Titration of mRNA Delivery: An Approach for Efficient Transfection with Modified or Unmodified mRNA

Application Note

In recent years, researchers have developed protocols for introducing mRNA into cells for reprograming, directed differentiation, and genome editing.

The ability to transfect a population of cells at high efficiency with mRNA offers many advantages over DNA transfection.

In this application note, we discuss the best approach to transfecting with mRNA to achieve biologically relevant levels of protein in a variety of cells, including cells lines that are considered difficult to transfect, while minimizing toxicity and creating a relatively homogeneous population of cells.

Transfection as a Means of Studying Biological Systems

Transfection is the process of introducing new nucleic acids into a cell. Quite often, the thought process in transfection is "more is better" – that is, higher levels of protein expression achieved following transfection are the goal. However, cells can be stressed by over-expression of specific proteins, especially when over-expression takes over the protein production machinery at the expense of producing other, necessary proteins for the cell. Quite often there is a tipping point at which over-expression of a protein actually leads to cell death, which interferes with obtaining and interpreting solid data. From a biological perspective, it is possibly more relevant to titrate in just as much protein as is needed to change a cell's activity, without using the "sledgehammer" approach that disrupts other important cellular activities.

mRNA Delivery as a Pathway to Studying Biology in a Nearly Homogenous Population of Cells

Transfection has traditionally been thought of as introducing new DNA into a cell's genome. However, in recent years this has expanded to include the introduction of mRNA directly into a cell. This approach has many advantages, including high percentage of cells transfected; promoter-independent expression; faster protein expression following transfection; and no risk of insertional mutagenesis. mRNA transfection also has the important advantage of providing control over the amount the of transgene introduced into the cell, as a way of driving protein production without stressing the cell and causing toxicity. However, mRNA transfection also has its drawbacks, as unmodified mRNA delivered at high efficiency causes toxicity by turning on innate immunity, and reducing overall expression.

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The Need to Optimize mRNA Transfection

When optimizing mRNA transfection, there are two issues to consider. The first is whether or not there is an advantage to modifying the mRNA to reduce its immunogenicity. Previous studies in mice showed that "naked" unmodified mRNA can elicit immune responses against the corresponding antigens and could even engender antitumor protective immunity. Karikó *et al.* reported that replacing the uridines in an mRNA backbone with pseudouridines not only enhanced nuclease stability and translatability of the modified mRNA but also reduced off-target effects. They showed that RNA signals through human Toll-like receptors (TLRs), but incorporation of modified nucleosides m5C, m6A, m5U, or pseudouridine ablated their triggered immune system activation.

The second issue to consider is how much protein expression is sufficient to study the biology of the system. Rather than approaching the transfection with a one-size-fits-all approach with the goal of maximizing protein expression, in many cases it is advantageous to titrate the expression levels, looking for a transfection efficiency that introduces enough protein into a high percentage of cells in order to study the system, and does not cause significant toxicity. The titration approach allows researchers to reduce the amount of mRNA needed to answer specific biological questions.

Data shown here demonstrates that **mRNA-In™ Transfection Reagent** can introduce both modified and unmodified mRNA into cells, achieving a very high percentage of cells transfected with minimal toxicity. Due to the exceptionally high efficiency of mRNA-In™, reduced mRNA amounts are required for maximum efficiency when using unmodified mRNA.

Experimental Design

In the following experiments, cells were plated in 24-well plates to give 70% confluence the day of transfection. Cells were transfected with various amounts of either modified 5meC, Ψ GFP mRNA (Trilink Cat #L-6101) or unmodified mRNA (Trilink Cat # L-6301) using 0.5-1 µl **mRNA-In™ Transfection Reagent**. Relative fluorescent intensities were measured 24 hours post-transfection using Tecan GENios at excitation 485nm and emission 535nm. Backgrounds were subtracted and the remaining values were plotted. Cells were also observed by fluorescence microscopy 24 hours post-transfection.

Toxicity Assays: Viable cells were measured using Promega Cell Titer 96® Aqueous One Solution Cell Proliferation Assay 24 hours post-transfection. Proliferation Assay reagent was then added to the wells. After incubating at 37°C for 30-60min, absorbance at 490nm was measured using BioRad Benchmark. Background absorbance was subtracted and percent viability was then calculated relative to wells containing non-transfected cells.

