Optimization of In Vitro Transcription for mRNA Production

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Abstract

In vitro transcription (IVT) is a biochemical reaction used for the enzyme-catalyzed synthesis of therapeutic RNA macromolecules, which are generally longer than 200 ribonucleotides and not readily synthesized by automated solid support. Reactions are buffered near neutrality with cations included to stabilize the mRNA and its interaction to enzyme. The T7 RNA polymerase has become the industry standard, and a typical reaction occurs at 37 degrees Celsius for several hours. The performance of the IVT reaction can be quantified as RNA output relative to DNA template input (turnover). Process steps following IVT include 5′-end capping and DNase treatment, followed by purification, TFF and mRNA lipid nanoparticle formulation. To optimize IVT we investigated the use of two alternative salts and surfactant achieving as much as a 208-fold turn-over. Moreover to increase economy of scale we were able to lower the enzyme:DNA ratio yet still achieve 183-fold turnover. To monitor mRNA at each stage of the process, we compared standard agarose gel electrophoresis versus a newer HPLC assay for the ability to resolve the transformed RNA species. We observed little to no influence on mRNA purity or integrity across multiple unit operations including capping, DNase treatment and purification by oligodT, and buffer exchange via TFF. Finally, to demonstrate the expression activity of our IVT produced mRNA encoding green fluorescent protein (GFP), we investigated its physical or chemical delivery into cells and expression analysis by fluorescence imaging and flow cytometry. Here, we outline our progress in mRNA production, aiming to develop a GMP-compatible process for clinically relevant mRNA. The data support IVT reactions at scale with optimized salt, surfactant and enzyme:DNA ratio. Even unpurified, the mRNA so produced exhibited comparable expression activity to purified mRNA reference standard. Currently the team is pursuing future directions including improvements in the downstream purification and concentration process, the in-process and release analytics, the incorporation of the mRNA into lipid nanoparticle, as well as the IVT-based production of other forms of macromolecular RNA.

RESULTS

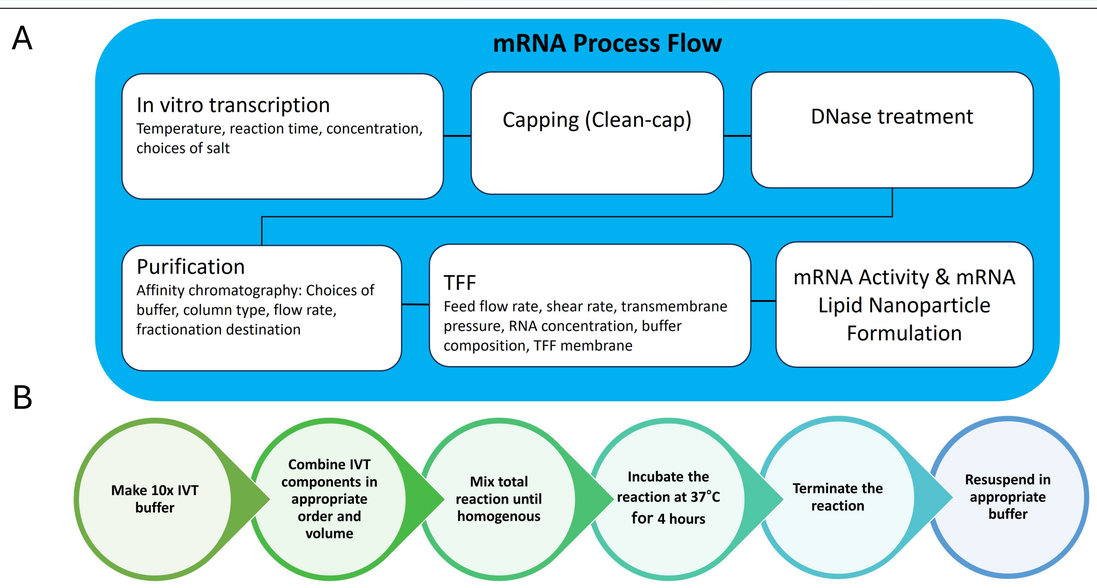

Figure 1. mRNA process flow. A. Flow diagram shows key steps including; in vitro transcription, capping, DNase treatment, mRNA purification, TFF, mRNA activity, and mRNA lipid nanoparticle formulation. B. Schematics of IVT reaction including IVT buffer preparation, addition and mixture of IVT reagents, solution incubation, reaction termination, centrifugation, aspiration, and resuspension in desired buffer.

