

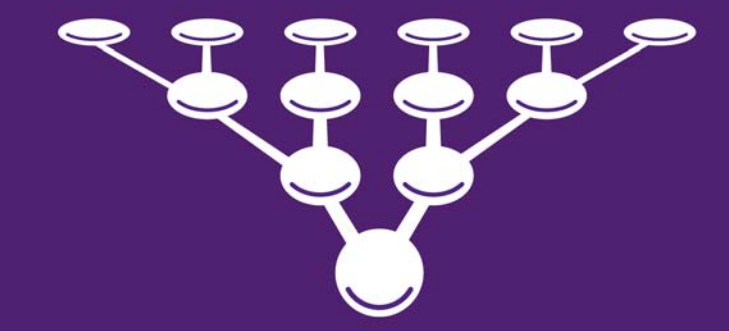
A Validated mRNA Reprogramming Protocol for the Reproducible Generation of Integration-free Human iPS Cells

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ABSTRACT

To date, the broad implementation of induced pluripotent stem (iPS) cells in regenerative medicine and drug screening applications has been limited by the inability to efficiently derive iPS cell lines that are free from genomic integration. Past publications have highlighted the use of DNA-based reprogramming technologies that are: 1) directly integrating into the target cell genome, 2) directly integrating into the target cell genome, but can be removed, or 3) minimally integrating into the target cell genome, but still require screening to ensure no genomic alteration. More recent works have utilized recombinant protein or mRNA to derive iPS cell lines that are inherently free from any genomic integration. However, these methods have been plagued by low efficiency and/or the lack of reproducibility. Here, we demonstrate a validated protocol utilizing the Stemgent® mRNA Reprogramming Factor Set for the efficient and reproducible generation of iPS cells from both BJ foreskin fibroblasts and patient derived dermal fibroblasts. This reproducibility is dependent on the use of Stemgent® Pluriton™ mRNA Reprogramming Medium that is essential for complete conversion to iPS. In addition, the highly efficient Stemfect® RNA Transfection Reagent was developed to ensure that mRNA can be delivered to a range of cell types, including fibroblasts and lymphocytes, with titratable control over expression. This methodology provides a reproducible, non-integrating method for generating iPS cells which has the potential to be expanded to multiple cell types.

INTRODUCTION

Several recent publications have demonstrated the potential of mRNA to reprogram somatic cells to induced pluripotent stem (iPS) cells, albeit with varying levels of efficiency, reproducibility and colony stability^{1,2,3}. Messenger RNA reprogramming is a desirable method for iPS cell generation for a number of reasons. First, it eliminates the need for DNA based reprogramming factor delivery, thereby eliminating any risk for insertional mutagenesis of the parental cell genome. Second, published methods have demonstrated an increased yield in the number of iPS colonies generated per defined starting cell population, thereby potentially reducing the number of target cells required to initiate the reprogramming process. Third, this method has generated iPS colonies in as little as over two weeks and in doing so has minimized the primary cell culturing time during the reprogramming process when compared to other DNA based delivery methods. In an effort to develop a more robust and reproducible method for iPS cell generation using this integration-free technology, our lab evaluated both the mRNA reprogramming protocol as whole as well as the individual materials and reagents employed.

Early experiments using the Stemgent® mRNA Reprogramming Factor Set and the mRNA reprogramming protocol as published¹, revealed that while we were able to detect properly localized protein expression from each mRNA delivered to our target fibroblasts (Figure 6), overall the culture was unable to sustain the morphological changes recognized very early in the reprogramming process. As a result, we incorporated five significant changes to generate a more streamlined and reproducible protocol (Figure 1). Most significantly we developed Pluriton™ mRNA Reprogramming Medium which allows for better mRNA reprogramming factor delivery and protein expression (data not shown) as well as exclusive support of iPS cell colony generation when compared to other commonly used human ES culture/reprogramming media (Figure 3). Second, we converted the protocol to a no-split format to minimize the inherent variability associated with enzymatic passaging of the reprogramming culture and thereby allow for an accurate reprogramming efficiency determination. Third, we included three different target cell plating densities to ensure that there is a cell culture density condition that is amenable to iPS cell colony generation regardless of the target cell's propensity for expansion. Thereby allowing for healthy, expanding cultures to enter the reprogramming protocol with as little as 10K starting cells and still generate significant numbers of iPS colonies. Fourth, we incorporated conditioned medium into the protocol to ensure that the culture environment remains healthy as the feeder layer benefit typically deteriorates after 6-7 days in culture. Lastly, we reduced the plating density of the human feeders from 300K/well to 250K/well to allow for more culture expansion and iPS cell colony growth. Incorporation of the above modifications has ensured that reprogramming with mRNA is not only reproducible, but also extremely efficient and rapid as demonstrated by a reprogramming efficiency as high as 5.88% (588 colonies from 10K starting BJ fibroblasts) after only sixteen transfections and nineteen days in culture. Importantly, the optimized protocol has also been validated for the derivation of iPS cell lines from patient derived fibroblast lines (Figure 1b).

