CleanCap® Co-transcriptional Capping Streamlines mRNA Manufacturing

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Abstract

Messenger RNA (mRNA) therapy is a popular platform technology for expressing proteins in cells or *in vivo* because there is minimal risk of insertional mutagenesis. mRNA transfection is used to express proteins for genome editing, protein replacement, vaccines and antibody expression. To avoid an innate immune response, transfected mRNAs should mimic the 5′ cap structure of non-immunogenic endogenous mRNAs.

During eukaryotic RNA capping, Cap 0 (^{m7}GpppN) is formed as an intermediate. Methylation of the 2′-O position of the first cap-proximal nucleotide forms Cap 1 (^{m7}GpppN$_m$N). In ~50% of transcripts, the 2′-O position of the second cap-proximal nucleotide is also methylated to form Cap 2 (^{m7}GpppN$_m$N$_m$). N6-methylation of adenosine at the first cap-proximal nucleotide (^{m7}Gppp^{m6}A$_m$N) is the second most frequently found modification in mRNA and occurs in conjunction with Cap 1 (and potentially Cap 2).

The immunogenic role of mRNA caps requires elucidation. Viral attenuation occurs after deleting methyltransferases that RNA viruses encode to convert Cap 0 to Cap 1. IFITs bind Cap 0 and activate antiviral translational repression. Thus, Cap 1 (and possibly Cap 2) marks endogenous mRNAs as "self" RNAs. The role of Cap 2 and Cap 1 (^{m6}A) is poorly understood because such capped mRNAs have not been produced synthetically at scale. In a recent study, Cap 1 (^{m6}A) caps may increase stability and translation while decreasing de-capping of mRNA (Mauer *et al.*, Nature 2017, 541, 371-375).

Traditional co-transcriptional capping utilizes ARCA (Anti-Reverse Cap Analog) to produce immunogenic Cap 0 with poor capping (~70%) and low yield. Post-transcriptional enzymatic capping to produce Cap 0 or Cap 1 is hindered by highly structured 5′ ends, requires further purification and is expensive. Methods to produce Cap 2 mRNAs have not been commercially available. We developed CleanCap®, a novel co-transcriptional capping method to yield Cap 0, Cap 1, Cap 2, Cap 1 (^{m6}A) or unnatural caps (Figure). Capping with CleanCap is reproducibly efficient (90-99%), less expensive than enzymatic capping and is done in a "one-pot" reaction without additional purification. In addition, CleanCap co-transcription method yields higher amount of capped mRNA than other methods including ARCA and enzymatic. Our studies in a THP-1 Dual monocyte cell line indicate that these various CleanCap mRNAs exhibit altered expression and immunogenicity. Further *in vivo* studies to characterize these mRNAs are ongoing.

Innate Immune Sensors Recognize mRNA

Transfection of cells with unmodified RNAs can lead to cell death due to activation of innate immune pathways

Toll-like receptors 3, 7, & 8 recognize different RNA forms
- Found in endosomes where some viruses enter cells

Cytosolic sensors
- Protein Kinase R (PKR): dsRNA
- MDA5: long dsRNA
- IFITs: unmethylated cap structures
- RIG-I: 5′-triphosphate

Background: Why mRNA Therapeutics?

mRNA is a popular new tool for gene expression
- Does not have a risk of insertional mutagenesis
- Can transfect difficult cells such as non-dividing cells
- Is transient

Applications
- Genome editing (Transposons, Cre, ZFNs, TALENs and CRISPR/Cas9)
- Gene replacement
- Vaccines

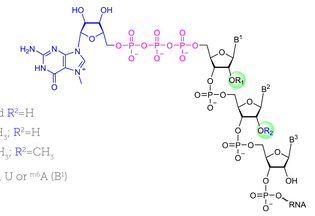
Limitations
- Innate immune response to unmodified mRNA

Solutions
- Proper capping
- Chemical modification of mRNA can prevent innate immune stimulation
- Removal of dsRNA

Figure 1: Cap 0, Cap 1 and Cap 2 Structures of 5′-Ends of mRNAs

Eukaryotic mRNAs have a Cap 1 or Cap 2 structure.

