TRILINK BIOTECHNOLOGIES PRESENTS

Next-generation mRNA design – increasing mRNA potency with a new cap analog

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Agenda

01	**History of capping technology**
02	Introduction of our newest cap analog - CleanCap® M6
03	Economics of CleanCap®

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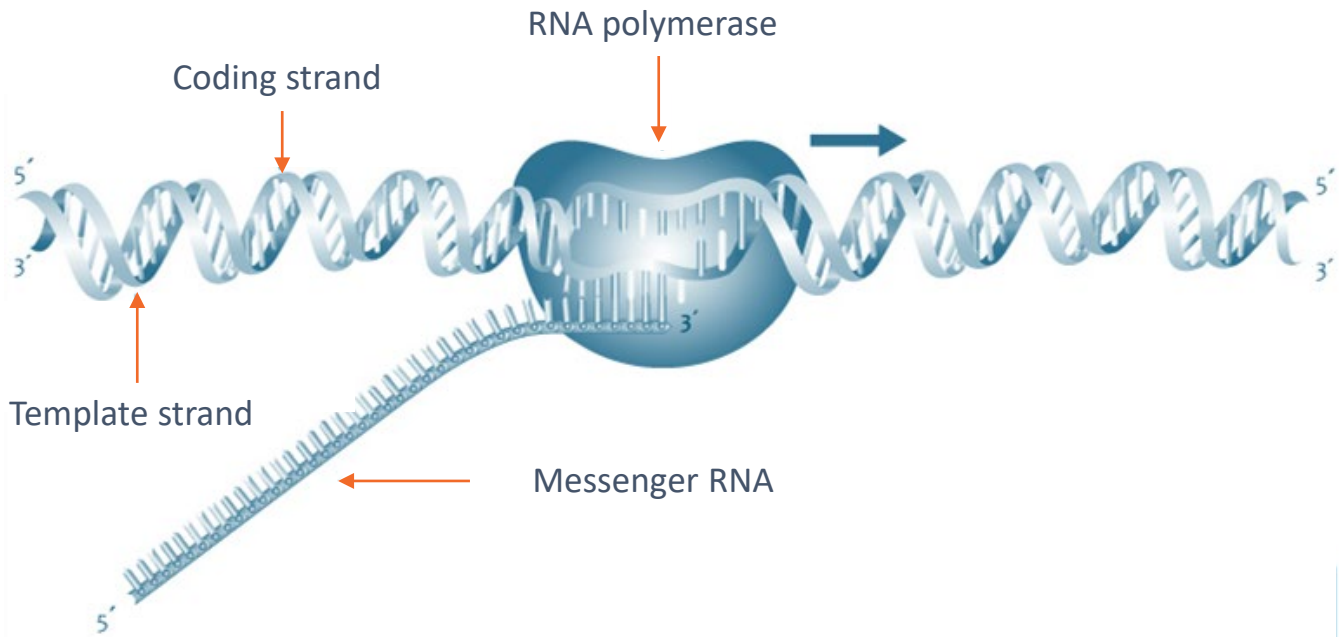
In Vitro transcription

IVT is the process of DNA template-directed RNA synthesis in a tube (or bioreactor)

- RNA polymerase
- Transcription template
- Standard and/or modified NTPs
- Cap analogs (optional)
- Inorganic pyrophosphatase
- RNase inhibitor
- Reaction buffer



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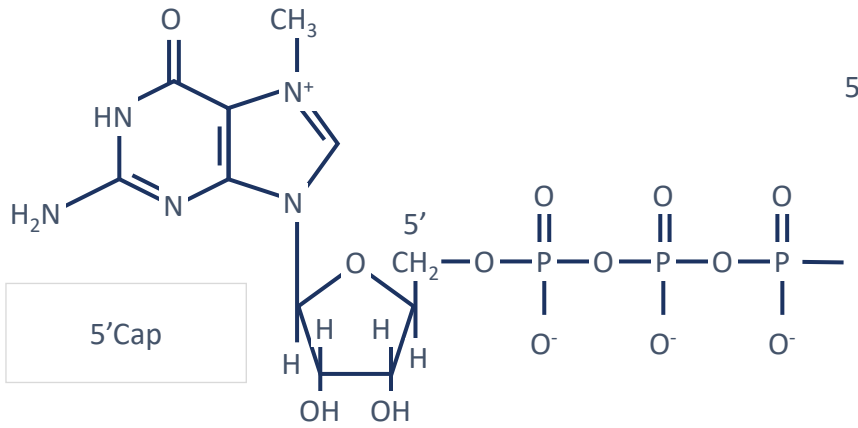
Mature mRNA structure

Nucleoside-modified mRNA

e.g., Ψ, m1Ψ, 5meC

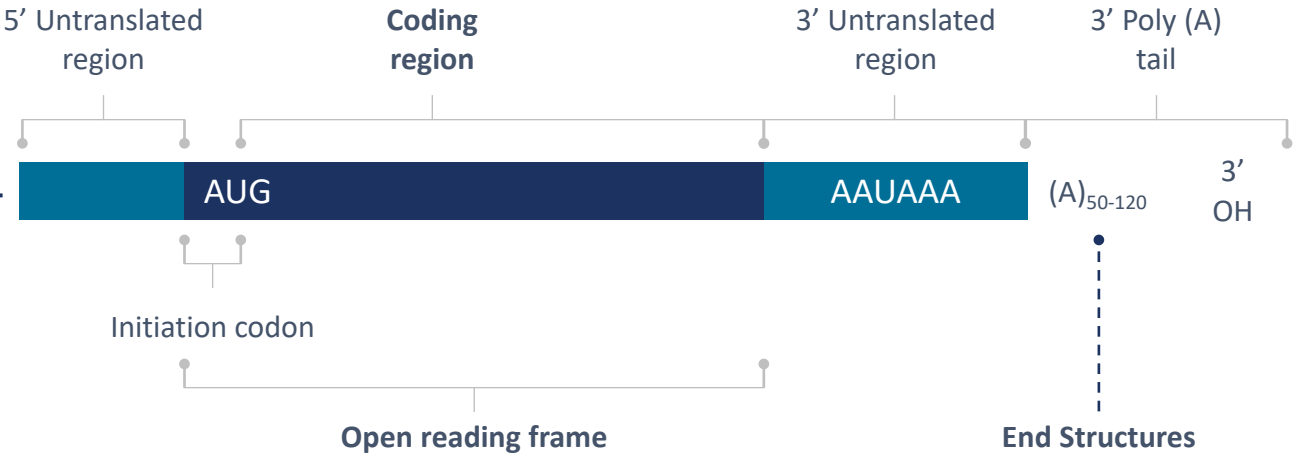
Sequence-engineered mRNA

e.g., GC motif, structural UTR elements



Cap Analogs

e.g., CleanCap reagents



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mRNA cap structure functions

mRNA cap structures are involved in modulating:

- Nuclear export
- Splicing
- **Translation initiation**
- **De-capping/turnover**
- **Self/non-self recognition**

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Endogenous cap structures are methylated

CAP 0

Not abundant in higher eukaryotes

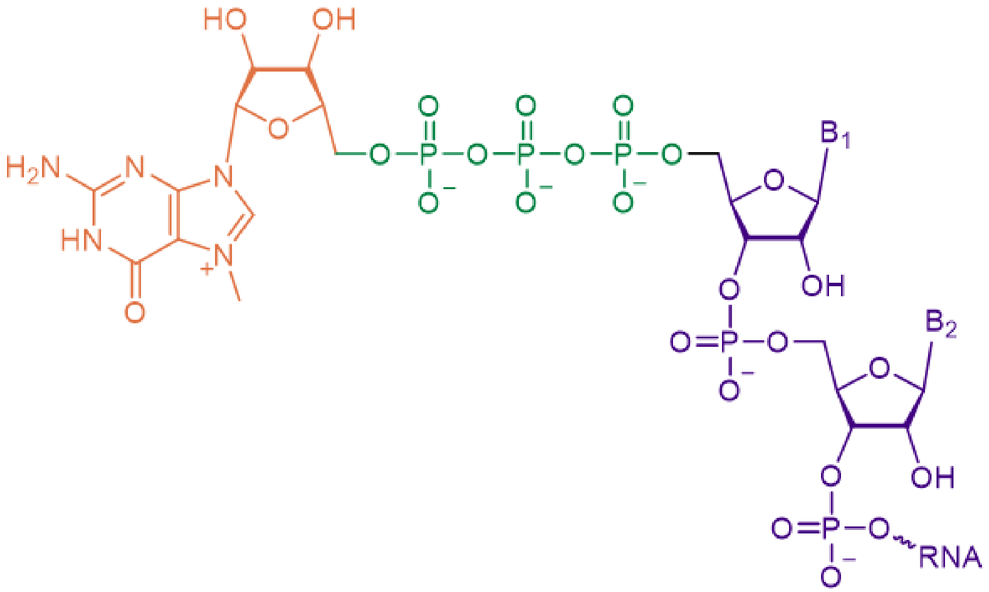
→ *Binding by cytosolic receptors triggers innate immune response*

CAP 1

Found in all eukaryotic mRNA

CAP 2

Found in ~50% of eukaryotic mRNA



Cap 0

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Endogenous cap structures are methylated

CAP 0

Not abundant in higher eukaryotes

→ Bound by cytosolic receptors triggers innate immune response

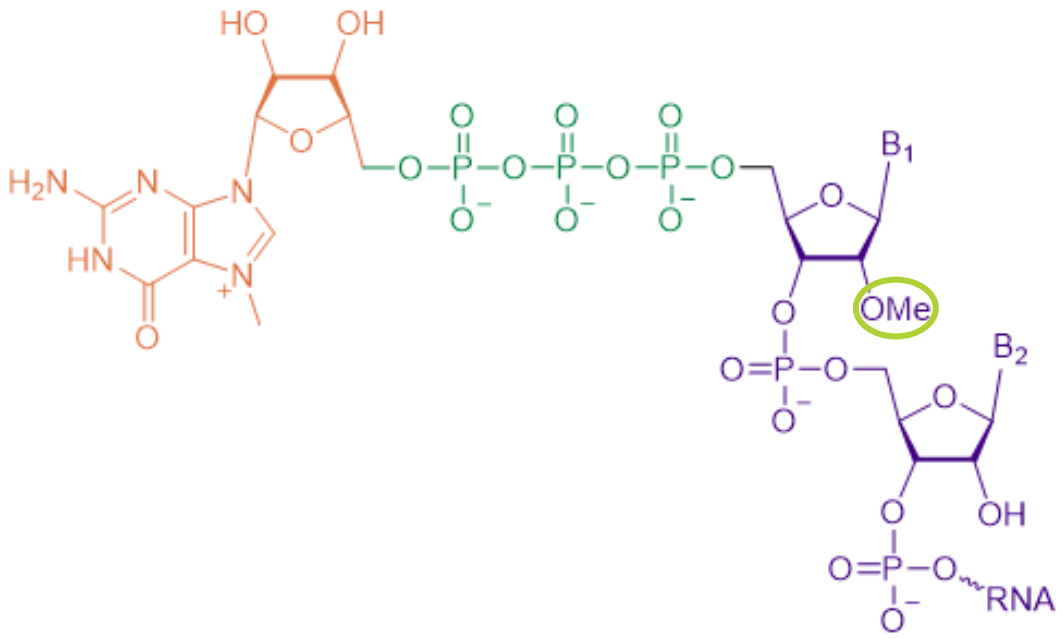
CAP 1

Found in all eukaryotic mRNA

→ Promotes Translation

CAP 2

Found in ~50% of eukaryotic mRNA



Cap 1

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Endogenous cap structures are methylated

CAP 0

Not abundant in higher eukaryotes

→ Bound by cytosolic receptors triggers innate immune response

CAP 1

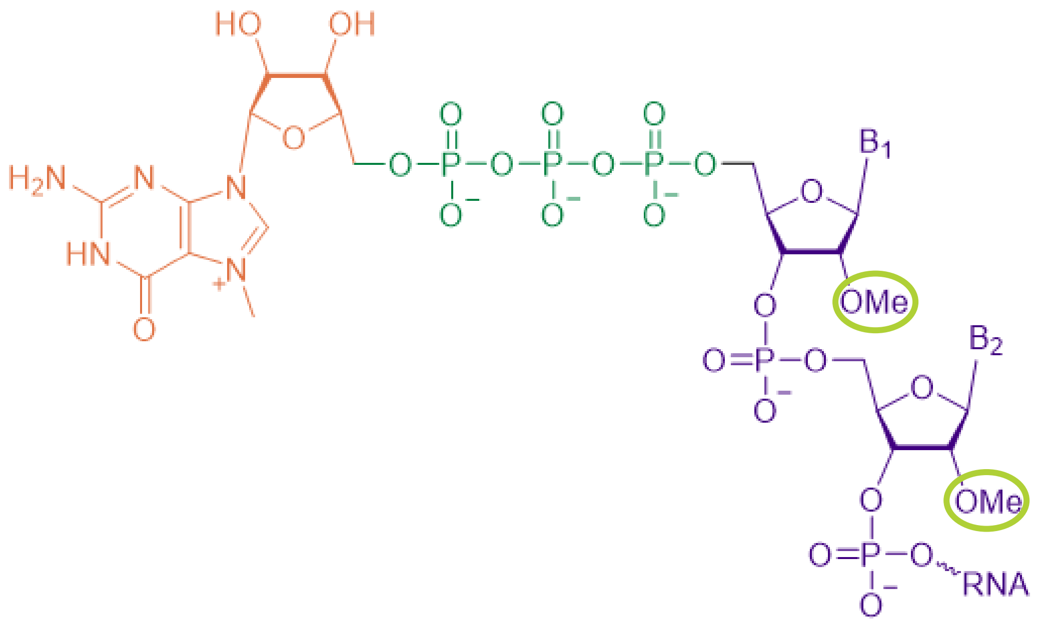
Found in all eukaryotic mRNA

→ Promotes Translation

CAP 2

Found in ~50% of eukaryotic mRNA

→ Dynamic aging marker



Cap 2

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Capping strategies for mRNA manufacturing

Enzymatic capping

Co-transcriptional capping

Generation	Cap Analog
1st	Cap and mCAP (Cap 0)
2nd	Anti-reverse cap analog (ARCA, Cap 0)
3rd	CleanCap® Reagents (Cap 1)

