Considerations for the Design and cGMP Manufacturing of mRNA Therapeutics

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Abstract

Recently, there has been significant interest in the use of messenger RNA (mRNA) as an *ex vivo* and in vivo therapeutic. Since mRNA is expressed in the cytoplasm it may be particularly useful for improving gene expression in difficult to transfect non-dividing cells. In contrast to plasmid or viral vectors, there is no risk of insertional mutagenesis or subsequent oncogenesis upon mRNA transfection and the transient nature of mRNA expression is desirable for genome editing (CRISPR/Cas9, ZFNs and TALENs) and vaccines. In each case, the goal is to produce a synthetic RNA that mimics a natural mRNA.

In anticipation of clinical testing, it is essential to use sequence designs, raw materials and manufacturing processes that are scalable. Early on, critical decisions must be made about 1) transcription template designs, 2) capping methodologies, 3) sequence optimization, 4) chemical modifications to evade innate immune responses, 5) polyadenylation methods and 6) purification methods.

For optimal expression in cells or target organs, transfected mRNAs must avoid detection by pattern recognition receptors (PRRs) that evolved to sense improperly capped RNAs and double stranded RNA. PRR activation leads to cytokine production, translational arrest and cell toxicity or death. Mammalian mRNAs are modified post-transcriptionally to contain modified nucleotides (e.g. pseudouridine and 5-methylcytidine). These modifications can reduce activation of PRRs and allow maximal translation.

During RNA capping, Cap 0 (m7GpppN) is formed as an intermediate. Methylation of the 2' position of the first nucleotide forms Cap 1 (found in 100% of transcripts) and Cap 2 (found in 50% of transcripts) of endogenous mRNAs. mRNAs generated with commercially available cap analogs (ARCA) contain Cap 0 structures that can be immunogenic. Recombinant enzymes used to generate Cap 1 mRNA are expensive, do not always go to completion and the RNA must be purified prior to capping. A novel co-transcriptional capping method that yields Cap 1 or Cap 2 with high efficiency and lower costs in a "one pot" reaction will be discussed.

First generation mRNAs were modified with pseudouridine or 5-methylcytidine/pseudouridine and had Cap 0 structures. Data from improved second generation Cap 1 mRNAs containing a combination of sequence engineering and chemical modifications will be presented. We will provide a broad roadmap for the application of these principles to the design and manufacturing of novel mRNA therapeutics.

Background: Why mRNA Therapeutics?

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

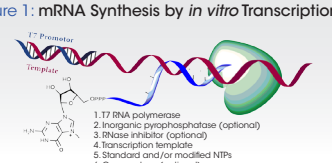
- **Applications**
  - Genome editing (Transposons, Cre, ZFNs, TALENs and CRISPR/Cas9)
  - Gene replacement
  - Vaccines
- **Limitations**
  - Innate immune response to unmodified mRNA
- **Solutions**
  - Proper capping
  - Chemical modification of mRNA can prevent innate immune stimulation
  - Removal of dsRNA

Chemical Modification and Sequence Optimization of mRNA Can Prevent Innate Immune Stimulation

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

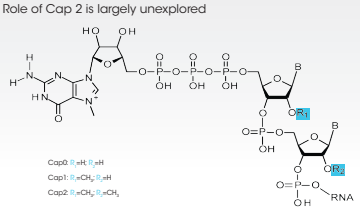
- **Toll-like receptors 3, 7 & 8 recognize different RNA forms**
  - Found in endosomes where some viruses enter cells
- **Cytosolic sensors**
  - Protein Kinase R (PKR): dsRNA
  - MDA5: dsRNA
  - IFITs: unmethylated cap structures
  - RIG-I: 5' triphosphate
  - cGAS/STING – cytosolic DNA

Figure 1: mRNA Synthesis by *in vitro* Transcription



Function of mRNA Cap Structures

- **mRNA cap structures are involved in modulating**
  - Nuclear Export - Splicing – RNA Turnover - Translational Regulation
- **Cap 1 and Cap 2 are important for self/non-self recognition by the innate immune system**
  - IFITs recognize non-methylated caps
  - Cap 0 recognized as foreign
  - Cap methylation reduces binding to pattern recognition receptors
  - Role of Cap 2 is largely unexplored



Capping Methods

- **Enzymatic capping characteristics**
  - Utilizes Vaccinia virus capping enzyme and 2' O-methyltransferase enzymes
  - Can potentially achieve 100 % capping with a natural unmodified cap structure
  - Very costly at scale with significant batch- to-batch enzymatic
  - Accessibility of 5' end important for efficient capping

