

# Highly efficient induced pluripotent stem cell reprogramming of cryopreserved lymphoblastoid cell lines

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Abbreviations used: DAPI, 4',6-Diamidino-2-Phenylindole, Dihydrochloride; EBV, Epstein-Barr virus; ESC, embryonic stem cell; hESC, human embryonic stem cell; iPSC, human pluripotent stem cell; iPSC, induced pluripotent stem cell; LCL, lymphoblastoid cell line; NIH, National Institutes of Health; PBS, phosphate buffered saline; PFA, para-formaldehyde

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## ABSTRACT

Tissue culture based *in-vitro* experimental modeling of human inherited disorders provides insight into the cellular and molecular mechanisms involved and the underlying genetic component influencing the disease phenotype. The breakthrough development of induced pluripotent stem cell (iPSC) technology represents a quantum leap in experimental modeling of human diseases, providing investigators with a self-renewing and thus unlimited source of pluripotent cells for targeted differentiation into functionally relevant disease specific tissue/cell types. The existing rich bio-resource of Epstein-Barr virus (EBV) immortalized lymphoblastoid cell line (LCL) repositories generated from a wide array of patients in genetic and epidemiological studies worldwide, many of them with extensive genotypic, genomic and phenotypic data already existing, provides a great opportunity to reprogram iPSCs from any of these LCL donors in the context of their own genetic identity for disease modeling and disease gene identification. However, due to the low reprogramming efficiency and poor success rate of LCL to iPSC reprogramming, these LCL resources remain severely underused for this purpose. Here, we detailed step-by-step instructions to perform our highly efficient LCL-to-iPSC reprogramming protocol using EBNA1/OriP episomal plasmids encoding pluripotency transcription factors (*i.e.*, OCT3/4, SOX2, KLF4, L-MYC, and LIN28), mouse p53DD (p53 carboxy-terminal dominant-negative fragment) and commercially available reprogramming media. We achieved a consistently high reprogramming efficiency and 100% success rate (> 200 reprogrammed iPSC lines) using this protocol.

**Keywords:** lymphoblastoid cell line, iPSC reprogramming, human, disease modeling, disease genetics

## BACKGROUND

Tissue culture based *in-vitro* experimental modeling of human inherited disorders have been used to investigate cellular and molecular mechanisms, and the underlying genetic component influencing the disease phenotype. However, relevant human tissue or cell samples, which are essential for these experimental approaches [1], are often difficult to obtain, sometimes requiring invasive surgery or only becoming available post-mortem. The stem cell-based system, which carries intrinsic capability for indefinite self-renewal and the potential to model the

tissue specific physiology through the use of differentiation protocols, enable us to study genotype-phenotype relationships in a broad range of human cell/tissue types and differentiation states, as well as obtain a large number of cells for additional purposes, including drug screening and stem cell based therapeutics [2].

Embryonic stem cell (ESC) lines were first established in mouse [3], and subsequently in human from *in-vitro* derived embryos [4]. However, the challenges related to bioethics, safety, and the limited availability of disease-specific human embryonic stem cell (hESC) lines have complicated the utilization of this approach to its full potential.

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This changed dramatically in 2006 when Takahashi and Yamanaka made the seminal discovery that mouse skin fibroblasts, using a simple cocktail of pluripotency transcription factors, can be reprogrammed into an induced pluripotent stem cell (iPSC) state that shares the indefinite self-renewal and pluripotent differentiation capacities of ESCs [5]. One year later, the same investigators, as well as groups headed by James Thomson and George Daley, succeeded in converting human fibroblasts into iPSCs [6-8].

Reprogramming to pluripotency has now been demonstrated starting with a variety of somatic cell types, including immortal cell lines such as LCLs [9-13]. However, due to the low reprogramming efficiency and poor success rate of the other cell types including LCLs, dermal fibroblasts isolated from skin biopsies remains the material of choice for reprogramming experiments. Therefore, the already existing rich bio-resource of numerous LCL repositories generated from a wide array of patients, many of them with extensive genotypic and phenotypic data already generated, remains severely underused for this purpose.

We developed and published a highly efficient iPSC reprogramming methodology for cryopreserved LCLs [14]. The two major changes we made in the cocktail of reprogramming factors (*i.e.*, the use of a mouse p53 carboxy-terminal dominant-negative fragment for cellular *TP53* suppression and the removing of *SV40LT* from our reprogramming factors cocktail) has significantly improved the efficiency and success rate of LCL to iPSC reprogramming compared to the previously published methods [12,13,15]. Using our efficient iPSC reprogramming methodology, we have achieved 100% success rate in reprogramming cryopreserved LCLs of more than 200 individuals of our San Antonio Family Heart Study cohort for disease modeling and disease gene identification approaches. We strongly believe that a step-by-step protocol of our methodology will be beneficial to many laboratories worldwide intending to utilize cryopreserved LCLs for iPSC generation. Furthermore, the majority of the media and other materials used in this protocol are available from commercial or public sources and requires minimal to no further re-optimization, making this protocol easily reproducible by other laboratories.

The differential gene expression analysis of the cellular and EBV viral genes, as well as the quantitative PCR analysis of the EBV DNA in the LCL reprogrammed iPSCs, shows that transcription and replication of the EBV genome are inhibited in the reprogrammed iPSCs, which ultimately results in the complete depletion of the EBV genome from the reprogrammed iPSCs [12-15], making the iPSCs and iPSC differentiated disease target cells much more suitable for disease modeling than the original LCLs.

## MATERIALS

### Reagents, media and solutions

- ✓ RPMI 1640 Medium (Cat. # 11875-085, Gibco, Thermo Fisher Scientific, USA)
- ✓ Fetal Bovine Serum (Cat. # 10082-147, Gibco, Thermo Fisher Scientific, USA)
- ✓ MEM Non-Essential Amino Acids (Cat. # 11140-050, Gibco, Thermo Fisher Scientific, USA)
- ✓ Sodium Pyruvate (Cat. # 11360-070, Gibco, Thermo Fisher Scientific, USA)
- ✓ HEPES (Cat. # 15630-080, Gibco, Thermo Fisher Scientific, USA)