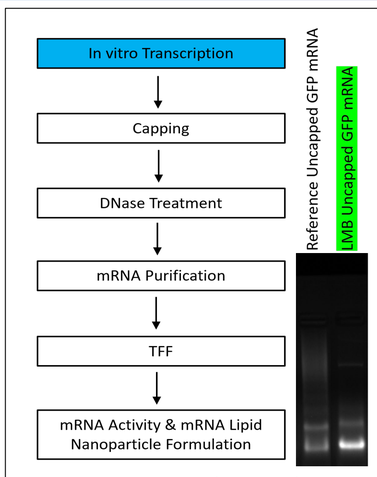

Table 1. IVT Reaction Performance

IVT rxn	Total Volume (mL)	DNA template (µg)	mRNA yield (mg)	Δ	Turnover (grams of RNA per grams of DNA)
Expected (Reference mRNA)	1	50	5		100
LMB 1	0.1	4	0.198	Salt 1	49.5
LMB 2	1	50	10.44	Salt 2	208.8
LMB 3	0.1	5	0.65	Surfactant 1	130
LMB 4	0.1	5	0.61	Surfactant 2	123
LMB 5	1	25	4.58	Reduced enzyme concentration	183.2
LMB 6	10	250	68.83	Optimized	275

Figure 2. IVT. The crude uncapped mRNA was analyzed by RNA agarose gel electrophoresis (RAGE) relative to uncapped mRNA reference standard. The crude material appeared to be primarily one species (mRNA) with a small amount of presumed double-stranded RNA (dsRNA) running just above it.
Table 1. IVT reaction performance. Table summarizes IVT parameters for reaction performance for input of linear DNA template to output crude RNA. The turn-over (grams of RNA per grams of DNA) could be increased by varying the salt but was constant for GMP versus non-GMP surfactant, and could be maintained upon scaling up and lowering T7 RNA polymerase. 275-fold turn over was generated at 10x scale.

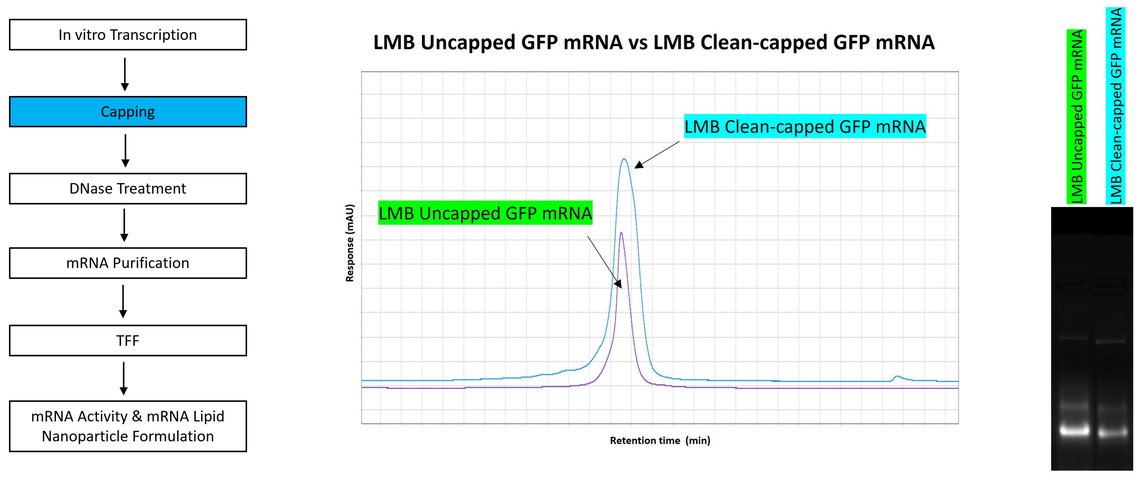

Figure 3. Capping. Comparison of LMB uncapped GFP mRNA vs LMB capped GFP mRNA by HPLC and gel electrophoresis.

