Essential media system for Reprogramming

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ABSTRACT

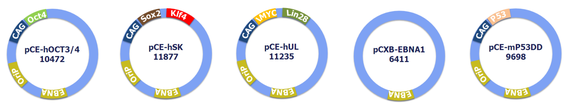
Essential 8™ media is a simple and cost effective media system for the expansion and maintenance of pluripotent human ESC and iPSC. To extend the use of this system for somatic reprogramming, the basal Essential 6™ media was supplemented with the two growth factors, bFGF and TGFb, at distinct stages of reprogramming.

The optimal timing of growth factor reconstitution to Essential 6™ media was determined for CytoTune® iPSC Sendai and Epi5™Episomal mediated reprogramming of human BJ fibroblasts and human CD34+ blood cells. CytoTune®-mediated reprogramming of BJ fibroblast requires Essential 6™ supplemented with bFGF at early stages for optimal reprogramming efficiencies. In contrast, Essential 8™ media was optimal for reprogramming of CD34+ blood cells both via CytoTune® and Epi5™ reprogramming methods. Further characterization of iPSC derived from BJ fibroblasts confirmed positive pluripotent marker expression, trilineage differentiation potential and normal karyotype. These results and the ability of Essential 8™ media to support fibroblast culture enables a modular feeder-free, defined media system for generation, expansion and maintenance of iPSC.

BACKGROUND

Induced pluripotent stem cells (iPSC) can be derived from diverse somatic cells using different methods in different media and matrix conditions. As technologies progress towards pharmaceutical and clinical applications, the need for non-integrating methods of reprogramming and the ability to culture the derived cells in chemically defined and feeder independent methods is important. We previously demonstrated the efficient derivation of iPSC using CytoTune® iPSC Sendai Reprogramming kits, a Sendai RNA-virus based non-integrating viral method of reprogramming, using traditional OSKM factors.[1]

An alternate method that is non-viral, non-integrating reprogramming methods that also enables efficient derivation of iPSC is the Epi5™ Episomal reprogramming kits, a non-integrating episomal plasmid DNA based method.



In addition to efficient and safe reprogramming methods, it is critical to develop versatile media/ matrix systems that supports different reprogramming systems from diverse somatic cell types. The Essential™ media systems provides the flexibility to be used not only for maintenance of pluripotent stem cells with Essential 8™ but also the modular Essential 6™ which allows the supplementation of bFGF and TGFβ growth factors for iPSC derivation.[2]

In this study we demonstrate the utility of these reprogramming technologies and media systems to efficiently reprogram both fibroblasts and CD34+ blood cells. The modular use of the essential medias can provide the flexibility to optimize reprogramming and culture conditions.

MATERIAL AND METHODS

All reagents were purchased from Life Technologies, unless noted.

Somatic Cell Culture: BJ strain neonatal foreskin fibroblasts (ATCC-2522) and DF1 adult dermal fibroblasts (donated and collected under all ethical and clinical regulations) were grown in DMEM (Cat# 10569-010) supplemented with ES-FBS (Cat# 16141-079) and Non-essential amino acids (Cat# 11140-050). CD34+ hematopoetic precursor cells were cultured using the supplied basal media and supplement in the StemPro® CD34+ Cell Kit (Cat# A14059), supplemented with SCF (Cat# PHC2111), GM-SCF (PHC2015), and IL3 (Cat# PHC0035) as per the manufacturer's instruction.

Reprogramming: CytoTune® iPSC Sendai Reprogramming kits (Cat# A1378001) were used to reprogram fibroblast at an MOI of 3, and CD34+ cells at an MOI of 5 , with the addition of polybrene. Epi5™ Episomal reprogramming kits (Cat# A15960) were used via the manufacturer's instructions using the Neon® Transfection System (Cat# MPK1025).

