Chapter 15

Evaluating Reprogramming Efficiency and Pluripotency of the Established Human iPSCS by Pluripotency Markers

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Abstract

The pluripotency of human induced pluripotent stem cells (HiPSCs) cannot be tested strictly in a similar way as we can do for the mouse ones because of ethical restrictions. One common and initial approach to prove the pluripotency of an established human iPSC line is to demonstrate expression of a set of established surface and intracellular pluripotency markers. This chapter provides procedures of immunocytochemistry of the established HiPSC lines for a set of the signature intracellular pluripotency proteins, OCT4, SOX2, NANOG, and LIN28. We also describe cell phenotyping by flow cytometry for the five established human pluripotency surface markers, SSEA3, SSEA4, TRA-1-60, TRA-1-81, and TRA2-49 (ALP). Numbers of ALP^+ and TRA-1-60^+ colonies are the most widely used parameters for evaluation of human iPSC reprogramming efficiency. Therefore, this chapter also provides detailed steps for substrate colorimetric reaction of the ALP activity, as well as the TRA-1-60 staining, of the iPSC colonies in the reprogramming population.

Key words Immunophenotyping, Human induced pluripotent stem cells, Pluripotency, Human iPSCs, Pluripotency surface markers, Pluripotency factors, iPSC reprogramming

1 Introduction

Both mouse and human somatic cells such as fibroblasts and blood cells can be converted into pluripotent stem cells (PSCs) by ectopic expression of reprogramming factors [1–6]. Whether the induced PSCs (iPSCs) are really pluripotent or not have to be tested. The pluripotency of iPSCs can be tested in various facets [7–10]. These include: (1) expression of pluripotency surface markers [9]; (2) expression of pluripotency transcription factors; (3) expression of other genes uniquely expressed in pluripotent stem cells; (4) genome-wide profiling of the pluripotent transcriptome [7]; (5) functional test by teratoma formation [7–9]; (6) functional test by formation of the embryoid bodies; (7) functional test by directed differentiations into various lineages [9]; and (8) test of developmental potentials by injection of iPSCs into early embryos

Kejin Hu (ed.), *Nuclear Reprogramming: Methods and Protocols*, Methods in Molecular Biology, vol. 2239,
https://doi.org/10.1007/978-1-0716-1084-8_15, © Springer Science+Business Media, LLC, part of Springer Nature 2021

and subsequent development in a foster mother. The last characterization is the most stringent test of pluripotency, but cannot be done for human iPSCs as for mouse ones because of ethical restrictions.

The pluripotency of the generated human iPSCs can be tested by all of the first seven approaches though. Among those characterizations, the initial and the easiest ones are expression of the pluripotency surface markers, the reigning transcriptional factors of pluripotency, and other signature pluripotency genes. Mouse iPSCs express the pluripotency surface marker SSEA1 and alkaline phosphatase (ALP). Human iPSCs, however, express five defining pluripotency surface markers SSEA3, SSEA4, TRA-1-60, TRA-1-81, and ALP, but not SSEA1 [10]. Like mouse iPSCs, human iPSCs are defined by three characteristic transcription factors, OCT4, SOX2 and NANOG. LIN28, an RNA-binding protein, is also an established pluripotency signature molecule.

In this chapter, we first describe procedures to examine the expression of the signature intracellular proteins of pluripotency, OCT4, SOX2, NANOG, and LIN28, by immunocytochemistry of the established human iPSC lines (Subheading 3.1). We then provide a protocol for immunophenotyping of human iPSCs by flow cytometry using the five established surface markers of human pluripotency, SSEA3, SSEA4, TRA-1-60, TRA-1-81, and TRA-2-49 (ALP) (Subheading 3.2). The reprogramming efficiency is commonly evaluated by the numbers of colonies positive for ALP and/or TRA-1-60 in the reprogramming vessels. Therefore, we provide additional protocols for substrate-based staining for ALP activity (Subheading 3.3), and TRA-1-60 staining of iPSCs (Subheading 3.4) in reprogramming experiments described in other chapters of this volume.