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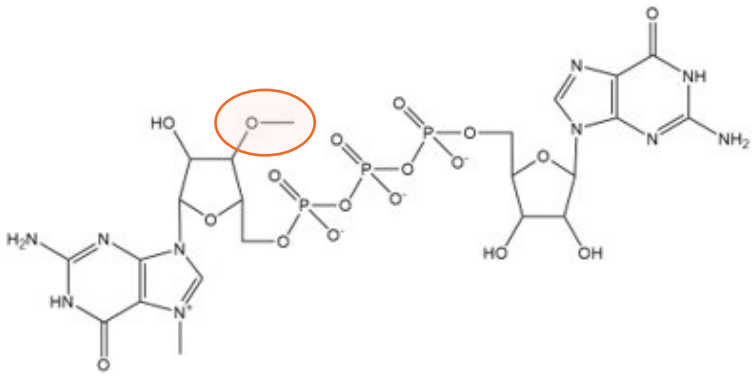
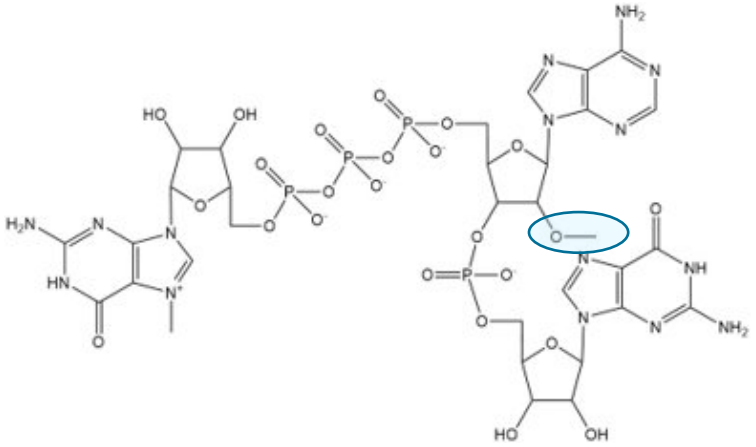
Co-transcriptional capping

ARCA vs CleanCap Reagent



VS



ARCA	CleanCap Analog
ARCA $_{3'Ome}$ ^{m7} GpppG ------	CleanCap Cap 1 AG ^{m7}GpppA$_m$G ------
Template 3' – A T A T C C C -5'	Template 3' – A T A T T C C -5'
T7 Promoter Region $^{-1}$ +1 +2 +3	T7 Promoter Region $^{-1}$ +1 +2 +3
~80% Capped	**~95%** Capped
GTP starvation in IVT = Low mRNA Yields per IVT reaction	**No NTP limitations required = Higher mRNA yields per reaction**

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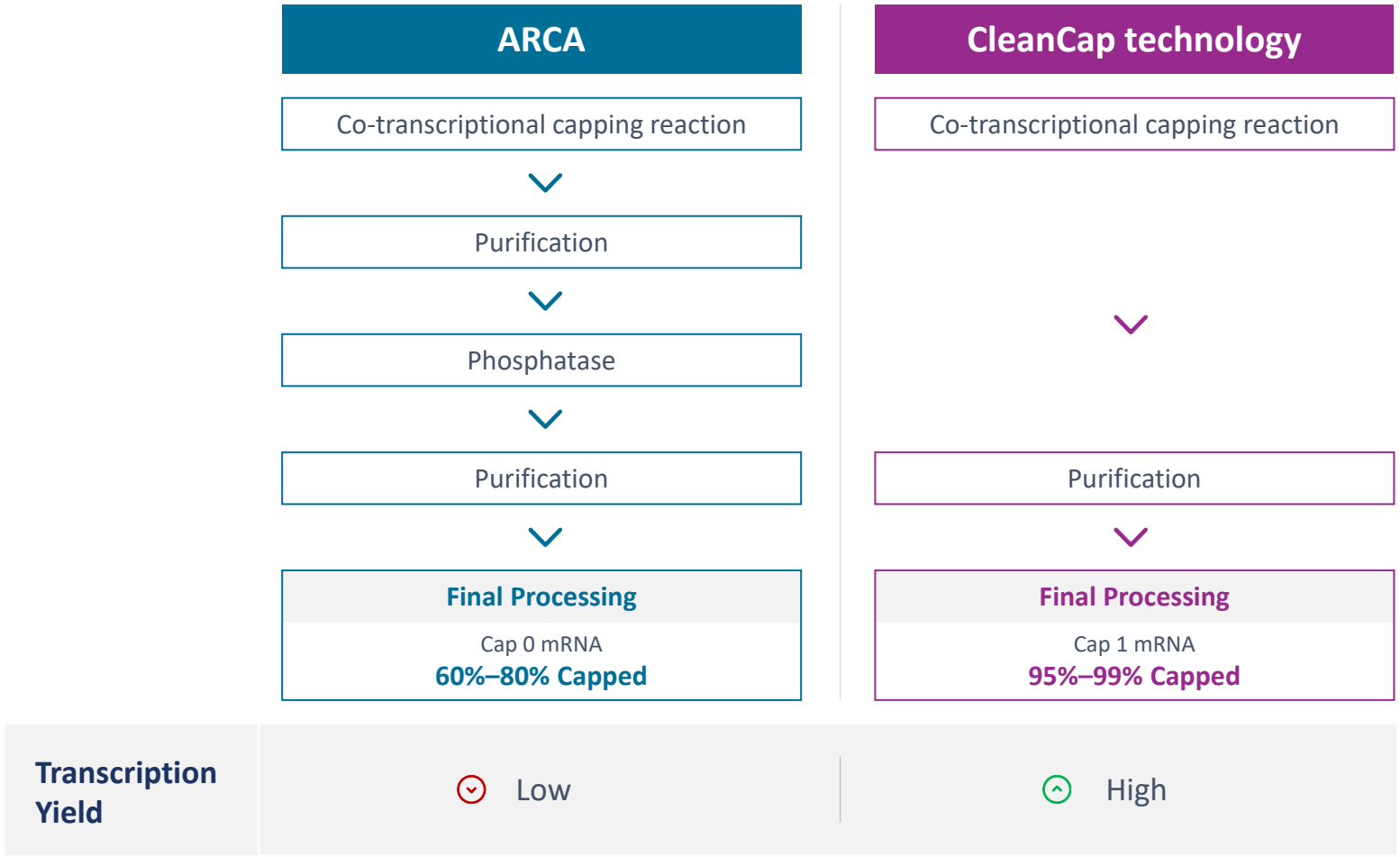
Simplified mRNA manufacturing with CleanCap® technology



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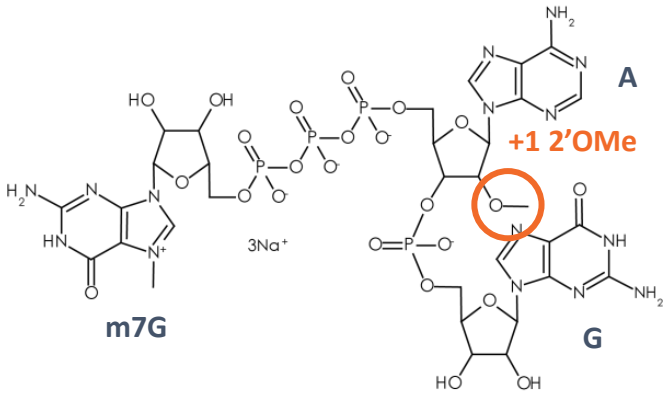
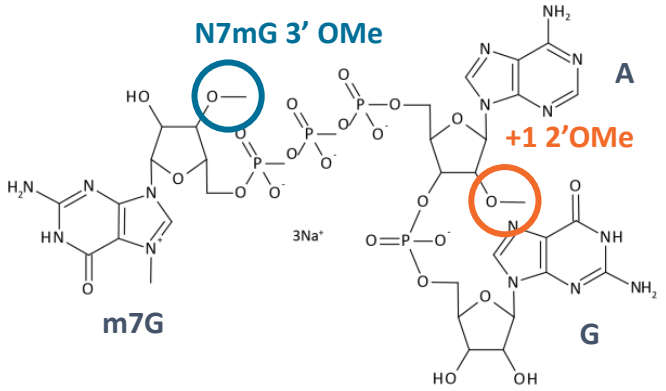
Expanding the CleanCap® family | Evolution of the technology

CleanCap AG



CleanCap AG 3'OMe



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Impact of cap structure on mRNA quality attributes

Sample	5'Cap	IVT Reaction Yield	Integrity	Capping Efficiency	Relative dsRNA	Residual DNA	Residual Protein
N1-methylpseudo-UTP modified Firefly luciferase (FLuc)	ARCA+* (Cap 0)	1.3 mg/mL	78.6%	80%	+++	1.54 ng/mg	< 2%
	CleanCapAG (Cap 1)	4.7mg/mL	93.3%	97%	++	1.27 ng/mg	< 2%
	CleanCapAG 3' OMe (Cap 1)	4.4 mg/mL	94%	96%	++	1.22 ng/mg	< 2%

mRNA integrity by IP-RP-HPLC; Capping efficiency by LC-MS; dsRNA by J2 blot; Residual DNA by qPCR; Residual Protein by NanoOrange

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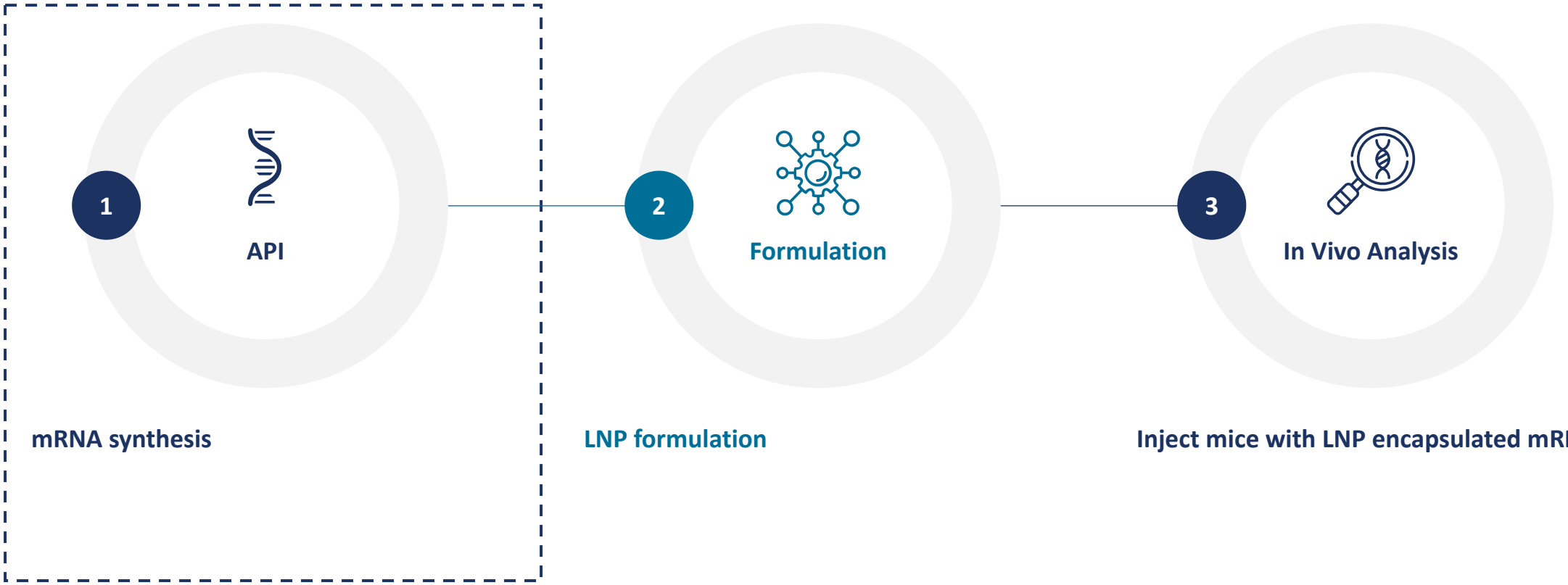
Capping Strategy Data

Luciferase mRNA Expression in Mice



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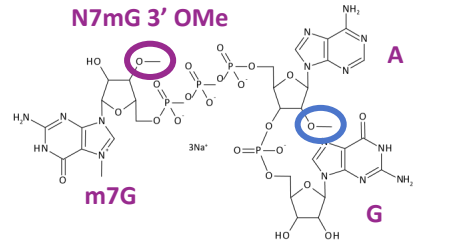
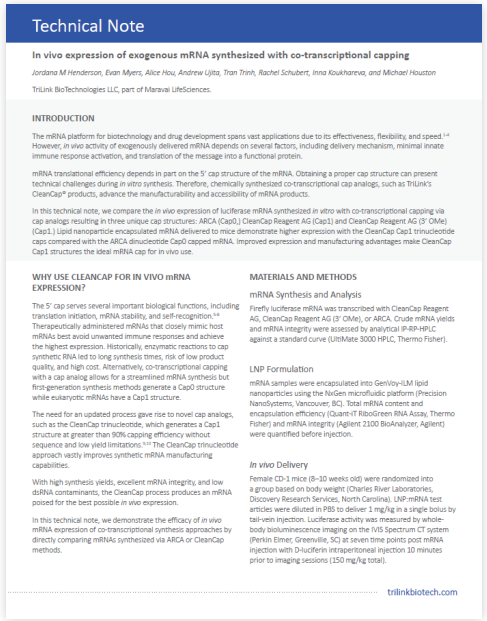
Improved protein expression with modification on m7G - CleanCap® AG 3’OMe





Technical Note

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

Jordana M Henderson, Evan Myers, Alice Hou, Andrew Lijita, Tran Trinh, Rachel Schubert, Inna Koukhareva, and Michael Houston

TriLink BioTechnologies LLC, part of Maravai LifeSciences.

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.[5,6] 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.[5,12] 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).

