Enhanced mRNA production by *in vitro* transcription and co-transcriptional capping

Increasing mRNA yields using CleanCap® M6 pulse-feed protocol

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Introduction

Recent innovations in mRNA design have accelerated the clinical application of several mRNA-based therapeutics. Included among these breakthroughs, TriLink's CleanCap® technology facilitated the development of BNT162b1, the highly successful Pfizer/BioNTech COVID-19 vaccine[1]. As the field of mRNA medicine evolves, continued advancement of CleanCap technology will be key to meeting the future needs of researchers, drug developers, and mRNA manufacturers.

TriLink's original CleanCap analog, CleanCap AG, was developed to overcome known challenges—such as low capping efficiencies, limited IVT yields, and the formation of Cap-0 structures—when using dinucleotide cap analogs (e.g. ARCA) for mRNA capping. By using a 2' O-methyl nucleoside that becomes the +1 base of mRNA in a trinucleotide cap analog to directly confer a Cap-1 structure (**Figure 1A**) and optimizing the cap analog with the DNA template start site, CleanCap AG can be co-transcriptionally incorporated to provide higher mRNA yields with improved capping efficiencies.

The CleanCap family was subsequently expanded with CleanCap AG 3' OMe (**Figure 1B**), which additionally features a 3' O-methylation on the inverted m7G ribose and enhances translation. When luciferase mRNA samples capped with CleanCap AG 3' OMe and encapsulated with lipid nanoparticles (LNP) were intravenously delivered to mice, the 3' OMe modification was shown to increase *in vivo* protein expression than CleanCap AG[2].

CleanCap M6 (**Figure 1C**), the latest addition to the CleanCap portfolio, was based on the hypothesis that an m6A modification adjacent to the m7G cap can further boost protein expression[3]. The structures of all three CleanCap analogs are shown in **Figure 1** and the increased mRNA potency afforded by CleanCap M6 can be seen in **Figure 2**. This technical note reviews the *in vitro* transcription (IVT) kinetics of CleanCap M6, including those associated with a supplementary pulse-feed protocol to increase IVT yields and help lower the costs associated with mRNA manufacturing.