2 Materials

2.1 Reagents for iPSC Culture

1. hESC-qualified Matrigel.
2. Essential 8 (E8) Medium for human PSCs, pH 7.4, 340 mOsm [11, 12]: 1× DMEM/F-12, 1.74 g/L $NaHCO_3$, 13.6 μg/L sodium selenium, 64 mg/L L-ascorbic acid 2-phosphate sesquimagnesium, 10 μg/mL transferrin, 20 μg/mL insulin, 4 ng/mL basic FGF (bFGF, also known as FGF2), 2 μg/L TGFβ1 (*see* **Note 1**).
3. iPSC EDTA dissociation buffer: 0.5 μM ethylenediaminetetraacetic acid (EDTA), 0.18% NaCl in PBS (without calcium, without magnesium, pH 7.4). Add 1 mL of 0.5 M EDTA (pH 8.0), and 1.8 g of NaCl into 1 L of PBS (calcium-/magnesium-free, pH 7.4). Sterilize the buffer by autoclaving or by filtration with a 0.22-μM filter. Store at room temperature or at 4 °C.

2.2 Antibodies

1. Human OCT4 antibody.
2. Human SOX2 antibody.
3. Human NANOG antibody.
4. Human LIN28 antibody.
5. Goat-anti-mouse IgG1, Alexa Fluor 568 conjugated.
6. Donkey-anti-rabbit IgG, Alexa Fluor 568 conjugated.
7. PE-TRA-1-60.
8. PE-TRA-1-81.
9. PE-SSEA4.
10. PE-SSEA3.
11. PE-SSEA1.
12. PE-TRA-2-49 (ALP antibody).
13. PE-mouse IgM isotype control antibody.
14. PE-rat IgM isotype control antibody.
15. PE-mouse IgG isotype control antibody.

2.3 Equipment and Software

1. Flow cytometer.
2. Fluorescent microscope with a camera.
3. CO_2 incubator.
4. Photo scanner (Epson perfection v700 or of your choice).
5. Test-tube cooler.
6. FlowJo.

2.4 Materials for Immunohistochemistry

1. Plastic chamber slides, 8-well (*see* **Note 2**).
2. PBS (without calcium and magnesium).
3. Fixation solution: 4% paraformaldehyde in PBS (pH 7.4). Dissolve 4 g of paraformaldehyde in 96 mL of PBS (pH 7.4). Put the solution inside a beaker water bath and boil the water inside a fume hood by a stirrer hot plate with gentle agitation until the solution becomes clear. Bring the volume to 100 mL with PBS. Filter the solution to remove any undissolved particles with a 0.45 μm filter. Aliquot the solution into 15-mL tubes at 5 mL each and freeze the aliquots at −20 °C.
4. Blocking buffer: 1% bovine serum albumin (BSA) in PBS with 0.3% Triton X-100. Dissolve 1 g BSA in 100 mL PBS. Add Triton X-100 at the final concentration of 0.3%, and mix well. Aliquot and store the buffers at −20 °C.
5. ProLong™ antifade mount with DAPI.

2.5 Materials for Flow Cytometry

1. Accutase.
2. 5-mL FACS tubes without caps.
3. 5-mL FACS tubes with cell strainer caps (mesh size 35 μm).
4. Cell strainers (mesh size, 70 or 100 μm).
5. FACS buffer: PBS (pH 7.4) supplemented with 2% fetal bovine serum (FBS), 1 mM EDTA, and 0.1% sodium azide.
6. 7-AAD solution: 1 mg/mL 7-Aminoactinomycin D (7-AAD). Dissolve 1 mg of 7-AAD powder in 50 μL methanol, and then add 950 μL PBS into the 7-AAD-methanol solution to achieve 1 mg/mL 7-AAD. Mix well, and then store at 4 °C. Protected from light.