- ✓ Antibiotic-Antimycotic (Cat. # 15240-062, Gibco, Thermo Fisher Scientific, USA)
- ✓ Phosphate buffered saline (PBS) without CaCl<sub>2</sub> and MgCl<sub>2</sub> (Cat. # 14190-144, Gibco, Thermo Fisher Scientific, USA)
- ✓ Myco Alert Detection Kit (Optional) (Cat. # LT07-318, Lonza, USA)
- ✓ Cell Culture Treated T-25 Flasks (Cat. # 430639, Corning, USA)
- ✓ Cell Culture Treated 35 mm dish (Cat. # 150318, Nunc, Thermo Fisher Scientific, USA)
- ✓ SE Cell Line 4D-Nucleofector X Kit S (32 RCT) (Cat. # V4XC-1032, Lonza, USA)
- ✓ Episomal plasmid pCE-hOCT3/4 (Cat. # 41813, Addgene, USA)
- ✓ Episomal plasmid pCE-hSK (Cat. # 41814, Addgene, USA)
- ✓ Episomal plasmid pCE-hUL (Cat. # 41855, Addgene, USA)
- ✓ Episomal plasmid pCE-mp53DD (Cat. # 41856, Addgene, USA)
- ✓ Countess Cell Counting Chamber Slides (Cat. # C10228, Invitrogen, Thermo Fisher Scientific, USA)
- ✓ Cell Culture Treated 6 well plate (Cat. # 353934, Corning, USA)
- ✓ DMEM/F-12 Medium (Cat. # 10565018, Gibco, Thermo Fisher Scientific, USA)
- ✓ Matrigel hESC Qualified Matrix (Cat. # 354277, Corning, USA)
- ✓ TeSR-E7 Medium (Cat. # 5910, Stem Cell Technologies, Canada)
- ✓ mTeSR-1 Medium (Cat. # 5850, Stem Cell Technologies, Canada)
- ✓ ROCK inhibitor Y-27632 (Cat. # 04-0012-02, Stemgent, USA)
- ✓ Stain Alive TRA-1-81 Antibody (Optional) (Cat. # 09-0069, Stemgent, USA)
- ✓ Alkaline Phosphatase Live Stain (Optional) (Cat. # A14353, Invitrogen, Thermo Fisher Scientific, USA)
- ✓ Cell Scraper (Cat. # 08-100-241, Fisher Scientific, USA)
- ✓ Dispase (Cat. # 7923, Stem Cell Technologies, Canada)
- ✓ ReLeSR dissociation reagent (Cat. # 5872, Stem Cell Technologies, Canada)
- ✓ mFreSR (Cat. # 05854, Stem Cell Technologies, Canada)
- ✓ CryoStem Freezing Medium (Cat. # 01-0013-50, Stemgent, USA)
- ✓ Tissue Culture Treated 4 well plate (Cat. # 229103, CELL-TREAT Scientific Products, USA)
- ✓ Paraformaldehyde (Cat. # 158127-100G, Sigma Aldrich, USA)
- ✓ 1.0 N NaOH solution (Cat. # S2770-100ML, Sigma Aldrich, USA)
- ✓ Triton™ X-100 (Cat. # T8787-100ML, Sigma Aldrich, USA)
- ✓ Bovine Serum Albumin (Cat. # A7906-100 G, Sigma Aldrich, USA)
- ✓ Donkey serum (Cat. # D9663-10ML, Sigma Aldrich, USA)
- ✓ TRA-1-60 Antibody Mouse (Cat. # 09-0010, Stemgent, USA)
- ✓ TRA-1-81 Antibody Mouse (Cat. # 09-0011, Stemgent, USA)
- ✓ Oct4 Antibody Rabbit (Cat. # 09-0023, Stemgent, USA)
- ✓ SSEA-4 Antibody Mouse (Cat. # 09-0006, Stemgent, USA)
- ✓ Donkey anti-Mouse Secondary Antibody, Alexa Fluor 488 (Cat. # A21202, Invitrogen, Thermo Fisher Scientific, USA)

- ✓ Donkey anti-Rabbit Secondary Antibody, Alexa Fluor 594 (Cat. # A21207, Invitrogen, Thermo Fisher Scientific, USA)
- ✓ 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) (Cat. # D9542-1MG, Sigma Aldrich, USA)
- ✓ Human Pluripotent Stem Cell Functional Identification Kit (Cat. # SC027B, R&D Systems, USA)
- ✓ Anti-Goat Secondary Antibodies, CF™ 488A antibody (Cat. # SAB4600032-50UL, Sigma Aldrich, USA)

Bovine serum albumin (BSA) in PBS.

- ✓ **Blocking buffer:** 2% w/v BSA, 5% v/v secondary antibodies host species serum or equivalent in PBS.

## Recipes

- ✓ **RPMI complete medium:** RPMI 1640 medium containing 15% heat inactivated fetal bovine serum, 1% MEM non-essential amino acids, 1 mM sodium pyruvate, and 10 mM HEPES buffer.
- ✓ **Paraformaldehyde (PFA) 4% solution:** 4% w/v paraformaldehyde in PBS. Heat to 55°C–60°C and add 1.0 N NaOH dropwise to dissolve PFA.
- ✓ **Permeabilization buffer:** 0.2% v/v Triton X-100, 1% w/v

## Equipment

- ✓ Countess Automated Cell Counter (Thermo Fisher Scientific)
- ✓ 4D-Nucleofector System (Lonza)
- ✓ Plate Vortexer (Electron Microscopy Sciences)
- ✓ Mr. Frosty Cryo 1°C freezing container (Nalgene, Thermo Fisher Scientific, USA)
- ✓ Inverted Microscope (Zeiss or any equivalent)
- ✓ Centrifuge (Eppendorf or any equivalent)
- ✓ CO<sub>2</sub> Cell culture Incubator (Panasonic or any equivalent)
- ✓ Magnetic hot plate stirrer (Any make)
- ✓ Pipet-Aid Pipette Controller (Drummond or any equivalent)
- ✓ Variable volume pipette set 0.5 µl to 1000 µl (Eppendorf or any equivalent)

## PROCEDURE

For iPSC reprogramming of cryopreserved LCLs, the LCLs are propagated and while still in logarithmic growth phase, nucleofected with episomal plasmids (pCEhOCT3/4, pCE-hSK, pCE-hUL, and pCE-mp53DD), encoding reprogramming factors (*i.e.*, *OCT3/4*, *SOX2*, *KLF4*, *LMYC*, and *LIN28*), and a mouse p53 carboxy-terminal dominant-negative fragment using 4D-Nucleofector X Kit and 4D-Nucleofector system. The plasmids are described in Okita *et al.* [11] and can be obtained from the Addgene plasmid repository. The Institutional Review Board of the University of Texas Rio Grande Valley has approved all methods described here.

## Lymphoblastoid cell culture

### 1. Culturing cryopreserved lymphoblastoid cell line(s)

- 1.1. Warm phosphate buffered saline (PBS) without CaCl<sub>2</sub> and MgCl<sub>2</sub> and RPMI complete medium to 37°C before starting the protocol to ensure that the thawing procedure is done as quickly as possible.
- 1.2. Label a T-25 cell culture flask, add 5 ml of RPMI complete medium and incubate in a CO<sub>2</sub> incubator at 37°C, 5% CO<sub>2</sub> and atmospheric O<sub>2</sub>.
- 1.3. Quickly thaw a vial of cryopreserved cells in a 37°C water bath by gently shaking the cryovial continuously until only a small frozen cell pellet remains.
- 1.4. Remove the cryovial from the water bath and wipe it with 70% ethanol or isopropanol.
- 1.5. Transfer cells (~1 ml) from the cryovial to a pre-labeled 15 ml tube and add 9 ml of pre-warmed (37°C) PBS without CaCl<sub>2</sub> and MgCl<sub>2</sub> with gentle mixing.
- 1.6. Centrifuge cells at 500 × g for 5 min at room temperature.
- 1.7. Aspirate supernatant, leaving the cell pellet intact. Gently re-suspend the cell pellet in 2 ml of pre-warmed RPMI complete medium by pipetting 2–3 times using a 5 ml pipette.
- 1.8. Transfer the cells to the T-25 cell culture flask prepared in step 1.2 and incubate in a CO<sub>2</sub> incubator at 37°C, 5% CO<sub>2</sub> and atmospheric O<sub>2</sub>.
- 1.9. Culture cells for 5–7 d or until the desired number of cells are achieved, changing medium every 2–3 d.

**TIP:** In general, one frozen vial containing 3–5 × 10<sup>6</sup> cells can be successfully thawed into one T-25 cell culture flask in 5–7 ml of medium. However, medium volume and culture vessel may need to be optimized as per the cell numbers.