In concert, to enhance this process, we have developed a new RNA delivery method, based on leading edge lipid-based delivery systems, that allows for fine-tuned control over the amount of mRNA delivered and expressed. This reagent, Stemfect™ RNA transfection kit, has been shown to deliver mRNA at >95% efficiency in a broad range of cell types (Figure 4) and could enable the reprogramming of cell lines that are normally refractory to transfection.

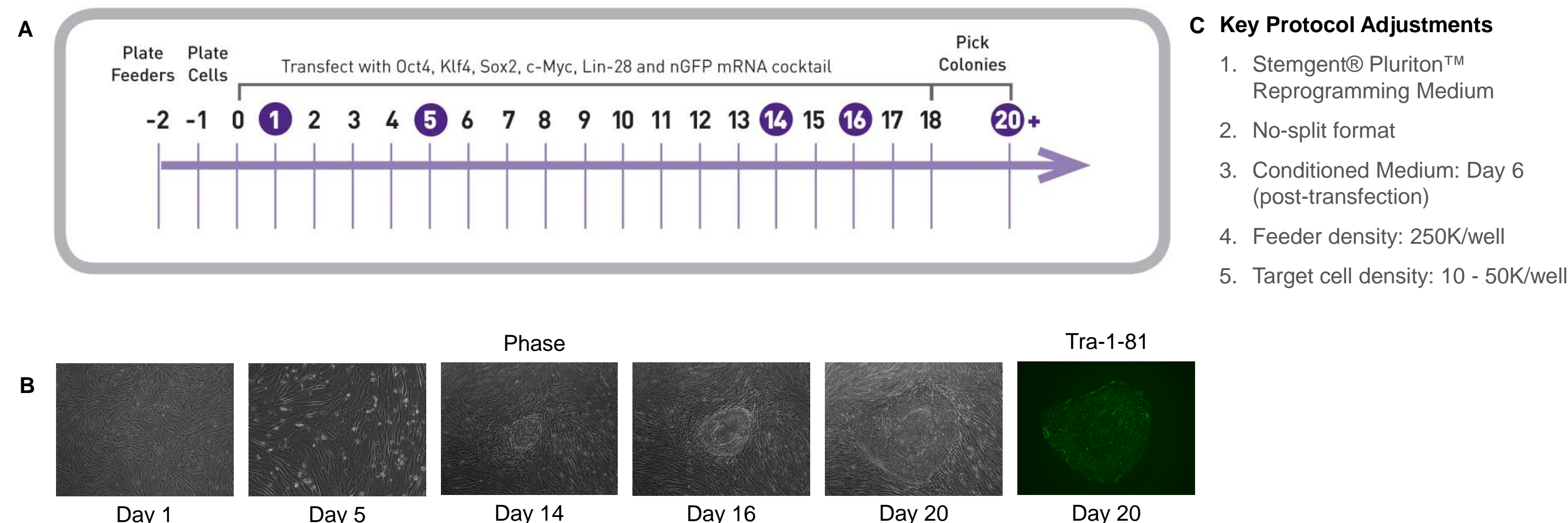


Figure 1. Modified protocol for mRNA reprogramming of human fibroblasts.