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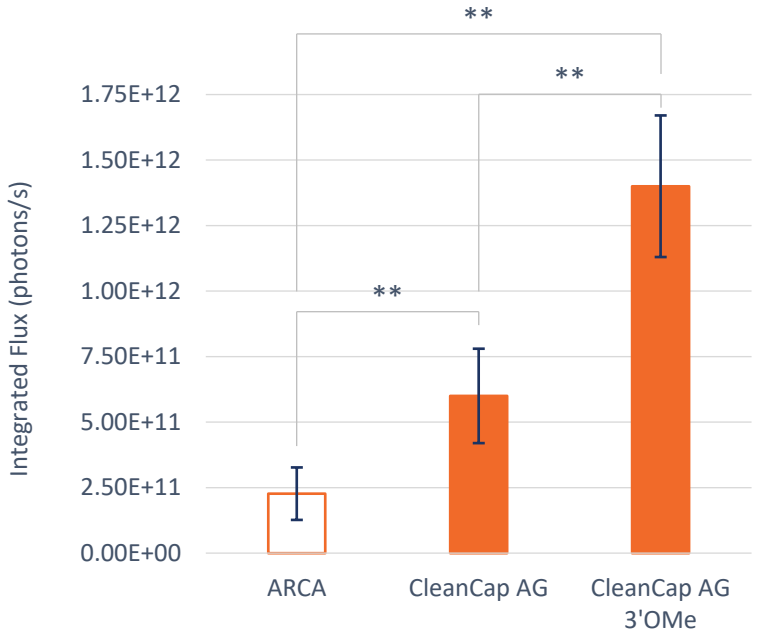
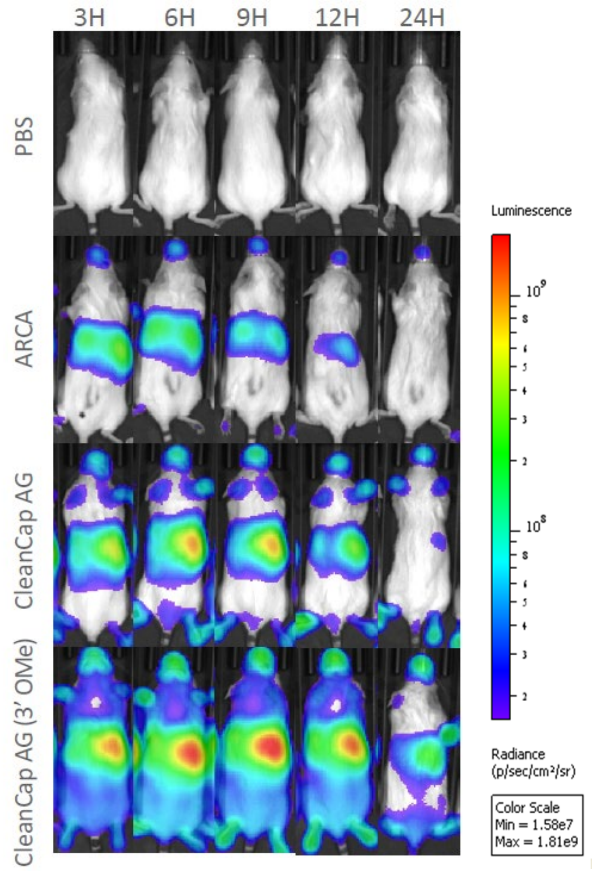


Integrated luminescent signal from N=5 animals across all time points displayed as mean with standard deviation. Two-tailed student’s t-test was performed for all comparisons. ARCA signal is significantly lower than all other mRNAs (***p<0.0001, *p<0.01). CleanCap Reagent AG (3’ OMe) is significantly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



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CleanCap® Analogs in COVID-19 Vaccines - CleanCap AG 3'OMe

A prefusion SARS-CoV-2 spike RNA vaccine is highly immunogenic and prevents lung infection in non-human primates

Annette B. Vogel, Isis Kanevsky, Ye Che, Kena A. Swanson, Alexander Muik, Mathias Vormehr, Lena M. Kranz,

Received: 12 May 2020 | Revised: 1 June 2020 | Accepted: 1 June 2020
DOI: 10.1002/amp2.10060

COMMENTARY

JOURNAL OF ADVANCED MANUFACTURING AND PROCESSING WILEY

Rapid development and deployment of high-volume vaccines for pandemic response

Zoltán Kis[1] | Cleo Kontoravdi[1] | Antu K. Dey[2] | Robin Shattock[3] | Nilay Shah[1]

nature

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Article | Published: 30 September 2020

COVID-19 vaccine BNT162b1 elicits human antibody and T_H1 T cell responses

Ugur Sahin ✉, Alexander Muik, [...] Özlem Türeci

Nature **586**, 594–599(2020) | Cite this article

Manufacturing of RNA

BNT162b1 incorporates a Good Manufacturing Practice (GMP)-grade mRNA drug substance that encodes the trimerized SARS-CoV-2 spike glycoprotein RBD antigen. The RNA is generated from a DNA template by in vitro transcription in the presence of 1-methylpseudouridine-5′-triphosphate (m1ΨTP; Thermo Fisher Scientific) instead of uridine-5′-triphosphate (UTP). Capping is performed co-transcriptionally using a trinucleotide cap 1 analogue ($(m_2^{7,3'-O}Gppp(m^{2'-O})ApG$; TriLink). The antigen-encoding RNA contains sequence elements that increase RNA stability and translation efficiency in human dendritic cells[13,14]. The mRNA is formulated with lipids to obtain the RNA–LNP drug product. The vaccine was transported and supplied as a buffered-liquid solution for intramuscular injection and was stored at −80 °C.

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Agenda

01	History of capping technology
02	**Introduction of our newest cap analog - CleanCap®M6**
03	Economics of CleanCap® technology

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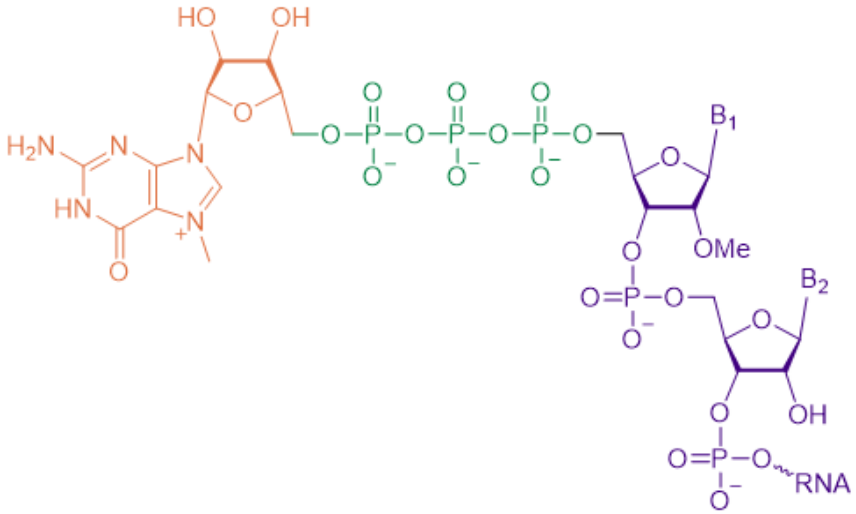
Synthesis, screening and testing of novel cap structures

Screening process

- **Cap analog synthesis** – Library of over 65 unique cap modifications
  - Modifications to m7G, modifications to 5'ppp5', modifications to +1A, etc.
- Screening those caps in IVT for capping efficiency with T7 RNA Pol.



In vivo testing

Firefly luciferase mRNA with over 20 new cap analogs

- LNP formulation
- Tail vein injection (1mg/kg)
- Body weights tracked out to 96 hours post mRNA delivery
- Imaging at 3, 6, 9, 12, 24, 36, and/or 48 hours post mRNA delivery by whole body bioluminescence

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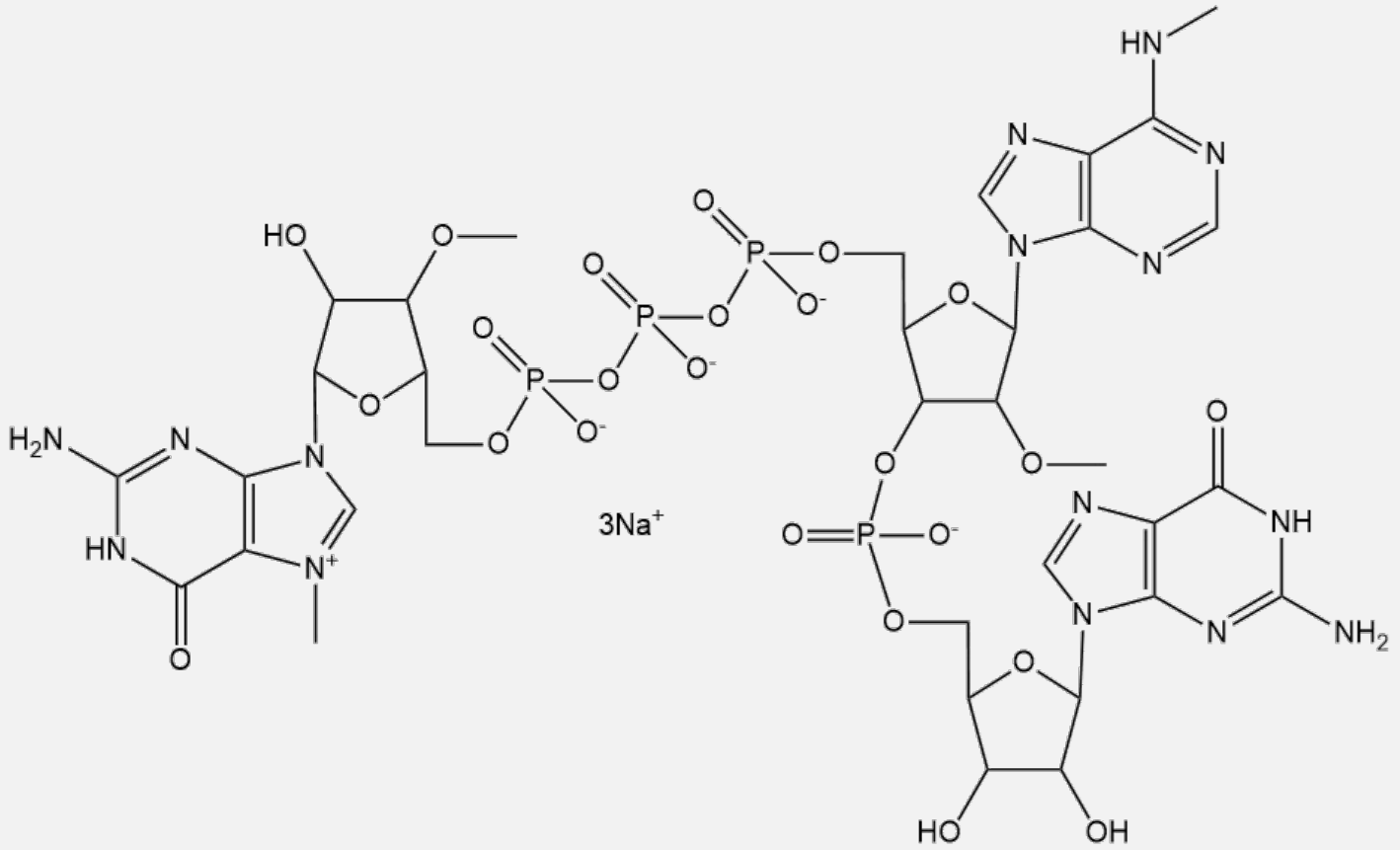
Our newest cap analog



CleanCap® Reagent M6

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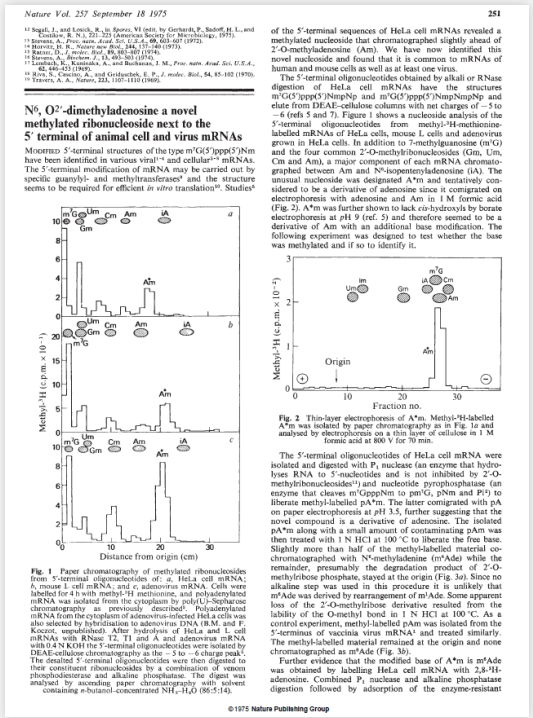
m6Am modified caps occur naturally – Applying rational design principals



Nature Vol. 257 September 18 1975 251

N6, O2'-dimethyladenosine a novel methylated ribonucleoside next to the 5' terminal of animal cell and virus mRNAs

Moomtro 5'-terminal structures of the type m7G(5')ppp(5')Nm have been identified in various viral[1-4] and cellular[5-9] mRNAs. The 5'-terminal modification of mRNA may be carried out by specific guanylyl- and methyltransferases[9] and the structure seems to be required for efficient *in vitro* translation[10]. Studies[8]

Fig. 1 Paper chromatography of methylated ribonucleosides from 5'-terminal oligonucleotides of: *a*, HeLa cell mRNA; *b*, mouse L cell mRNA; and *c*, adenovirus mRNA. Cells were labelled for 4 h with methyl-^{3}H methionine, and polyadenylated mRNA was isolated from the cytoplasm by poly(U)-Sepharose chromatography as previously described[5]. Polyadenylated mRNA from the cytoplasm of adenovirus-infected HeLa cells was also selected by hybridisation to adenovirus DNA (B.M. and F. Kozzot, unpublished). After hydrolysis of HeLa and L cell mRNAs with RNase T2, T1 and A and adenovirus mRNA with 0·4 N KOH the 5'-terminal oligonucleotides were isolated by DEAE-cellulose chromatography as the −5 to −6 charge peak[1]. The desalted 5'-terminal oligonucleotides were then digested to their constituent ribonucleosides by a combination of venom phosphodiesterase and alkaline phosphatase. The digest was analysed by ascending paper chromatography with solvent containing n-butanol–concentrated NH_4OH–H_2O (86:5:14).

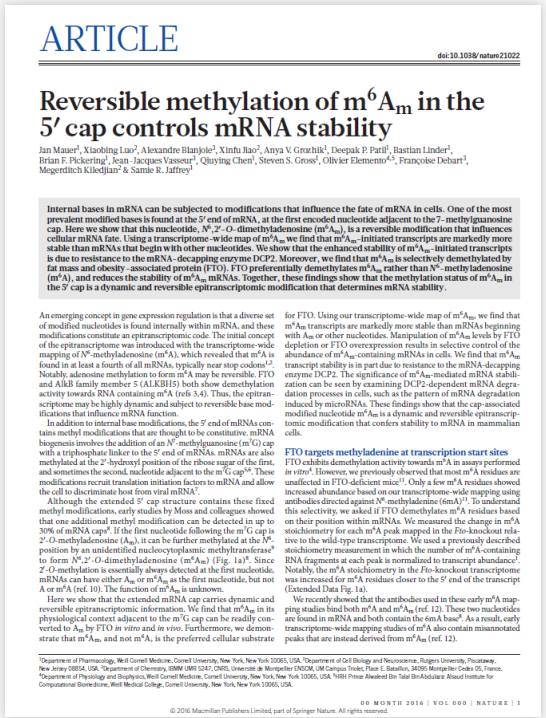
Fig. 2 Thin-layer electrophoresis of A*m. Methyl-^{3}H-labelled A*m was isolated by paper chromatography as in Fig. 1a and analysed by electrophoresis on a thin layer of cellulose in 1 M formic acid at 800 V for 70 min.

