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From Reprogramming to Differentiation -

Transfection Applications for Stem Cell Research

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Introduction

Advances in the fields of stem cell differentiation and reprogramming have accelerated drug development in recent years by providing homogenous cell populations for cell-based testing. Several of these breakthroughs rely on the use of transfection for non-viral delivery of nucleic acids into different cell types. Mirus Bio provides high efficiency nucleic acid delivery tools through a suite of *Trans*IT® Transfection Reagents and an Ingenio® Electroporation Kit that have been validated for many of these applications.

Mirus Bio for Stem Cell Reprogramming

Reprogramming of somatic cells such as mouse and human fibroblasts to generate induced pluripotent stem (iPS) cells provides a limitless and homogenous supply of source material for the generation of differentiated cell types of various tissue lineages. Human iPS cells provide a viable alternative for the use of human embryonic stem cells (hESCs) that are embroiled in legal and ethical issues.

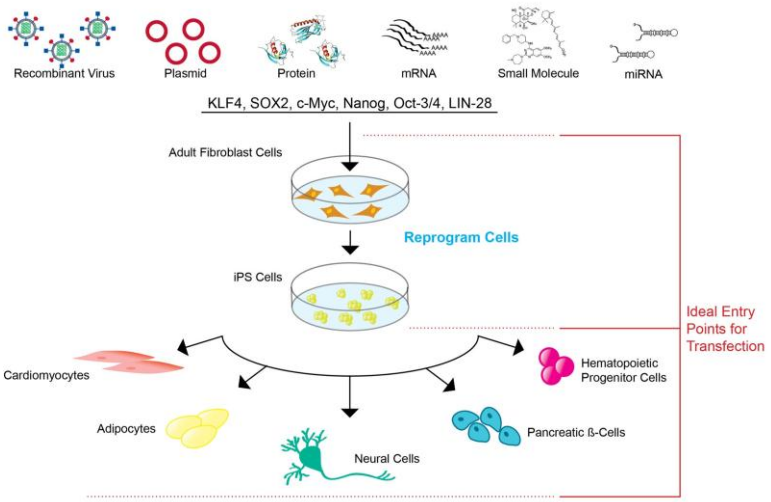

Figure 1. Entry Points for Transfection. Adult fibroblast cells can be transfected or transduced via several methods (e.g. recombinant virus, plasmid, protein, mRNA, small molecule and miRNA) with a combination of transcription factors including KLF4, SOX2, c-MYC, NANOG, OCT-4 and LIN-28 to reprogram the cells to a pluripotent state. iPS cells can then be differentiated to a myriad of cell types through growth factor addition and/or transfection of selection markers driven by cell type specific promoters. Stem cell derived cell types such as cardiomyocytes, adipocytes, neural cells, pancreatic β-cells, and hematopoietic progenitor cells provide researchers with relevant models for their experiments.

Additionally, iPS cells can be generated from specific genetic backgrounds for disease modeling and a variety of other applications including toxicological testing.

Reprogramming of somatic cells into iPS cells can be achieved by introducing a combination of key transcription factors through recombinant virus, small molecules or transfection of plasmid, protein, mRNA, or miRNA [Fig. 1]. This cocktail of transcription factors can vary depending on the cellular species, for example, Klf4, Sox2, c-Myc, and Oct-3/4 are needed for reprogramming mouse cells[1] whereas SOX2,

[1] Takahashi and Yamanaka. Cell 126: 663-676 (2006)

OCT4, NANOG and LIN28 can provide an alternative combination for reprogramming human somatic cells[2]. These reprogramming factors were initially introduced into somatic cells via retroviral transduction, which affords reproducible reprogramming through high transduction efficiency; however, caveats to the use of viruses include genomic integration, associated oncogenic effects and induction of immune response. The issues with viral transduction can be circumvented through the use of chemical transfection and electroporation to deliver reprogramming factors.