Results

Modified mRNA Produced Greater GFP Protein Expression Across Cell Lines

In the first experiment, a variety of cell types were transfected with either modified or unmodified mRNA. The cells included HeLa cells, a cell line frequently used as a model system due to its ease of culture and transfection; and three hard-to-transfect cell types: human umbilical vein cells (HUVEC), a commonly used *in vitro* model for studying endothelial cell function and dysfunction; and human iPSC-derived neural stem cells and adipose-derived mesenchymal cells. All four cell types exhibited a similar pattern, where modified mRNA transfection resulted in strong GFP fluorescence and high number of transfected cells, compared to much unmodified mRNA which showed high toxicity and variable expression (Figure 1).

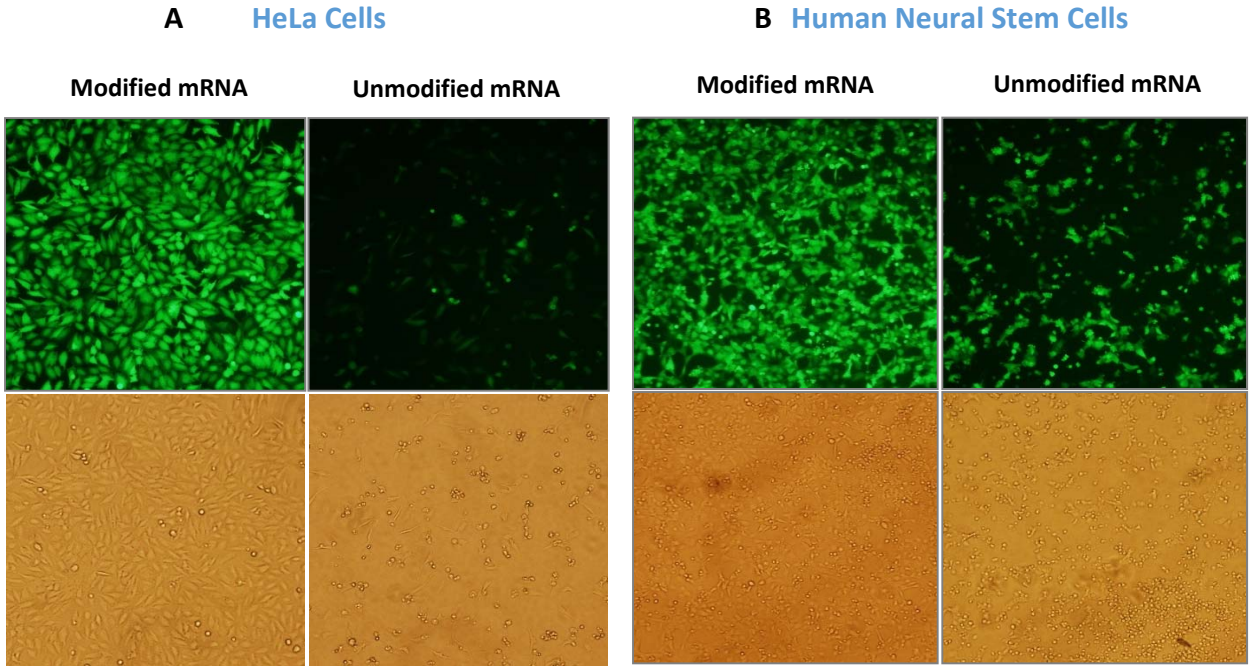

Figure 1. HeLa cells (A) and human iPSC-derived neural stem cells (B) were transfected with either 250 ng modified or 250 ng unmodified mRNA using **mRNA-In® Transfection Reagent**. Modified mRNA produced higher GFP expression and lower toxicity in all cells tested, including HUVEC and adipose-derived mesenchymal cells (data not shown).

A close examination of the phase contrast pictures in Figure 1 suggests that transfection with unmodified mRNA created cells that were transfected yet caused rapid cell death, while modified mRNA causes minimal toxicity. To further explore this observation, a range of mRNA concentrations were tested in all four cell types. In all cell types examined, there was a strong dose-response relationship between mRNA concentration and cellular toxicity. Very high transfection levels were achieved at the higher concentrations of modified mRNA, achieving >95% transfection efficiency with minimal toxicity at amounts of up to 500 ng mRNA (Figure 2).

Based on this data, it can be concluded that transfection with modified mRNA is possible at higher mRNA concentrations as compared to unmodified mRNA, and also results in greater protein expression levels when using modified mRNA.