## iPSC reprogramming

### 2. Pre-nucleofection

- 2.1. Inspect cell culture microscopically for any sign of infection. A mycoplasma test is optional but recommended.
- 2.2. To keep the cell in logarithmic growth phase, passage LCL culture 24 h before nucleofection at a density of about  $0.2$  to  $0.5 \times 10^6$  cells/ml.

**NOTE:** To obtain optimal nucleofection efficiency cells must be in logarithmic growth phase. The plasmid DNA should be highly purified, preferably by using endotoxin-free kits; A260:A280 ratio should be at least 1.8.

### 3. Nucleofection

Nucleofection is performed using SE Cell Line 4D-Nucleofector X Kit and 4D-Nucleofector DN-100 program on a 4D-Nucleofector system.

- 3.1. Label a 35 mm cell culture dish and add 1.4 ml RPMI complete medium. Incubate the dish in a CO<sub>2</sub> incubator at 37°C, 5% CO<sub>2</sub> and atmospheric O<sub>2</sub>.
- 3.2. Warm PBS without CaCl<sub>2</sub> and MgCl<sub>2</sub> and RPMI complete medium to 37°C.
- 3.3. Start 4D-Nucleofector system and create or upload the file with following experimental parameters: SE Cell Line solution and 4D-Nucleofector DN-100 program.
- 3.4. Add 3.6 µl of nucleofector supplement to 16.4 µl of nucleofector solution per sample nucleofected. Alternatively, add entire supplement supplied in the kit to the nucleofector solution, and use 20 µl of this mix per sample nucleofected. When stored at 4°C, the nucleofector solution and supplement mix is stable for 3 months.
- 3.5. Thaw plasmid DNA on ice and prepare plasmid mix by adding equal amounts of pCEh-OCT3/4, pCE-hSK, pCE-hUL, and pCE-mp53DD plasmid DNA (250 ng each/sample) in a 0.5 ml tube. Keep the tube on ice.
- 3.6. To prepare LCLs for nucleofection, gently pipette LCL culture 3–5 times to make single cell suspension, then collect 5 ml of cell suspension in a 15 ml tube and centrifuge at  $500 \times g$  for 5 min at room temperature.
- 3.7. Aspirate the supernatant and resuspend cells in 3–5 ml of pre-warmed PBS without CaCl<sub>2</sub> and MgCl<sub>2</sub>.
- 3.8. Determine the cell density using preferred cell-counting method and aliquot  $4 \times 10^5$  cells in a 1.5 ml microcentrifuge tube.
- 3.9. Centrifuge the LCL aliquot at  $500 \times g$  for 5 min at room temperature. Remove supernatant completely.
- 3.10. Add 1 µg of the plasmid DNA mix prepared in step 3.5 and resuspend the cell pellet gently in 20 µl of room temperature nucleofector solution mix prepared in step 3.4.

**NOTE:** The volume of plasmid DNA mix should not exceed 10% of the total nucleofection reaction volume.

- 3.11. Transfer 20 µl of the cells and plasmid DNA mix into a well of 16-well nucleocuvette strip (supplied with nucleofection kit).
- 3.12. Gently tap the nucleocuvette strip to make sure the sample covers the bottom of the cuvette.
- 3.13. Place nucleocuvette strip into the retainer of the 4D-Nucleofector X Unit and start nucleofection.
- 3.14. After run completion, remove the nucleocuvette strip from the retainer, add 80 µl of pre-warmed (37°C) RPMI complete medium, and incubate for 5 min at room temperature.
- 3.15. Mix cells by gently pipetting 2–3 times and transfer to the 35 mm cell culture dish prepared in step 3.1.
- 3.16. Transfer the culture dish to a CO<sub>2</sub> incubator at 37°C, 5% CO<sub>2</sub> and atmospheric O<sub>2</sub> and allow the cells to recover overnight (16–18 h).

**NOTE:** Leaving cells in nucleofector solution for extended periods of time may lead to reduced transfection efficiency and cell viability.

### 4. Maintenance of iPSC reprogramming culture

- 4.1. Day 1, after overnight recovery, plate the nucleofected cells into two wells of a Matrigel coated six well plate (~750 µl per well) and add 750 µl of iPSC reprogramming medium (TeSR-E7) in each well. Place the plate in a CO<sub>2</sub> incubator at 37°C, 5% CO<sub>2</sub> and atmospheric O<sub>2</sub>.

- 4.2. Day 3 and 5, add 0.5 ml pre-warmed (37°C) TeSR-E7 medium to each well.
- 4.3. Day 7 and 9, replace 1 ml of spent medium with pre-warmed (37°C) TeSR-E7 medium.

**CAUTION:** Take all precautions; do not discard cells during the medium change up to this stage. Use a pipette to remove spent medium rather than an aspirator.

**TIP:** By days 7–9, cells will start to adhere to the well surface and changes in cellular morphology can be observed (**Fig. 1**).

- 4.4. On day 11, replace spent medium with 2 ml of pre-warmed (37°C) TeSR-E7 medium.

**TIP:** The cells that are still in suspension (not adhered to the well surface) can be discarded from this stage onwards.

- 4.5. Day 13 to 14 when iPSC like cell colonies start to appear (**Fig. 1**), transition the culture to human pluripotent stem cell maintenance medium (mTeSR-1) by replacing spent medium with fresh 1 ml mTeSR-1 medium.

- 4.6. From day 15 onwards, change spent medium with 2 ml of pre-warmed (37°C) mTeSR-1 medium daily.

**NOTE:** From day 15 onward, an aspirator may be used to remove spent media.

- 4.7. On days 18–21, 10 to 15 colonies morphologically similar to human ESCs (**Fig. 1** and **Fig. 2A**) are manually picked for further expansion. Refer to following section for detailed protocol.

**NOTE:** The number of reprogrammed iPSC colonies differs significantly from sample to sample. On an average we have observed 20 to 100 iPSC like colonies per well of a six well plate.