2.6 Reagents for Substrate Colorimetric Staining of ALP Activity

1. ALP reaction buffer, pH 9.5: 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, and 5 mM $MgCl_2$. Dissolve 12.114 g Tris-HCl, 5.844 g NaCl, and 1.0165 g $MgCl_2$ in 800 mL dH_2O. Adjust pH to 9.5 and bring the volume to 1000 mL.
2. 5-Bromo-4-chloro-3-indolyl-phosphate (BCIP) stock (50 mg/mL): Dissolve 0.5 g of BCIP in 10 mL of 100% DMF. Aliquot at 1 mL per tube into 1.5 mL microcentrifuge tubes. Wrap the tube with aluminum foil to protect the BCIP from light. This stock solution is stable for 1 year at 4 °C.
3. 4-Nitro blue tetrazolium chloride (NBT) stock (50 mg/mL): First, prepare 70% *N*,*N*-dimethylformamide (DMF) as follows: add dH_2O into 70 mL of 100% DMF and bring the volume to 100 mL. Then, prepare NBT stock as follows: Dissolve 0.5 g of NBT in 10 mL 70% DMF. Aliquot at 1 mL per tube into 1.5 mL microcentrifuge tubes. Wrap the tubes with aluminum foil to protect the BCIP solution from light. This stock solution is stable for 1 year at 4 °C.
4. BCIP/NBT working solution: Add 30 μL of BCIP (0.15 mg/mL) and 60 μL of NBT (0.30 mg/mL) into 10 mL of ALP reaction buffer. The working solution should be prepared and used freshly.
5. 100% methanol, pre-cooled at –20 °C.
6. Preservation buffer for the stained iPSC colonies: PBS (calcium-free and magnesium-free) supplemented with 0.05% sodium azide.

2.7 Reagents for TRA-1-60 Staining of iPSC Colonies

1. Biotin-conjugated human TRA-1-60 antibody.
2. Streptavidin-Horseradish Peroxidase (HRP).
3. 3,3′-Diaminobenzidine (DAB) peroxidase (HRP) substrate kit (vector Laboratories, SK-4100).
4. Phosphate-buffered saline (PBS) without calcium and magnesium, pH 7.4.

5. Staining buffer: 3% fetal bovine serum (FBS), 0.3% Triton X-100 in PBS without calcium and magnesium.
6. Blocking solution: 1% bovine serum albumin (BSA) in PBS.

3 Methods

3.1 Test Pluripotency by Immunocytochemistry for the Intracellular Pluripotency Proteins

3.1.1 Establishing Human iPSCs on Chamber Slides from the Conventional Culture Vessels for Immunocytochemistry

1. Thaw an aliquot of Matrigel inside a test-tube cooler inside a 4 °C refrigerator overnight (*see* **Note 3**).
2. The next day dilute the Matrigel on ice with ice-cold DMEM/F-12 to a final concentration of 75–150 μg/mL. Coat one well of a 6-well plate with 1.5 mL of diluted Matrigel at 37 °C for at least 1 h.
3. Take one vial of human iPSCs from the liquid N_2 storage tank (*see* **Note 4**) and thaw quickly in a 37 °C water bath. Transfer the iPSCs into a 15-mL sterile tube containing 10 mL of E8 medium pre-warmed to room temperature. Centrifuge at 400 × *g* for 5 min.
4. Remove the wash medium and resuspend the iPSCs in 2 mL of the E8 medium pre-warmed to room temperature supplemented with the ROCK inhibitor Y-27632 at a final concentration of 10 μM.
5. Remove the Matrigel from the well and seed the iPSCs into the coated well of a 6-well plate.
6. The next day remove the ROCK inhibitor by changing media.
7. Grow iPSCs with daily medium change.
8. When the neighboring iPSC colonies are about to merge, treat the cells with 1.5 mL of iPSC dissociation buffer at 37 °C for 5 min (*see* **Note 5**). Add 2 mL of the E8 medium and wash to detach the cells with a 1-mL pipette tip. Transfer the cells into a sterile 15-mL tube containing 10 mL of the E8 medium pre-warmed to 37 °C. Centrifuge at 400 × *g* for 5 min.
9. Remove the dissociation buffer/media from the cells and seed the cells in the E8 medium supplemented with ROCK inhibitors (10 μM) into a Matrigel coated 6-well plate (or 12-well plates, or 4-well plates).
10. The next day remove ROCK inhibitors by changing media.
11. Grow human iPSCs with daily medium change to 80% confluence in a 6-well plate (*see* **Note 6**).
12. Coat all the 8 wells of a chamber slide (*see* **Note 2**) with 200 μL per well of Matrigel pre-diluted 100× in cold DMEM/F-12 for at least 1 h at 37 °C (*see* **Note 7**).
13. Treat the cells with iPSC EDTA dissociation solution at 37 °C for 5 min.

