Chapter 12

An Improved Method for Generating and Identifying Human Induced Pluripotent Stem Cells

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Abstract

This chapter describes the methods we use to derive and characterize human induced pluripotent stem (iPS) cells. We describe in order, first our culture techniques for the starting fibroblast populations and methods for retrovirus preparation and concentration. Subsequently, a detailed iPS derivation protocol suitable for human fibroblast populations is discussed using standard retroviral vectors expressing the classic four or three reprogramming genes. Finally, we elaborate a robust technique for monitoring and identification of potential iPS cells through live staining of reprogrammed cells. We also outline steps for characterization of the resulting iPS cell lines.

Key words: Embryonic stem cells, Induced pluripotent stem cells, Retroviruses, Genetic modification, Transformation, Self-renewal, Pluripotency, Embryoid bodies, Teratoma

1. Introduction

Human embryonic stem (hES) cells have the ability to self renew and differentiate into cell types of all germ layers and thus have the potential to serve as an unlimited source for cell-replacement therapy (1, 2). Recent advances in generating induced pluripotent stem (iPS) cells which circumvent the ethical and source issues associated with the derivation of hES cells by directly converting an easily accessible somatic tissue cell to a pluripotent state, have made the dream of making patient-specific pluripotent cell lines and eventually transplantable tissues for therapy a distinct possibility (3–8).

In this chapter, we describe our protocol for the generation of iPS cells from human fibroblasts of adult or fetal origin. A basic proficiency of hES cell culture and genetic modification techniques

is assumed: details of those protocols may be found elsewhere (9, 10). We expect a period of about 6 months to derive and completely characterize a new iPS cell line and this protocol walks the user through the various steps involved therein. We highlight in particular the critical junctures and also areas where user cell specific changes may be desired. While the protocol below for derivation using four transgenes (encoding Oct4, Sox2, Klf4, and Myc) is very robust, derivation using only the first three factors may require further user cell type specific optimization.

2. Materials

2.1. Tissue Culture

1. Dulbecco's Modified Eagles Medium (DMEM), High Glucose.
2. Minimum Essential Medium with Earle's salts (Invitrogen).
3. KNOCKOUT ™ D-MEM: Optimized D-MEM for ES Cells (Invitrogen).
4. Fetal Bovine Serum (Hyclone defined).
5. Knockout Serum Replacement (KSR) (Invitrogen).
6. L-glutamine, (200 mM) (Invitrogen).
7. MEM Nonessential amino acids (NEAA), 10 mM (100×) (Invitrogen).
8. 1× Phosphate-buffered saline (PBS) Ca^{+2} and Mg^{+2} free (Invitrogen).
9. β-mercaptoethanol (EM Science).
10. Penicillin–Streptomycin (100×) (Invitrogen).
11. Antibiotic–Antimycotic solution (100×) (Invitrogen).
12. Basic fibroblast growth factor (bFGF) (Peprotech).
13. 0.05% Trypsin-EDTA (Invitrogen).
14. Collagenase Type IV (Sigma): used at 1 mg/ml in KNOCKOUT ™ D-MEM.
15. Stericup™ (Millipore).
16. Trypan blue 0.4% solution (Invitrogen).
17. Hemocytometer.
18. 6-well, 12-well tissue culture plates (BD).
19. 10-cm tissue culture dish (Corning).
20. Gelatin (Sigma).
21. 25-cm cell scrapers (Sarstedt).
22. Matrigel™ matrix (BD).
23. 5-ml, 15-ml and 50-ml polystyrene tubes (Sarstedt).

24. 10% buffered formalin (Fischer Scientific).

25. Mouse (IgM) anti-human antibody TRA-1-60 (Millipore).

26. Secondary Alexa Fluor 555 anti-mouse IgM (Invitrogen)

27. Polybrene (Sigma).

2.2. Retrovirus Production

1. 293 T cells (ATCC).

2. Opti-MEM I medium (Invitrogen).

3. Lipofectamine 2000 (Invitrogen).

4. Poly-D-Lysine (Sigma).

5. Reprogramming retroviral vectors: pMX-Oct4, pMX-Sox2, pMX-Klf4, and pMX-c-Myc (Addgene).

6. Helper plasmids: one expressing Vesicular stomatitis virus (VSV) G protein such as MD.G, and one expressing MLV (retroviral) gag-pol (Addgene).

7. Centricon (Plus-20, 20 ml from Millipore) with a cutoff 100,000 NMWL.

8. 15-cm tissue culture dish (Corning).