Chemical transfection for reprogramming has been most successful with DNA and RNA delivery. DNA mediated reprogramming is possible through the use of integrative or non-integrative methods. Integrative methods such as the use of *PiggyBac* transposons[3, 4] or linear DNA fragments flanked by *loxP* sites[5] require integration into the cellular genome. For a majority of applications including biotherapeutics, it is desirable to generate iPS lines free of genomic integration that may introduce mutations, impact subsequent differentiation or alter tissue functions. Non-integrative alternatives for transgene expression include episomes and DNA minicircles that can be delivered via transfection. While transfection of these non-viral expression vectors reduces the potential for integration, very high transfection efficiencies are required for reprogramming to occur. For these applications, high efficiency and low toxicity transfection reagents such as Mirus Bio's *Trans*IT®-2020 and *Trans*IT®-LT1 can provide an alternative to deliver DNA successfully. For DNA delivery with even higher efficiencies, electroporation can be performed using the Ingenio® Electroporation Solution.

A novel stem cell reprogramming approach that completely eliminates integration concerns and has been employed with great success in recent years is the transfection of fibroblasts with modified base containing mRNA to generate iPS cells.[6] Incorporation of modified bases such as pseudouridine and 5-methylcytosine within *in vitro* transcripts has been shown to enhance mRNA stability while simultaneously reducing innate immune activation in transfected cells[7, 8]. Repeated transfection of such modified mRNA leads to high efficiencies of reprogramming without the worry of genomic integration. Furthermore, cellular toxicity due to repeated transfection is alleviated by using low toxicity RNA transfection reagents such as the Mirus *Trans*IT®-mRNA Transfection Kit.[9,10]

As a reiteration of these findings, *Trans*IT®-mRNA Transfection Kit was used to transfect BJ and MRC-5 fibroblast cell lines which are commonly used for reprogramming [Fig. 2]. In these experiments, cells were transfected with pseudouridine and 5-methylcytosine modified GFP encoding RNA transcripts to show high efficiency transfection. In addition, transfection also showed minimal toxicity via propidium iodide staining.

[2] Yu, J. *et al*. Science 318: 1917-1920 (2007)
[3] Woltjen, K. *et al*. Nature 458: 766–770 (2009).
[4] Yusa, K. *et al*. Nature Methods 6: 363–369 (2009)
[5] Kaji, K. et al. Nature 458: 771-774 (2009)
[6] Warren, L. *et al*. Cell Stem Cell 7: 618-630 (2010)
[7] Kariko K. *et al*. Immunity 23: 165-175 (2005)
[8] Kariko K. *et al*. Mol Ther. 15: 1833-184 (2008)
[9] Angel and Yanik. PLoS one 5: e11756 (2010)
[10] Kariko, K. *et al*. Nucl. Acids Res. 39: e142 (2011)