The 5'-terminal oligonucleotides of HeLa cell mRNA were isolated and digested with P_1 nuclease (an enzyme that hydrolyses RNA to 5'-nucleotides and is not inhibited by 2'-O-methylribonucleosides[11]) and nucleotide pyrophosphatase (an enzyme that cleaves m7GpppNm to pm7G, pNm and Pi[1]) to liberate methyl-labelled pA*m. The latter comigrated with pA on paper electrophoresis at *p*H 3.5, further suggesting that the novel compound is a derivative of adenosine. The isolated pA*m along with a small amount of contaminating pAm was then treated with 1 N HCl at 100 °C to liberate the free base. Slightly more than half of the methyl-labelled material co-chromatographed with N6-methyladenine (m6Ade) while the remainder, presumably the degradation product of 2'-O-methylribose phosphate, stayed at the origin (Fig. 3a). Since no alkaline step was used in this procedure it is unlikely that m6Ade was derived by rearrangement of m1Ade. Some apparent loss of the 2'-O-methylribose derivative resulted from the lability of the O-methyl bond in 1 N HCl at 100 °C. As a control experiment, methyl-labelled pAm was isolated from the 5'-terminus of vaccinia virus mRNA[4] and treated similarly. The methyl-labelled material remained at the origin and none chromatographed as m6Ade (Fig. 3b).

Further evidence that the modified base of A*m is m6Ade was obtained by labelling HeLa cell mRNA with 2,8-^{3}H-adenosine. Combined P_1 nuclease and alkaline phosphatase digestion followed by adsorption of the enzyme-resistant

of the 5'-terminal sequences of HeLa cell mRNAs revealed a methylated nucleoside that chromatographed slightly ahead of 2'-O-methyladenosine (Am). We have now identified this novel nucleoside and found that it is common to mRNAs of human and mouse cells as well as at least one virus.

The 5'-terminal oligonucleotides obtained by alkali or RNase digestion of HeLa cell mRNAs have the structures m7G(5')ppp(5')NmpNp and m7G(5')ppp(5')NmpNmpNp and elute from DEAE-cellulose columns with net charges of −5 to −6 (refs 5 and 7). Figure 1 shows a nucleoside analysis of the 5'-terminal oligonucleotides from methyl-^{3}H-methionine-labelled mRNAs of HeLa cells, mouse L cells and adenovirus grown in HeLa cells. In addition to 7-methylguanosine (m7G) and the four common 2'-O-methylribonucleosides (Gm, Um, Cm and Am), a major component of each mRNA chromatographed between Am and N6-isopentenyladenosine (iA). The unusual nucleoside was designated A*m and tentatively considered to be a derivative of adenosine since it comigrated on electrophoresis with adenosine and Am in 1 M formic acid (Fig. 2). A*m was further shown to lack *cis*-hydroxyls by borate electrophoresis at *p*H 9 (ref. 5) and therefore seemed to be a derivative of Am with an additional base modification. The following experiment was designed to test whether the base was methylated and if so to identify it.



ARTICLE

doi:10.1038/nature21022

Reversible methylation of m^6A_m in the 5′ cap controls mRNA stability

Jan Mauer[1], Xiaobing Luo[2], Alexandre Blanjoie[3], Xinfu Jiao[2], Anya V. Grozhik[1], Deepak P. Patil[1], Bastian Linder[1], Brian F. Pickering[1], Jean-Jacques Vasseur[3], Qiuying Chen[1], Steven S. Gross[1], Olivier Elemento[4,5], Françoise Debart[3], Megerditch Kiledjian[2] & Samie R. Jaffrey[1]

Internal bases in mRNA can be subjected to modifications that influence the fate of mRNA in cells. One of the most prevalent modified bases is found at the 5′ end of mRNA, at the first encoded nucleotide adjacent to the 7-methylguanosine cap. Here we show that this nucleotide, N^6,2′-*O*-dimethyladenosine (m^6A_m), is a reversible modification that influences cellular mRNA fate. Using a transcriptome-wide map of m^6A_m we find that m^6A_m-initiated transcripts are markedly more stable than mRNAs that begin with other nucleotides. We show that the enhanced stability of m^6A_m-initiated transcripts is due to resistance to the mRNA-decapping enzyme DCP2. Moreover, we find that m^6A_m is selectively demethylated by fat mass and obesity-associated protein (FTO). FTO preferentially demethylates m^6A_m rather than N^6-methyladenosine (m^6A), and reduces the stability of m^6A_m mRNAs. Together, these findings show that the methylation status of m^6A_m in the 5′ cap is a dynamic and reversible epitranscriptomic modification that determines mRNA stability.

An emerging concept in gene expression regulation is that a diverse set of modified nucleotides is found internally within mRNA, and these modifications constitute an epitranscriptomic code. The initial concept of the epitranscriptome was introduced with the transcriptome-wide mapping of N^6-methyladenosine (m^6A), which revealed that m^6A is found in at least a fourth of all mRNAs, typically near stop codons[1,2]. Notably, adenosine methylation to form m^6A may be reversible. FTO and ALkB family member 5 (ALKBH5) both show demethylation activity towards RNA containing m^6A (refs 3,4). Thus, the epitranscriptome may be highly dynamic and subject to reversible base modifications that influence mRNA function.

In addition to internal base modifications, the 5′ end of mRNAs contains methyl modifications that are thought to be constitutive. mRNA biogenesis involves the addition of an N^7-methylguanosine (m^7G) cap with a triphosphate linker to the 5′ end of mRNAs. mRNAs are also methylated at the 2′-hydroxyl position of the ribose sugar of the first, and sometimes the second, nucleotide adjacent to the m^7G cap[5,6]. These modifications recruit translation initiation factors to mRNA and allow the cell to discriminate host from viral mRNA[7].

Although the extended 5′ cap structure contains these fixed methyl modifications, early studies by Moss and colleagues showed that one additional methyl modification can be detected in up to 30% of mRNA caps[8]. If the first nucleotide following the m^7G cap is 2′-*O*-methyladenosine (A_m), it can be further methylated at the N^6-position by an unidentified nucleocytoplasmic methyltransferase[9] to form N^6,2′-*O*-dimethyladenosine (m^6A_m) (Fig. 1a)[8]. Since 2′-*O*-methylation is essentially always detected at the first nucleotide, mRNAs can have either A_m or m^6A_m as the first nucleotide, but not A or m^6A (ref. 10). The function of m^6A_m is unknown.

Here we show that the extended mRNA cap carries dynamic and reversible epitranscriptomic information. We find that m^6A_m in its physiological context adjacent to the m^7G cap can be readily converted to A_m by FTO *in vitro* and *in vivo*. Furthermore, we demonstrate that m^6A_m, and not m^6A, is the preferred cellular substrate for FTO. Using our transcriptome-wide map of m^6A_m, we find that m^6A_m transcripts are markedly more stable than mRNAs beginning with A_m or other nucleotides. Manipulation of m^6A_m levels by FTO depletion or FTO overexpression results in selective control of the abundance of m^6A_m-containing mRNAs in cells. We find that m^6A_m transcript stability is in part due to resistance to the mRNA-decapping enzyme DCP2. The significance of m^6A_m-mediated mRNA stabilization can be seen by examining DCP2-dependent mRNA degradation processes in cells, such as the pattern of mRNA degradation induced by microRNAs. These findings show that the cap-associated modified nucleotide m^6A_m is a dynamic and reversible epitranscriptomic modification that confers stability to mRNA in mammalian cells.

FTO targets methyladenine at transcription start sites

FTO exhibits demethylation activity towards m^6A in assays performed *in vitro*[3]. However, we previously observed that most m^6A residues are unaffected in FTO-deficient mice[11]. Only a few m^6A residues showed increased abundance based on our transcriptome-wide mapping using antibodies directed against N^6-methyladenine (6mA)[1,2]. To understand this selectivity, we asked if FTO demethylates m^6A residues based on their position within mRNAs. We measured the change in m^6A stoichiometry for each m^6A peak mapped in the *Fto*-knockout relative to the wild-type transcriptome. We used a previously described stoichiometry measurement in which the number of m^6A-containing RNA fragments at each peak is normalized to transcript abundance[2]. Notably, the m^6A stoichiometry in the *Fto*-knockout transcriptome was increased for m^6A residues closer to the 5′ end of the transcript (Extended Data Fig. 1a).

We recently showed that the antibodies used in these early m^6A mapping studies bind both m^6A and m^6A_m (ref. 12). These two nucleotides are found in mRNA and both contain the 6mA base[6]. As a result, early transcriptome-wide mapping studies of m^6A also contain misannotated peaks that are instead derived from m^6A_m (ref. 12).

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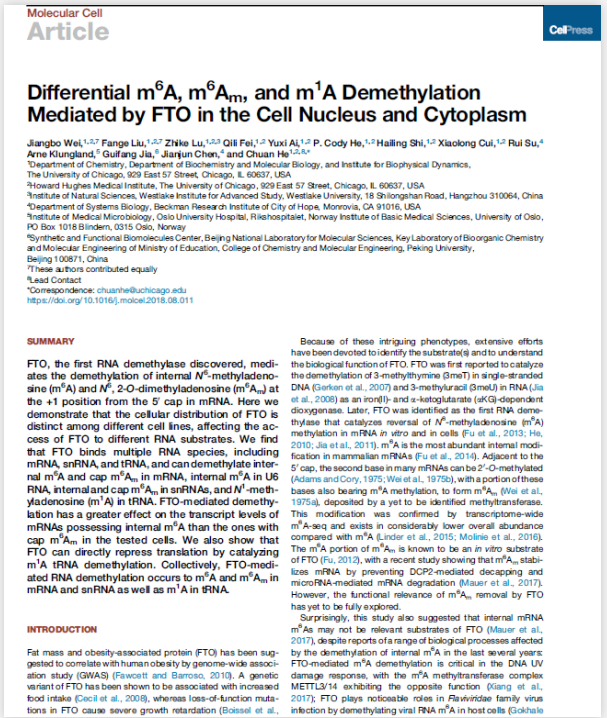

Molecular Cell
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CellPress

Differential m^6A, m^6A_m, and m^1A Demethylation Mediated by FTO in the Cell Nucleus and Cytoplasm

Jiangbo Wei[1,2,7], Fange Liu[1,2,7], Zhike Lu[1,2,3], Qili Fei[1,2], Yuxi Ai[1,2], P. Cody He[1,2], Hailing Shi[1,2], Xiaolong Cui[1,2], Rui Su[4], Arne Klungland[5], Guifang Jia[6], Jianjun Chen[4], and Chuan He[1,2,8,*]

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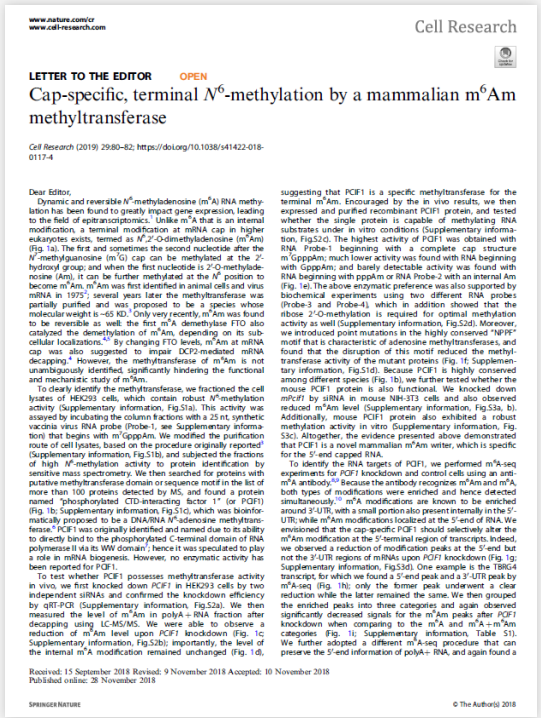
SUMMARY

FTO, the first RNA demethylase discovered, mediates the demethylation of internal N^6-methyladenosine (m^6A) and N^6, 2-O-dimethyladenosine (m^6A_m) at the +1 position from the 5′ cap in mRNA. Here we demonstrate that the cellular distribution of FTO is distinct among different cell lines, affecting the access of FTO to different RNA substrates. We find that FTO binds multiple RNA species, including mRNA, snRNA, and tRNA, and can demethylate internal m^6A and cap m^6A_m in mRNA, internal m^6A in U6 RNA, internal and cap m^6A_m in snRNAs, and N^1-methyladenosine (m^1A) in tRNA. FTO-mediated demethylation has a greater effect on the transcript levels of mRNAs possessing internal m^6A than the ones with cap m^6A_m in the tested cells. We also show that FTO can directly repress translation by catalyzing m^1A tRNA demethylation. Collectively, FTO-mediated RNA demethylation occurs to m^6A and m^6A_m in mRNA and snRNA as well as m^1A in tRNA.

INTRODUCTION

Fat mass and obesity-associated protein (FTO) has been suggested to correlate with human obesity by genome-wide association study (GWAS) (Fawcett and Barroso, 2010). A genetic variant of FTO has been shown to be associated with increased food intake (Cecil et al., 2008), whereas loss-of-function mutations in FTO cause severe growth retardation (Boissel et al., Because of these intriguing phenotypes, extensive efforts have been devoted to identify the substrate(s) and to understand the biological function of FTO. FTO was first reported to catalyze the demethylation of 3-methylthymine (3meT) in single-stranded DNA (Gerken et al., 2007) and 3-methyluracil (3meU) in RNA (Jia et al., 2008) as an iron(II)- and α-ketoglutarate (αKG)-dependent dioxygenase. Later, FTO was identified as the first RNA demethylase that catalyzes reversal of N^6-methyladenosine (m^6A) methylation in mRNA *in vitro* and in cells (Fu et al., 2013; He, 2010; Jia et al., 2011). m^6A is the most abundant internal modification in mammalian mRNAs (Fu et al., 2014). Adjacent to the 5′ cap, the second base in many mRNAs can be 2′-O-methylated (Adams and Cory, 1975; Wei et al., 1975b), with a portion of these bases also bearing m^6A methylation, to form m^6A_m (Wei et al., 1975a), deposited by a yet to be identified methyltransferase. This modification was confirmed by transcriptome-wide m^6A-seq and exists in considerably lower overall abundance compared with m^6A (Linder et al., 2015; Molinie et al., 2016). The m^6A portion of m^6A_m is known to be an *in vitro* substrate of FTO (Fu, 2012), with a recent study showing that m^6A_m stabilizes mRNA by preventing DCP2-mediated decapping and microRNA-mediated mRNA degradation (Mauer et al., 2017). However, the functional relevance of m^6A_m removal by FTO has yet to be fully explored.