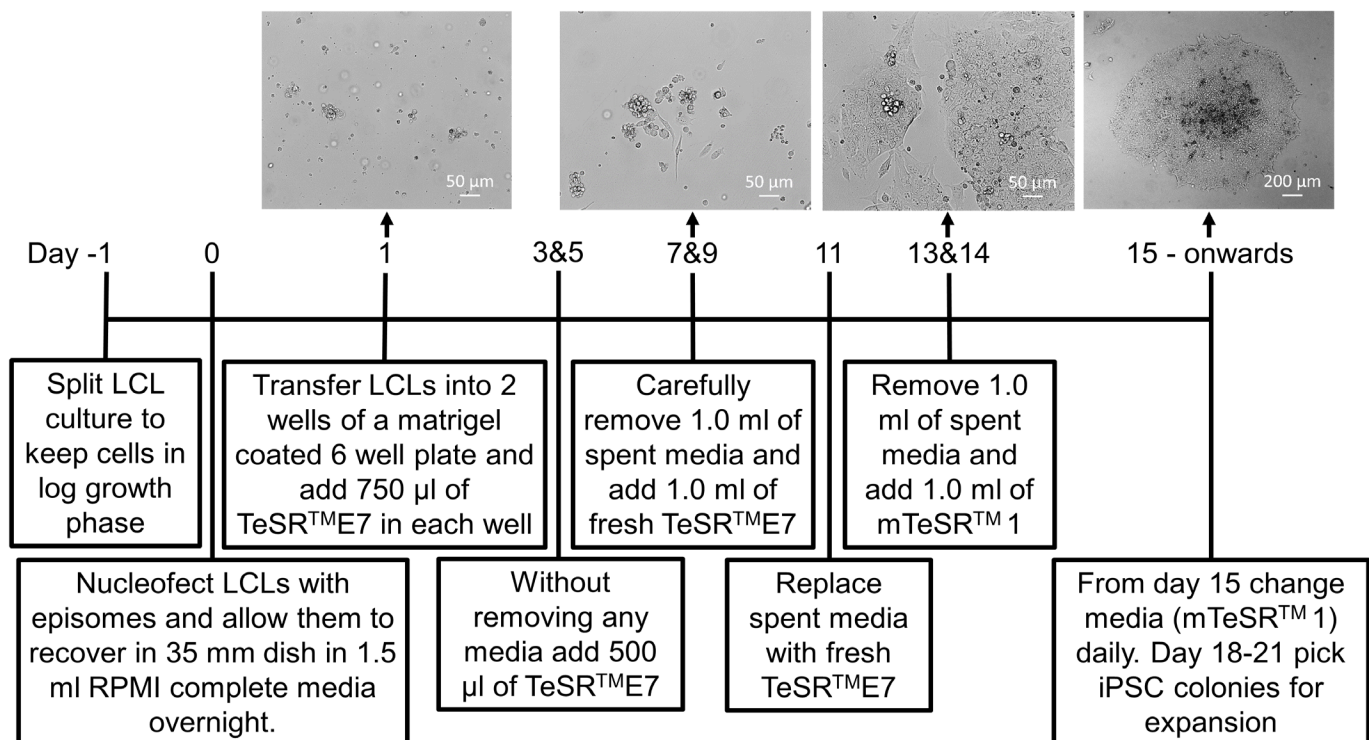


Figure 1. Schematic diagram of LCL to iPSC reprogramming methodology.

## 5. iPSC colony selection and expansion

- 5.1. At least 1 h before picking the iPSC colonies from the reprogramming plate, coat two wells of a six well plate with Matrigel hESC qualified matrix. Follow the manufacturer protocol.

- 5.2. Warm a 4 ml aliquot of mTeSR-1 medium to room temperature (15°C–25°C) and add 10 µM ROCK inhibitor (Y-27632).
- 5.3. Aliquot 1 ml of mTeSR-1 medium supplemented with ROCK inhibitor in a 15 ml tube and 1.5 ml in a Matrigel coated well for each iPSC clone to be established. Place the plate in CO<sub>2</sub> incubator at 37°C, 5% CO<sub>2</sub> and atmospheric O<sub>2</sub>.
- 5.4. Place the reprogramming plate on an inverted microscope kept in a laminar flow hood and pick 10–15 iPSC colonies that are morphologically similar to human ESCs (**Fig. 1** and **Fig. 2A**) using a stretched glass Pasteur pipette or 200 µl pipette tip. Collect the picked colonies in the 15 ml tube(s) prepared in step 5.3. Alternatively, colonies may be live stained for TRA-1-81 or alkaline phosphatase for easy identification.
- 5.5. After picking the desired number of colonies, gently pipette the medium containing iPSC colonies 2–3 times with a 1 ml pipette to achieve desired iPSC aggregate size.
- 5.6. Plate the iPSC aggregates (0.5 ml in each well) in two wells of the six well plate prepared in step 5.3.
- 5.7. Place the plate in a CO<sub>2</sub> incubator at 37°C, 5% CO<sub>2</sub> and atmospheric O<sub>2</sub>. Move the plate in several quick, short, back-and-forth and side-to-side motions to evenly distribute the cell aggregates. Do not disturb the plate for next 24 h.
- 5.8. Change medium (mTeSR-1 without ROCK inhibitor) daily and visually assess cultures to monitor growth.
- 5.9. On day 3 or 4 after plating, when iPSC colonies are easily distinguishable from differentiated cells. Remove the differentiated areas using a stretched glass Pasteur pipette or 200 µl pipette tip.
- 5.10. On day 5 to 7 when iPSC colonies are ready for passaging, coat appropriate number of six well plate(s) with Matrigel hESC qualified matrix.

**NOTE:** At this stage iPSCs can be passaged using either enzymatic (using Dispase enzyme) or non-enzymatic method. Here we describe a commonly used enzymatic method.

- 5.11. Warm a 12 ml aliquot/six well plate of mTeSR-1 medium to room temperature (15°C–25°C) and add 10 µM ROCK inhibitor (Y-27632).
- 5.12. Add 1.5 ml per well to the Matrigel coated six well plate. Place the plate in CO<sub>2</sub> incubator at 37°C, 5% CO<sub>2</sub> and atmospheric O<sub>2</sub>.
- 5.13. Warm sufficient volumes of DMEM/F12 and Dispase to 37°C in a water bath.
- 5.14. Aspirate spent medium from the iPSC well and wash with 2 ml of pre-warmed (37°C) DMEM/F12.
- 5.15. Add 1 ml of Dispase (1 U/ml) solution and incubate for 6–8 min in a CO<sub>2</sub> incubator at 37°C, 5% CO<sub>2</sub> and atmospheric O<sub>2</sub>.

**NOTE:** The incubation time may need to be optimized for a new iPSC line. To optimize incubation time, after adding Dispase observe iPSC colonies under microscope in frequent interval until the outer edge of the iPSC colonies starts to fold inwards. Do not expose cells to Dispase for an extended period.

- 5.16. Aspirate Dispase and wash each well 3 times with 2 ml of pre-warmed (37°C) DMEM/F12.

**CAUTION:** At this stage, the iPSC colonies are loosely attached to the well surface. The washing steps should be performed with gentle dispensing of medium to the side wall of the well.