9. Whatman 0.45 micron, cellulose acetate filters (F8677).

3. Methods

3.1. Preparation of Cell Culture Media

3.1.1. Media and Feeder Cells for hES and iPS Cells

Cells are maintained in the standard hES cell culture condition, i.e., KNOCKOUT™ D-MEM, 20% KSR, 1× NEAA, 1× L-Glutamine & 1×-Antibiotic/antimycotics, 0.1 mM β-mercaptoethanol and 10 ng/ml of basic fibroblast growth factor (bFGF, purchased from Peprotech). Mouse embryonic fibroblasts (MEFs) were used as feeder cells or a source of conditioned media as previously described (9, 10).

3.1.2. Media for Human Fibroblasts

We propagate human fibroblasts in DMEM (low glucose) with Earle's Salts, 10% FBS, 1× NEAA, 1× L-Glutamine, and 1×-Antibiotic/antimycotics. Derivation and propagation of human mesenchymal stem cells (MSCs) have been described previously (10). Addition of low levels of bFGF (1 ng/ml) was used for culture of hMSCs. For propagating fibroblasts (IMR90 and hMSCs), the split ratios were never more than 1:4, with cells passaged about every 6 days (see Note 1).

3.2. Retroviral Production and Usage

We have successfully used both lentiviral and retroviral vectors for reprogramming human adult and fetal fibroblasts (6). For retroviral vectors expressing the four standard Yamanaka four factors Oct4 (O), Sox2 (S), Klf4 (K), and c-Myc (M), we used the original pMX-based vectors. These and other retroviral vectors (such

as pMIG-based) expressing the four genes are available via Addgene. Transgenes derived from mouse coding sequences are fine for human cell preprogramming, likely due to the high levels of homology between mouse and human genes for the four factors (6). In the following, we describe a detailed and optimized protocol based on the method and pMX-based vectors that Yamanaka and colleagues first described for human cells (3). Making high titer retroviruses is absolutely critical for successful reprogramming of human cells, but cotransduction of ecotropic (mouse) receptor gene is not necessary (3). Others and we instead use VSV.G pseudo-typed retroviral or lentiviral vectors for transducing efficiently both human and mouse cells (4–8). We also detail below a protocol for retroviral production, concentration, storage, and usage at appropriate concentrations. Protocols for the use of lentiviral vectors by a similar method using 293 T cells have been previously published (11).

3.2.1. Production of Recombinant Retroviruses

1. Day 0: Coat 15-cm dishes with 50 µg/ml poly-D-lysine dissolved in PBS (12 ml/dish) for a period of 1 hour. Wash twice with PBS and then dispense 8–10 million 293 T cells in standard DMEM (high glucose) + 10% FBS to a total volume of 20 ml.

2. Day 1: After 24 h the plates should be about 70–80% confluent. Proceed to make the transfection cocktail (one for each viral vector encoding Oct4, Sox2, Klf4, and Myc). Our modified formula (to reduce Lipofectamine and DNA amounts) for cells seeded in a 15-cm plate is as follows: Add 36 µl of Lipofectamine to 1.2 ml of OPTI-MEM-I in a 15-ml polystyrene tube, and incubate for 5 min at room temperature in a 5 ml polystyrene tube. In parallel, mix 24 µg of total DNA (i.e., 3 µg VSV.G, 6 µg of retro-gag/pol, and 15 µg of retroviral vector, see Note 2) into 1.2 ml of OPTI-MEM in another 5 ml polystyrene tube. After 5 min, mix the diluted DNA with diluted Lipofectamine and incubate for 20 min at room temperature. In the mean time, change to fresh media in the 293 T cultures now using DMEM (with 1%FBS) to a total volume of 20 ml. Finally, add the DNA–lipid complexes drop wise onto the 293T cultures. Gently swirl the plates and shake back and forth and sideways to mix uniformly and place dishes in the incubator. Culture overnight.

3. Day 3: After 48 hours, Harvest the supernatant from the plates in 50-ml tubes and store at 4°C. Add fresh DMEM with 1% FBS to a total volume of 20-ml to the plates for another harvest later.

4. Day 4: Harvest the second round of supernatant and collect into the original 50-ml tubes. There should now be a total of about 40 ml supernatant per each vector. Typical titers of

unconcentrated viruses obtained using this procedure are in the range of $\sim10^5$–5×10^5 transducing units/ml. To eliminate cell contamination, filter the supernatant using a 0.45-μm filter unit (low protein binding).

3.2.2. Usage of Recombinant Retroviral Vectors

1. To concentrate viruses by 50–100-folds the Centricon (Plus-20, 20 ml from Millipore) with a cutoff 100,000 NMWL is our method of choice (11). The filter filtration method also helps to reduce the free form of VSV.G proteins that are toxic to target cells. Each device concentrates ~18 ml to ~200 μl each time with a spin for 20–30 min at 3,000 rpm, and repeated loading is fine. Using this procedure starting from a volume of ~40 ml of supernatant we typically concentrate down to about 300–500 μl. Designate this final volume amount as V. A practical guide is provided below to use an appropriate amount of concentrated viruses without the need to measure the viral titers precisely.