Surprisingly, this study also suggested that internal mRNA m^6A may not be relevant substrates of FTO (Mauer et al., 2017), despite reports of a range of biological processes affected by the demethylation of internal m^6A in the last several years: FTO-mediated m^6A demethylation is critical in the DNA UV damage response, with the m^6A methyltransferase complex METTL3/14 exhibiting the opposite function (Xiang et al., 2017); FTO plays noticeable roles in *Flaviviridae* family virus infection by demethylating viral RNA m^6A in host cells (Gokhale



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LETTER TO THE EDITOR OPEN

Cap-specific, terminal N^6-methylation by a mammalian m^6Am methyltransferase

Cell Research (2019) 29:80–82; https://doi.org/10.1038/s41422-018-0117-4

Dear Editor,

Dynamic and reversible N^6-methyladenosine (m^6A) RNA methylation has been found to greatly impact gene expression, leading to the field of epitranscriptomics.[1] Unlike m^6A that is an internal modification, a terminal modification at mRNA cap in higher eukaryotes exists, termed as N^6,2′-O-dimethyladenosine (m^6Am) (Fig. 1a). The first and sometimes the second nucleotide after the *N*7-methylguanosine (m^7G) cap can be methylated at the 2′-hydroxyl group; and when the first nucleotide is 2′-O-methyladenosine (Am), it can be further methylated at the N^6 position to become m^6Am. m^6Am was first identified in animal cells and virus mRNA in 1975[2], several years later the methyltransferase was partially purified and was proposed to be a species whose molecular weight is ~65 KD.[3] Only very recently, m^6Am was found to be reversible as well: the first m^6A demethylase FTO also catalyzed the demethylation of m^6Am, depending on its subcellular localizations.[4,5] By changing FTO levels, m^6Am at mRNA cap was also suggested to impair DCP2-mediated mRNA decapping.[4] However, the methyltransferase of m^6Am is not unambiguously identified, significantly hindering the functional and mechanistic study of m^6Am.

To clearly identify the methyltransferase, we fractioned the cell lysates of HEK293 cells, which contain robust N^6-methylation activity (Supplementary information, Fig.S1a). This activity was assayed by incubating the column fractions with a 25 nt, synthetic vaccinia virus RNA probe (Probe-1, see Supplementary information) that begins with $m^7GpppAm$. We modified the purification route of cell lysates, based on the procedure originally reported[6] (Supplementary information, Fig.S1b), and subjected the fractions of high N^6-methylation activity to protein identification by sensitive mass spectrometry. We then searched for proteins with putative methyltransferase domain or sequence motif in the list of more than 100 proteins detected by MS, and found a protein named "phosphorylated CTD-interacting factor 1" (or PCIF1) (Fig. 1b; Supplementary information, Fig.S1c), which was bioinformatically proposed to be a DNA/RNA N^6-adenosine methyltransferase.[6] PCIF1 was originally identified and named due to its ability to directly bind to the phosphorylated C-terminal domain of RNA polymerase II via its WW domain[7], hence it was speculated to play a role in mRNA biogenesis. However, no enzymatic activity has been reported for PCIF1.

To test whether PCIF1 possesses methyltransferase activity in vivo, we first knocked down *PCIF1* in HEK293 cells by two independent siRNAs and confirmed the knockdown efficiency by qRT-PCR (Supplementary information, Fig.S2a). We then measured the level of m^6Am in polyA+ RNA fraction after decapping using LC-MS/MS. We were able to observe a reduction of m^6Am level upon *PCIF1* knockdown (Fig. 1c; Supplementary information, Fig.S2b); importantly, the level of the internal m^6A modification remained unchanged (Fig. 1d), suggesting that PCIF1 is a specific methyltransferase for the terminal m^6Am. Encouraged by the in vivo results, we then expressed and purified recombinant PCIF1 protein, and tested whether the single protein is capable of methylating RNA substrates under in vitro conditions (Supplementary information, Fig.S2c). The highest activity of PCIF1 was obtained with RNA Probe-1 beginning with a complete cap structure $m^7GpppAm$; much lower activity was found with RNA beginning with GpppAm; and barely detectable activity was found with RNA beginning with pppAm or RNA Probe-2 with an internal Am (Fig. 1e). The above enzymatic preference was also supported by biochemical experiments using two different RNA probes (Probe-3 and Probe-4), which in addition showed that the ribose 2′-O-methylation is required for optimal methylation activity as well (Supplementary information, Fig.S2d). Moreover, we introduced point mutations in the highly conserved "NPPF" motif that is characteristic of adenosine methyltransferases, and found that the disruption of this motif reduced the methyltransferase activity of the mutant proteins (Fig. 1f; Supplementary information, Fig.S1d). Because PCIF1 is highly conserved among different species (Fig. 1b), we further tested whether the mouse PCIF1 protein is also functional. We knocked down *mPcif1* by siRNA in mouse NIH-3T3 cells and also observed reduced m^6Am level (Supplementary information, Fig.S3a, b). Additionally, mouse PCIF1 protein also exhibited a robust methylation activity in vitro (Supplementary information, Fig. S3c). Altogether, the evidence presented above demonstrated that PCIF1 is a novel mammalian m^6Am writer, which is specific for the 5′-end capped RNA.

To identify the RNA targets of PCIF1, we performed m^6A-seq experiments for *PCIF1* knockdown and control cells using an anti-m^6A antibody.[8,9] Because the antibody recognizes m^6Am and m^6A, both types of modifications were enriched and hence detected simultaneously.[10] m^6A modifications are known to be enriched around 3′-UTR, with a small portion also present internally in the 5′-UTR; while m^6Am modifications localized at the 5′-end of RNA. We envisioned that the cap-specific PCIF1 should selectively alter the m^6Am modification at the 5′-terminal region of transcripts. Indeed, we observed a reduction of modification peaks at the 5′-end but not the 3′-UTR regions of mRNAs upon *PCIF1* knockdown (Fig. 1g; Supplementary information, Fig.S3d). One example is the TBRG4 transcript, for which we found a 5′-end peak and a 3′-UTR peak by m^6A-seq (Fig. 1h); only the former peak underwent a clear reduction while the latter remained the same. We then grouped the enriched peaks into three categories and again observed significantly decreased signals for the m^6Am peaks after *PCIF1* knockdown when comparing to the m^6A and m^6A-m^6Am categories (Fig. 1i; Supplementary information, Table S1). We further adopted a different m^6A-seq procedure that can preserve the 5′-end information of polyA+ RNA, and again found a

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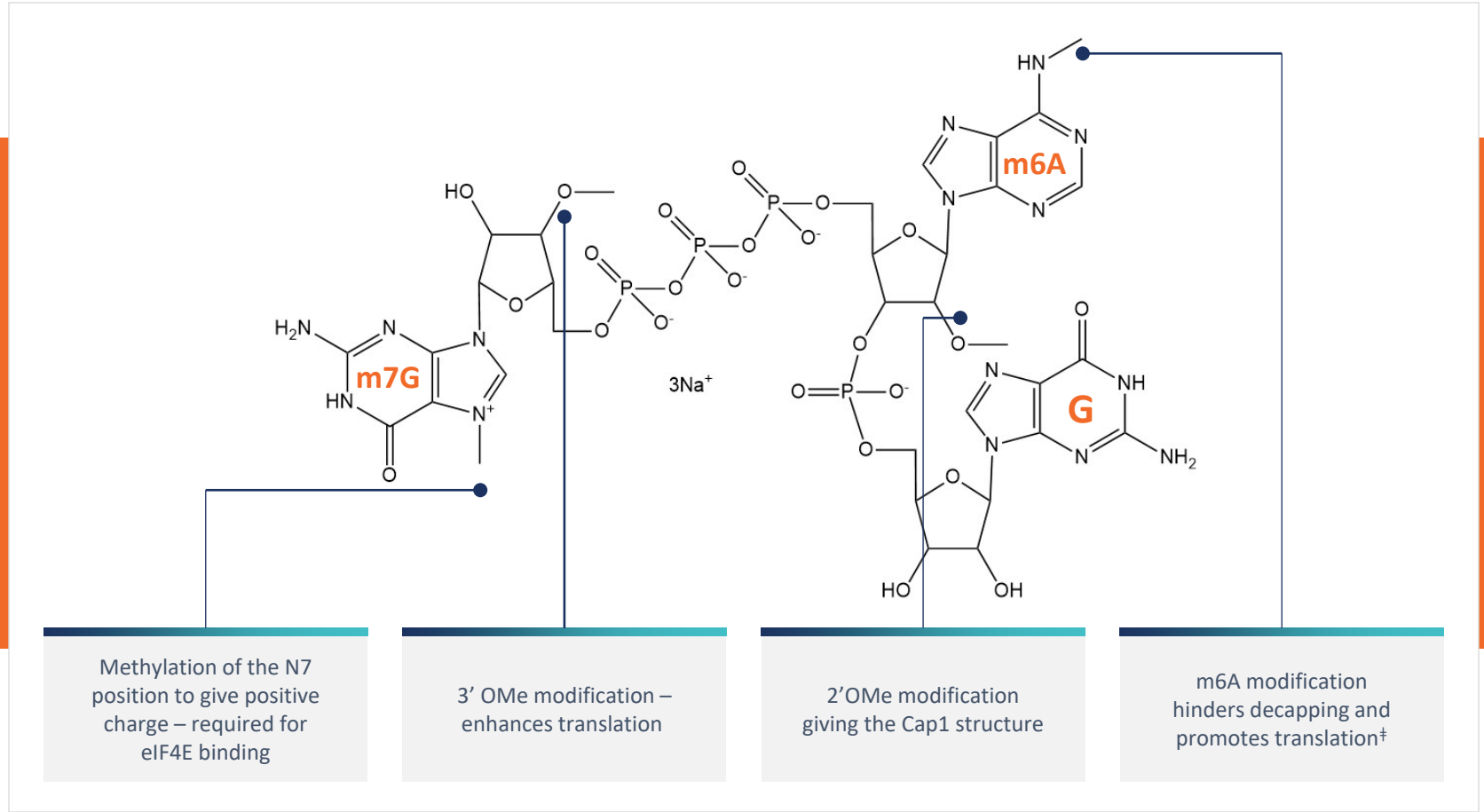
CleanCap® M6 – the newest cap analog in the CleanCap® portfolio

Synonyms

- CleanCap m6AG (3’OMe)
- m7(3’OMeG)(5’)ppp (5')m6(2'OMeA)pG



‡ Mauer, J., Luo, X., Blanjoie, A. et al. Reversible methylation of m6Am in the 5’ cap controls mRNA stability. Nature 541, 371–375 (2017). https://doi.org/10.1038/nature21022

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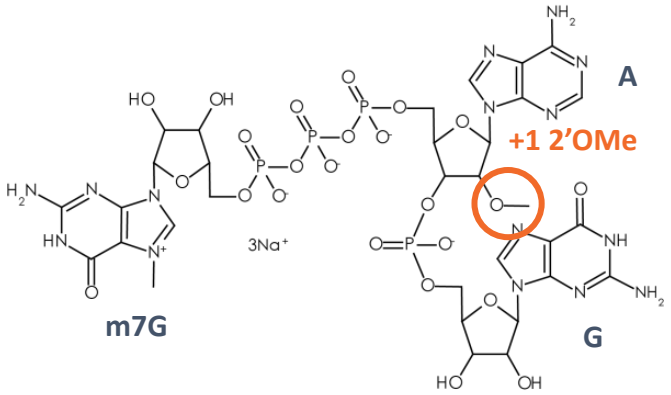
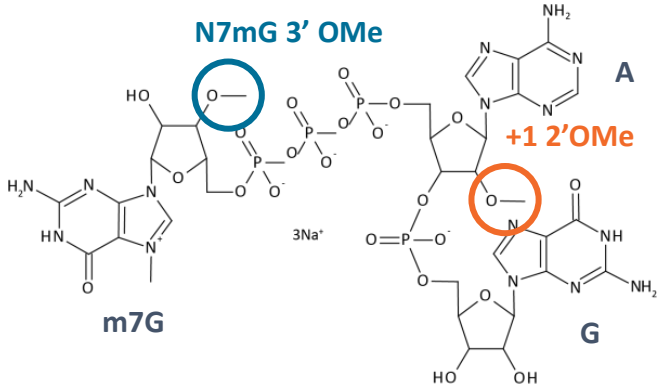
Growing the CleanCap® family | CleanCap® AG technology portfolio

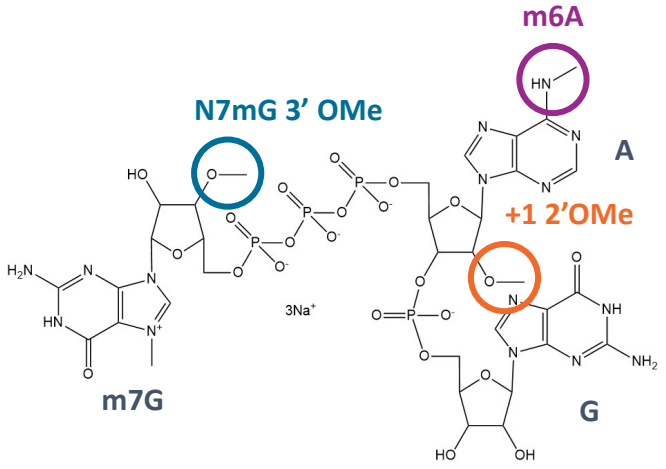
CleanCap AG



CleanCap AG 3'OMe



CleanCap M6



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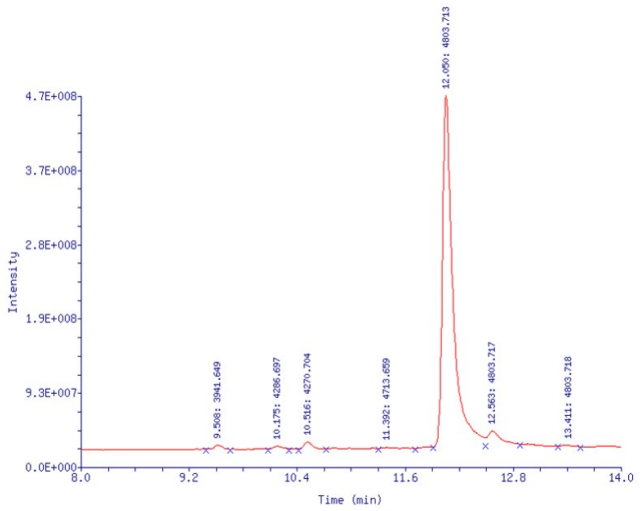
Maintenance of capping efficiency across CleanCap® family members