A) Timeline for the reprogramming of human fibroblasts by repeated mRNA transfection using Stemgent's optimized no-split mRNA Reprogramming protocol. **B)** Primary culture morphology progression (Phase - 10x) during the emergence of a primary iPS cell colony resulting from the reprogramming of Parkinson's disease patient dermal fibroblasts with Stemgent® mRNA Reprogramming Factor Set in combination with Pluriton™ mRNA Reprogramming Medium. Last panel: Day 20 primary iPS colony identification using the Stemgent® StainAlive™ Tra-1-81 antibody. **C)** Key adjustments/optimizations, by Stemgent, to the mRNA reprogramming protocol as published in Cell Stem Cell¹. For the full protocol, please visit www.stemgent.com.

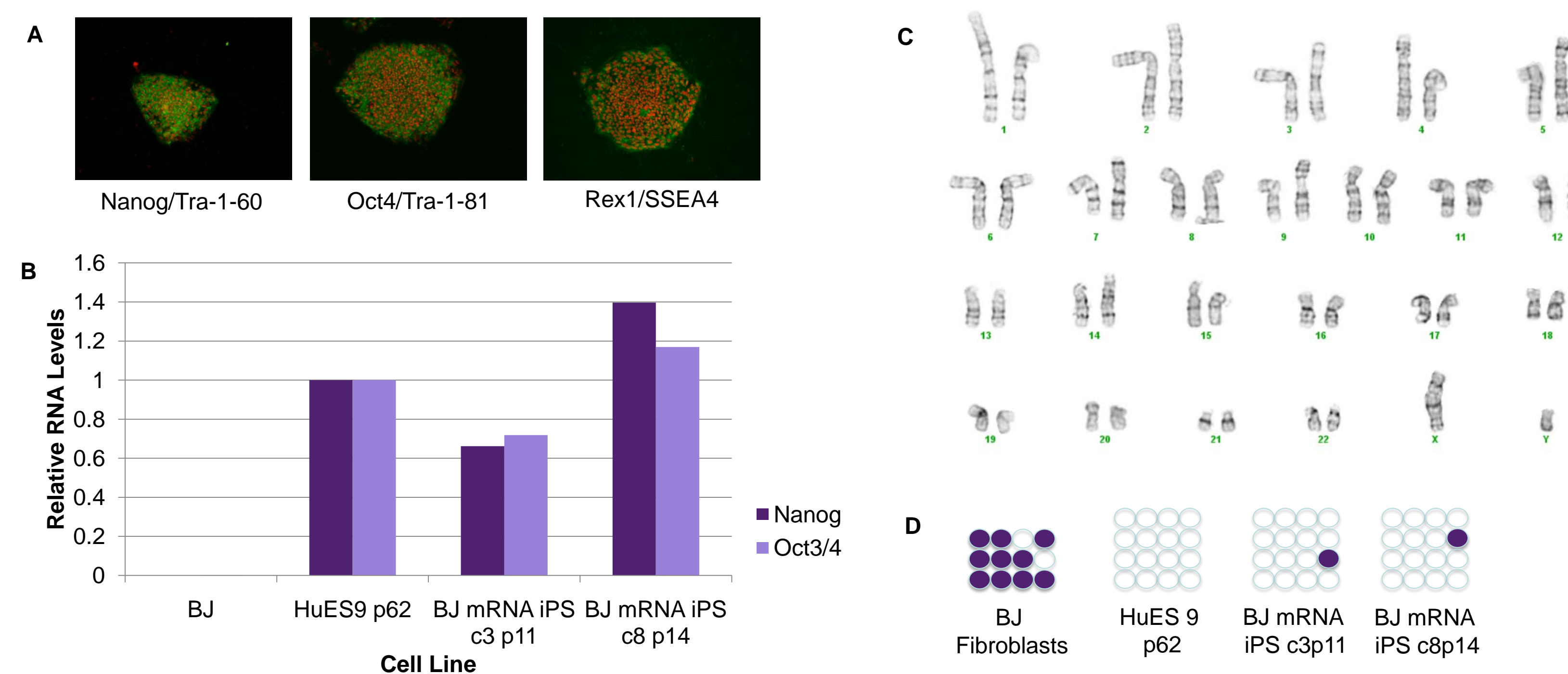


Figure 2. Characterization of BJ mRNA iPS cell lines.

