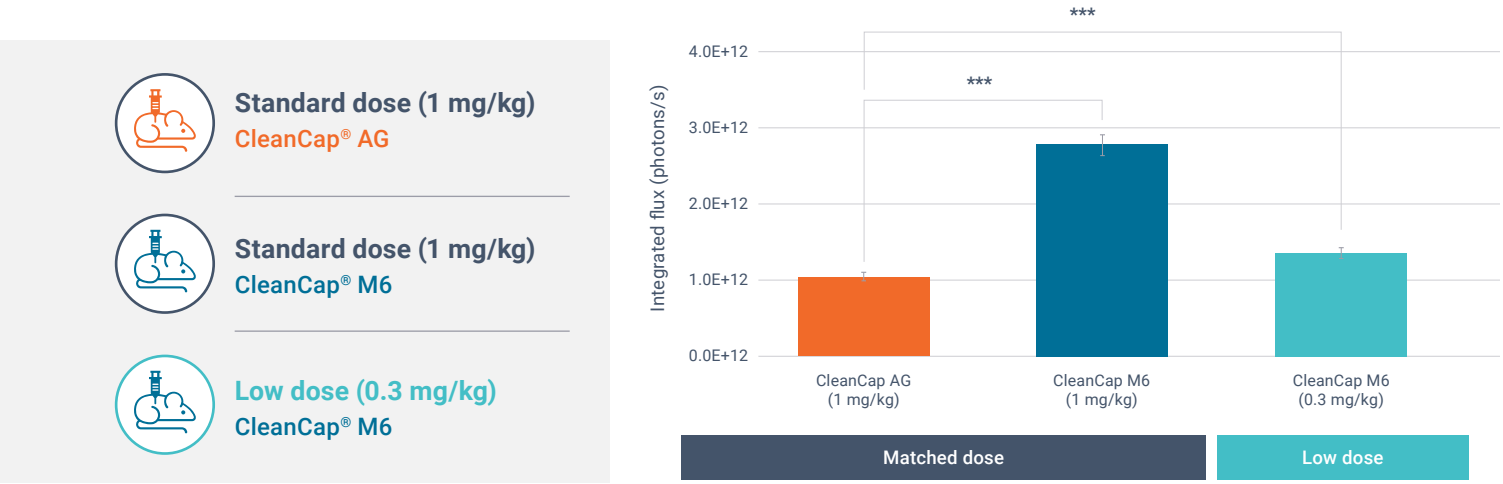

Figure 5. M6 modifications allow for lower mRNA dosing regimes to obtain similar protein expression levels. (*** $p < 0.001$, one-way ANOVA. Error bars are standard error of mean. n = 7/group).

Comparing capping methods for protein replacement

To evaluate the impact of capping on protein replacement therapy (a major application of mRNA therapeutics), we tested CleanCap AG 3' OMe and CleanCap M6 with human erythropoietin (hEPO) mRNA[9]. We encapsulated hEPO mRNA with either cap into LNPs and administered them to C57BL/6NCrl mice. We then measured serum hEPO levels by ELISA. The results indicated that CleanCap M6 mRNA consistently produced higher levels of hEPO protein compared to CleanCap AG 3' OMe mRNA (**Figure 6**), suggesting its potential superiority for protein replacement applications.

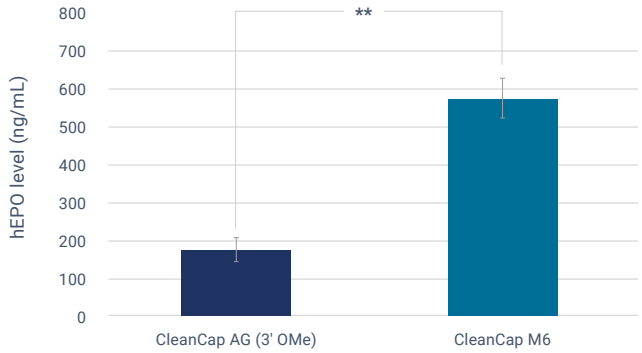

Figure 6. hEPO serum level in mice after hEPO mRNA delivery. LNP-formulated mRNA was tail vein–delivered to the mice at 0.1 mg/kg (n = 5 per group). Serum was collected 24 hrs post delivery and measured for hEPO by ELISA. (** $p < 0.005$, student's *t*-test. Error bars are standard error of mean.)

Conclusion

This technical note evaluates the performance of mRNAs with CleanCap cap analogs and enzymatic capping. CleanCap technology, which enables co-transcriptional capping and chemical modifications, demonstrates several advantages.

- **Improved mRNA quality:** mRNAs capped with CleanCap technology demonstrate comparable or improved quality attributes to enzymatically capped mRNAs. Low dsRNA levels were also observed with CleanCap M6.
- **Enhanced protein expression:** CleanCap M6 modification consistently leads to higher protein expression *in vitro* and *in vivo*, outperforming enzymatic capping and other CleanCap analogs.
- **Dose-saving potential:** CleanCap M6 mRNA can achieve comparable *in vivo* expression at one-third mRNA per dose compared to CleanCap AG mRNA.
- **Promising for protein replacement:** CleanCap M6 mRNA outperforms CleanCap AG 3' OMe mRNA in protein replacement applications, producing higher levels of hEPO protein.

Overall, CleanCap technology, particularly with the M6 modification, offers a promising approach for improving mRNA therapeutics through low dsRNA, enhanced protein expression, and potential for reduced dosing.

References

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