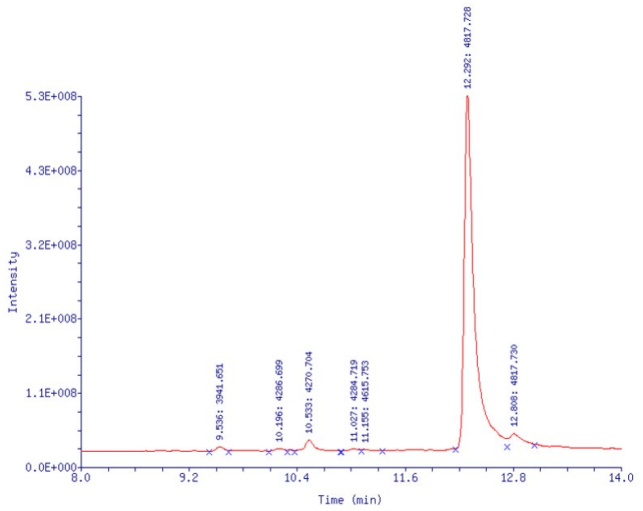
CleanCap AG

97.9%



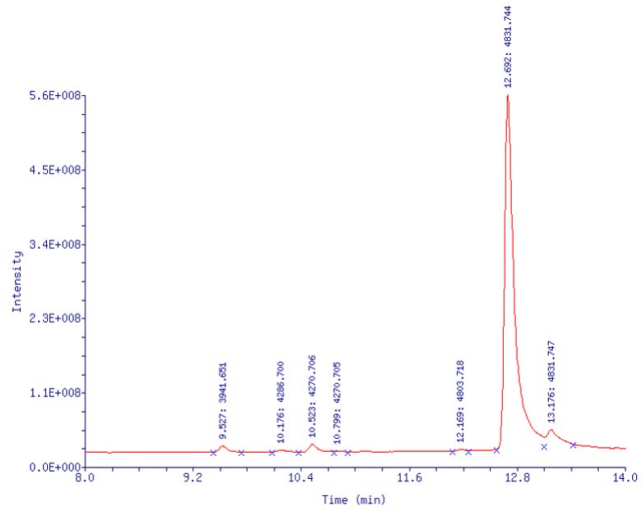
CleanCap AG 3'OMe

97.3%



CleanCap M6

97.6%



Capping efficiency by LC-MS – all cap analogs produce >95% capping efficiency

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CleanCap® M6 analog quality attributes are maintained using different sequences and lengths

5’ Cap	Constructs	Length	IVT Reaction Yield	Capping Efficiency	Relative dsRNA
CleanCap M6 N1-methylpseudo-UTP modified bases	eGFP	~ 1kb	4.4 ± 0.1 mg/mL	97.9 ± 0.4%	++
	FLuc	~2 kb	5.15 ± 0.1 mg/mL	99.6 ± 0.1%	+
	Cas9	~4.5 kb	5.1 ± 0.1 mg/mL	97.2 ± 1.2%	++

Capping efficiency by LC-MS; dsRNA by J2 blot

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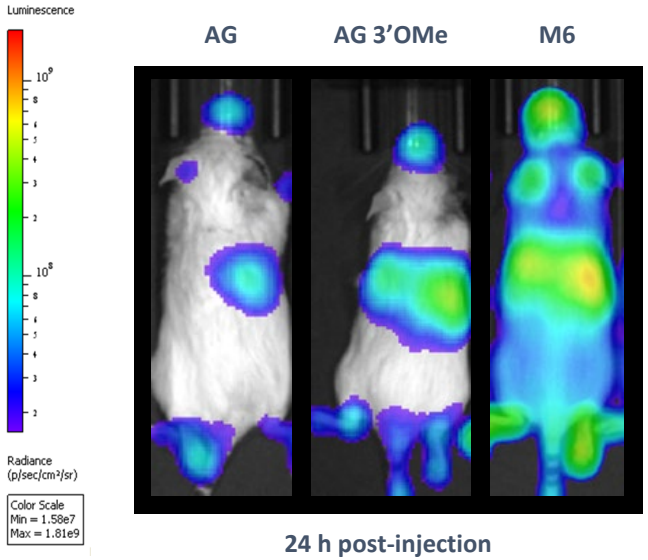
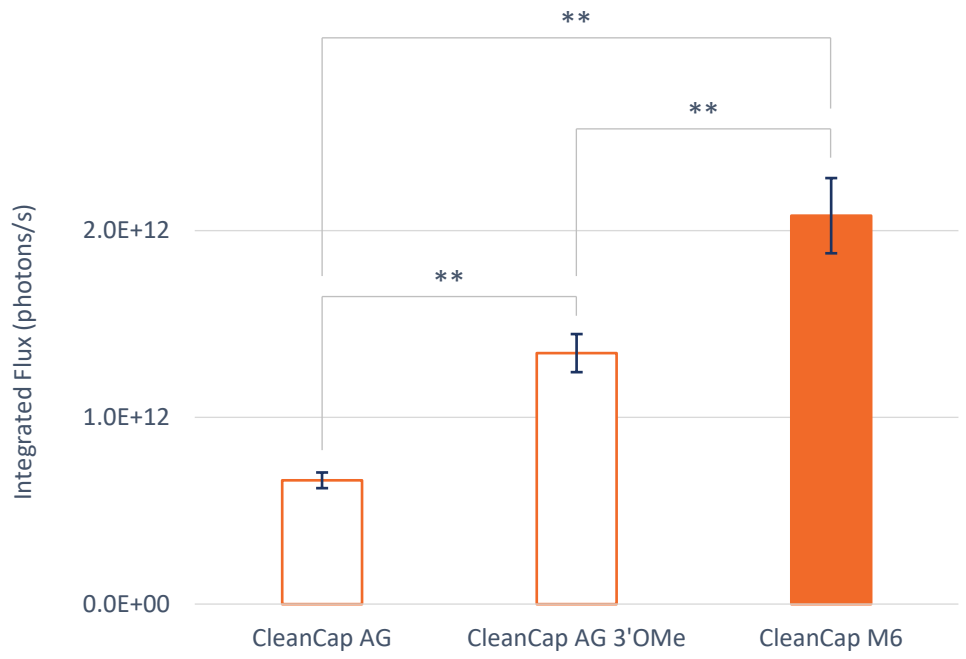
CleanCap® M6 analog significantly improves protein expression levels

M6 modification promotes higher protein translation (FLuc) than previously observed

Whole body luminescence from FLuc mRNA manufactured with different cap analogs



Performance of FLuc mRNA in an LNP-formulated, tail vein delivered mouse model. 1 mg/kg dose per group. Luciferase activity, as photons per second, is measured after luciferin injection. The difference between groups is cap analog structure. All other variables are controlled.



All groups of significantly different, ** $p < 0.01$, one-way ANOVA
Error bars are standard error of mean. n = 5/group

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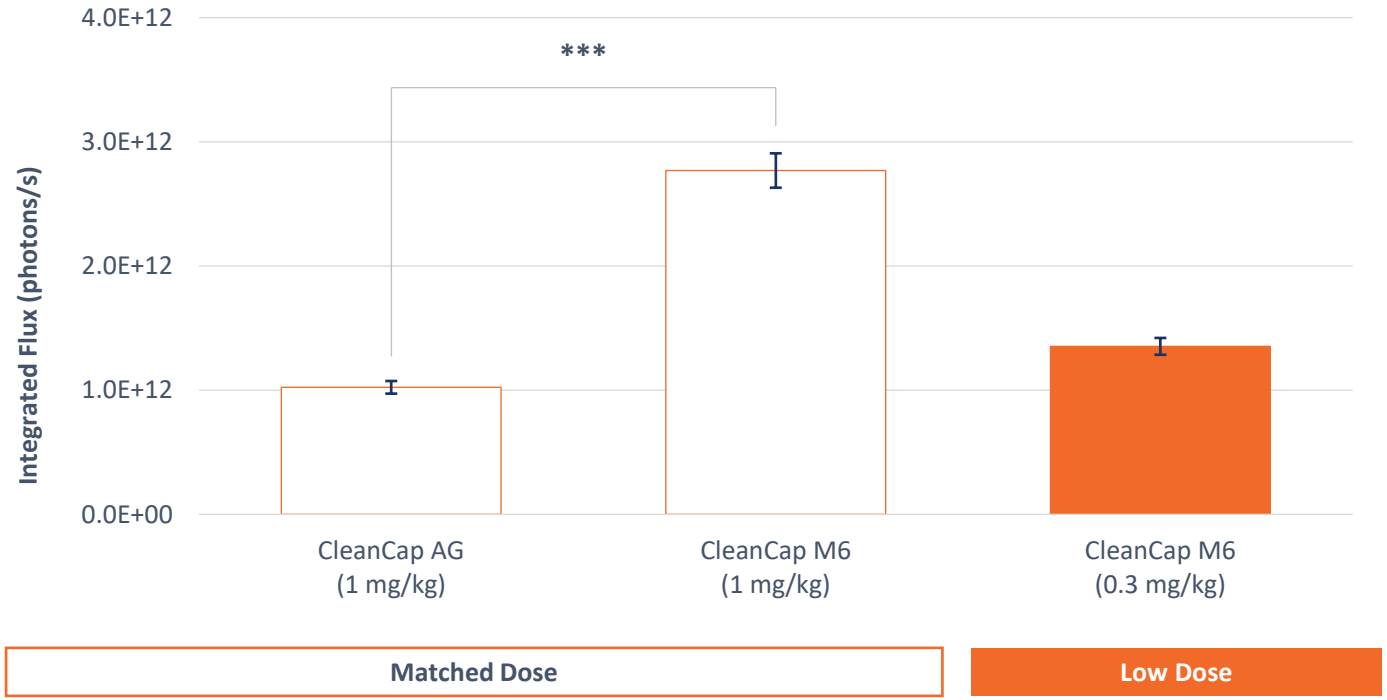
CleanCap® M6 analog enables potential lower dosing scenarios

M6 modifications allows for lower mRNA dosing regimes to get similar protein expression levels

Standard dose (1 mg/kg)
CleanCap AG

Standard dose (1 mg/kg)
CleanCap M6

Low dose (0.3 mg/kg)
CleanCap M6



All groups of significantly different, *** p < 0.001, one-way ANOVA Error bars are standard error of mean. n = 7/group

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Capping strategy comparison for potent mRNA manufacturing

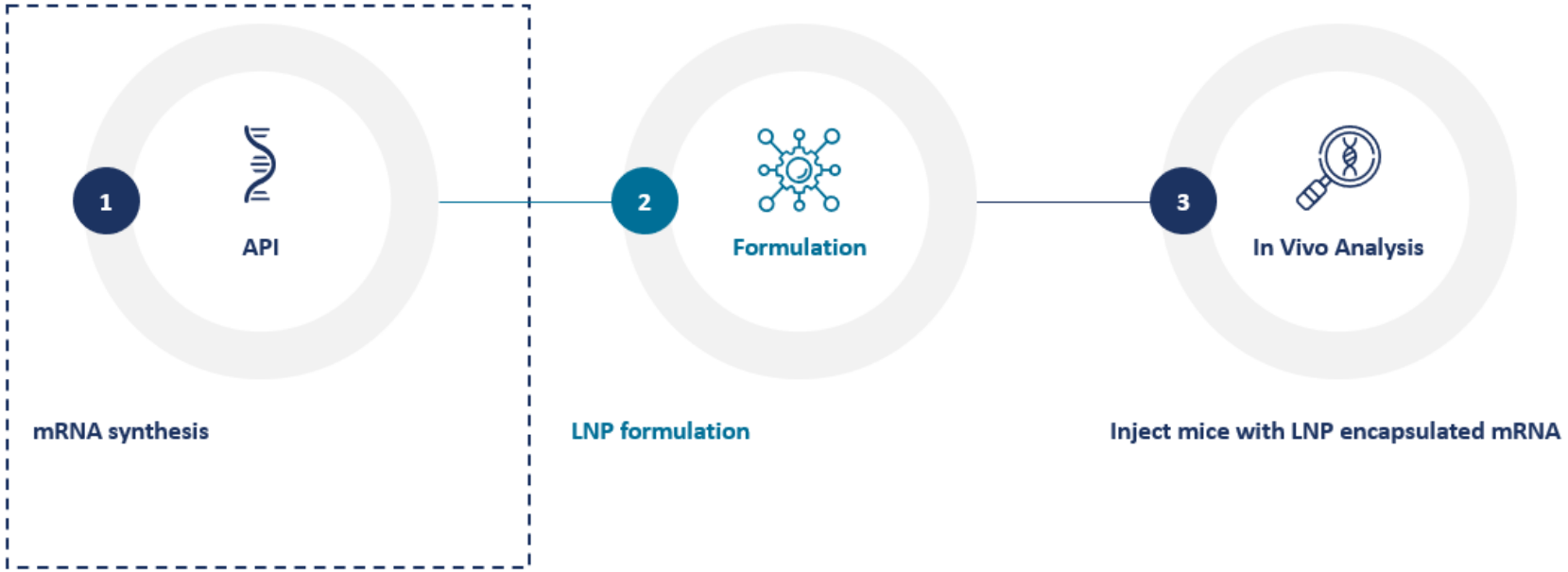
Enzymatic capping (Post-transcriptional)

VS

Co-transcriptional capping

Generation	Cap Analog
4th	CleanCap M6

Luciferase mRNA Expression in mice



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mRNA quality attributes comparing CleanCap® technology to enzymatic capping

Sample	5’ Capping	IVT Reaction Yield	Integrity	Capping Efficiency	Relative dsRNA	Residual DNA	Residual Protein
N1-methylpseudo-UTP modified Firefly luciferase (FLuc)	Enzymatic (Cap 1)	4.4 mg/mL	92.8%	99%	++	1.27 ng/mg	< 2%
	CleanCap AG (Cap 1)	4.7 mg/mL	93.3%	97%	++	1.12 ng/mg	< 2%
	CleanCap AG 3’OMe (Cap 1)	4.4 mg/mL	94%	96%	++	1.22 ng/mg	< 2%
	CleanCap m6 (Cap 1)	4.3 mg/mL	97%	97%	+	0.44 ng/mg	< 2%

mRNA integrity by IP-RP-HPLC; Capping efficiency by LC-MS; dsRNA by J2 blot; Residual DNA by qPCR; Residual Protein by NanoOrange