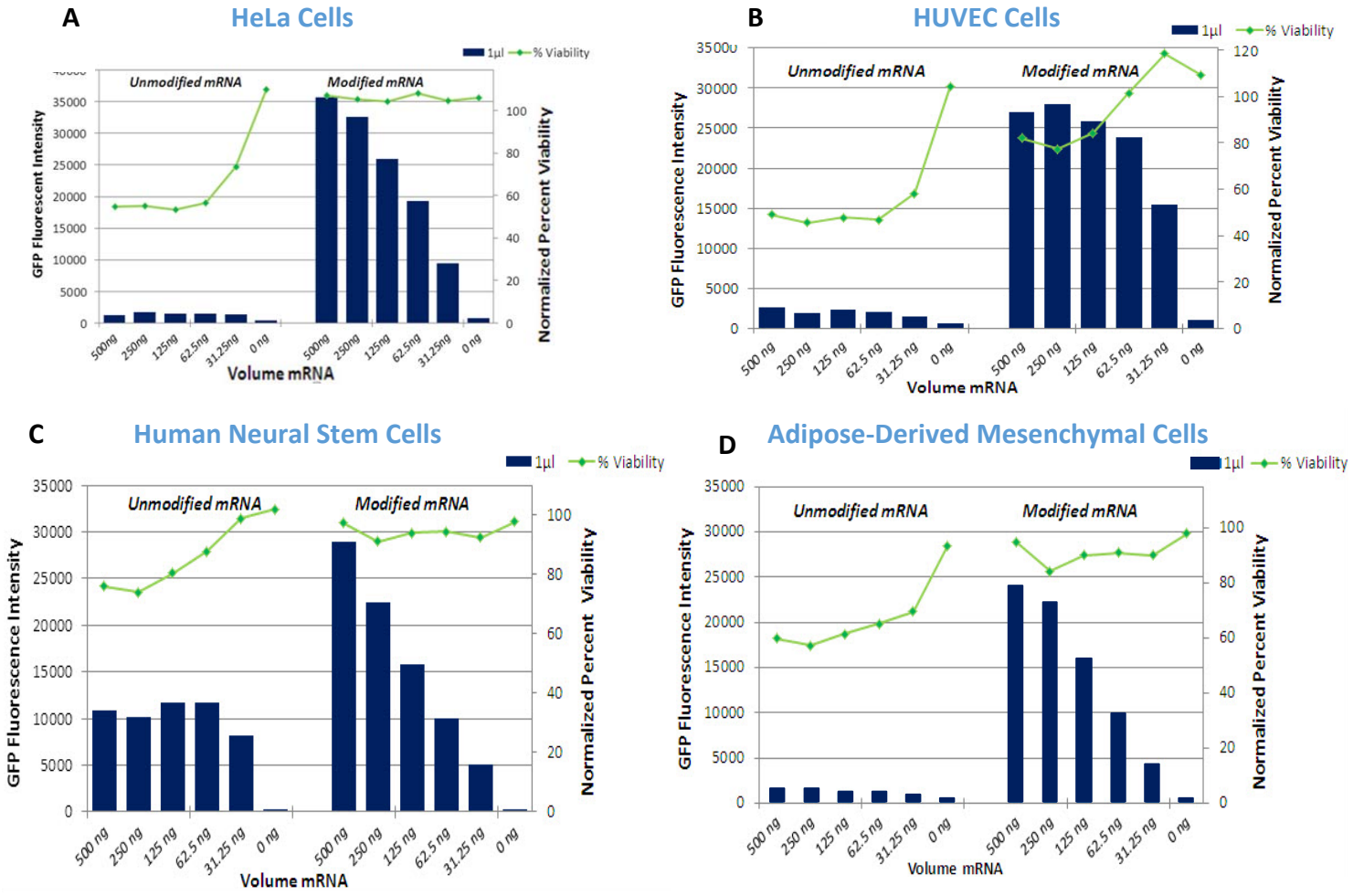

Figure 2. HeLa cells (A), HUVEC cells (B), human iPSC-derived neural stem cells (C) and adipose-derived mesenchymal cells (D) were transfected with unmodified mRNA or modified mRNA with **mRNA-In™ Transfection Reagent**. Both types of cells transfected with modified mRNA show high expression (blue bars) with little to no toxicity (green line), maintaining excellent cell viability and cell health across a large volume range. By contrast, significant toxicity was observed with increasing concentrations of unmodified mRNA and varying transfection rates.