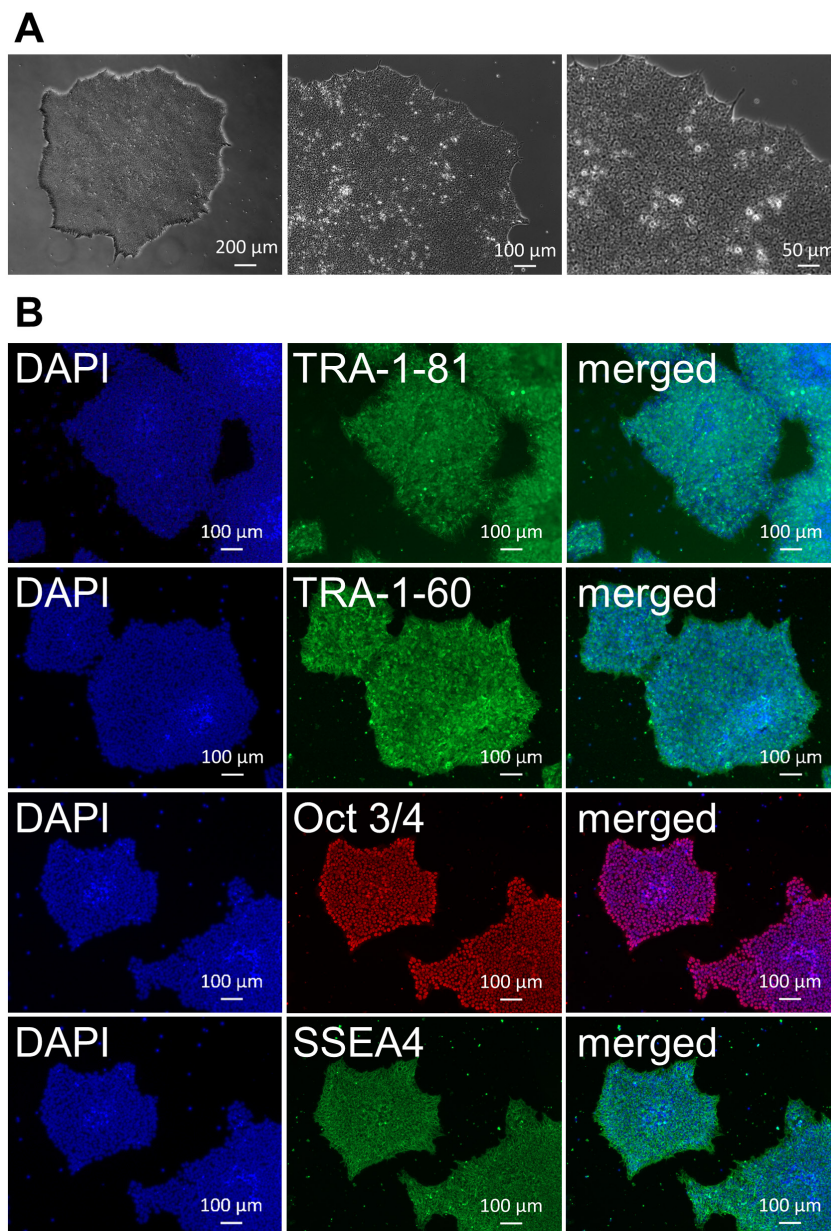
- 5.17. Add 1 ml of pre-warmed (37°C) DMEM/F12 to each well and detach iPSC colonies by gentle scraping with a cell scraper.
- 5.18. Collect the scrapped iPSC colonies in a 15 ml tube and wash each well with an additional 1 ml of pre-warmed (37°C) DMEM/F12 and combine with the iPSC colonies collected in the 15 ml tube.

**TIP:** Perform step 5.18 with a wide bore pipette to minimize the breakage of cell aggregates.

- 5.19. Centrifuge the 15 ml tube containing cell aggregates at 90 × g for 5 min at room temperature.
- 5.20. Remove supernatant and resuspend cell aggregates in 3 ml of mTeSR-1 medium supplemented with 10 µM ROCK inhibitor (Y-27632) prepared in step 5.11.

**TIP:** Pipetting 4–5 times with a wide bore pipette should achieve appropriate size of iPSC aggregates for plating. However, this step may need optimization based on individual’s pipetting habit.

- 5.21. Plate the iPSC aggregates at a density of 1:6 to 1:10 depending upon the confluency of the iPSC wells, into the six well plate prepared in step 5.12.
- 5.22. Place the plate in a CO<sub>2</sub> incubator at 37°C, 5% CO<sub>2</sub> and atmospheric O<sub>2</sub>. Move the plate in several quick, short, back-and-forth and side-to-side motions to evenly distribute iPSC aggregates. Do not disturb the plate for 24 h.
- 5.23. Change medium (mTeSR-1 without ROCK inhibitor) daily and visually assess cultures to monitor growth.
- 5.24. The cultures can be split every 4–7 d upon maturity of the iPSC colonies. The iPSCs should be passaged at least 3–5 times before characterization, functional validation, and experimental use.



**Figure 2. Characterization of LCL generated iPSCs. A.** Morphology of a reprogrammed iPSC colony at 50×, 100×, and 200× original magnifications, respectively. **B.** Immunocytochemistry analysis of reprogrammed iPSC colonies showing expression of pluripotency markers TRA-1-81, TRA-1-60, Oct3/4 and SSEA4.

## Cryopreservation

6. Cryopreserving generated iPSC clones/lines
  - 6.1. Warm sufficient volumes of DMEM/F12 and Dispase to 37°C in a water bath.
  - 6.2. Using an inverted microscope identify and remove any differentiated areas by scrapping with a stretched glass Pasteur pipette or 200 µl pipette tip.
  - 6.3. Follow steps 5.14 through 5.19.
  - 6.4. Remove supernatant and resuspend cell aggregates in an appropriate volume (1 ml/harvested well) of iPSC freezing media (CryoStem or mFreSR freezing media).

**TIP:** Gentle pipetting with a wide bore pipette 2–3 times should achieve desired resuspension of cell aggregates.

- 6.5. Immediately aliquot resuspended cell aggregates into pre-labelled cryovial(s) (1 ml/cryovial).
- 6.6. Transfer the cryovials into Mr. Frosty Cryo 1°C freezing container and store at –80°C for 4–6 h, followed by long-term storage in liquid nitrogen.

**CAUTION:** Do not leave cells in – 80°C storage for more than 24 h.

## iPSC characterization/validation

7. Immunocytochemistry analysis of pluripotency markers (TRA-1-60, TRA-1-81, Oct 3/4, SSEA4)
  - 7.1. Using the methodology described above grow iPSCs on a Matrigel coated four well plate.
  - 7.2. On day 3–4 after plating iPSCs on Matrigel coated four well plate or when iPSC colonies acquire characteristic morphology.
  - 7.3. Wash the cells with 0.5 ml ice-cold PBS and then fix with 0.5 ml 4% PFA, for 15 min at room temperature.
  - 7.4. Briefly wash with 0.5 ml of ice-cold PBS 2 times. If not immediately proceeding to immunostaining, add fresh 0.5 ml PBS and seal the culture plate with parafilm. The fixed cells can be stored at 4°C for a week.
  - 7.5. Permeabilize cells with 0.5 ml of permeabilization buffer for 12 min at room temperature.

**NOTE:** The permeabilization step is not required for surface antigens.

- 7.6. Aspirate permeabilization buffer and add 0.5 ml of blocking buffer. Incubate at room temperature for 1 h.
- 7.7. During the incubation period prepare 1× working solution of each primary antibodies (*i.e.*, anti TRA-1-60, anti TRA-1-81, anti Oct 3/4 and anti SSEA4) in blocking buffer and keep on ice.

**TIP:** Follow manufacturer instructions to prepare 1× working solutions of the primary antibodies.

**NOTE:** Optimization of primary antibodies working concentration may be required.

- 7.8. After an hour aspirate blocking buffer and add 250 µl of respective primary antibodies to each well.
- 7.9. Incubate at 4°C overnight in a humidifying chamber.
- 7.10. Next day prepare respective fluorochrome-conjugated secondary antibodies 1× working solution(s). Keep the solution on ice protected from light.
- 7.11. Wash each well 3 times with 0.5 ml of blocking buffer for 5 min each.
- 7.12. Add 250 µl of respective fluorochrome-conjugated secondary antibodies to each well and incubate for 1 h at room temperature protected from light.
- 7.13. Dilute DAPI stock solution (5 mg/ml) 1:10 in H<sub>2</sub>O and store protected from light.
- 7.14. Wash each well with 0.5 ml of PBS for 5 min.
- 7.15. Add 5 µl of DAPI working solution in 0.5 ml of PBS to each well, incubate 2 min at room temperature.
- 7.16. Wash each well for 5 min twice with 0.5 ml PBS. Add 0.5 ml of fresh PBS and seal the plate with parafilm.