2. We recommend the use of freshly made viruses for transduction, but if it is not possible, we make small aliquots and store them at –80°C where it is stable for many years (see Note 3).

3. Using the appropriate amount of virus is critical for successful reprogramming. We find that both too much and too little virus amount adversely affects reprogramming efficiency. Consequently our recommendation for the appropriate dosage based on the above viral production protocol is as follows: If V is the total amount of virus obtained per viron type per 15-cm plate (see step 1), then we recommend as a starting amount V/12 for each virus per 100 Kcells/10 cm² all dissolved together in a total volume of 2 ml to be transduced. The ratio of retroviral viruses per cell (per each vector) is typically ~4–5. It gives at least 70–80% transduction efficiency, as measured by a compatible GFP-expressing viral vector in both IMR90 fetal fibroblasts and adult MSCs (the virus amount for the latter may need to be increased to V/8). In case transduction efficiency is too poor we recommend increasing the ratio to V/10, V/8 or V/4 of each virus type, until a positive reprogramming result is obtained. This is one of the reasons why concentrated viruses are used.

3.3. Basic Reprogramming Procedure

The overall reprogramming procedure is highlighted in Fig. 1a. Specific details are as follows.

1. Day-1: In general, fibroblasts are seeded at a density in the range of 50–100 K/10 cm². For this protocol we will consider the specific case for 100 K cells per one well of a six-well
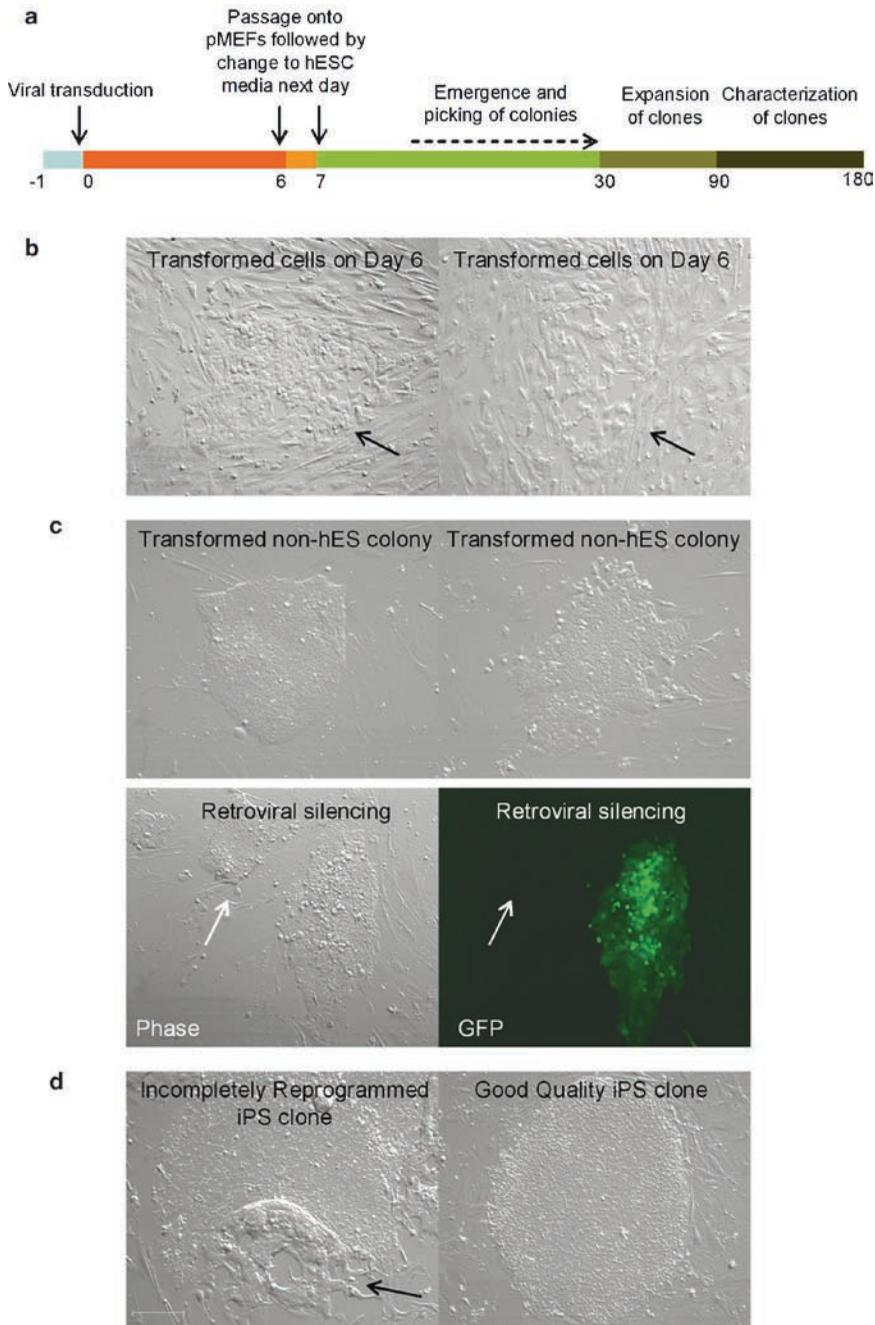
Fig. 1. (**a**) Timeline for the overall iPS cell derivation protocol is outlined. (**b**) By Day 6, "transformed" cell clusters are visible due to retroviral vector-mediated gene expression. (**c**) As transforming/reprogramming proceeds, a myriad of colonies are observed that are visible starting around day 9-12. Two examples of transformed but non-hES-like colony morphologies is highlighted. If the retroviruses also co-express GFP then Silencing of retroviral-mediated transgene expression, assayed by a loss of GFP expression, is a good indication of "correct transformation" or reprogramming. (**d**) Not all hES-like colonies that are picked (based on morphology) grow and expand equally well. Among those that proliferate, one cannot assume that they are clonal either genetically or epigenetically. Some clones self renew normally after picking, resembling hES cell colonies (*right*). However, others may occasionally bud-off transformed cells (*left*), likely due to either incomplete reprogramming or contaminated cells near the hES-like colony originally picked. These latter clones usually do not show good growth and differentiation ability in the longer term.