Co-transcriptional capping

- **ARCA capping characteristics**
  - Capping fairly reproducible (60-80 % capping, 20-40% uncapped)
  - There is a significant amount of transcriptional "stuttering"
  - Cap contains an unnatural 3' O-methyl group
  - Transcript must start with G
- **CleanCap™ 3rd generation cap analog**
  - Capping efficiencies typically 90-99%
  - Can produce Cap 0, Cap 1, or Cap 2
  - Yields a natural unmodified cap structure
  - Allows any base (A, C, G, or U) at 5' end
  - Allows ^{m6}A or $^{m6}A_m$ at 5' end
  - More affordable than enzymatic capping

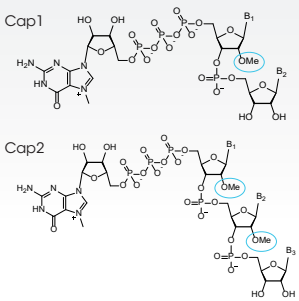


Figure 2: CleanCap™, Enzymatic and ARCA, Capping Comparison

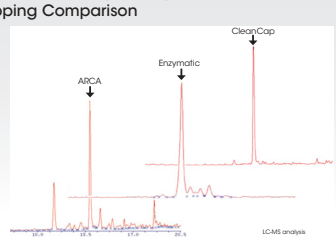
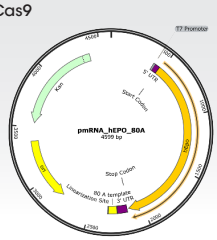


Figure 3: Template Design Cas9

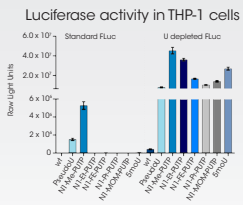
- Endotoxin free
- Kanamycin resistance
- Epitope tags may be undesirable
- Is GMP plasmid required?
- Cell banking
- Think about plasmid sourcing early



Chemical Modification in Combination with Sequence Optimization

- TriLink offers over 200 modified NTPs
- Uridine depletion of template in combination with chemical modification increases activity

Luciferase activity in THP-1 cells



- Primary sequence affects selection of optimal chemical modifications
- Uridine depletion of transcription template frequently improves incorporation by T7 RNA polymerase as well as transcription quality
- In numerous uridine-depleted sequence contexts, 5-methoxyuridine yields high cell activity, low toxicity, and reduced interferon induction (Luciferase, Renilla, mCherry, ß-gal and others)

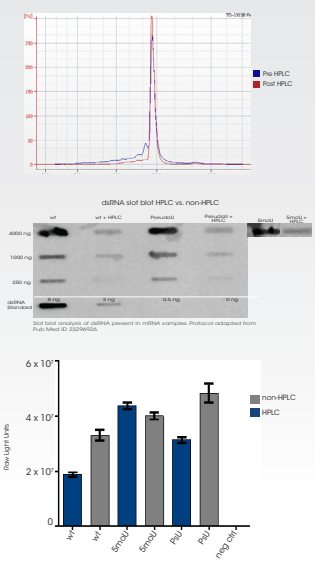
mRNA Purification

Purification is required to remove: salts, NTPs, cap analogs, proteins, truncated mRNA products, residual DNA and sometimes double stranded RNA

- **TriLink has developed two complementary chromatographic mRNA purification methods:**

- LC-Isolation
  - Removes residual proteins, DNA, and NTPs
  - Highly scalable
- Reverse phase HPLC (RP-HPLC)
  - Decreases double-stranded RNA
  - Enriches for full length mRNA
  - Longer sequences more challenging to purify

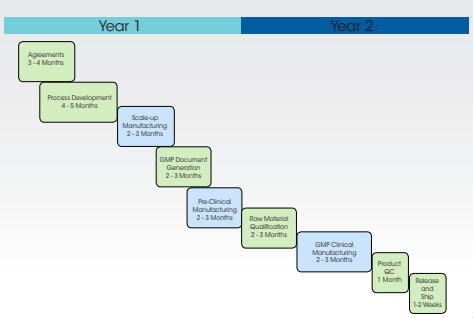
Figure 4: RP-HPLC Purification Yields More Homogenous Luciferase mRNA and Depletes dsRNA (Slot Blot)



Purification Conclusions

- For some applications, LC-Isolation may be of sufficient quality especially if using uridine-depleted 5-methoxyuridine-substituted mRNAs. May not require RP-HPLC
- For other applications, RP-HPLC purification may be required to remove double-stranded RNA
- RP-HPLC purification reduces interferon induction in THP-1 cells in many cases
- When selecting synthesis scale, account for purification yield

Figure 5: Phases GMP mRNA Drug Development Program



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