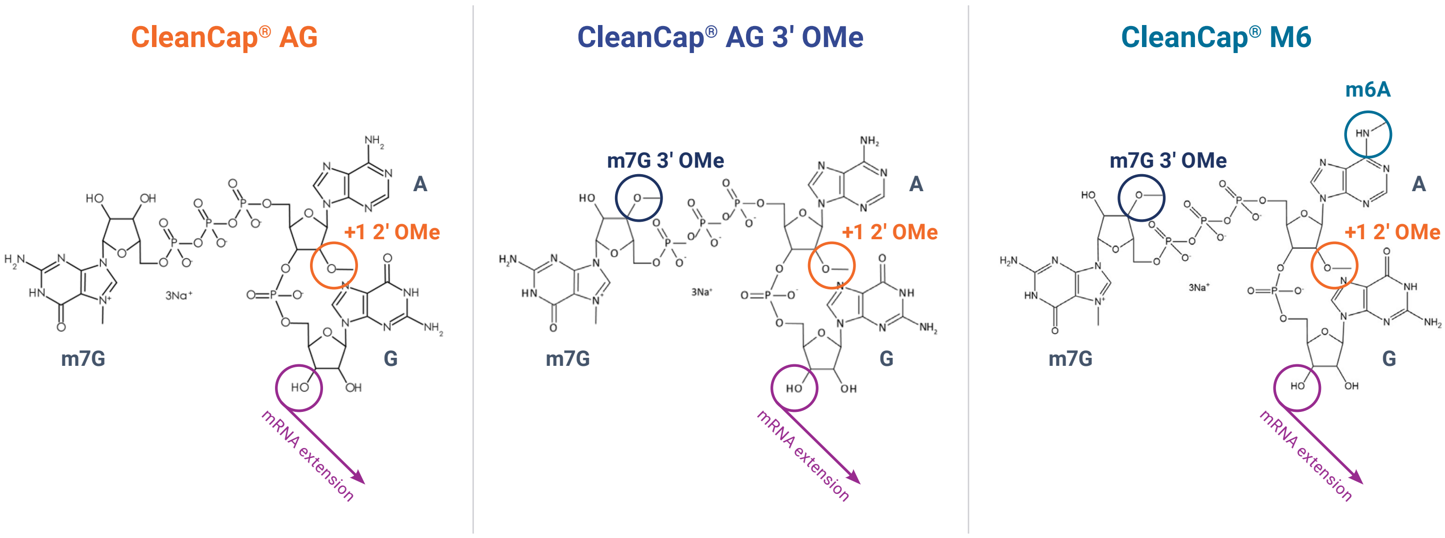

Figure 1. The CleanCap portfolio. CleanCap AG has a 2' OMe modification on adenosine which confers the Cap-1 structure. CleanCap AG 3' OMe additionally features a 3' OMe modification on the inverted m7G and enhances translation. CleanCap M6 includes an m6A modification on the +1 base of the mRNA and further boosts potency in certain applications.

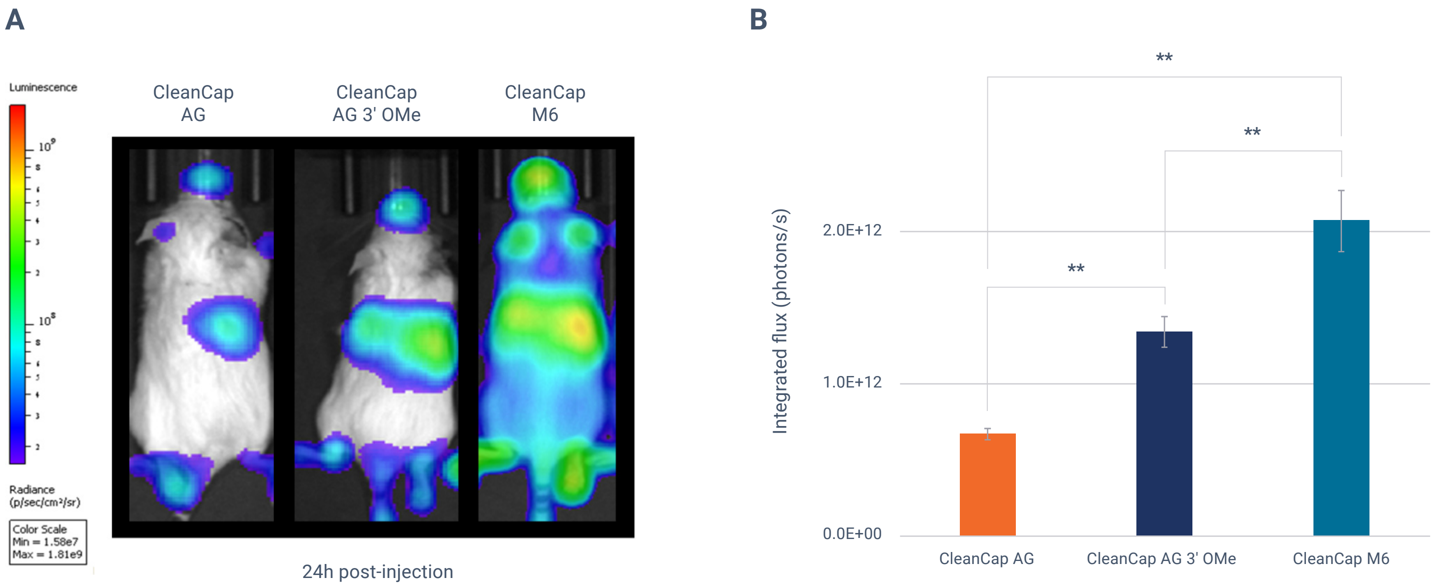

Figure 2. More potent mRNA from CleanCap M6 capping. (A) N1-methylpseudouridine (N1MePsU)-modified, uridine-depleted firefly luciferase (FLuc) mRNA was transcribed with the indicated cap analogs. 1 mg/kg dose of mRNA:LNP was delivered to mice by tail vein injection (n = 5/group). Luciferase activity (photons per second) of the whole body was measured 24h post mRNA:LNP delivery following the luciferin substrate injection. (B) Quantitation of areas under the curve for total luciferase from 6 timepoints over 3-48h post mRNA:LNP delivery (*** p < 0.01, one-way ANOVA; error bars are standard error of mean.)

