HPLC Purified Wild-type Cap1 EGFP mRNAs Perform as Well as Pseudouridine Modified mRNAs in Cultured Cells

Richard I Hogrefe[1], Krist T. Azizian[1], Alexandre Lebedev[1], Dongwon Shin[1], Jordana M. Henderson[1], Hiroko Yokoe[2], Anton P. McCaffrey[1] and Joel Jessee[2]

[1]TriLink BioTechnologies, San Diego, CA 92121, USA. [2]MTI-GlobalStem, Gaithersburg MD 20877, USA.

Hypothesis: Optimal capping and purification of mRNA may reduce the need for chemical modification to decrease innate immune stimulation by transfected mRNAs. Innate immune response may be further influenced by mRNA primary sequence, transfection system, and cell line.

Abstract

Messenger RNA (mRNA) therapeutics have gained attention as a platform technology for genome editing, gene replacement therapy, immunotherapy and vaccines. New drug candidates can be delivered to cells or organisms using standard nucleic acid delivery strategies. However, crude preparations of mRNA containing wild-type (wt) bases induce strong innate immune responses, leading to translational shutdown and cell toxicity. This has been partly overcome by introducing modified bases such as pseudouridine (Ψ) which reduce cytotoxicity and may enhance expression.

Kariko *et al.* showed that in wheat germ extracts, which lack the dsRNA sensor protein kinaseR (PKR), wt mRNA showed higher translation compared to modified mRNAs. This suggests that Ψ modification may suppress translation. The observation that Ψ modified mRNAs have higher expression in cells suggests that there may be multiple competing mechanisms.

PKR activation leads to phosphorylation of eIF2alpha and suppression of translational initiation. *In vitro* transcription is known to produce dsRNA side products. Notably, Ψ substitution has been shown to reduce activation of PKR *in vitro* and in cells. In PKR deficient cells, wt and Ψ modified RNAs were translated equivalently.

Recently, it has emerged that the cap structure of mRNAs can influence activation of innate immune sensors. Messages methylated at the N7 position of the guanosine cap are referred to as Cap0. Eukaryotic messages are further methylated at the 2′ position of the first nucleotide (Cap1) or the first and second nucleotide (Cap2). Cap1 and Cap2 are thought to be important for self/non-self RNA recognition.

Recently, Kariko *et al.* reported that HPLC purification of mRNA could deplete dsRNA from *in vitro* transcripts and increased expression of both wt and Ψ modified RNAs. However, this method was scalable to only a few milligrams. We have developed an HPLC method that is scalable to hundreds of milligrams.

Given that wt mRNA has potential for comparable activity to Ψ in the absence of innate immune inhibition of translation, we sought to determine if wt Cap1 HPLC purified mRNA could perform comparably to Ψ modified mRNA. We generated a series of EGFP modified mRNAs and tested them in A549, C2C12, HDF, HEKa, HeLa and HuVec cells. In agreement with previous results, HPLC purification increased the activity of both wt and Ψ mRNAs. Surprisingly, the activity of wt Cap1 HPLC and Ψ Cap1 HPLC mRNAs were comparable in these cell lines. This suggests with proper capping and purification, chemical modification may not be absolutely required.

Innate Immune Recognition

- Pathogenic non-self RNAs are recognized by pattern recognition receptors (PRRs).
- Endosomal PRRs
  -Toll-like receptors 3, 7 & 8 recognize single stranded or double stranded RNA.
- Cytosolic sensors
  - PKR - dsRNA
  - MDA5 - dsRNA
  - RIG-I - 5′ phosphate
  - IFITs - cap structures

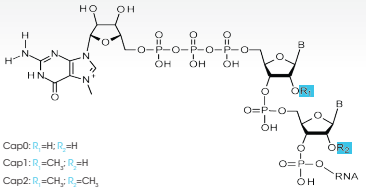
Chemical Modification Reduces Activation of PRRs

- Pseudouridine modification of mRNAs reduces activation of:
  - TLRs
  - RIG-I
  - PKR

(PMID:20457754)

Cap0, Cap1 and Cap2 structures of 5′-Ends of mRNAs

- Eukaryotic mRNAs have a Cap1 or Cap2 structure.
- Sensing of proper cap structure is thought to be involved in self/non-self RNA recognition.



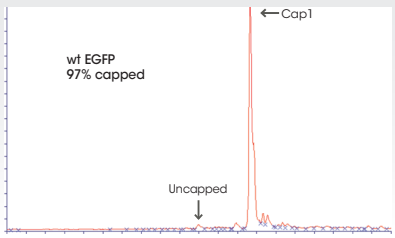
Cap Structure Influences Activation of PRRs

- RIG-I is activated by Cap0 RNAs but not Cap1 mRNAs (PMID:18426922 and 20457754).
- IFIT1 binds Cap0 RNAs more tightly than Cap1 mRNAs (PMID:24371270).