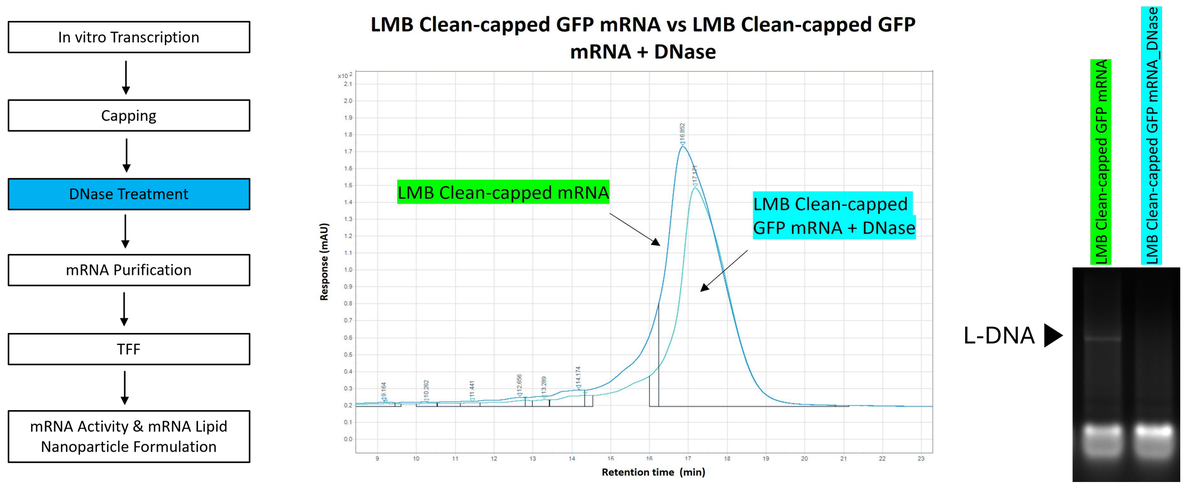

Figure 4. DNAse. DNase treatment was performed after IVT and the material before and after DNase treatment analyzed by HPLC and gel electrophoresis.

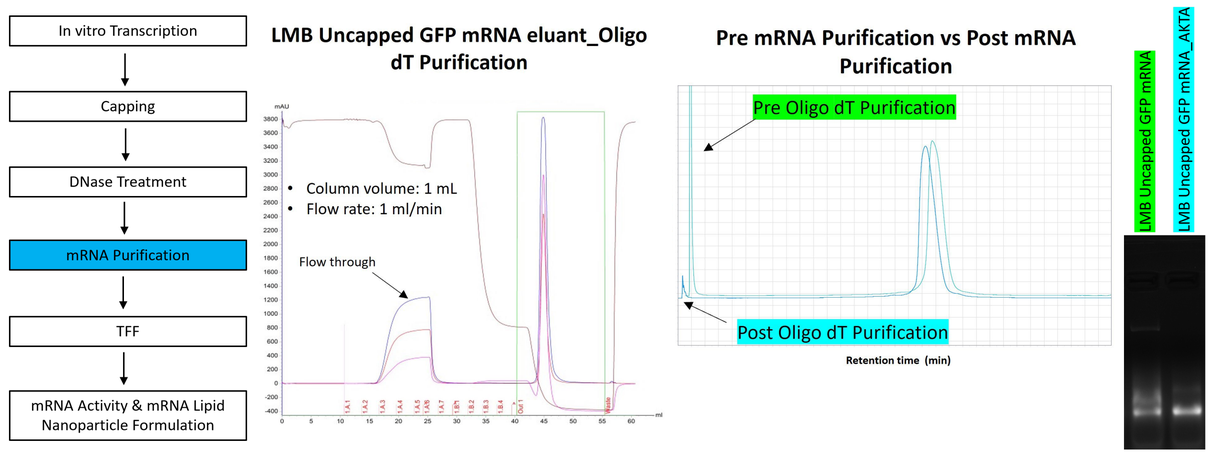

Figure 5. Purification. LMB uncapped GFP mRNA was purified by OligodT on the AKTAavant and analyzed by HPLC and by gel electrophoresis before and after.

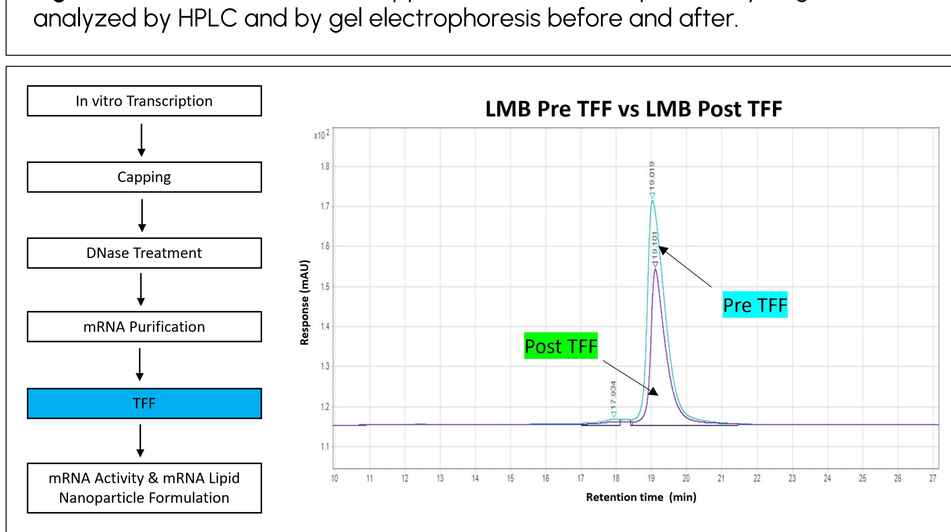

Figure 6. TFF. Unit operation. Analysis of the mRNA before and after TFF reveals no change in mRNA integrity after buffer exchange.