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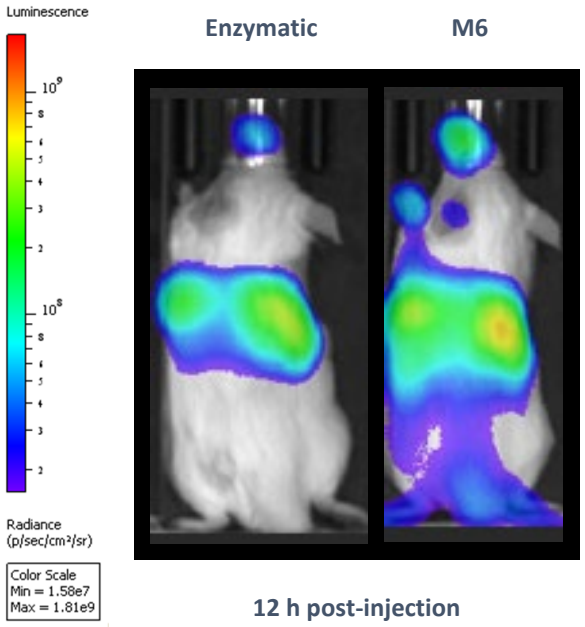
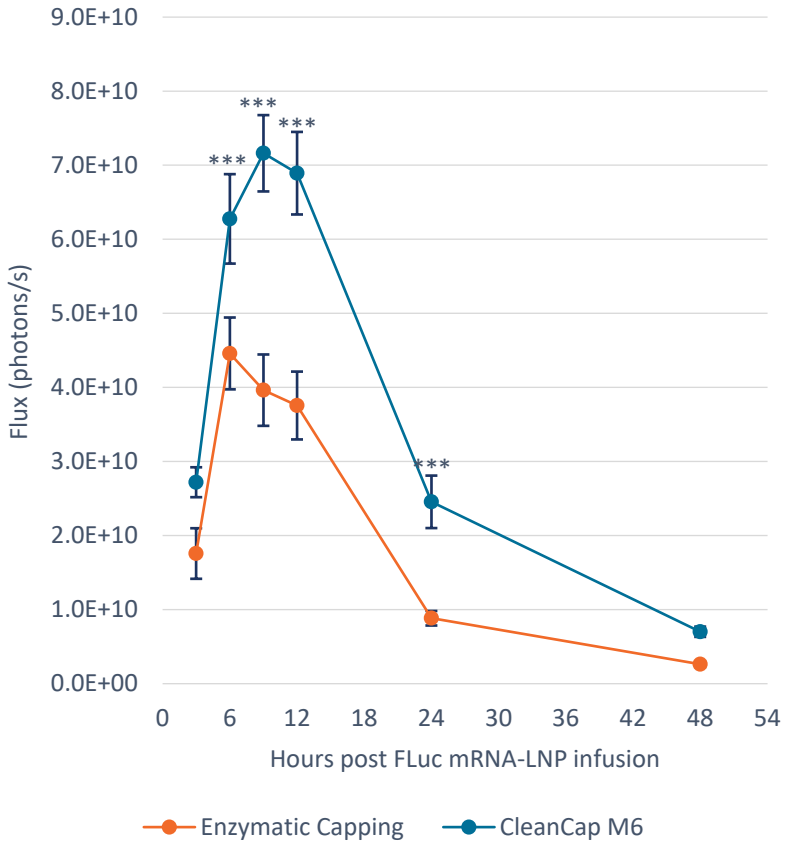
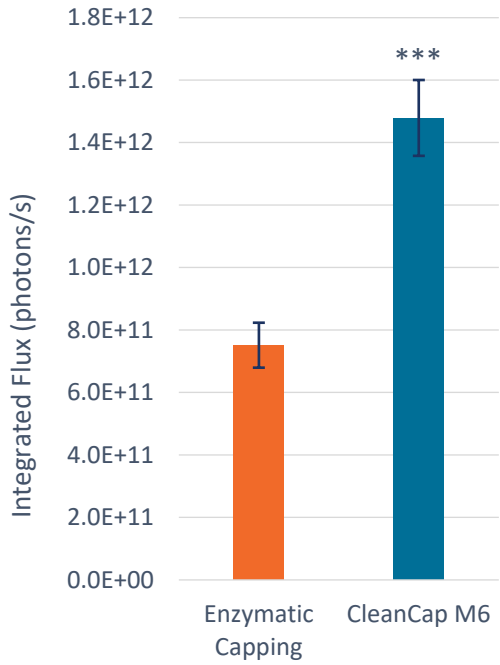
CleanCap® M6 makes mRNA more potent – superior protein expression compared to enzymatically capped mRNA



Performance of FLuc mRNA in an LNP-formulated, tail vein delivered mouse model. 1 mg/kg dose in each group. Luciferase activity, as photos per second, is measured after luciferin injection. The difference between groups is the capping strategy. All other variables are controlled.





*** p < 0.001, two-tailed T test
Error bars are standard error of mean
n = 9/group

*** p < 0.001, two-tailed T test. Error bars are standard error of mean. n = 9/group

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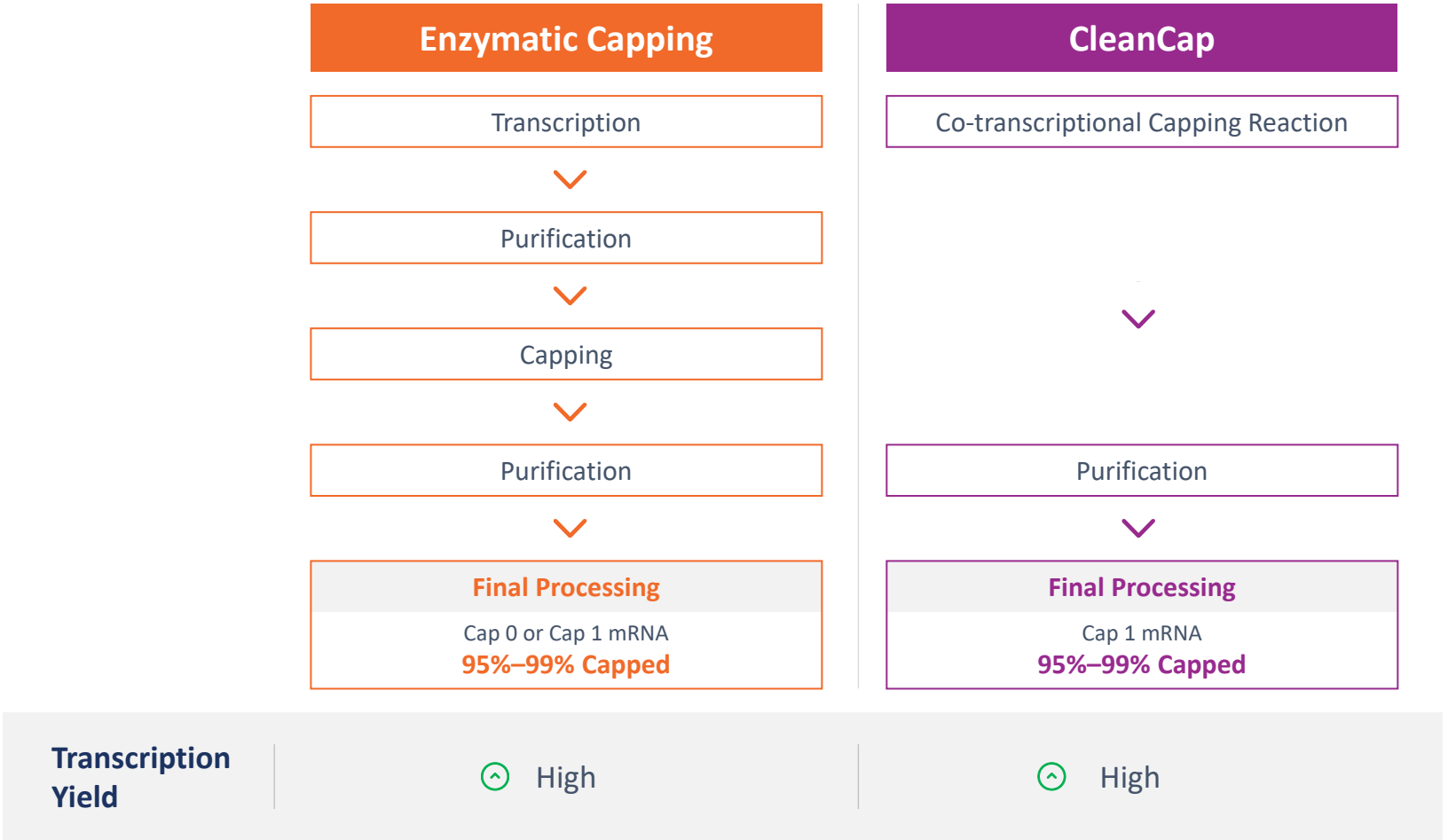
Simplified mRNA manufacturing with CleanCap® technology



Transcription Yield	High	High

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Available now

Standalone cap analog	Size	SKU
CleanCap Reagent M6	1 µmol	N-7453-1
	5 µmol	N-7453-5
	10 µmol	N-7453-10
	100 µmol	N-7453-100

mRNA service	Modification	SKU
CleanCap M6 capped custom mRNA	Unmodified Bases	L-7507
	Custom Base Modified	L-7508
	PsU	L-7509
	N1-Me-PsU	L-7510

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Clean Cap® M6 analog| The Clear Choice

Higher protein expression

- New cap structure can product 30%+
- Potential to increase potency of mRNA drug substance
- Lower doses results in higher manufacturing yield

Capping efficiency

Provides category leading capping efficiency of >95%

- Increased IVT efficacy resulting in high manufacturing yield
- Demonstrates reduced immunogenicity compared to other cap analogs

Manufacturing ease

Maintains the one-pot workflow benefit of CleanCap technology

- Simplified manufacturing process, decreasing process risk
- Lowers time, labor, and cost to manufacture

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Agenda

01	History of capping technology
02	Introduction of novel cap analog – CleanCap® M6
03	**Economics of CleanCap® technology**

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Economics considerations of mRNA capping strategies

- Third party study on economics of mRNA manufacturing and cost drivers
- 30 interviews of Biopharmaceutical experts in North America and Europe
- Quantified the raw material, labor and opportunity costs and benefits of each strategy

Reagent costs

Labor costs

Purification costs

Total manufacturing time

Process development time

Challenges at large scale manufacturing

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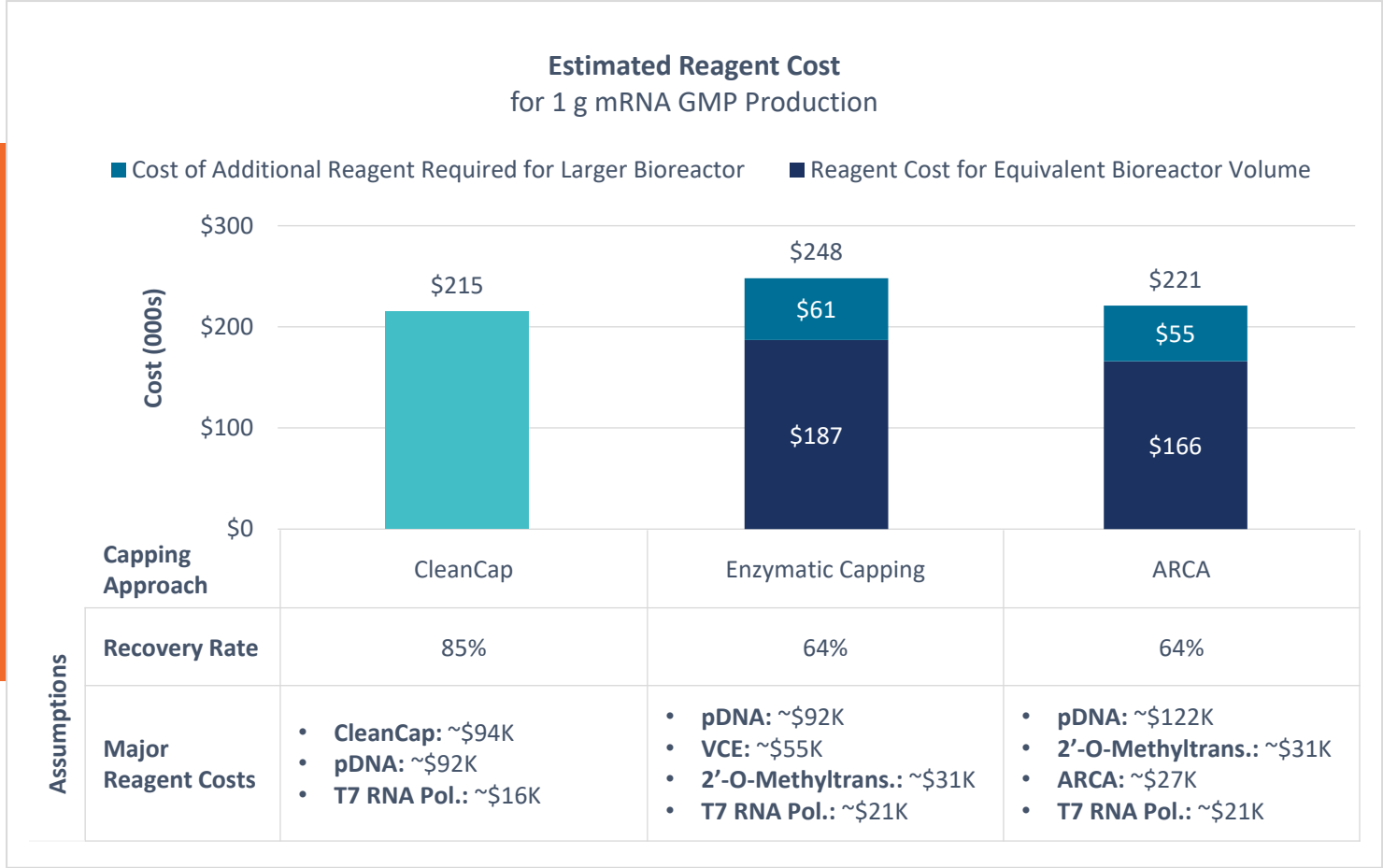
Reagent cost comparison

Estimated both enzymatic capping and ARCA technologies lead to higher total reagent cost per 1 g of produced mRNA, GMP-grade:

- ~$248k for enzymatic,
- ~$221k for ARCA
- ~$215k for CleanCap® technology



Assumptions	CleanCap	Enzymatic Capping	ARCA
Capping Approach			
Recovery Rate	85%	64%	64%
Major Reagent Costs	• **CleanCap:** ~$94K • **pDNA:** ~$92K • **T7 RNA Pol.:** ~$16K	• **pDNA:** ~$92K • **VCE:** ~$55K • **2'-O-Methyltrans.:** ~$31K • **T7 RNA Pol.:** ~$21K	• **pDNA:** ~$122K • **2'-O-Methyltrans.:** ~$31K • **ARCA:** ~$27K • **T7 RNA Pol.:** ~$21K

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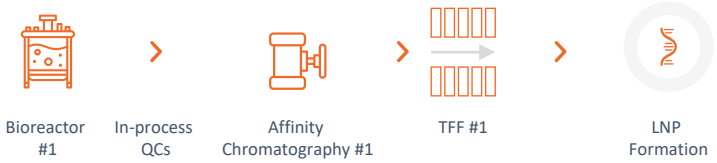
Process development time

