RNA Replicon Platform to Enable Long-lasting Transient Expression in Primary and Stem Cells

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ABSTRACT
RNA replicons are an emerging platform for delivering complex genetic content into mammalian cells. Replicons are synthetic mRNA molecules that include viral nonstructural proteins (nsP1-4) from Alpha viruses such as Semliki Forest Virus and Venezuelan Equine Encephalitis Virus (VEE). Replicons can be transfected into cells and can self-amplify by virtue of a self-encoded RNA-dependent RNA polymerase. These molecules provide long-lasting, high-level gene expression from a few initial RNA molecules, making them use ideal for gene transfer applications needing sustained expression.

Current mRNA generation kits are suited for smaller (5kb or less) transcripts sizes and thus are not ideal for RNA replicons that are typically over 10 kb. In addition, the 5' end is less amenable to capping, necessitating enrichment and quantification of the capped functional replicon. Here we propose construction of a robust self-replicating vector platform with intrinsic structures that enable enrichment of functional mRNA. As a proof of principle we constructed a vector encoding self-replicating EmGFP and sustained GFP expression was verified in human dermal fibroblast and resting as well as activated T cells. The utility of this platform was further extended in other primary cell types and stem cells as well. Applications that benefit from replicon use include RNA Chimeric Antigen Receptor – T cell immunotherapy, iPSC cell reprogramming strategies using multiple factors to modulate cell fate, and engineering via synthetic gene circuits.

INTRODUCTION
The robust Replicon gene expression vector system with accompanying enrichment and QC solutions will provide a platform for generation and purification of self replicating mRNA (srRNA). This molecule provides prolonged expression in mammalian cells thus overcoming current constraints with synthetic mRNA that requires repeated transfections for sustained expression.

RESULTS

Figure 1. Vector Construction

Figure 2: Insertion of GFP using MultiSite Gateway Cloning

PCR of GFP > BP Cloning GFP into pZ21
LR Cloning pZ21 pcDNA3 pDEST VEE & pDEST VEE Cas6

Figure 3: mRNA Generation

VEE plasmid > Linearize plasmid > In Vitro Transcription > 5’ Capping

To test the functionality of the generated VEE vectors, GFP from a plasmid cloning and the resulting expression vector was confirmed via restriction endonuclease digestion and sequencing.

Figure 4. SrRNA Delivery and expression in primary & stem cells

HDFa

HUMEC

Heka

Ad5

AdSC

HCEC

Sr-GFP RNA efficiently expresses in multiple cell types. Cells were transfected with Lipofectamine™ MessengerMAX™ Transfection Reagent and imaged 24 hours after transfection. Percentage of GFP expression was obtained by flow analysis of sister well with AttoMax™ NIT Flow Cytometer from Thermo Fisher Scientific 24 hours post transfection.

Figure 5. SrRNA Expression in T cells

Sr-GFP or Sr-GFP Cas6

Figure 6. Persistence of SrRNA expression in T cells

CONCLUSIONS
- We were able to create a self-replicating gene expression technology platform that exhibits persistent expression in a variety of primary cells as well as resting and activated T cells.
- Large Sr-GFP RNA has been efficiently transfected into a variety of primary cell lines with minimal cytotoxicity.
- This platform is suitable for gene delivery both by lipid-based transfection as well as electroporation.
- Strategic incorporation of Cas6 cleavage site near 5’ of the construct ensures quality control issues for large-scale manufacturing as uncapped mRNA induces cytotoxicity.
- This technology shows promise and has potential future applications in modulating cell fate (IPS cell-reprogramming) and T cell immunotherapy (CAR-T cell generation).

MATERIALS AND METHODS
All materials are from Thermo Fisher Scientific unless specified otherwise.

Primary cell transfection:
Cells were sensitized with B1R8 containing Opti-MEM for at least 2 hours before transfection. Approximately 100 thousand cells (at about 70-80% confluence) were transfected with 500ng SrRNA using Lipofectamine™ MessengerMAX™. Media was changed 4 hours post transfection. Cells were maintained with B1R8 containing media for the rest of the experiment and fed every alternative day. Images were captured using EVOS™ XL Core Imaging System. Sr-GFP expression was analyzed using AttoMax™ NIT Flow Cytometer.

T cell culture and electroporation:
Purified CD8+ cells were cultured with Dynabeads™ Human T-Expander CD3/CD28 and 1000/mL recombinant human IL-2 - CTS™ OptiTec™ T Cell Expansion SFM for 12 days. Three million cells at density of 20 million/mL in a 100 uL tip was electroporated with 3 ug of RNA in T buffer using Neon Electroporation System.