Sensing of proper cap structure is thought to be involved in self/non-self RNA recognition.



Cap 0: R^1 and R^2=H
Cap 1: R^1=CH_3; R^2=H
Cap 2: R^1=CH_3; R^2=CH_3
$B^{1\text{-}3}$ = A, C, G, U or ^{m6}A (B^1)

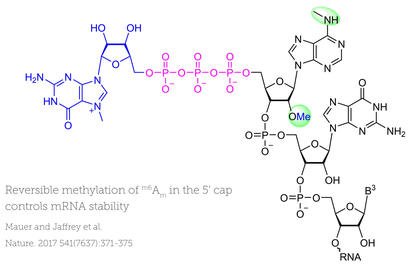
Co-transcriptional capping with CleanCap (Cap 1) helps evade an immune response

Function of mRNA Cap Structures

mRNA cap structures are involved in modulating
- Nuclear export
- Splicing
- Turnover
- De-capping

Cap 1 and Cap 2 are important for self/non-self recognition by the innate immune system
- Cap 0 recognized as foreign
- IFITs recognize non-methylated caps
- Cap 1 methylation reduces binding to pattern recognition receptors
- Role of Cap 2 is largely unexplored because it was not possible to easily generate Cap 2 RNAs until now

Figure 2: N6-methyladenosine Methylated Caps: Regulate Translation and mRNA Stability

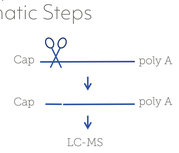


Reversible methylation of ^{m6}A$_m$ in the 5′ cap controls mRNA stability

Mauer and Jaffrey et al.
Nature. 2017 541(7637):371-375

Figure 3: mRNA Capping Assay: Enables Quantitation of Multiple Enzymatic Steps

- Guanyl transfer reaction (Cap)
- N7 methylation of 5′-Guanosine (Cap 0)
- 2′-O-methylation (Cap 1)
- Phosphatase step



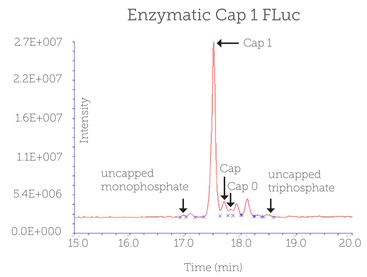
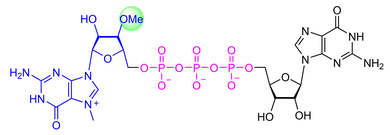


Figure 4: Anti-Reverse Cap Analog (ARCA) Capping is Inefficient



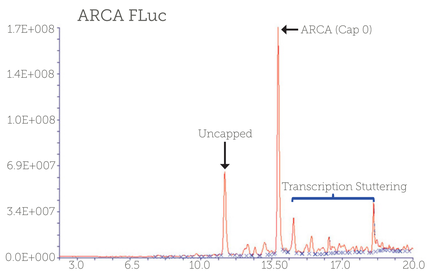
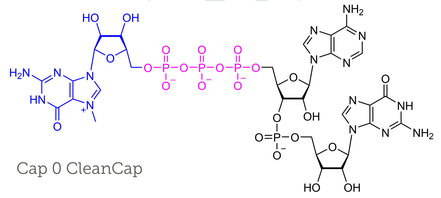
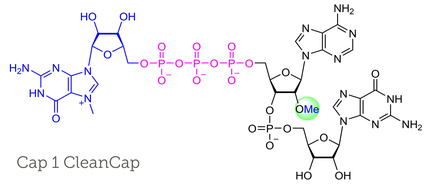