plate. It is important that cells must be dispensed evenly across the well (see Note 4).

2. Day 0: Cells are transduced using a combination of the retroviruses as per the concentrations recommended in the retroviral usage section. The retroviral amount for the four-factor (OSKM) and three-factor (OSK) conditions is the same. Pre-mix the viruses with the standard fibroblast media and 6 μg/ml polybrene to a total of 2 ml per well before dispensing.

3. Day 1: Supplement the wells with an additional 1 ml of fibroblast media. Note that the concentration of polybrene now is reduced to 4 μg/ml and the total media per well is 3 ml.

4. Day 2: Aspirate the retrovirus containing media and add fresh 3 ml fibroblast media to the well.

5. Day 4: Aspirate the old media off the well and add fresh 3 ml media as specified on day 2. The cells should be proliferating well by now, and will begin to get confluent. We find that increased cell–cell contact and continued proliferation during these initial days is favorable for the reprogramming process.

6. Day 5: Plate irradiated MEF feeder cells into gelatin-coated dishes. For the above specific case, we recommend one six-well plate, and two 10-cm dishes per one well of reprogramming cells. The former plate serves as a monitoring dish useful for day-to-day observations and analysis of efficiency and other assays during the course of reprogramming. The larger 10-cm dishes are a convenient format for the purpose of colony picking.

7. Day 6: By now the reprogramming well will be confluent with cells, and occasional clusters of transformed cells will be visible (Fig. 1b). Several regions of the well will show cells growing rapidly without any contact inhibition (see Note 5). On this day the cells are passaged onto MEFs and the procedure is outlined next.

First, prepare the plates preseeded with MEF feeder cells as follows. Change the media in the MEFs plates or dishes (after inspection) with fresh fibroblast media at 2 ml per well of six-well plates (or 12 ml per 10-cm dish).

Next, aspirate the media off the reprogramming well, and wash once with PBS and add 1 ml trypsin to the well. In about 5–10 min, the cells will detach and breakdown into small clumps. Add 2 ml of fresh fibroblast media to the well to neutralize the trypsin. Pipet up and down to mix the cells and breakdown any remaining clumps.

When using the OSKM condition proceed as follows: dilute the cell suspension (3 ml) by transferring them into a new tube containing an additional 7 ml of media to bring the total to 10 ml. Dispense 3 ml of the total volume of cells into each

10-cm dish, and 0.5 ml/well of a six-well plate with feeder cells (*see below*). Note that the split ratio used here is effectively 1:20 (see Note 6). Typically this corresponds to 10–25 K/10 cm² density of cell plating.

When using the OSK condition proceed as follows: the 3 ml of harvested cells will be transferred into a new tube. Single cell suspension will be dispensed 0.5 ml/well of six-well plates with feeder cells. Note that the split ratio used here is effectively 1:6 (see Note 6).

Finally, disperse the cells evenly and place into the incubator for overnight attachment of the cells.