A) Pluripotency immunocytochemistry for mRNA iPS cell line (c8) derived from BJ fibroblasts. Nuclear pluripotency for Nanog, Oct4, and Rex1 (Red). Cell surface pluripotency for Tra-1-60, Tra-1-81, and SSEA4 (Green). **B)** Relative endogenous mRNA expression level comparison for Nanog and Oct4. qRT-PCR calculations and data normalization against HuES9 p62 human ES positive control cell line. **C)** Normal karyotype analysis of BJ mRNA iPS cell line (c8 p14) after fourteen culture passages on mouse embryonic fibroblast feeders. **D)** Bisulfite sequence analysis of Oct4 promoter for BJ mRNA iPS cell lines c3 p11 and c8 p14.

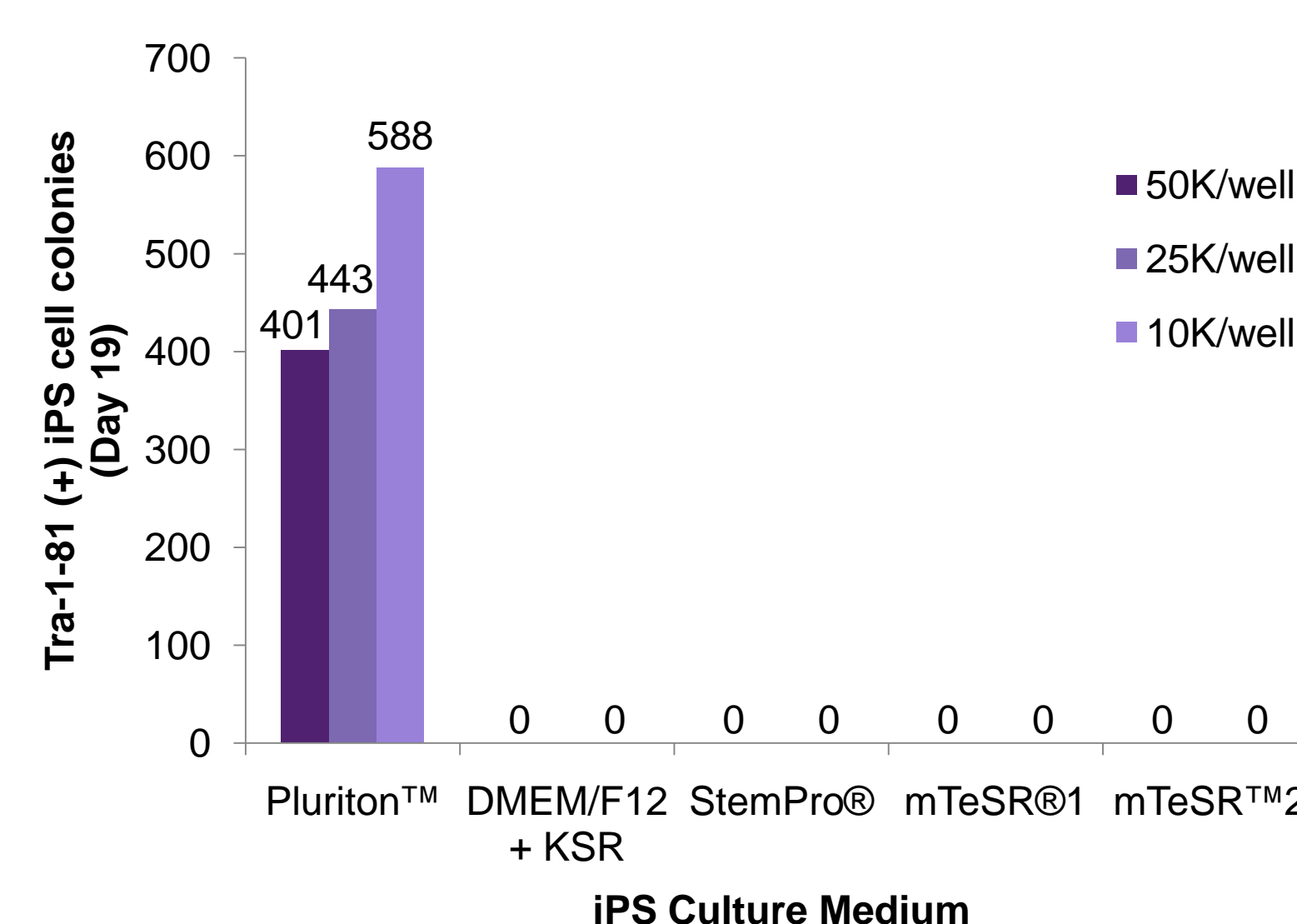


Figure 3. Comparison of Pluriton™ mRNA Reprogramming Medium and other common human ES culture media for iPS cell colony generation during mRNA based reprogramming.