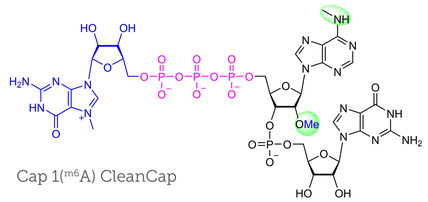
Figure 5: CleanCap Analogs Expand the Range of 5′ Sequences that can be Used to Initiate T7 RNA Polymerase



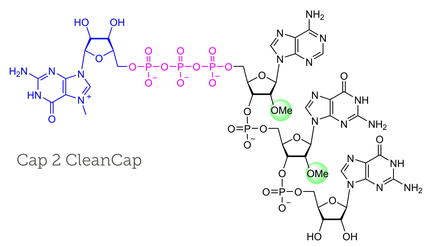
Cap 0 CleanCap



Cap 1 CleanCap



Cap 1(^{m6}A) CleanCap



Cap 2 CleanCap

Figure 6: Comparison of Capping Methods

Capping Method	Capping Efficiency	Cost/mg Capped RNA	Cap 2 Achievable	Cap 1 (^{m6}A) Achievable	Capping Inhibited by 5′ End Structure
Enzymatic	Variable	High	No	No	Yes
ARCA	~70 %	Moderate	No	No	No
CleanCap	~90-99%	Low	Yes	Yes	No

Figure 7: Cap 1 Capping Assay

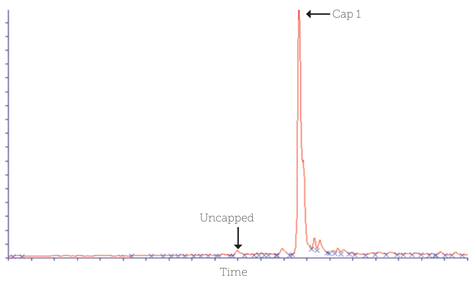


Figure 8: Cap 1 (^{m6}A) Capping Assay

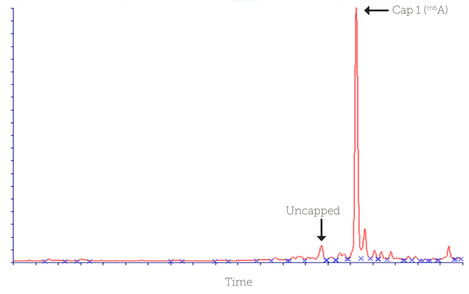
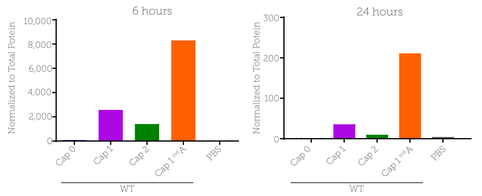


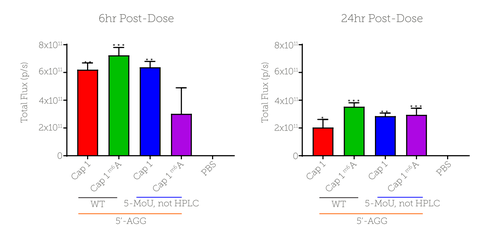
Figure 9: Protein Expression for Cap 0, Cap 1, Cap 2 or Cap 1 (^{m6}A) HPLC Purified Luciferase mRNAs in Mice

a) At 6 and 24 hours Luciferase Protein was Measured in Liver by Western Blot



Cap 0 is inferior to Cap 1, Cap 2, and Cap 1 (^{m6}A) at 6 and 24 hours. Note: ARCA also yields Cap 0.
^{m6}A$_m$ mRNAs are significantly superior to Cap 1 and Cap 2 mRNAs at 6 and 24 hours.