8. Day 7: Aspirate off the fibroblast media and directly change it to hES cell culture media. Use 3 ml/well for one well of a six-well plate and 18 ml for the 10-cm dishes.

9. Day 9: Aspirate off the old media and add fresh hES cell culture media at the amounts mentioned on Day 7. Carefully scan the plates to check for transformation characterized by growth of cell clumps. Although colonies of "transformed" cells may emerge at day 9 and onward (Fig. 1c, see Note 7), however most of these epithelial colonies are typically not correctly reprogrammed cells. The candidate iPS cells emerge a little later and resemble hES cells in both morphology and expression of pluripotency markers such as TRA-1-60. (Fig. 2b).

10. Days 11 and 13: Repeat the feeding and cell-monitoring procedure from day 9.

11. Day 14: Recommended but optional: Collect the reprogramming cells from one well of a six-well plate to do FACS analysis for TRA-1-60 expression after trypsin digestion. We recommend repeating this procedure again on Days 18, 21, 24, and 27. Detection of a positive population at these time points and an increase in their percentages over time reflects that reprogrammed cells have emerged (refer Fig. 2a).

12. Day 15: As the MEF feeder cells are now more than a week old, from this day on MEF-conditioned media is used for feeding the reprogramming plates. The feeding and cell monitoring procedure remains the same as on Day 9. It is normal to observe both an increase in size of some transformed cell clusters as well as a loss of cells by apoptosis in others. A lot of the transformed cells are semiadherent and can get dislodged and reattach in different parts of a reprogramming plate. Consequently, especially when running multiple reprogramming experiments it is highly recommended that aspirating tips and feeding pipettes should be changed between different experimental conditions.

13. Days 17, 19: Repeat the feeding and cell-monitoring procedure from Day 15. Closely monitor, in particular, if the media color changes to even a pale yellow during these days. If so, from then on, it is important to switch to daily media changes
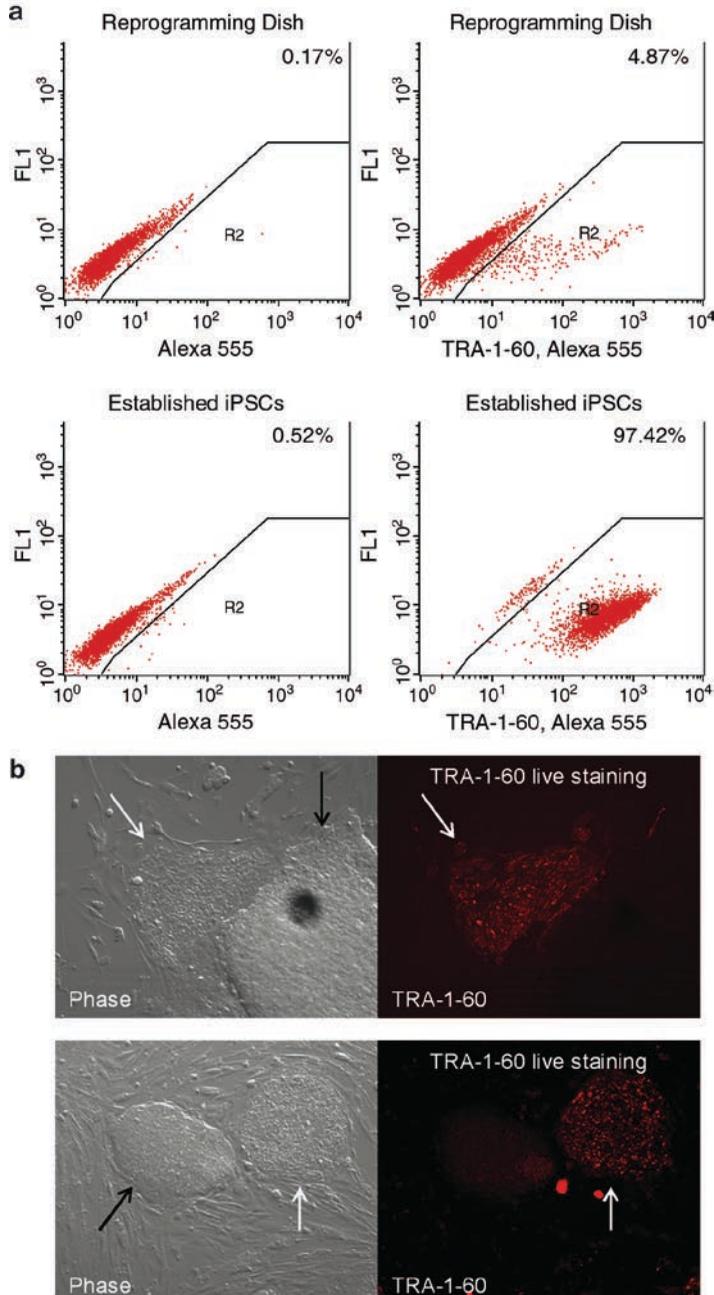
Fig. 2. (**a**) TRA-1-60 expression analysis by FACS is a useful method for measuring the kinetics of reprogramming. The *upper* panel gives the pattern of TRA-1-60 expression in cells from the whole reprogramming dish as compared to a fully reprogrammed iPS clone (*lower panel*). (**b**) Further, TRA-1-60 live staining is a very efficient way for distinguishing hES-like colonies (*white arrow*) from transformed cell clusters (*black arrows*). Two representative examples are provided.