Different target cell densities (50K, 25K, or 10K per well) of BJ fibroblasts were plated on human fibroblast feeders (250K/well) in a single well of 6-well plate. Each condition was incubated at 5% O₂ and transfected with 1.2 ug of mRNA reprogramming cocktail for 16 consecutive days without enzymatic passaging. Primary cultures were assayed with the Stemgent® StainAlive™ Tra-1-81 antibody (1:100) on Day 19 and Tra-1-81 positive colonies were counted using a fluorescent microscope. Each bar in the graph is individually labeled with the number of iPS cell colonies generated.

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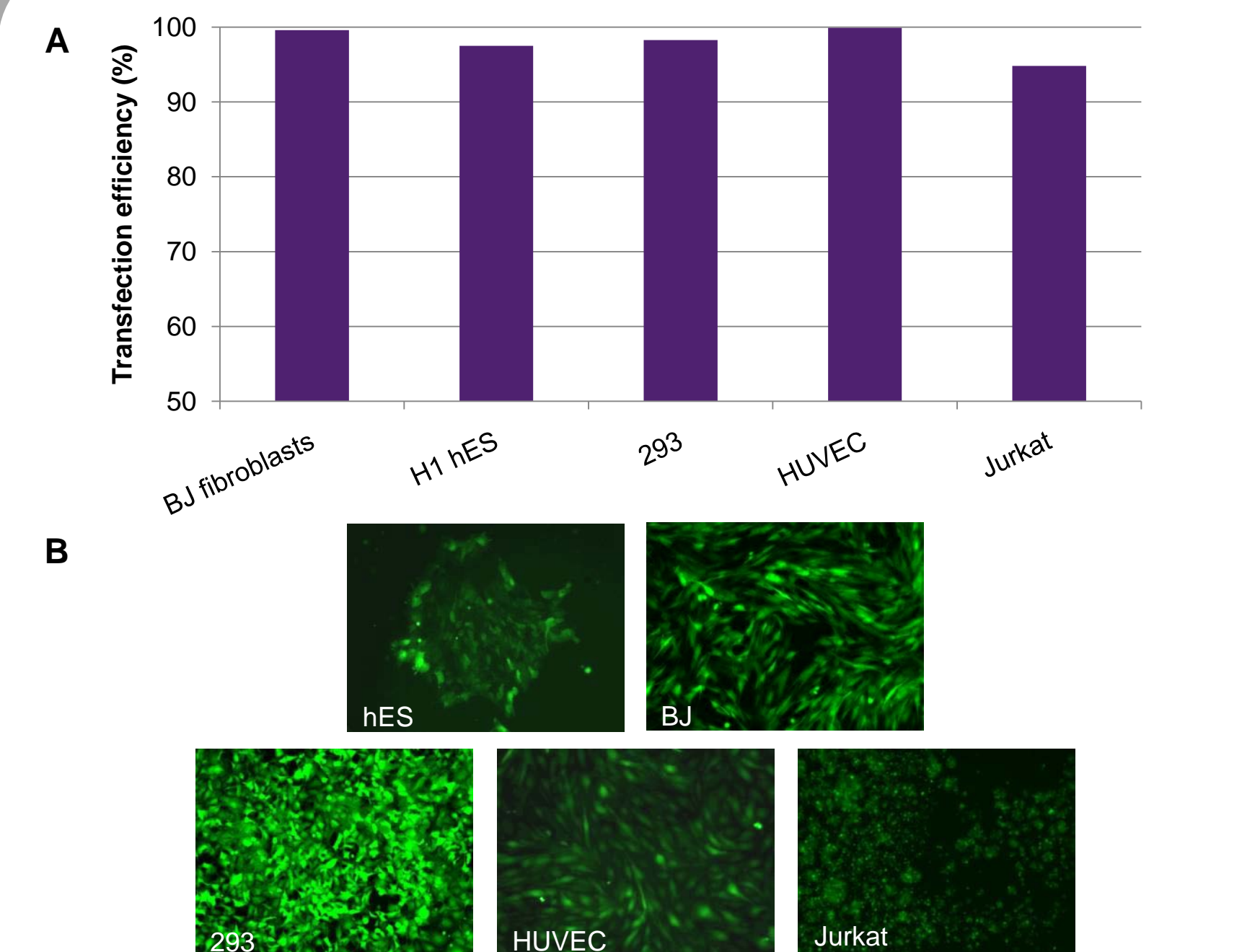