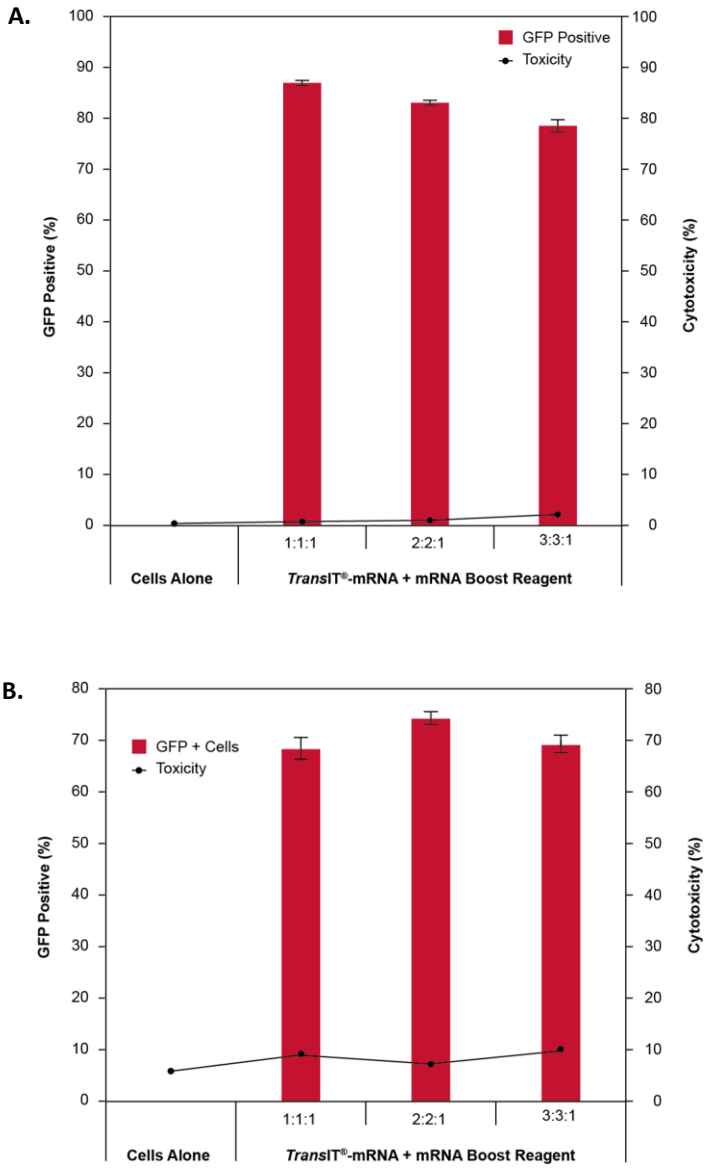

Figure 2. Fibroblast Transfection with *Trans*IT®-mRNA. The *Trans*IT®-mRNA Transfection Kit was used to transfect BJ human neonatal foreskin fibroblasts (A) and MRC-5 human lung fibroblasts (B) with a pseudouridine and 5mC modified based GFP mRNA (Trilink Biotechnologies, Inc.). Transfections were performed in 12-well plates using 1-3 µl of *Trans*IT-mRNA Transfection Reagent and mRNA Boost Reagent to deliver 1 µg of RNA (1:1:1, 2:2:1 and 3:3:1; reagent: boost: RNA ratio). Cells were assayed 18 hours post-transfection on a BD LSR II Flow Cytometer. Cell viability was measured using propidium iodide stain.

Mirus Bio for iPS Cell Differentiation

iPS cells generated by reprogramming somatic cells can be further differentiated into biologically relevant cell lines of diverse tissue lineages. Defined culture conditions may be used to differentiate iPS cells into specific cell lineages, and this methodology can be further streamlined by introducing selectable markers driven by cell type specific promoters via transfection.