instead of the every alternate day procedure. When changing media daily only 2 ml per well of a six-well plate or 12 ml per 10-cm dish may be used. If, however, one observes that even with this daily feeding the media gets acidic too soon, we recommend increasing the media amounts progressively in steps

of 1 ml per well of a six-well plate (i.e., 2 ml to 3 ml to 4 ml and so on), and in steps of 6 ml for the 10-cm dish (i.e., 12 ml to 18 ml to 24 ml and so on). Usually a rapid change in media color is a reflection of high confluence of the reprogramming wells and is not a desired phenomenon (see Note 8).

14. Day 21 onward: Repeat the feeding and cell-monitoring procedure from above.

3.4. Human iPS Colony Identification and Picking

Note that in general by Day 21 for most starting fibroblast populations both a large number and varying morphology of colonies are visible in different regions of the plate. This is a reflection of a remarkable mesenchymal to epithelial transition that occurs during the reprogramming process from fibroblasts. However, not all epithelial-like colonies are hES like – which are characterized by a flatter cobblestone like morphology with individual cells clearly demarcated from each other in the colonies. Moreover, a lot of the non-hES-like colonies form closely resembling but not identical compact clusters of cells and are capable of sustained self-renewal and successive passaging for long periods under hES cell culture conditions. This makes identification of the successfully reprogrammed colonies a very critical step in this process. In our hand the most reliable method for selecting a reprogrammed colony is live staining by the TRA-1-60 antibody that also recognizes undifferentiated hES cells.

1. The primary and secondary antibodies are both used at a 1:200 dilution (see Note 9) and are premixed together into hES cell media. After aspirating the existing media off the reprogramming dish we add this antibodies containing media at 1 ml per one well of a six-well plate or 6 ml per 10-cm dish. This amount is sufficient to safely cover the surface of the dishes without the need for a shaker. The plates are then directly placed into the tissue culture incubator for about 1 h ($37°C$, 5% CO_2).

2. Following this period the media is aspirated, washed once with PBS and finally fresh hES cell medium is added. The plates are left in the incubator for 15 min and then imaged under a standard fluorescence microscope. Successful antibody staining can very specifically delineate reprogrammed colonies from just plain transformed counterparts (refer Fig. 2b), and can be detected for up to 24–36 h. This aspect is particularly useful since it helps in identification and tracking of the candidate iPS colonies both before picking and also the day after they are picked and transferred into a new well.

3. For the purpose of picking we follow this procedure: First, we fabricate our colony-picking tool, which is made by drawing the thin end of a glass Pasteur pipette into a J-shaped fine curve (about 10–50 microns in thickness). For this the pipette is held

over a Bunsen flame, and when the thin end starts to melt, the glass is slowly pulled apart, and just before it melts off completely, it is in a (critical step) quick motion pulled apart and away from the flame. This usually leaves a very thin curved end on one or both of the two separated parts of the pipettes. Second, the TRA-1-60 positive stained colonies are identified under the fluorescence microscope at a 10× magnification. We prefer to select colonies at least 100–500 microns in diameter. These can usually be discerned by the naked eye when the plate is held up to light. Once a positive colony is identified, it is brought to the center of the viewing field and the magnification is switched down to 4×. Third, the dish cover is removed and the picking tool is immersed in the media. Looking through the microscope at this 4× resolution it is easy to view the approach trajectory of tool as it is brought next to the colony, and then it is used to gently scrape the colony off the surface until it is completely detached and floating in solution. At the same 4× magnification, a 10-µl micropipette tip is brought next to the colony and which is then sucked up in a volume no more than 5 µl to avoid carry over of additional floating/dislodged cells in the dish. Fourth, this drawn volume can be either dispensed directly into a single well of a 96-well MEF feeder plate, or (preferred alternative) it is dispensed into 50 µl of hES media in an eppendorf tube, following which a 200-µl pipette tip is used to break the picked colony into 3–5 smaller clumps by a few gentle pipetting motions and then dispensed in a single well of a 96-well plate.