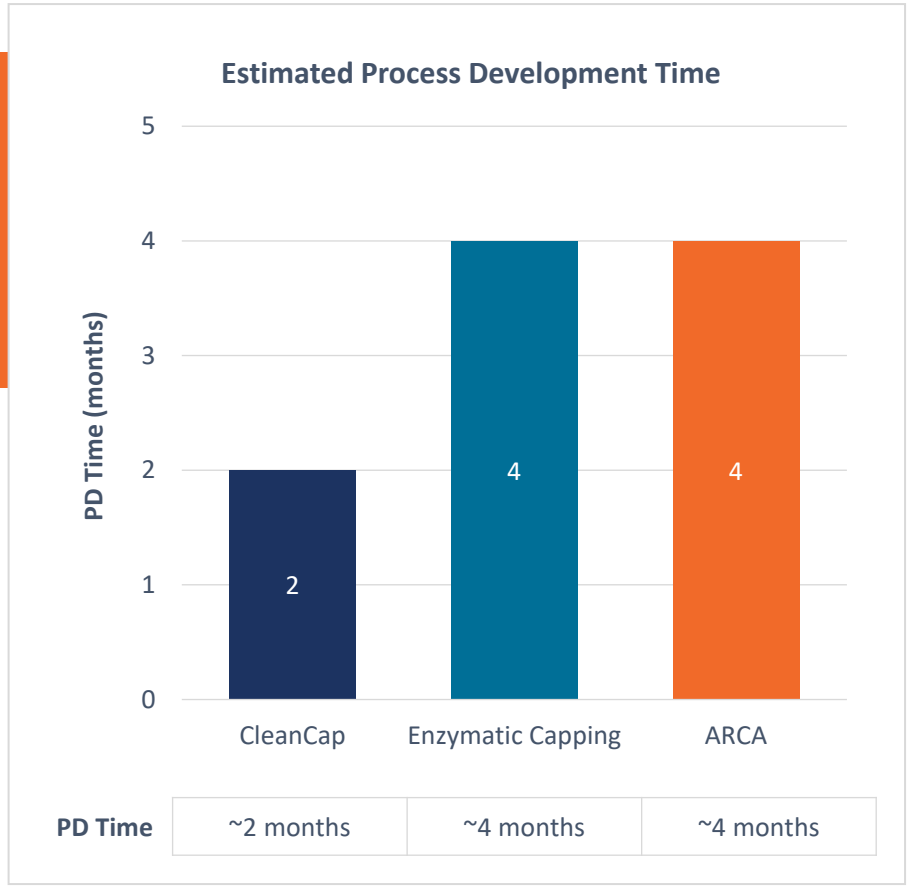
Process development time utilizing CleanCap co-transcriptional technology is estimated to be about **½ the time required** to optimize enzymatic or ARCA capping

- Simplified manufacturing process
- Fewer failure points/lower risk profile

CleanCap® Technology Capping Workflow Overview



Enzymatic Capping Workflow Overview



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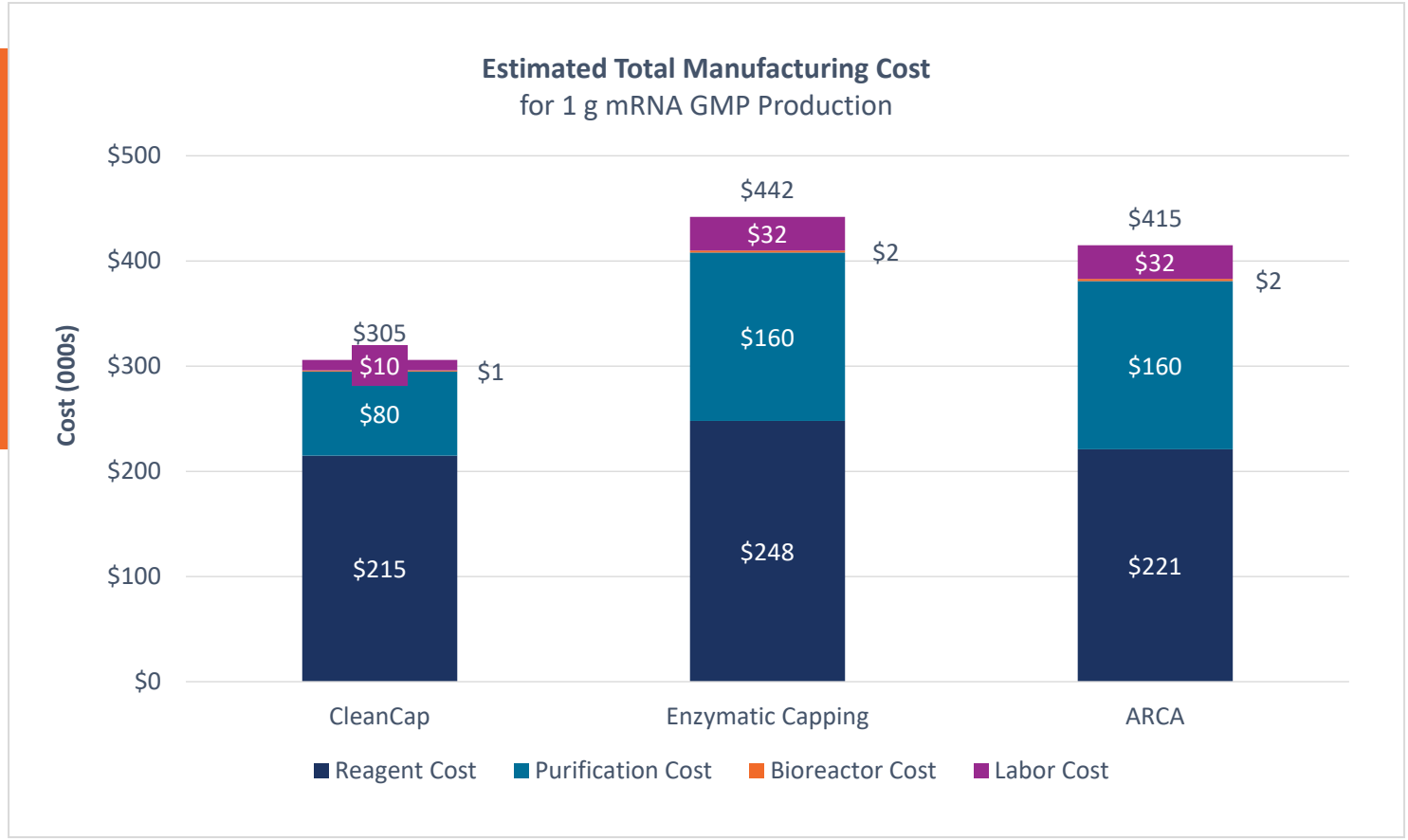
Total manufacturing cost comparison

When comparing the overall manufacturing costs of the three capping strategies, CleanCap® technology is expected to be **30% less than enzymatic capping and 20% less than ARCA.**

For 1 gram of GMP grade mRNA batch, this CleanCap technology showed an estimated savings of:

- ~$135,000 compared to enzymatic capping
- ~$110,000 compared to ARCA capping



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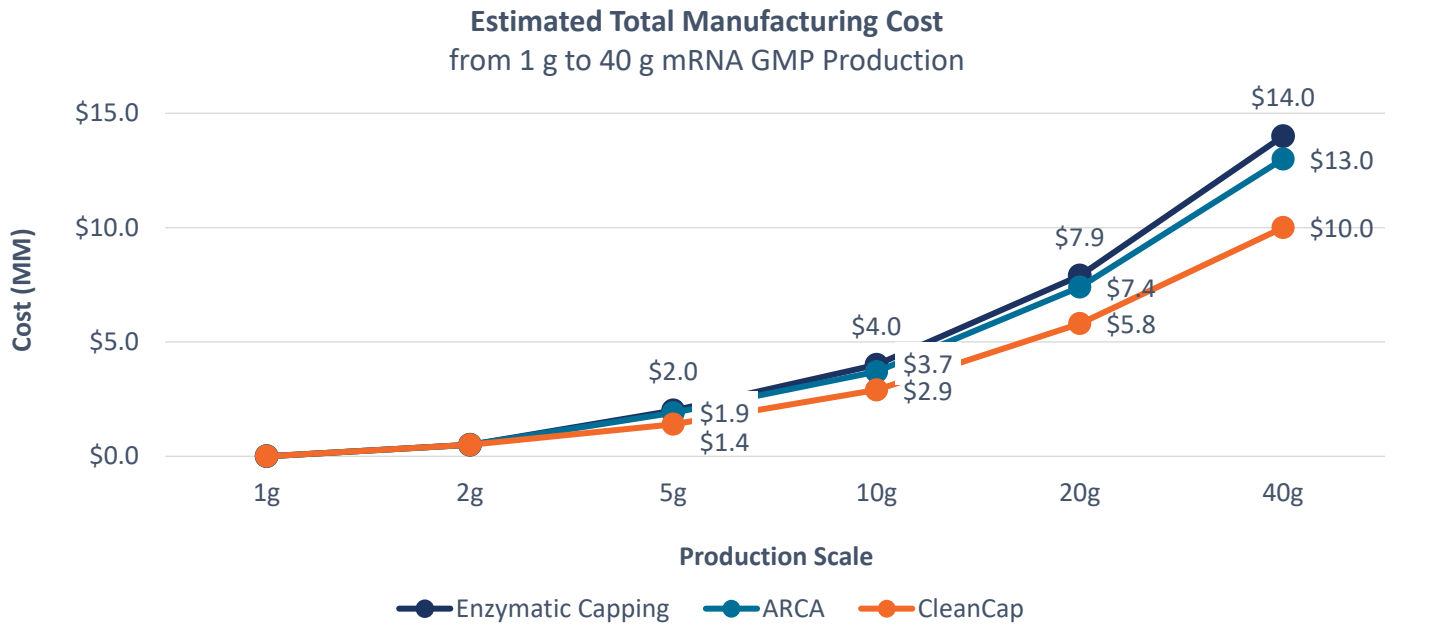
Manufacturing Costs at Large Scale

Costs are non-linear in the range of 1 to 40 gram

At the greatest amount of production as predicted for Phase III studies,

ARCA and enzymatic capping are estimated to be 30% and 40% more expensive than CleanCap



Corresponding Development Stage for mRNA Therapeutics

Ph I → Ph II → Ph III

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Conclusion slide – Benefits of using CleanCap® technology

Reduce both reagent and labor costs

- Simplified and streamlined manufacturing process limited excess raw material consumption and labor costs
- Cost savings increase significantly as manufacturing processes scale into late clinical and commercial development

Cap1 structure – no extra steps

- CleanCap technology achieves Cap1 structure without the need for multiple enzymatic and purification processes
- Extra bioreactor and purification steps are a fixed cost per batch, even with column reuse

Process development time is cut in half

- Simple processes decrease process risk and accelerate development timelines
- Preclinical velocity is key to bringing new therapies to market

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Who we are | TriLink BioTechnologies

Determined to deliver innovative nucleic acid tools and services to help you bring transformative nucleic acid therapies from research to patients.

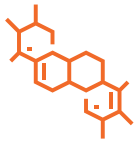
Technical expertise to support your programs:

- Capping analogs, NTPs, and modified UTPs
- Custom oligonucleotides
- Custom chemistry
- Plasmid manufacturing for mRNA
- Discovery mRNA Services (>25 years, >975 customers served)
- GMP mRNA Services (>100 batches to date)
- Process Development, Quality Systems, and Analytical Services

Products & Technology

CleanCap®

NTPs



Services

pDNA → IVT reaction → mRNA → Analytical

Expert Support

Discovery → Clinical GMP manufacturing

Process Development, Analytics, Quality Systems

Innovation

Rational R&D → Technology advances

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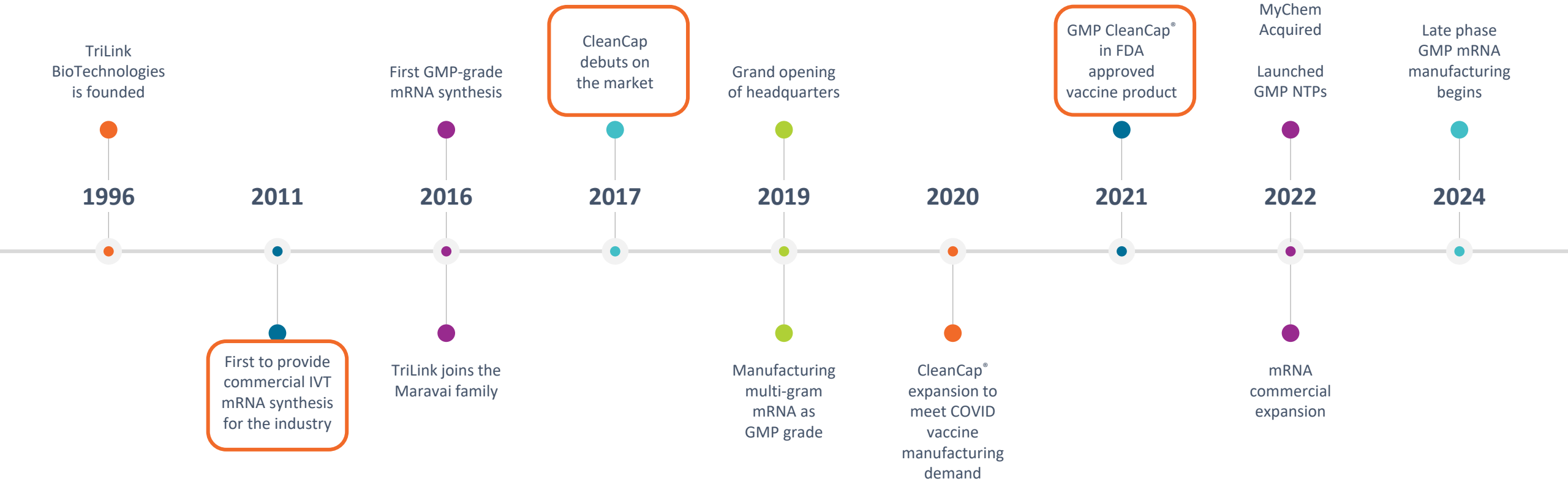
Our history | Over 25 years of innovation in mRNA



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Facilities to meet the demand in mRNA products and services

Wateridge facility

mRNA & related raw materials

- 118,000 ft^2 (10,963 m^2)
- mRNA services for development and early clinical
- CleanCap® reagents and NTP innovation and scale up

Flanders building 1

Nucleic acid production

- 32,000 ft^2 (2,973 m^2)
- CleanCap Reagents and NTP manufacturing
- GMP raw materials for clinical and commercial use
- BARDA Award

Flanders building 2

Late phase clinical mRNA manufacturing

- 32,000 ft^2 (2,973 m^2)
- mRNA development and manufacturing space
- Phase 1 clinical and beyond

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Natalie Hauser

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Contact us

To order CleanCap® M6 analog

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CleanCap® Reagent M6 | TriLink BioTechnologies

Thank you

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