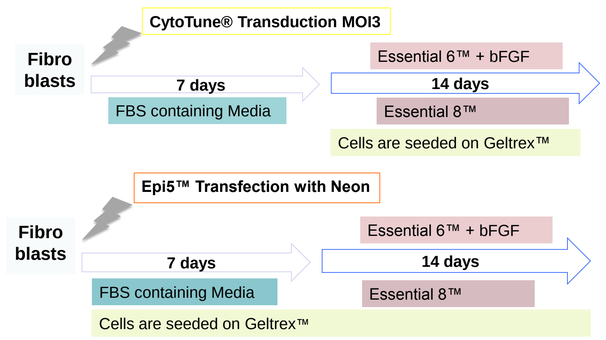
Reprogramming/ PSC Culture Medias: Essential 6™ (Cat# A1516401) was utilized, supplemented with 100 ng/mL of bFGF (Cat# PHG0264). Essential 8™ media (Cat# A1517001) was utilized as per the manual. N2/B27 media was made by supplementing DMEM/F-12 basal media (Cat# 10565-018) with N2 supplement (Cat# 17502-048), B27 supplement (Cat# 17504-044), Non-essential amino acids (Cat# 11140-050), 2-mercaptoethanol (Cat# 21985-023), and 10 ng/mL bFGF (Cat# PHG0264).

Matrices: iPSC were derived and maintained on either Vitronectin (VTN-N) Recombinant Human Protein, Truncated (Cat# A14700) used at 1 µg/cm^2 or Geltrex™ LDEV-Free hESC-qualified Reduced Growth Factor Basement Membrane Matrix (Cat# A1413302) at a 1:100 dilution.

Characterization Tools: Reprogramming efficiencies were determined via Vector Red Alkaline Phosphate staining (Vector Labs).
Pluripotent reagent and antibodies used were Alkaline Phosphatase Live Stain (Cat# A14353), SSEA4-Alexa Fluor®647 Ab (Cat# SSEA421), Oct4 Ab (Cat# A13998), DAPI (Cat# D1306), AFP Ab (Cat# 180003), SMA Ab (Cat# 180106), β-III Tubulin (Cat# 480011). Molecular analysis was performed using the TaqMan® hPSC Scorecard™ Kit 384w (Cat# A15872) on the QuantStudio™ 12K Flex System (Cat # 4471134). Karyotype analysis was performed by Cell Line Genetics.

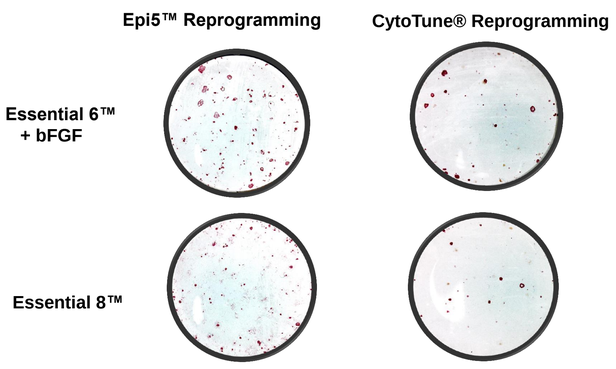
RESULTS

FIGURE 1. Workflow Schematic for Reprogramming of Fibroblasts



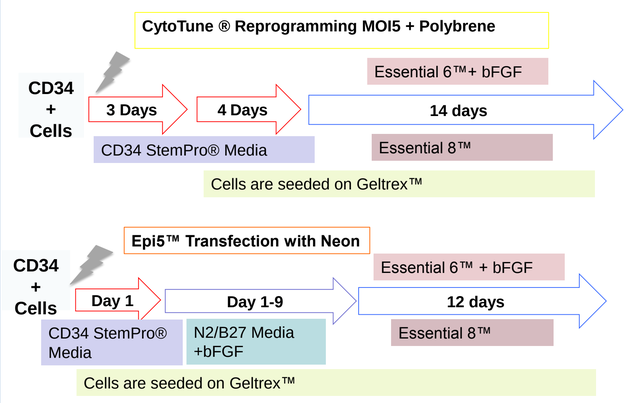
For fibroblast reprogramming, the timelines are very similar in length and times at which medias are exchanged. The difference is that using the CytoTune® reprogramming kit, the fibroblasts are transduced for 24hr after already being adherent, and are not re-seeded on the matrix until days 7 post transduction. The Epi5™ transfection occurs when the fibroblasts are in suspension and hence the cells are directly plated onto the desired matrix at the time of transfection.