4. The colonies thus picked are allowed to attach for 48 h before media is changed, and subsequently these are treated like normal hES colonies and passaged, expanded, and maintained using standard culture procedures. We recommend picking at least 10 distinct colonies by the end of each reprogramming experiment.

3.5. Initial iPS Clone Expansion and Characterization

Until proven that they are truly pluripotent, these TRA-1-60+ colonies we picked are referred to as hES-like colonies or potential iPS colonies. These potential iPS cell colonies in their early passages must be constantly monitored. Some of the colonies will be more fragile and prone to rapid apoptosis and/or differentiation, while some others may show more robust growth, and while yet some others may occasionally bud-off transformed non-hES-like cells (refer Fig. 1d). All these phenotypes are normal and we observe them on a regular basis, and may characterize, respectively, transient, stable, or unstable reprogramming states of the individual clones. Usually from starting ten colonies we are able to derive four to five stable hES-like colonies that display normal growth patterns and remain a pure population during subsequent expansion. These are the clones we focus on for subsequent characterization.

Typically expansion of a clone from a single colony stage to a confluent one-well of a six-well plate takes about 5–7 weeks. We strongly recommend only 1:1.5 to 1:2 passaging of clones during this initial critical and slow period of expansion, and we often use the 50:50 ratio of plain hES cell medium and MEF-conditioned medium for feeding the cells. Cell passaging is either by mechanical means or by the use of collagenase (but not trypsin), and especially when working with very small wells and colonies, we strongly recommend the use of 1,000-μl pipette tip (and not smaller) for gentle scraping/breaking of colonies during cell splitting.

To facilitate subsequent rapid characterization of the cells, we typically expand the iPS colonies uninterrupted (with only occasional freezing of cells) while using a faction of cells for the following assays (in the order of preference). We estimate that we need cells from a total of 24 wells (in six-well plates) for the assays or tasks listed below.

1. Pluripotency markers (iPS cells from two wells can be replated into smaller well with MEF feeder cells): We fix cells after iPS cells reach an optimal size. Cells can be stained for surface markers such as TRA-1-60 (and/or TRA-1-81) and SSEA4 (and/or SSEA3) or nuclear antigens such as NANOG (human and endogenous) and OCT4. Alkaline phosphatase staining can be done by standard histochemistry.

2. Karyotype analysis (two wells): cells are harvested after appropriate treatments required for karyotyping analysis.

3. DNA and RNA extraction (two wells): Gene expression analysis and fingerprinting is carried out using RNA or DNA derived in this step.

4. Embryoid body (EB) formation (three wells): Cells are collected by collagenase passaging and resuspended in FBS-containing media (standard procedures) and allowed to form EBs for up to 2 weeks. Formation of cystic structures is typically observed within 6–10 days varying from clone to clone. After 2 weeks, the EBs are broken down into smaller clumps using a 200 μl pipet tip and allowed to attach onto gelatin-coated plates for an additional 2 days followed by fixing and staining for the three embryonic germ layers and trophectoderm.

5. Teratoma formation (12–15 wells): Cells are collected from the plates into a 50-ml tube by directly scraping them in their native media using a cell-scraper. After spinning down, the cell pellet is resuspended on ice (critical step) in 400 μl of a 1:1 mixture of matrigel and knockout DMEM and collected in an eppendorf tube and stored on ice. This volume of suspension is suitable for intramuscular injection into the hind limb of two immunodeficient mice (200 μl each). We

prefer to use immunodeficient mice with further reduced NK activities, such as SCID/Beige or RAG$^{-/-}$IL2RG(γ_c)$^{-/-}$ mice. Palpable tumors can be detected as early as 6 weeks postinjection with the improved method, but up to 4 months is also normal.

Typically, full characterization of an iPS clone takes between 5 and 6 months. There is clone to clone variation in differentiation ability and expansion/survival potential, so identification of a good quality normal karyotype clone is a tedious but essential aspect of reprogramming (see Note 10).

4. Notes

1. Poorly growing primary fibroblast cultures can be improved by addition of bFGF and relatively confluent passaging (1:1.5 to 1:2).

2. The overall protocol for production of retroviral vectors by transfection of 293 T cells is similar to that for lentiviral vectors (11). However, the ratio of the gag-pol plasmid and a transducing vector plasmid is different between the two systems. More retroviral vector plasmid should be used (2–2.5-fold) as compared to the MLV (retro-) gag-pol helper plasmid. The total amount of DNA (three plasmids) is kept the same (24 µg).

3. For a short-term storage (up to a week), it is better to store retroviral virions at 4°C. Note that the virions do not survive well after repeated freez and thaw.