**7.17.** Image stained iPSC colonies immediately or it can also be stored at 4°C in the dark for 1–2 d.

## Functional validation of reprogrammed iPSCs

The functional competence of reprogrammed iPSC lines to differentiate into the cells of all the three germ layers (*i.e.*, endoderm, mesoderm, and ectoderm) is assessed using commercially available hPSC functional identification kit or alternatively using the traditional teratoma formation assays. The procedure to perform monolayer differentiation into cells of all three germ layers using the hPSC functional identification kit is described below.

### 8. Culturing iPSCs for differentiation assays

- 8.1.** Coat 2 wells in each of three 4 well plates with Cultrex PathClear BME supplied with the kit.
- 8.2.** Warm a 3 ml aliquot of mTeSR-1 medium supplemented with 10  $\mu$ M ROCK inhibitor (Y-27632) to room temperature (15°C–25°C).
- 8.3.** Warm an aliquot of PBS and DMEM/F12 medium to 37°C in the water bath.
- 8.4.** Wash iPSC culture plate(s) with pre-warmed (37°C) PBS.
- 8.5.** Add appropriate amount of pre-warmed (37°C) Accutase to iPSC well(s) and incubate at 37°C for 5–7 min.
- 8.6.** Collect the dissociated cells in a 15 ml tube and dilute 1:4 with pre-warmed DMEM/F12 medium.
- 8.7.** Centrifuge the cells at 200  $\times$  g for 4 min at room temperature. Aspirate the supernatant and resuspend cell pellet in 5 ml pre-warmed DMEM/F12 medium.
- 8.8.** Determine cell density using preferred cell-counting method. Aliquot  $1.2 \times 10^6$  cells in a fresh 15 ml tube.
- 8.9.** Centrifuge at 200  $\times$  g for 4 min at room temperature. Aspirate the supernatant and resuspend cell pellet in 3 ml of mTeSR-1 medium supplemented with ROCK inhibitor.
- 8.10.** Plate 0.5 ml cell suspension ( $1.1 \times 10^5$  cells/cm<sup>2</sup>) in each Cultrex PathClear BME coated wells of the 4 well plates prepared in step 8.1.
- 8.11.** Place the plate in a CO<sub>2</sub> incubator at 37°C, 5% CO<sub>2</sub> and atmospheric O<sub>2</sub>. The next day cells should be approximately 50% confluent. If cells are not 50% confluent, replace medium with fresh mTeSR-1 medium and continue iPSC culture until 50% confluency is achieved.
- 8.12.** Label the plates as “endoderm”, “mesoderm” and “ectoderm” and proceed for respective differentiation.

### 9. Endoderm differentiation

- 9.1.** Prepare 2 ml of endoderm differentiation medium-I, as described in the kit’s manual.
- 9.2.** Replace mTeSR-1 with endoderm differentiation medium-I (1 ml/well) in the plate labeled as “endoderm”. Return the plate to CO<sub>2</sub> incubator.
- 9.3.** On day 2 about 16–24 h after replacing endoderm differentiation medium-I, prepare endoderm differentiation medium-II.
- 9.4.** Replace endoderm differentiation medium-I with endoderm differentiation medium-II. Return the plate to CO<sub>2</sub> incubator.
- 9.5.** Replace spent medium with fresh endoderm differentiation medium-II on day 3.
- 9.6.** On day 4, cells are ready of immunocytochemistry analysis of endoderm markers. Proceed to immunocytochemistry analysis of germ layer markers.

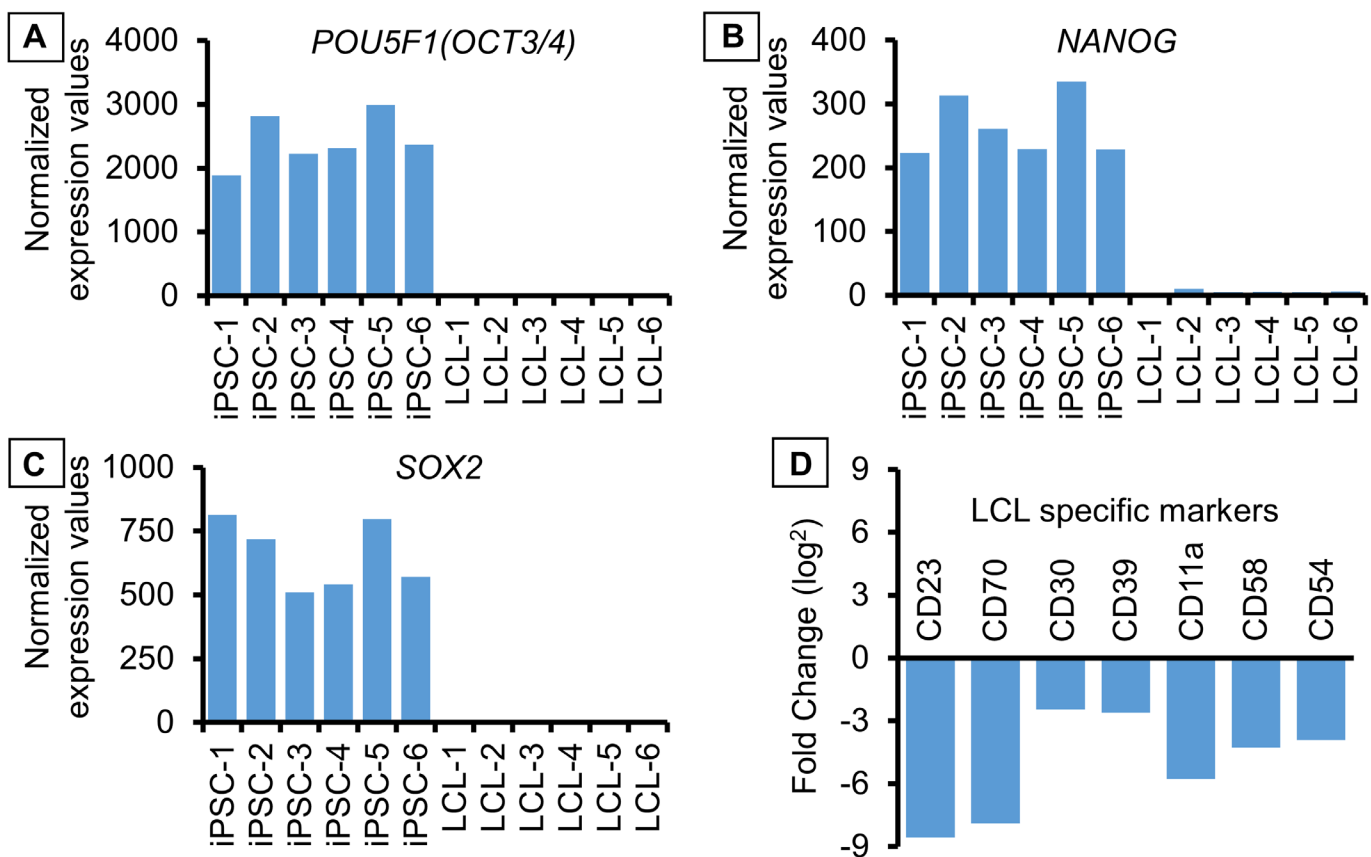
### 10. Mesoderm differentiation

- 10.1.** Prepare 2 ml of mesoderm differentiation medium, as described in the kit’s manual.
- 10.2.** Replace mTeSR-1 with mesoderm differentiation medium (1 ml/well) in the plate labeled as “mesoderm”. Return the plate to CO<sub>2</sub> incubator.
- 10.3.** After 12 to 16 h, replace spent medium with fresh mesoderm differentiation medium.
- 10.4.** Cells are ready for immunocytochemistry analysis of mesoderm markers within 24–36 h of differentiation. Proceed to immunocytochemistry analysis of germ layer markers.