Figure 4. Transfection efficiency of Stemfect RNA Transfection Kit on various cell lines. Stemfect RNA transfection reagent was complexed with mRNA encoding eGFP and added to cells plated in a 24-well format. The cells were cultured at 37°C / 5% CO₂ overnight and analyzed at 18-24 hrs. post transfection. **A)** Graph of transfection efficiency as determined by flow cytometry. **B)** Fluorescence images of cells transfected with eGFP mRNA.

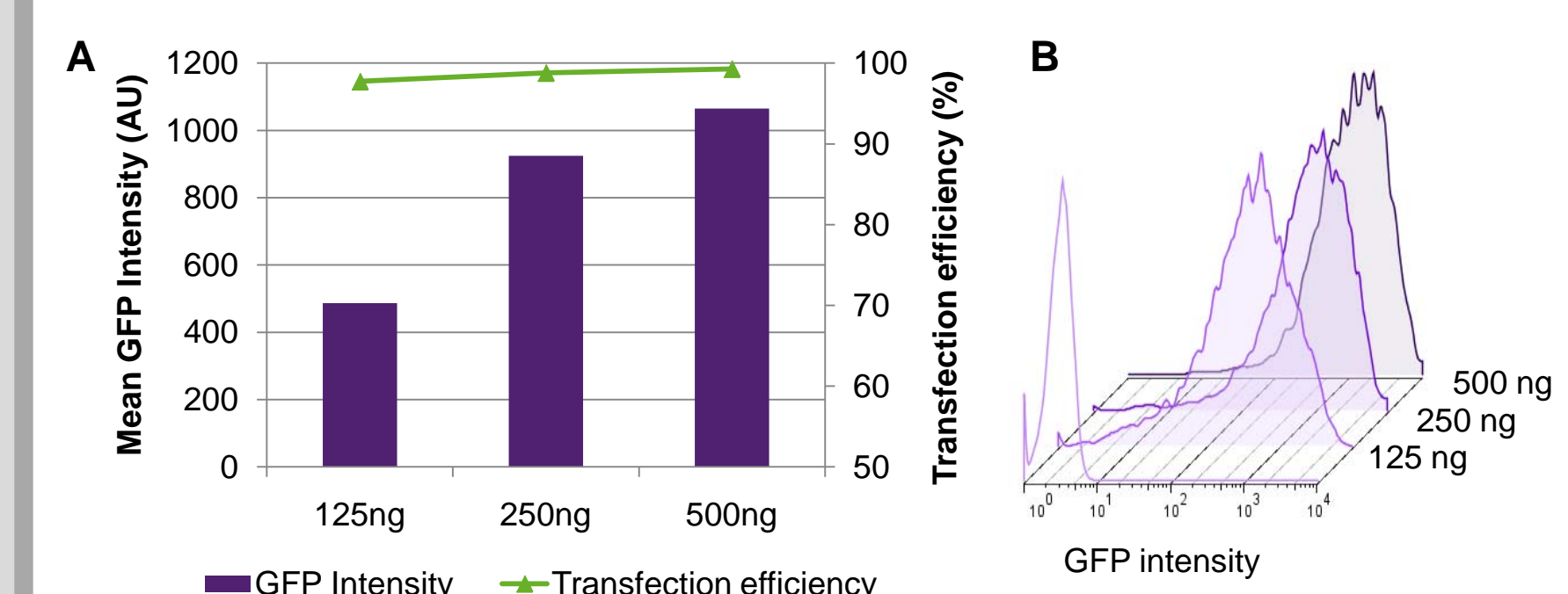


Figure 5. Titration of protein expression using the Stemfect RNA transfection kit. BJ fibroblast cells were seeded in a 24-well format and transfected with 125, 250 or 500 ng of eGFP mRNA. The cells were cultured at 37°C / 5% CO₂ and analyzed at 18-24 hrs. post transfection. **A)** Graph of mean fluorescence intensity and transfection efficiency as determined by flow cytometry. **B)** Representative histograms.