FIGURE 2. Fibroblast reprogramming in Essential™ medias



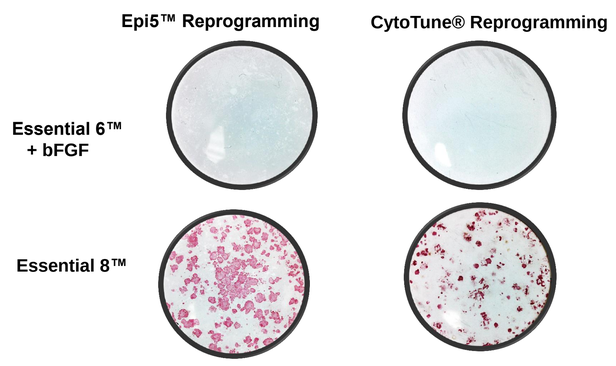
BJ fibroblasts were transduced with CytoTune® at an MOI of 3 in FBS containing media and re-seeded onto Geltrex™ at a density of 50,000 cells per media condition at day7. BJ Fibroblasts were transfected with the Epi5™ as per the manual with FBS containing media and directly seeded ont Geltrex™ at a density of 50,000 cells per media condition. At Day 21 post transduction/transfection the cells were stained with Vector Red for alkaline phosphatase as a measure of reprogrammed colonies. Essential 6™ + bFGF yielded more colonies that are larger and more typical of iPSC morphologies in comparison to Essential 8™ media.

FIGURE 3. Workflow schematic for reprogramming of CD 34+ hematopoetic precursor cells



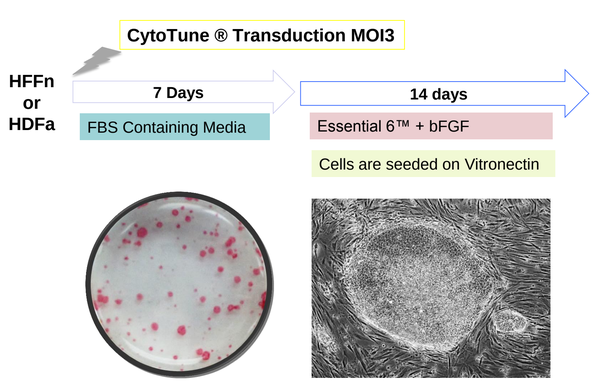
CD34+ blood reprogramming requires different protocol as the blood cells are non-adherent to begin with. Both CytoTune® transductions, at MOI5, and Epi5™ transfections occur while the cells are in suspension. The use of N2/B27 media during the Epi5™ reprogramming is necessary for efficient colony formation prior to switching to the Essential medias.

FIGURE 4. Blood reprogramming in Essential™ medias



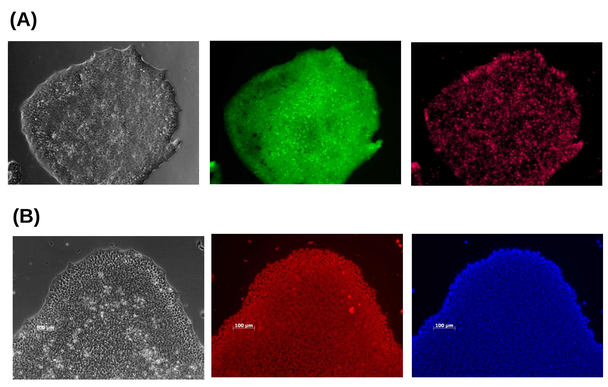
CD34+ blood cells were transduced with CytoTune® at an MOI of 5 and seeded onto Geltrex™ at a density of 50,000 cells per media condition. CD34+ cells were transfected with the Epi5™ as per the manual directly seeded onto Geltrex™ at a density of 50,000 cells per media condition. At Day 21 post transduction/transfection the cells were stained with Vector Red for alkaline phosphatase as a measure of reprogrammed colonies. Essential 6™ + bFGF yielded zero colonies for both reprogramming methods, but the use of Essential 8™ yielded robust iPSC generation for both methods.