Results and discussion

Optimized CleanCap M6 IVT

In traditional IVT reactions, CleanCap M6 faces challenges in transcription efficiency compared to CleanCap AG and CleanCap AG 3' OMe. Specifically, CleanCap M6 does not outcompete ATP or GTP as effectively during initiation, resulting in capping efficiency of <70%. With low capping efficiency, the potency of transcribed mRNA in cells is expected to decrease[4], leading to fewer protein molecules translated per mRNA dose. Therefore, we performed a series of optimization IVT experiments using our catalog mRNA sequences to identify optimal manufacturing conditions.

The optimization process resulted in ~5 mg/mL crude IVT reaction yields with desirable critical quality attributes or CQAs, including high mRNA integrity, high capping efficiency, and low double-stranded (dsRNA). dsRNA is an unwanted by-product of IVT reactions with the potential to trigger innate immune responses. The crude mRNA can be purified by scalable downstream processes such as oligo dT capture and tangential flow filtration (TFF).

One of the critical goals of this optimization was to avoid the need for nucleoside triphosphate (NTP) starvation when transcribing co-transcriptionally capped M6 mRNAs because NTP starvation produces low yields similar to ARCA reactions. The optimized process resulted in capping efficiency of ≥95% when using 10 mM of CleanCap M6 with HCl in reaction buffer. Final optimized IVT conditions can be found on the **CleanCap M6 product insert** and highlighted in **Table 1**.

Table 1. Highlights of the optimized IVT reaction conditions of CleanCap M6 compared to CleanCap AG and CleanCap AG 3' OMe analogs.

Same	Different
• Start site on the DNA template • T7 RNA polymerase (wildtype) • Scalable (1 mg to ≥20 g) • ≥95% capping efficiency	• 10x reaction buffer • CleanCap analog to NTP ratio

IVT kinetics using CleanCap M6 and its supplementary pulse-feed protocol

In order to maximize the use of CleanCap M6 and increase the mRNA yield, a pulse-feed IVT reaction was developed as a user-friendly way to continue transcribing the chosen mRNA while leveraging the excess cap analog required to drive high cap initiation (**Figure 3**).

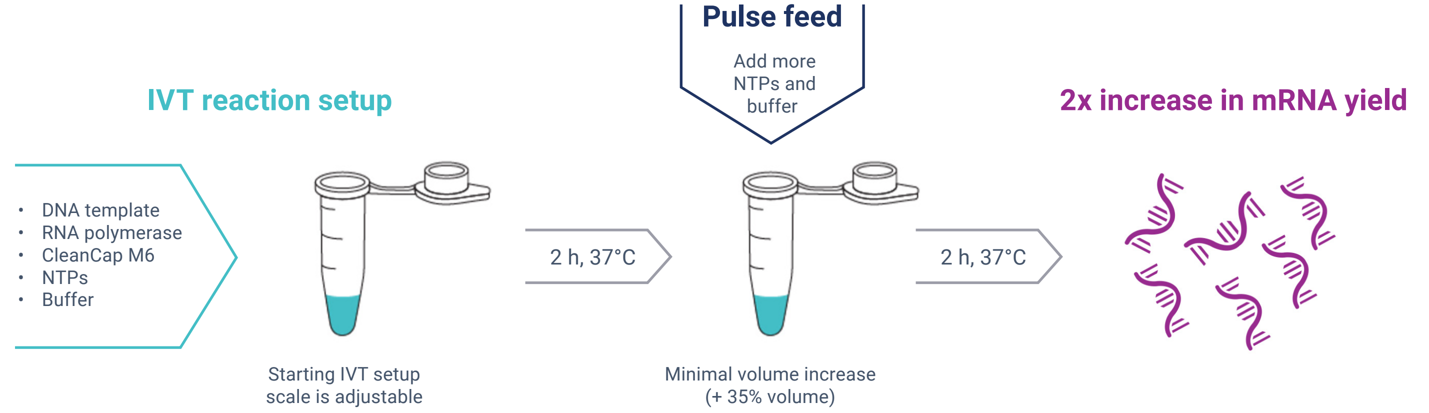

Figure 3. Pulse-feed IVT process flow for high-yield reactions.