### 11. Ectoderm differentiation

- 11.1.** Prepare 2 ml of ectoderm differentiation medium, as described in the kit’s manual.

- 11.2. Replace mTeSR-1 with ectoderm differentiation medium (1 ml/well) in the plate labeled as “ectoderm”. Return the plate to CO<sub>2</sub> incubator.
- 11.3. Repeat medium change with fresh ectoderm differentiation medium on day 2 and 3.
- 11.4. On day 4 cells are ready of immunocytochemistry analysis of ectoderm markers. Proceed to immunocytochemistry analysis of germ layer markers.
12. Immunocytochemistry analysis of germ layer markers
  - 12.1. Follow steps 7.3 to 7.5 to fix and permeabilize cells.
  - 12.2. Aspirate permeabilization buffer and add 0.5 ml of blocking buffer and incubate at room temperature for 1 h.
  - 12.3. During incubation period, dilute the following primary antibodies supplied with the kit in blocking buffer to a final concentration of 10 µg/ml and keep on ice: Goat anti-human SOX17 (Endoderm marker); Goat anti-human Brachyury (Mesoderm marker); Goat anti-human Otx2 (Ectoderm marker)
  - 12.4. After 1 h incubation, aspirate blocking buffer and add 300 µl of respective primary antibodies to 1 well of the endoderm, mesoderm and ectoderm plates. Use the second well as a negative control by adding 300 µl of blocking buffer.
  - 12.5. Incubate at 4°C overnight in a humidifying chamber.
  - 12.6. Next day prepare 1× working solution of fluorochrome-conjugated Donkey Anti-Goat secondary antibodies. Keep the solution on ice protected from light.
  - 12.7. Repeat steps 7.11 to 7.16.
  - 12.8. Image stained cells immediately or it can also be stored at 4°C in the dark for 1–2 d.



**Figure 3. Differential gene expression analysis of pluripotency and LCL specific markers in LCL and their reprogrammed iPSCs. A-C.** Graphs showing gene expression of core pluripotency markers (*POU5F1*, *NANOG*, *SOX2*) in LCLs and their reprogrammed iPSCs. **D.** The differential gene expression graph showing significant down-regulation of LCL specific markers (CD23, CD70, CD30, CD39, CD11a, CD58 and CD54).

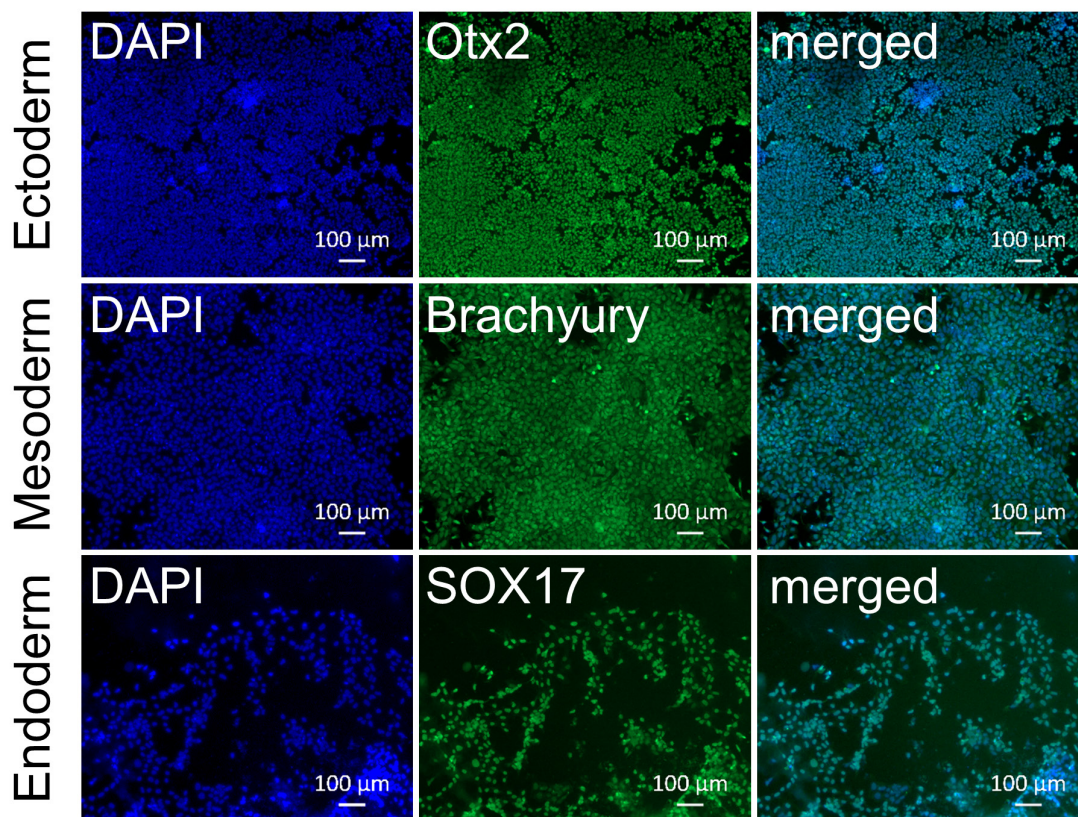
Genomic integrity of reprogrammed iPSCs

Prolonged culture and cellular manipulation involved in iPSC reprogramming may affect the genomic integrity of the reprogrammed iPSCs. Therefore, a test of genomic integrity is recommended. The most commonly used technique, by far, is the traditional karyotype analysis. Karyotyping is based on arresting the cells in metaphase stage of the cell division, followed by their staining, Giemsa staining (G-banding) is recommended. Alternatively, array-based molecular analyses of genomic integrity using commercially available microarray and kits may be employed.

ANTICIPATED RESULTS

The LCL reprogrammed iPSCs formed flat and compacted colonies and showed high nucleus-to- cytoplasm ratio, the typical morphology of human ESCs (Fig. 2A). The immunocytochemistry and differential gene expression analysis showed that LCL reprogrammed iPSCs express pluripotency markers (Fig. 2B and Fig. 3A-3C). The LCL

reprogrammed iPSCs also showed potential to differentiate into cells of all the three germ layers by *in-vitro* monolayer differentiation assays (Fig. 4). Apart from the routine validation, we have successfully generated terminally differentiated cells of all the three germ layers such as neurons, cardiomyocytes and hepatocytes, using these LCL reprogrammed iPSCs (Fig. 5).