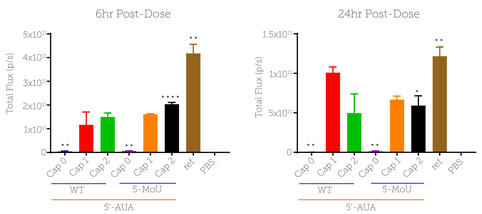
b) *In Vivo* Time-course of Luciferase Expression in Liver-Bioluminescence Imaging



Dunnett's multiple comparisons test
* p<0.05 **p<0.01 ***p<0.001

Not HPLC purified 5-MoU is showed similar activity to HPLC purified WT mRNA

c) *In Vivo* Time-Course of Luciferase Expression in Liver-Bioluminescence Imaging



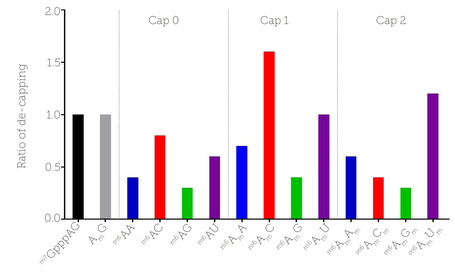
ref. Cap 1 WT ^{m7}GpppA$_m$GG
Dunnett's multiple comparisons test
* p<0.05 **p<0.01 ***p<0.001

Cap 0 is inferior to Cap 1 and Cap 2 at 6 and 24 hours.

Figure 10: *In Vitro* De-Capping with Dcp2 is Decreased with ^{m6}A Capped RNAs. Identity of First and Second Cap-Proximal Influences De-capping

^{32}P labeled capped oligonucleotides with different cap forms and 5′ sequences were de-capped *in vitro* with purified capping enzymes

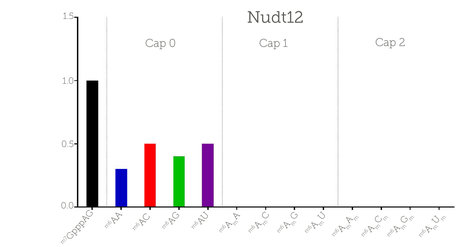
Data courtesy of Samie Jaffrey (Cornell) and Mike Kiledjian (Rutgers)

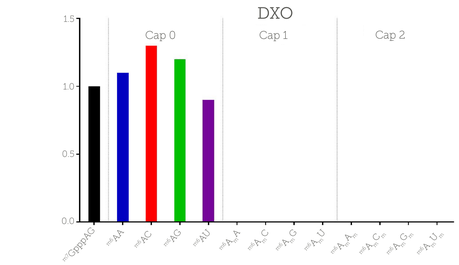


Conclusions for Cap 1 ^{m7}Gppp^{m6}A$_m$G is de-capped more slowly than Cap 1 ^{m7}GpppA$_m$G
- The identity of the second cap proximal nucleotide ^{m7}Gppp^{m6}A$_m$N influences the rate of Dcp2 mediated de-capping
- ^{m6}AG displays the lowest de-capping rate for all capped forms

Figure 11: Nudt12 and DXO Selectively De-Cap Cap 0 but Not Cap 1 or Cap 2 RNAs

Data courtesy of Samie Jaffrey (Cornell) and Mike Kiledjian (Rutgers)





Conclusions

- CleanCap is a novel co-transcriptional capping method
- Very high and consistent capping efficiencies obtained with CleanCap
- CleanCap is an attractive, cost effective alternative to enzymatic or ARCA capping of mRNA
- CleanCap allows novel cap forms that were not previously accessible such as Cap 2 and Cap 1 (^{m6}A)
- Cap 1 and Cap 1 (^{m6}A) Cap RNAs are more active than Cap 0 RNAs *in vivo*
- Cap 1 (^{m6}A) Cap alters activity *in vivo* and may extend persistence of ^{m6}A$_m$ capped RNAs
- ^{m6}A$_m$G RNAs are de-capped more slowly than A$_m$G RNAs
- The identity of the second cap proximal nucleotide (^{m7}Gppp^{m6}A$_m$N) influences the rate of Dcp2 mediated de-capping
- ^{m6}A$_m$G displays the lowest de-capping rate for all capped forms
- Nudt12 and DXO selectively de-cap Cap 0 but not Cap 1 or Cap 2 RNAs

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