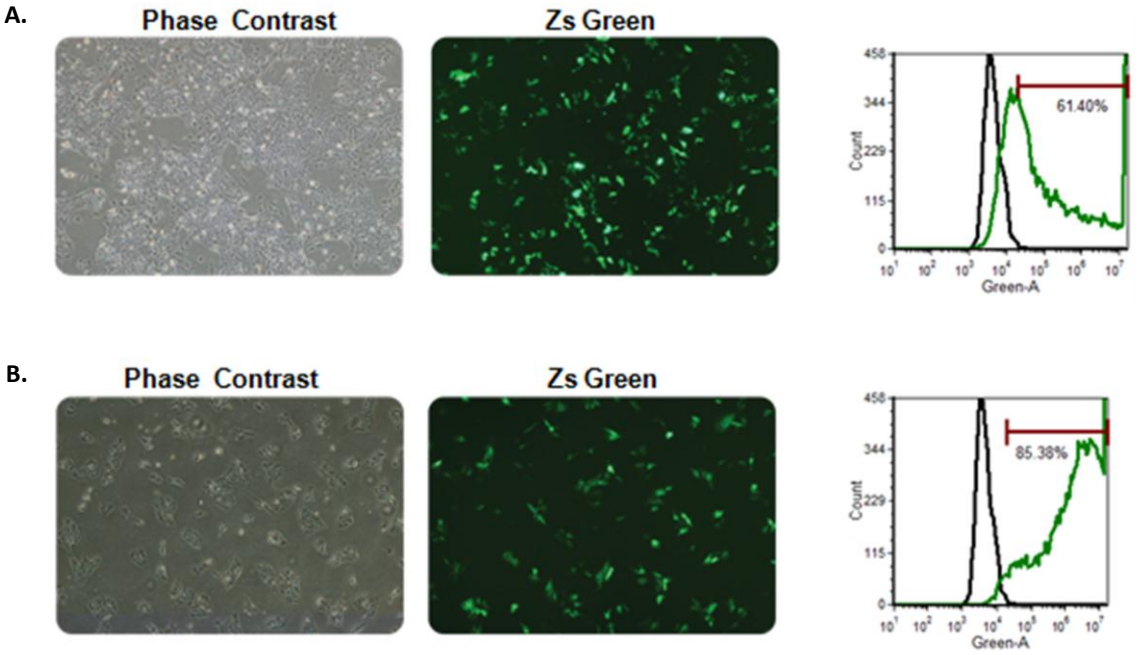

Figure 3. High Efficiency Transfection and Electroporation of Human iPS Cells. The *Trans*IT®-2020 Transfection Reagent was used to transfect 0.5 x 10^6 iPS cells with a ZsGreen™ expression plasmid (Clontech) (A). Transfections were performed in 6-well plates using 7.5 µl of *Trans*IT-2020 Transfection Reagent to deliver 2.5 µg of DNA (3:1; reagent: DNA). The Ingenio® Electroporation Kit was used to transfect 2 x 10^6 iPS cells on the Amaxa® Nucleofector® II Device (B). Cells were electroporated with 8 µg ZsGreen expressing plasmid (Clontech) in 100 µl and plated in 6-well plates at 0.33 x 10^6 cells/well. Cells were visualized 24 hours post-transfection and imaged at 4X objective with an Olympus IX71® Inverted Microscope. Images were acquired using phase contrast and green fluorescence. Cells were assayed 24 hours post-transfection on an Accuri® Cytometer. The histogram shows untransfected cells (black line) compared to cells transfected with plasmid (green line).

The feasibility of high efficiency transfection of iPS cells has been demonstrated successfully by scientists at Cellular Dynamics International (CDI – www.cellulardynamics.com) by transfecting iPS cells with a ZsGreen™ expressing plasmid (Clontech) [Figure 3]. The data shows that iPS cells can be transfected very efficiently with either the use of *Trans*IT-2020 Transfection Reagent or through electroporation with the Ingenio Electroporation Kit. These two alternatives give researchers the ability to choose between effective nucleic acid delivery methods and to determine which may work best in their platform.

Mirus Bio and iPS Cell Derived Cell Lines

Cell lineages derived from iPS cells provide a more biologically relevant cell source than immortalized cell lines. These populations of cells are also more homogenous than primary cells for studying specific disease models or for performing drug or toxicity screening. Additionally, the use of iPS cell derived models serve as a humane substitute for costly animal testing. iPS cell derived cardiomyocytes and neuronal cell types have recently gained attention for their application in drug discovery and toxicity testing.[11]

Transfection can also be used to establish reporter systems in iPS cell derived cell types for applications to screen compounds or to knockdown gene expression for pathway analysis. Both of these applications were validated in iCell® Cardiomyocytes from CDI. As demonstrated in Figure 4, iPS cell derived cardiomyocytes were transfected using *Trans*IT-LT1 Transfection Reagent to incorporate a luciferase system responsive to isproterenol, a known cardiomodulator [Fig. 4]. In addition, *Trans*IT-TKO® Transfection Reagent was successfully used to knockdown endogenous gene expression of the housekeeping gene GAPDH in iCell cardiomyocytes [Fig 5].