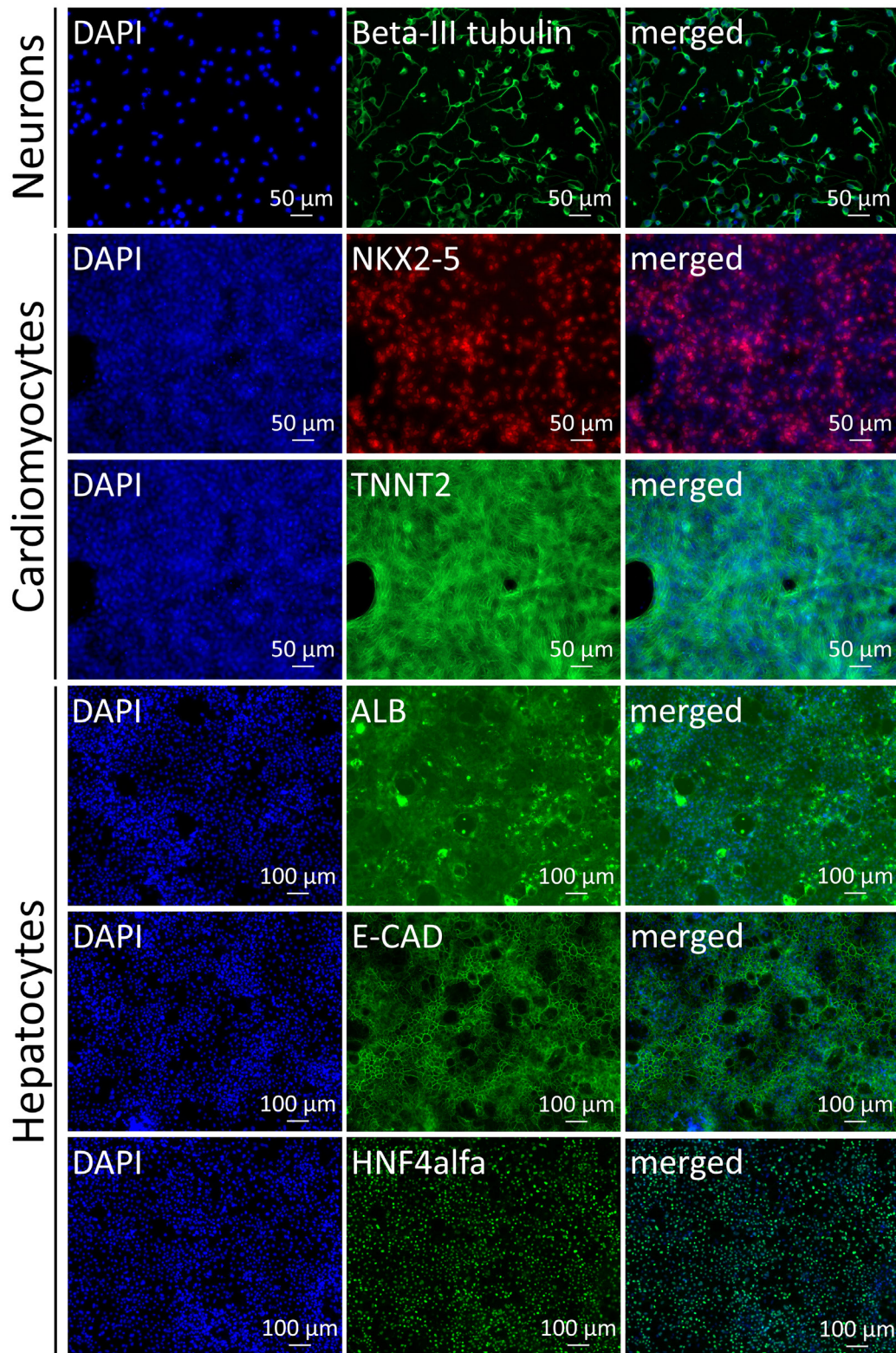


**Figure 4. Functional validation of LCL generated iPSCs.** Immunocytochemistry analysis of the cells of three embryonic germ layers differentiated from reprogrammed iPSCs using monolayer differentiation assay.

Overall, we achieved success in reprogramming cryopreserved LCLs of more than 200 San Antonio Family Heart Study participants in first attempt, except in two cases (~1.33%), the first attempt failed due to bacterial contaminations of the reprogramming culture. In these 200 successful reprogramming experiments more than 95% colonies out of 10 to 15 colonies screened per sample expressed pluripotency markers. One established iPSC clone per participant was tested for function competence and all tested iPSC clones successfully differentiated into cells of all the three germ layers.

LCLs show high expression of the B-cell activation markers (CD23, CD70, CD30, and CD39) and cellular adhesion molecules (CD11a,

CD58, and CD54) [16]. These markers are usually absent or expressed at very low levels in resting B-cells, but their expression is significantly upregulated by EBV encoded nuclear antigens (EBNA2, EBNA3C) and latent membrane proteins (LMP1, LMP2A) when EBV infection is used to generate the LCLs [17-19]. The differential gene expression analysis of LCLs and their reprogrammed iPSCs (at 10 to 15 passage) shows significant downregulation of B-cell activation markers and cellular adhesion molecules in the reprogrammed iPSCs (Fig. 3D), which supports previous findings that EBV transcriptional activity is inhibited in the reprogrammed iPSCs [12-14].



**Figure 5.** Generation of terminally differentiated cells of all the three germ layers from LCL reprogrammed iPSCs. Immunocytochemistry analysis of neurons (ectoderm), cardiomyocytes (mesoderm) and hepatocytes (endoderm) differentiated from LCL reprogrammed iPSCs.

**TROUBLESHOOTING**

Possible problems and their troubleshooting solutions are listed in **Table 1**.

**Table 1. Troubleshooting.**

Step #	Problem	Cause	Suggestions
1.9	Poor LCL viability/growth	<ul style="list-style-type: none"> <li>Cell culture contaminants</li> <li>Variable cell growth</li> <li>Poor genomic integrity</li> </ul>	<ul style="list-style-type: none"> <li>Test for mycoplasma and other cell culture contaminants</li> <li>Optimize culture vessel and medium volume as per cell number</li> <li>Use cell aliquot of early passages and test for genomic integrity</li> </ul>
4.1	Low cell survival upon nucleofection	<ul style="list-style-type: none"> <li>Cells were kept in nucleofector solution too long</li> <li>Poor cell quality</li> <li>Poor plasmid DNA quality</li> </ul>	<ul style="list-style-type: none"> <li>Perform nucleofection steps quickly and add pre-warmed medium immediately after nucleofection</li> <li>Make sure the cells are in log growth phase and cell viability is &gt; 90%</li> <li>Use endotoxin-free preparation of the plasmid DNA. DNA should be of high purity and integrity</li> </ul>
4.7	Poor iPSC reprogramming efficiency	<ul style="list-style-type: none"> <li>Poor cell quality</li> <li>Cell number too high or too low</li> <li>Poor plasmid DNA quality</li> </ul>	<ul style="list-style-type: none"> <li>Make sure the cells are in log growth phase and cell viability is &gt; 90%</li> <li>Optimize cell number, too high or too low cell number affects nucleofection and reprogramming efficiency</li> <li>Use endotoxin-free preparation of the plasmid DNA</li> </ul>
5.4 & 5.24	Poor iPSC quality	<ul style="list-style-type: none"> <li>Poor iPSC colony selection</li> <li>Differentiated or partially re-programmed cells in the iPSC culture</li> </ul>	<ul style="list-style-type: none"> <li>Select/pick only iPSC colonies that are morphologically similar to human ESCs; Live staining for TRA-1-81 or alkaline phosphatase may be performed for easy identification of fully reprogrammed iPSC colonies</li> <li>Before passaging iPSCs remove differentiated/unwanted areas using a stretched glass Pasteur pipette or 200 µl pipette tip</li> </ul>
9.6,10.4 & 11.4	Impaired potential to differentiate into cells three germ layers	<ul style="list-style-type: none"> <li>Poor quality iPSCs</li> <li>Suboptimal cell density/confluency</li> </ul>	<ul style="list-style-type: none"> <li>Make sure the starting iPSCs are of good quality; remove any spontaneously differentiated areas</li> <li>Make sure iPSCs are about 50% confluent before starting the differentiation</li> </ul>

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