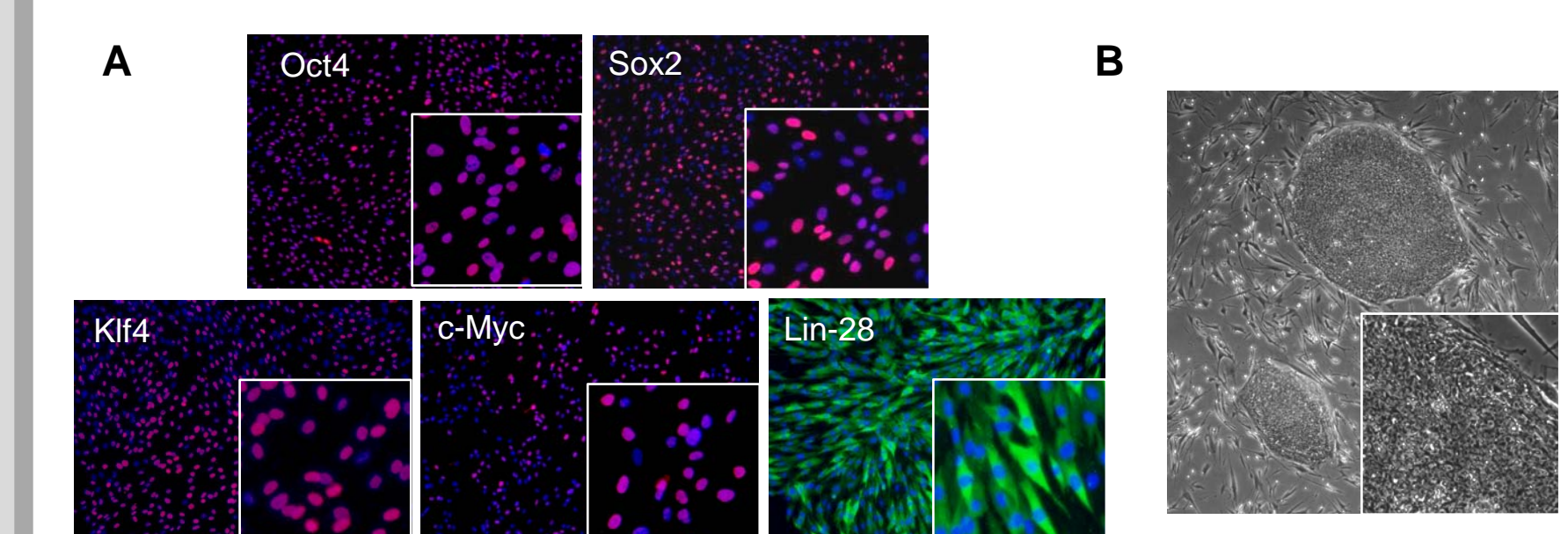


Figure 6. Expression of reprogramming factors and generation of iPS with Stemfect RNA Transfection Kit. **A)** BJ fibroblasts were transfected with mRNA encoding the transcription factors Oct4, Sox2, Klf4, c-Myc, or Lin-28 and fixed with 4% paraformaldehyde after incubation for ~18 hours. The cells were then stained with the appropriate antibody and DAPI for visualization. Merged images are shown. **B)** iPS cell line (p9) derived from BJ fibroblasts using 0.8 µg mRNA cocktail per transfection.

SUMMARY

1. Developed functionally validated mRNA reprogramming protocol
 - Integration-free
 - Increased reprogramming efficiency
 - Faster iPS cell colony generation – colony isolation within 3 weeks
 - Demonstrated on diseased patient fibroblasts
 - Validated at 5% O₂ and 21% O₂
2. Developed novel, xeno-free media essential for mRNA reprogramming success
3. Developed highly efficient RNA transfection reagent for broadened cell application