4. To ensure cells are uniformly dispersed, especially for smaller wells (i.e., in 12-well plates or smaller), it is recommended that one pre-mix the cells into the total volume of media (to be used per well) before dispensing.

5. Early signs of transformed growth of cells on day 6 are typically a very good sign that the viral transduction procedure is working well.

6. This split ratio can be further increased to up to 1:40 for cells that grow well in hES cell media – for example, in our experience, hMSCs can be passaged at this increased dilution. Again, it is important to assess the growth rate and viability of a user's cell type before making these adjustments.

7. A large number and type of "transformed" (epithelial-like) colonies are typically visible in a reprogramming plate. In the examples provided here (Fig. 1c), the colonies do not have the characteristic hES cell morphology and serve to help the user identify and familiarize oneself with partially or incorrectly reprogrammed cells.

8. Over confluence of cells during the reprogramming proce-
dure typically inhibits both emergence and expansion of the
true iPS colonies, and also makes identification and clean
picking of colonies difficult. In addition, the cells risk peeling
off from the surface over time.

9. A similar approach has been previously described with TRA-
1-81 antibody (7). We exclusively used TRA-1-60 antibody
that enables the identification of reprogrammed cells from a
variety of human cell types. The TRA-1-60 live staining has
little adverse effects on the growth of TRA-1-60+ or negative
colonies even after multiple rounds of staining. To conserve
antibody usage further (1:300 to 1:400) dilutions of the
TRA-1-60 antibody may be used too; however, the stained
colonies will appear dimmer but still discernible under the
microscope.

10. When troubleshooting for failure to obtain *bona fide* iPS cells
using the above procedure we recommend that the user
checks the following steps (in order of importance): first, the
titer of the unconcentrated retroviral supernatant (at least
~10^5 transducing units); second, transduction efficiency of
the target cell type (at least 60–80%); third, poor quality of
the pMEFs used during reprogramming (can affect repro-
gramming efficiency by tenfold or even more); and fourth,
refractory or senescent nature or late passage of the target cell
type (use earlier passage of the cells).

Acknowledgments

References

1. Thomson JA, Itskovitz-Eldor J, Shapiro SS,
Waknitz MA, Swiergiel JJ, Marshall VS,
Jones JM. (1998) Embryonic stem cell lines
derived from human blastocysts. *Science*
282(5391):1145-1147.

2. Reubinoff BE, Pera MF, Fong C, Trounson A,
Bongso A. (2000) Embryonic stem lines from
human blastocysts: somatic differentiation
in vitro. *Nat Biotechnol* 18:399-404.

3. Takahashi K, Tanabe K, Ohnuki M, Narita M,
Ichisaka T, Tomoda K, Yamanaka S. (2007)

Induction of pluripotent stem cells from adult
human fibroblasts by defined factors. *Cell*
131: 861-872.

4. Yu J, Vodyanik MA, Smuga-Otto K,
Antosiewicz-Bourget J, Frane JL, Tian S, Nie
J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin
II, Thomson JA. (2007) Induced pluripotent
stem cell lines derived from human somatic
cells. *Science* 318:1917-1920.

5. Park IH, Zhao R, West JA, Yabuuchi A, Huo
H, Ince TA, Lerou PH, Lensch MW, Daley

GQ. (2008) Reprogramming of human somatic cells to pluripotency with defined factors. *Nature* 451:141-146.

6. Mali P, Ye Z, Hommond HH, Yu X, Lin J, Chen G, Zou J, Cheng L. (2008) Improved efficiency and pace of generating induced pluripotent stem cells from human adult and fetal fibroblasts. *Stem Cells* 26:1998-2005.

7. Lowry WE, Richter L, Yachechko R, Pyle AD, Tchieu J, Sridharan R, Clark AT, Plath K. (2008) Generation of human induced pluripotent stem cells from dermal fibroblasts. *Proc Natl Acad Sci U S A* 105:2883-2888.

8. Park IH, Arora N, Huo H, Maherali N, Ahfeldt T, Shimamura A, Lensch MW, Cowan C, Hochedlinger K, Daley GQ. (2008) Disease-specific induced pluripotent stem cells. *Cell* 134:877-886.

9. Dravid G, Hammond H, Cheng L. (2006) Culture of human embryonic stem cells on human and mouse feeder cells. *Methods Mol Biol (Clifton, N.J.)* 331:91-104.

10. Cheng L, Hammond H, Ye Z, Zhan X, Dravid G. (2003). Human adult marrow cells support prolonged expansion of human embryonic stem cells in culture. *Stem Cells* 21:131-142.

11. Ye Z, Yu X, Cheng L. (2008) Lentiviral gene transduction of mouse and human stem cells. *Methods Mol Biol (Clifton, N.J.)* 430:243-53.