A. **B.** **C.**

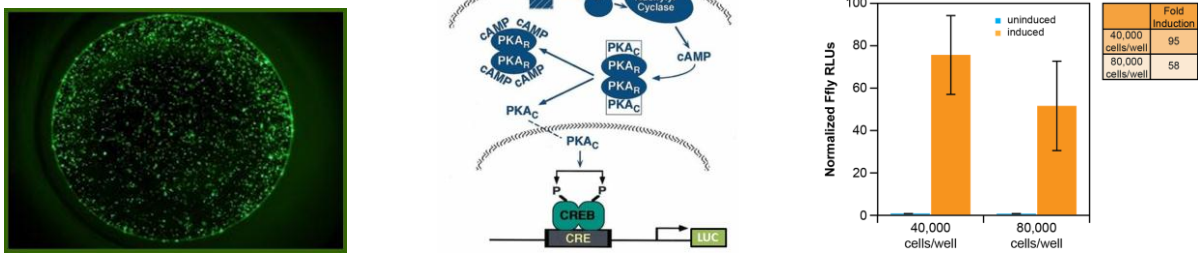

Figure 4. Plasmid DNA Delivery to iCell® Cardiomyocytes using *Trans*IT-LT1®. *Panel A* illustrates high efficiency transfection of a GFP encoding plasmid. iCell Cardiomyocytes were plated at 20,000 cells/well in a 96 well tissue culture plate coated with 0.1% gelatin. After allowing the cells to recover from thaw, cells were transfected with 100 ng/well of pMAXGFP™ (Amaxa™) using *Trans*IT-LT1 Transfection Reagent with a 2:1 (reagent:DNA) ratio according to the manufacturer's instructions. Fluorescent images were taken 3 days post transfection.

Panel B is a schematic of agonist binding inducing G protein (Gs) mediated activation of adenylyl cyclase which converts ATP to cAMP. The second messenger is able to bind to protein kinase A (PKA) and lead to phosphorylation of the cAMP response element-binding protein (CREB) protein. Upon translocation to the nucleus CREB is able to bind the cAMP response element (CRE) and initiate expression of the luciferase reporter.

Panel C illustrates cAMP induction measured via a luciferase reporter plasmid. iCell Cardiomyocytes were plated for 5 days and subsequently replated using 40,000 or 80,000 cells/well in a 96 well plate pre-coated with gelatin. Three days post-replating cells were transfected using *Trans*IT-LT1 and the CRE-luciferase reporter plasmid pGL4.29 (Promega). After 18 hours the cAMP pathway was induced using 10 μM isoproterenol for 6 hours. Luciferase activity was measured using the Promega Dual Glo® Luciferase Assay. Data is normalized to the control reporter plasmid pGL4.75 (Promega).

[11] Ebert and Svendsen. Nature Reviews Drug Discovery 9: 367-372 (2010)