Figure 5. Feeder free derivation of human iPSCs from fibroblasts using Essential™ medias



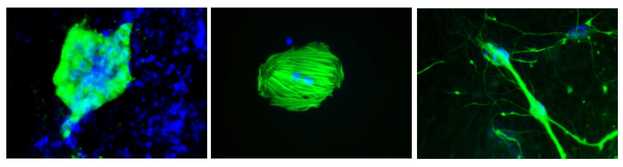
Fibroblasts were reprogrammed with CytoTune® Reprogramming kits using the schematic above, utilizing Vitronectin and Essential 6™ media supplemented with bFGF, Cells seeded on a 6 well plate were stained with Vector Red to demonstrate efficient iPSC colony formation (bottom left image) at Day 21. iPSC colonies were also manually selected on day 21 (bottom right image) and propogated on Vitronectin with Essential 8™ media.

Figure 6. Feeder free derived human iPSCs from BJ fibroblast demonstrate pluripotency

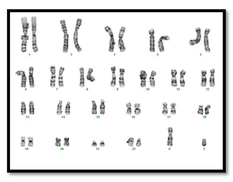


iPSC clones derived from BJ fibroblasts on feeder free conditions continued to demonstrate pluripotency when continually cultured on Vitronectin and Essential 8™ medium. Panel (A) shows a colony at 50X magnification (from left to right) seen at phase contrast, stained with Alkaline Phosphatase Live Stain (green) and SSEA4 (purple). Panel (B) shows a colony at 100X magnification (from left to right) seen at phase contrast, probed with anti-Oct4 antibody (red) and DAPI (blue).

Figure 7. Human iPSCs derived feeder-free from BJ exhibit tri-lineage differentiation potential



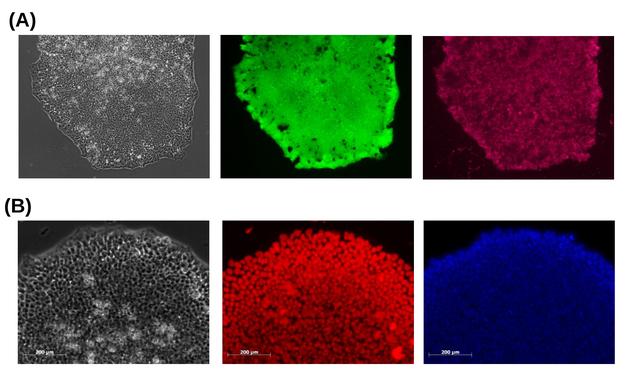
AFP / DAPI (200X) SMA / DAPI (200X) β-III-Tub/ DAPI (200X)



BJ Fibroblast derived iPSC *demonstrating normal* karyotype at P9 XY, 46 [20] Normal,

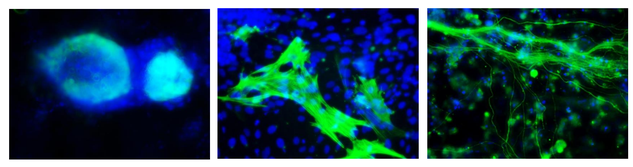
iPSC lines derived on Vitronectin and Essential medias have the potential to become cell types typical of the 3 germ layers, as depicted from left to right on the top panel: Endoderm (AFP), Mesoderm (SMA), and Ectoderm (β-III Tubulin). iPSC cells were spontaneously differentiated via in-vitro embryoid body formation. In addition, expanded lines continued to demonstrate normal karyotypes through passage.

Figure 8. Feeder free derived human iPSCs from adult dermal fibroblasts (DF1) demonstrate pluripotency

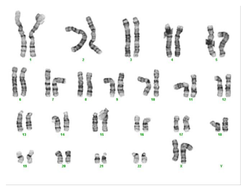


iPSC clones derived from DF1 adult dermal fibroblasts on feeder free conditions continued to demonstrate pluripotency when continually cultured on Vitronectin and Essential 8™ medium. Panel (A) shows a colony at 50X magnification (from left to right) seen at phase contrast, stained with Alkaline Phosphatase Live Stain (green) and SSEA4 (purple). Panel (B) shows a colony at 100X magnification (from left to right) seen at phase contrast, probed with anti-Oct4 antibody (red) and DAPI (blue).