Successful Transfection with Unmodified mRNA is Possible at Lower mRNA Concentrations

The previous set of experiments verified that in the four types of cells we examined, modified mRNA was less toxic while delivering high levels of protein expression. In some cases, however, it may be desirable or even necessary to use an unmodified mRNA. To this end, we tested very low amounts of unmodified mRNA for the ability to transfect cells while minimizing toxicity. In all four cell types tested, expression was detected after transfection using as low as 3.125ng

mRNA (Figure 3A), as confirmed by labeling transfected cells with anti-GFP antibody (Figure 3B). This data suggests that while high levels of expression are achievable using modified mRNA, unmodified mRNA transfection is also very efficient in terms of the percentage of cells successfully expressing protein, which can be a more biologically relevant approach in some experimental systems.

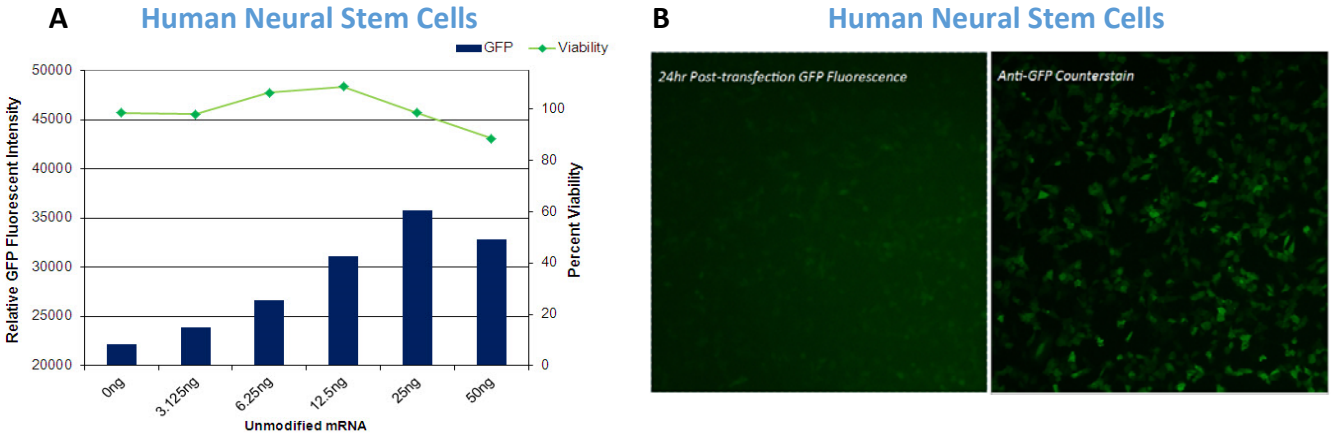

Figure 3. A) Human iPSC-derived Neural Stem Cells were transfected with very low amounts of unmodified mRNA for minimum toxicity using 1µl **mRNA-In™ Transfection Reagent**. B) Cells were then fixed and counterstained with an anti-GFP antibody (right) to confirm high transfection efficiency using **mRNA-In™ Transfection Reagent**. Similar results were observed in all four cell types examined (data not shown).

Summary

The data shows that various cell types consistently show significantly higher transgene expression and lower toxicity with modified mRNA (5meC, Ψ GFP mRNA) as compared to unmodified mRNA at both high and low amounts of mRNA. The data further shows that in order to maintain maximum cell viability when transfecting with unmodified mRNA, exceptionally lower amounts of mRNA are required to avoid toxicity. It is important to note that while the overall expression is lower, it is still possible to deliver to a high percentage of the cells in the population, making studying biological process with native mRNA possible. Unmodified mRNA will have shorter half-life as compared to modified mRNA. Each mRNA, depending on size, will require testing at various amounts to empirically determine the amount of mRNA that is non-toxic to the cell.

References

Karikó, K., Buckstein, M., Ni, H., & Weissman, D. (2005). Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA. Immunity, 23(2), 165-175.

Karikó, K, Muramatsu, H, Welsh, FA, Ludwig, J, Kato, H, Akira, S et al. (2008). Incorporation of pseudouridine into mRNA yields superior non-immunogenic vector with increased translational capacity and biological stability. Mol Ther 16: 1833–1840.