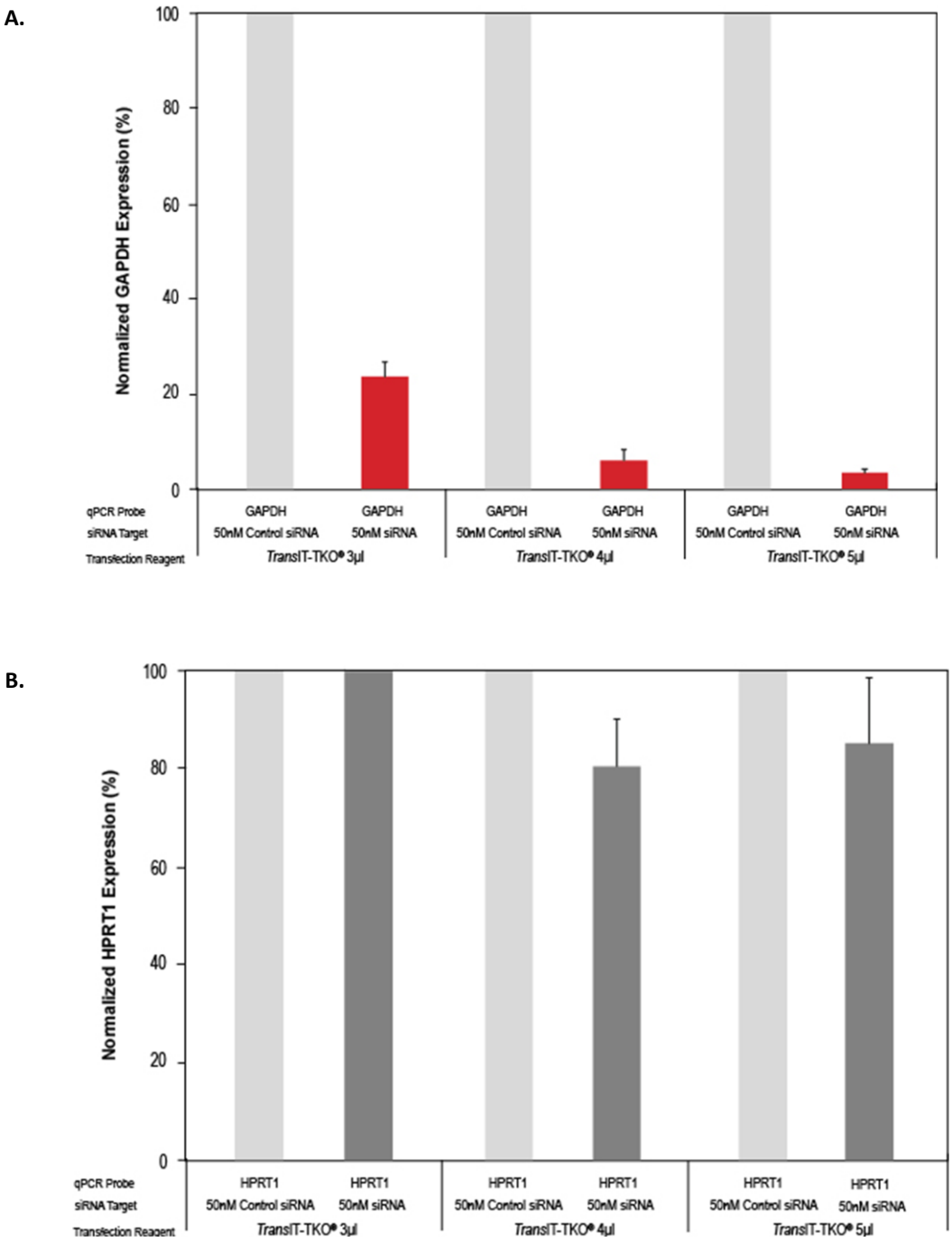

Figure 5. Efficient siRNA-mediated Gene Silencing by *Trans*IT®-TKO in iCell® Cardiomyocytes. Panels A and B show the effect of GAPDH-targeted siRNA on GAPDH (targeted) and HPRT1 (non-targeted) mRNA expression, respectively. iCell Cardiomyocytes were cultured for 7 days in a 12-well cell culture plate before transfection with either control (scrambled) or GAPDH siRNA (sense: GCUCAUUUCCUGGUAUGACUU; antisense: GUCAUACCAGGAAAUGAGCUU) using *Trans*IT-TKO (3 - 5 µl/well). 72 hours post-transfection the GAPDH and HPRT1 (non-targeted) mRNA levels were measured relative to 18s rRNA levels and normalized to the mRNA levels obtained following transfection of the control siRNA in each experiment. The bar graphs show the mean with standard error of the mean (SEM) of 3 independent transfection complexes.

Conclusion

As the list of potential applications for stem cell biology continues to grow, so does the need to develop new methodologies to produce homogenous cell populations that are free of integrated transgenes. Transfection via chemical reagents or through electroporation has emerged as a powerful tool for genetic manipulation of somatic cells, iPS cells and their derived cell lineages. Although plasmid delivery can be laborious due to necessary selection for non-integrative cell lines and modified mRNA transfection can be time consuming due to multiple, repeated transfections, the issues associated with transfection are miniscule compared to the more serious and longstanding implications of viral transduction. In applications for drug development and cell therapies, these key differences favor the emerging use of transfection for stem cell applications.