We reasoned that because free NTPs are depleted significantly faster than free CleanCap M6 within an IVT reaction, a "second round" of transcription can occur *in vitro* with minimal substrate replacements. By monitoring NTP consumption over the course of the reaction, we observed that ATP, CTP, and GTP were depleted to very low levels (0.5 to 1 mmol) by 2 hours (**Figure 4A**). For uridine-depleted constructs, the UTP depletion was less pronounced. This global free NTP depletion corresponded to a plateau in mRNA production.

As conjectured, by spiking in additional NTPs and 10x reaction buffer at the 2-hour timepoint and further incubating at 37°C, the IVT reaction continued a linear trajectory, resulting in a 2-fold increase in the mRNA yield without the need for additional CleanCap M6, DNA template, or T7 polymerase (**Figure 4B**).

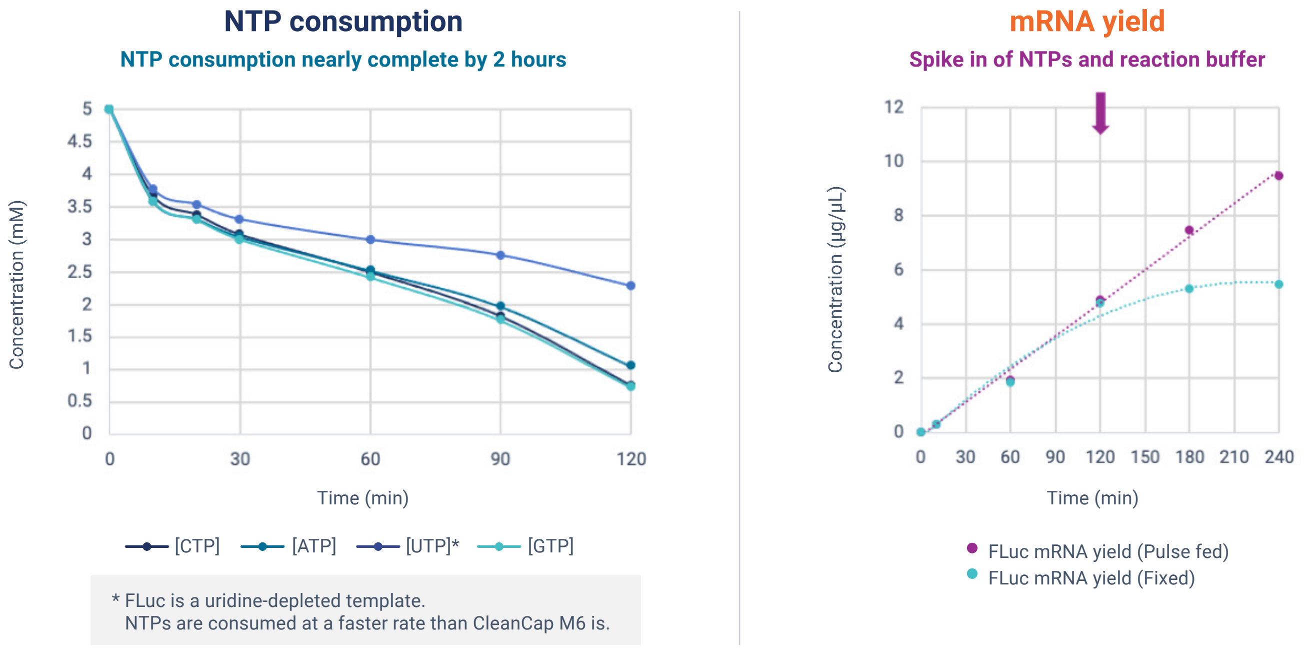

Figure 4. IVT kinetics with CleanCap M6. (A) NTP depletion rate within a uridine-depleted FLuc IVT reaction using optimized CleanCap M6 conditions at 37°C. Aliquots were taken at the indicated time points and analyzed for free NTPs by analytical anion exchange HPLC. (B) FLuc mRNA yield over time during an optimized FLuc M6 IVT reaction; fixed indicates no addition of NTPs and reaction buffer. Aliquots were taken at indicated time points and LiCl precipitated for a UV-vis concentration reading to determine mRNA yields.