Figure 9. Human iPSCs derived feeder-free from DF1 exhibit tri-lineage differentiation potential



AFP / DAPI (200X) SMA / DAPI (200X) β-III-Tub/ DAPI (200X)

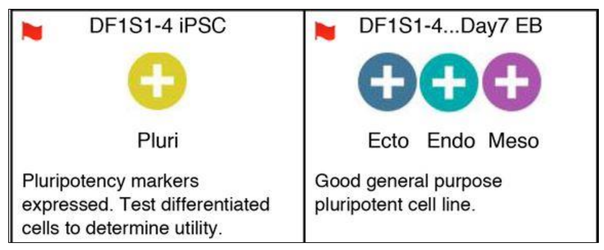


DF1 Fibroblast derived iPSC *demonstrating normal* karyotype at P10 XX, 46 [20] Normal,

DF1 iPSC lines derived on Vitronectin and Essential medias have the potential to become cell types typical of the 3 germ layers, as depicted from left to right on the top panel: Endoderm (AFP), Mesoderm (SMA), and Ectoderm (β-III Tubulin). iPSC cells were spontaneously differentiated via in-vitro embryoid body formation. In addition, expanded lines continued to demonstrate normal karyotypes through passage.

Figure 10. TaqMan® hPSC Scorecard™ analysis of feeder-free iPSC derived in Essential medias

Sample Name	Cell Type	Pluripotent	Ectoderm	Mesoderm	Endoderm
DF1S1-4 iPS	Pluripotent	0.00	-1.43	-0.05	-1.19
DF1S1-4 Day7EB	Trilineage	-2.22	6.26	4.03	1.61



DF1 fibroblast -derived iPSC undifferentiated can cells differentiated via EB formation for 7 days, were analyzed using the TaqMan® hPSC Scorecard™. Analysis of the resulting data using the accompanying software resulted in score relative to a reference standard. The values of undifferentiated cells shows pluripotency scores close to zero and lineage scores closer to or less than zero indicating high similarity to the reference pluripotent. The Day7 differentiated EBs show decreased pluripotency scores and increased lineage scores indication trilineage differentiation of the iPS line without any detectable lineage bias.

CONCLUSIONS

Fibroblasts can efficiently be reprogrammed via the non-integrating technologies of CytoTune® Sendai viruses and Epi5™ episomal vectors. iPSC can also be generated using both methods in feeder independent conditions using essential media systems and matrix combinations. The use of Essential 6™ supplemented with FGF is optimal for fibroblast reprogramming.

CD34 positive blood cells can also be efficiently reprogrammed via CytoTune® Sendai viruses and Epi5™ episomal vectors The matix and Essential media systems can also be used to derive feeder independant iPSC. In the case of reprogramming blood, the use of Essential 8™ media yields robust colony formation.

iPSC derived, isolated and expanded in feeder free conditions with esssential medias via CytoTune® mediated reprogramming of fibroblasts demonstrate all characteristics of pluripotent stem cells. They demonstrate karyotipic stability, expression of mature pluripotent markers, and the potential of tri-lineage differentiation.

The TaqMan® hPSC ScoreCard™ analysis can be used as a sensitive, fast and accurate measure to determine pluripotency and differentiation into the three germ layers: endoderm, ectoderm and mesoderm. In combination with efficient reprogramming methods, CytoTune® and Epi5™, and the versatility of the Essential™ media systems permits iPSC derivation in feeder independent methods from varying somatic cells.

ACKNOWLEDGMENTS

We thank Davis Kunninger for providing the DF1 adult fibroblast line and Chad MacArthur for scientific discussions and review. The gene content for the TaqMan® hPSC ScoreCard™ array and final reference data was a collaborative effort with team at Harvard lead by Alex Meissner.

REFERENCES

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2. Chen G, et al (2011) **Journal Nat Methods 8**:424-429ID:

TRADEMARKS/LICENSING