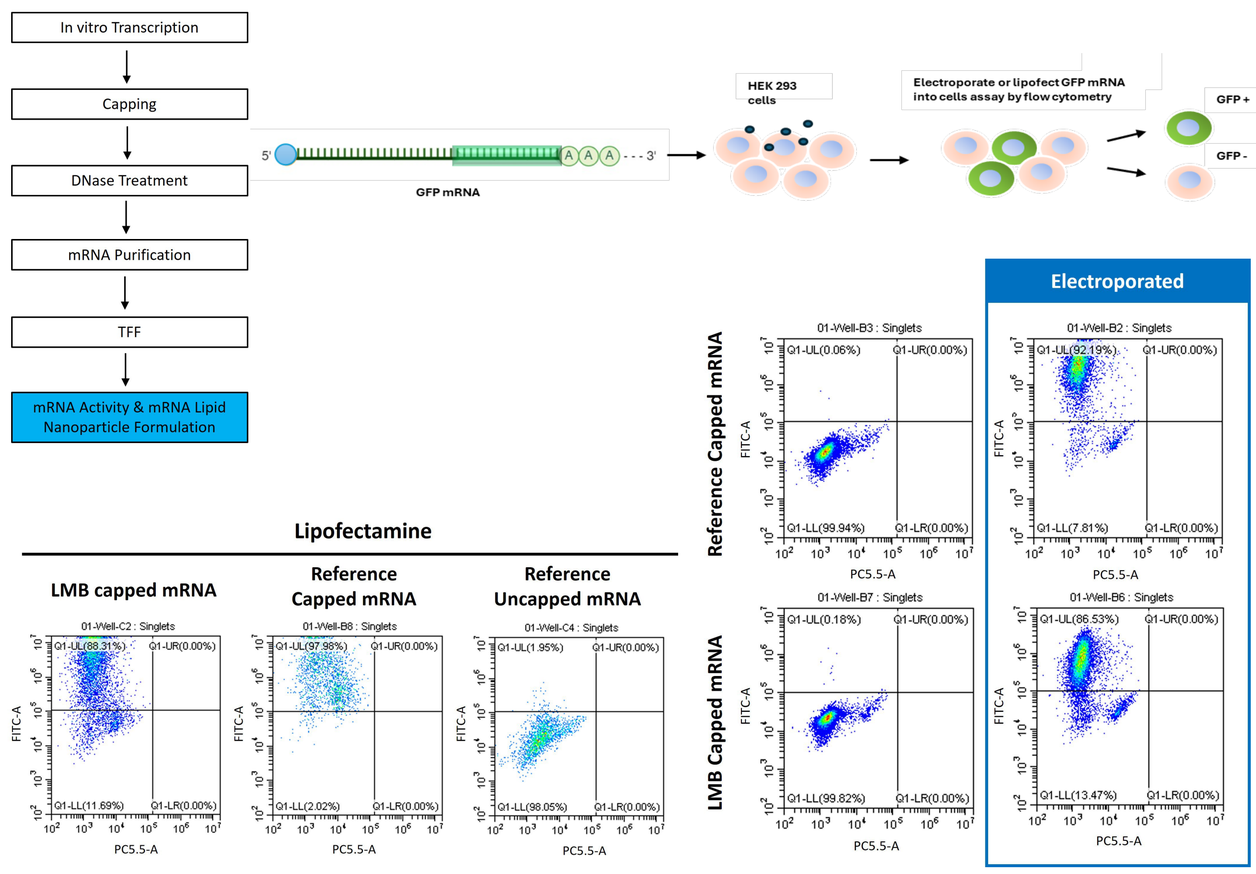

Figure 7. mRNA activity. Flow cytometry of electroporated or lipofected mRNA comparing activity of uncapped or capped reference material to LMB generated unpurified material. (FITC-A: GFP protein, PC5.5-A: PI cell viability marker)

CONCLUSIONS AND FUTURE DIRECTIONS

- In these studies, the IVT components, particularly salt, impacted performance, expressed as turn-over or mass crude RNA yield produced per linear DNA template mass input with a 208.8-fold turn-over achieved after optimization.
- Anticipating scale-up cost, lowering DNA template/enzyme could still obtain 183-fold turnover, with a 275-fold turn-over yielding 68 milligrams of crude mRNA at 10x scale.
- Using CleanCap for one pot chemical capping reaction, gel and HPLC analysis gave a slight shift in the band or peak after capping.
- Gel analysis revealed the removal of DNA from the reaction after DNase treatment with a slight shift in the HPLC peak.
- As expected oligodT purification removed unincorporated rNTPs which eluted early in the HPLC gradient early, and also generated a cleaner band in the gel analysis.
- Preliminary TFF data indicated no change in the HPLC elution time of the main peak before or after buffer exchange by TFF.
- Future directions include improving downstream processes, scaling up production, encapsulating mRNA into lipid nanoparticles, and exploring other IVT-based methods for producing macromolecular RNA.