Co-transcriptional Capping Using CleanCap™ Yields Cap1 or Cap2 mRNAs

- Traditional co-transcriptional capping with Anti-Reverse Cap Analog (ARCA) yields Cap0 with 50-80 % capping.
- Enzymatic capping can produce Cap1.
  - Requires multiple purifications
  - Requires two capping enzymes
  - Expensive
  - Variable capping achieved (typically 50-100 %)
- CleanCap™
  - Capping occurs in single reaction during transcription
  - Capping efficiencies of ~95 % are typical
  - Can achieve Cap1 or Cap2
  - More economical than enzymatic capping
  - Higher yields than ARCA capping

Capping Efficiency Assay Shows CleanCap™ Yields High Levels of Cap1

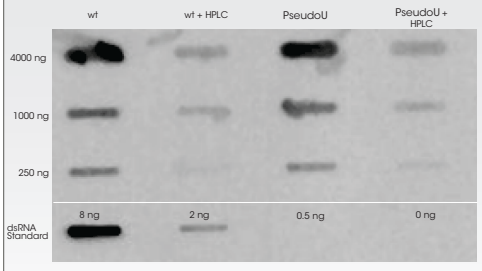


Capping efficiencies are consistently above 95% depending on reaction conditions.

HPLC Purification Increases Relative Expression Levels

Kariko *et al.* demonstrated that analytical HPLC purification of mRNA reduced dsRNA content and increased expression levels. This HPLC method was further optimized, scaled, and transferred to a prep HPLC platform enabling the purification of hundreds of milligrams of crude mRNA per run. This scalable purification method depletes dsRNA and enables the processing of up to 1 gram of mRNA per day.

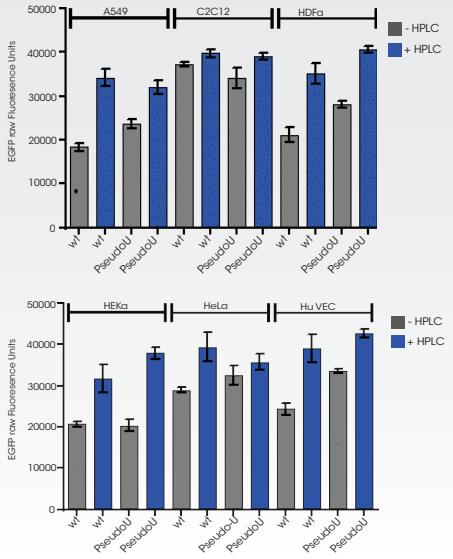
Slot Blot Demonstrates that HPLC Purification Depletes dsRNA



Slot blot analysis of dsRNA present in mRNA samples. Reference adapted from Pub Med ID 23296926.

Indicated amounts of mRNA and dsRNA standard were applied to a positively charged nylon membrane by vacuum suction using a slot blot apparatus. Blocking buffer (0.5% non-fat milk in TBST) was applied by vacuum followed by incubation of primary mouse monoclonal anti-dsRNA antibody (J2; 1:1500) for 20 min with the vacuum off. Membrane was then washed four times with a total of 30 ml of TBST by vacuum suction. Donkey anti-mouse HRP conjugated secondary antibody (1:1500) was incubated for 20 min with the vacuum off. Afterwards, secondary antibody was pulled through the membrane by vacuum suction, the membrane was washed again four times with a total of 30 ml of TBST, and signal was detected using ECL reagent and a chemiluminescence imager.

Expression of Wild-type Cap1 HPLC EGFP is Comparable to Pseudouridine Cap1 HPLC EGFP in Cultured Cells



We generated a series of EGFP WT and Pseudouridine modified Cap 1 mRNAs and tested them in human lung carcinoma (A549), mouse myoblasts (C2C12), adult human dermal fibroblasts (HDFa), adult human epidermal keratinocytes (HEKa), human cervical carcinoma (HeLa) and human vascular endothelial (HuVEC) cells. Cells were plated in 96-well format according to cell type in order to achieve 60-70% confluency at the time of transfection 20-24 hours later. Messenger RNAs were transfected in triplicate (25 ng mRNA/transfection) using mRNA-In® transfection reagent (MTI-GlobalStem; 0.75 ul reagent/transfection). Cells were assayed for EGFP expression 21 hours post-transfection. Data were graphed as the mean of three transfections, +/- standard deviation from the mean.

Conclusion

- HPLC purified Cap1 wt EGFP has comparable activity to Cap1 Pseudouridine EGFP.
- mRNA-In® reagent (MTI-GlobalStem) efficiently transfects numerous cell lines with low toxicity.
- As previously reported, HPLC purification depletes double stranded RNA and leads to increased expression.
- Optimized HPLC method scalable to hundreds of milligrams.

Contact
Richard Hogrefe marketing@trilinkbiotech.com

The Modified Nucleic Acid Experts™
www.trilinkbiotech.com

OTS_2016
Montreal QC
09/16