Critical quality attributes for standard and pulse-feed CleanCap M6 protocols

To determine if the extra incubation time at 37°C or the addition of more reaction buffer and NTPs impacted the synthesized mRNA, three constructs were tested for the same CQAs measured in the initial IVT optimization. Our results showed all CQAs were maintained while the pulse-feed protocol increased the reaction yield by approximately 2x (**Table 2**). Additionally, a J2 immunoblot displayed no observable differences in relative dsRNA levels between the two protocols. (**Figure 5**).

Table 2. Comparison of CQAs between standard and pulse-feed CleanCap M6 IVT protocols. The pulse-fed protocol almost doubles the reaction yield for all three constructs tested, with no significant differences in product quality.

		Average IVT results (n ≥ 3)							
		IVT reaction yield		Crude mRNA integrity (before oligo dT capture)		Capping efficiency		Crude relative dsRNA	
Constructs	Length	Standard (2-3 hr)	Pulse-fed* (4 hr)	Standard (2-3 hr)	Pulse-fed (4 hr)	Standard (2-3 hr)	Pulse-fed (4 hr)	Standard (2-3 hr)	Pulse-fed (4 hr)
eGFP	1 kb	~4.4 mg/mL	~8.5 mg/mL	~90%	~87%	~98.4%	~97.8%	<0.5 ng/µg	<0.5 ng/µg
FLuc	2 kb	~5.1 mg/mL	~9.2 mg/mL	~83%	~82%	~98.9%	~97.2%	<0.5 ng/µg	<0.5 ng/µg
Cas9	4.5 kb	~4.0 mg/mL	~9.5 mg/mL	~70%	~67%	~97.6%	~98.4%	<0.5 ng/µg	<0.5 ng/µg

- *mg RNA per mL starting IVT reaction volume; mRNA integrity by IP-RP-HPLC; capping efficiency by LC-MS; relative dsRNA by J2 immunoblot.
- Crude indicates LiCl precipitation of mRNA prior to analysis. No affinity chromatography or RP-HPLC purification was applied.
- Results were averages from WT and N1MePsU-modified reactions for yield and capping efficiency, WT only for integrity, and N1MePsU for dsRNA.
- All IVT reactions were run at 37°C for indicated incubation time.

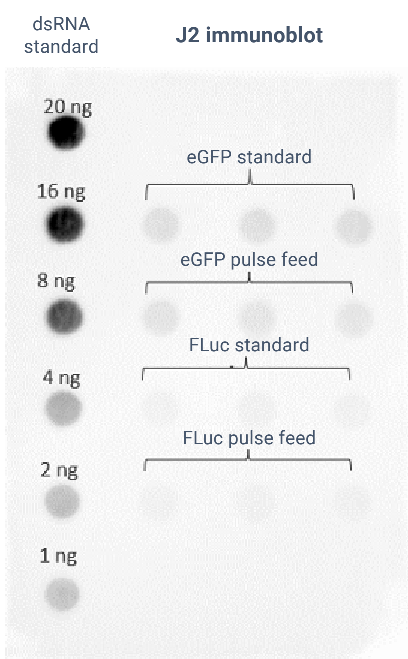

Figure 5. J2 immunoblot showing relative dsRNA levels between standard and pulse-feed IVT protocols for CleanCap M6. There are no observable differences for either of the constructs tested.

Conclusion

CleanCap M6 builds on the advantages of its predecessors, CleanCap AG and CleanCap AG 3' OMe, to boost *in vitro* protein expression. IVT yields can be further increased using its supplementary pulse-feed protocol that requires only additional NTPs while using the same amount of CleanCap M6, DNA template, and T7 polymerase as in the standard protocol. Importantly, by maintaining CQAs including crude mRNA integrity, capping efficiencies, and dsRNA levels, the CleanCap M6 pulse-feed protocol represents a viable strategy for reducing mRNA manufacturing costs.

Materials and methods

Experiments were performed as described in the results section of this technical note. The detailed protocol for CleanCap M6 is located at **trilinkbiotech.com/cleancap-reagent-m6**. It is important to note that the CleanCap analog to NTP ratio and the 10x reaction buffer are specific to CleanCap M6 (**Table 1**).

References

1. Sahin U, Muik A, Derhovanessian E *et al*. (2020). COVID-19 vaccine BNT162b1 elicits human antibody and T_H1 T cell responses. *Nature* 586, 594–599. **https://www.nature.com/articles/s41586-020-2814-7**
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