14. Add 2 mL of E8 medium into the cells undergoing EDTA dissociation.
15. Use pipette tip to gently wash cells so as to detach the colonies and then transfer the cell suspension into a sterile 15-mL conical tube containing 10 mL of the E8 medium pre-warmed to 37 °C.
16. Centrifuge the cells at 300 × *g* for 5 min.
17. Resuspend the cell pellet with 1 mL of the E8 medium.
18. Remove Matrigel from the wells of the slide chambers and add 300 μL of the pre-warmed E8 medium into each well.
19. Mix the cell suspension prepared at **step 17** above. Immediately after mixing, take 30 μL of resuspended iPSCs and put the iPSCs into each well of the chamber slide. Mix gently (*see* **Note 8**).
20. Grow the cells at 37 °C, 5% CO_2.
21. The next day change the medium and make sure the cells attach to the slides as small colonies.
22. Continue to culture the iPSCs with daily change of media until the culture reaches around 80% of confluence and proceed with immunostaining as described below in Subheading 3.1.2 (*see* **Note 9**).

3.1.2 Examine Expression of the Intracellular Pluripotent Markers by Immunostaining of iPSCs Grown on Slides

1. Warm up the E8 medium, and an aliquot of fixation buffer to 37 °C (*see* **Note 10**), and the blocking buffer to room temperature.
2. Rinse the cells one time with 300 μL of the pre-warmed E8 medium.
3. Fix the cells with 200 μL of the pre-warmed fixation buffer for 15 min at room temperature.
4. Aspirate the fixation solution and rinse three times with 300 μL of room temperature PBS each time.
5. Add 200 μL of blocking buffer and incubate for 1 h at room temperature.
6. Dilute the primary antibodies in the blocking buffer according to Table 1 or based on pre-testing results if you are using antibodies from a different vendor.
7. Aspirate the blocking buffer and add 200 μL of the diluted primary antibodies.
8. Incubate overnight at 4 °C.
9. The next day aspirate the antibody solution and rinse the cells with room temperature PBS (*see* **Note 11**).
10. Wash three times with room temperature PBS for 5 min each.

Table 1
Antibodies for characterization of human iPSCs

Antibody	Brand	Cat. #	Dilution (1:X)
Anti-OCT4	Cell Signaling	2840S	200
Anti-SOX2	BD Pharmingen	561469	200
Anti-NANOG	BD Pharmingen	560109	200
Anti-LIN28	Millipore	MABD53	200
Alexa568-Goat-Anti-Mouse IgG1	Life Technologies	A11004	1000
Alexa568-Donkey-Anti-Rabbit IgG	Life Technologies	A10042	1000
PE-Anti-TRA1-60 antibody	BD Pharmingen	560193	5
PE-Anti-TRA1-81 antibody	BD Pharmingen	560161	5
PE-Anti-SSEA4 antibody	BD Pharmingen	560128	5
PE-Anti-SSEA3 antibody	BD Pharmingen	560237	5
PE-Anti-SSEA1 antibody	BD Pharmingen	560142	5
PE-Anti-ALP (TRA2-49) antibody	R&D Systems	FAB1448P	10
PE-Anti Rat IgM isotype control antibody	BD Pharmingen	553943	10

11. Dilute the secondary antibody in the blocking buffer at a 1:1000 dilution.
12. Incubate the cells in the secondary antibody solution for 1 h at room temperature, protected from light.
13. Aspirate the second antibody and rinse with PBS.
14. Wash three times with PBS at room temperature for 5 min each.
15. Remove the chambers from the slide carefully and add one drop of ProLong antifade mount containing DAPI.
16. Incubate 10 min at room temperature, protected from light.
17. Cover the sample with a coverslip.
18. Cure the sample overnight at room temperature.
19. Examine expression of the pluripotency proteins using an epifluorescence or confocal microscope. Take images (*see* Fig. 1).

3.2 Detection of Pluripotency Surface Markers by Flow Cytometry

1. Culture each cell line of iPSCs with the E8 feeder-free system in two T25 cell culture flasks until the cells reach 80% confluence in a similar way as described in Subheading 3.1.
2. When iPSCs reach 80% confluence, aspirate the medium and add 2 mL of Accutase pre-warmed to room temperature into each flask. Incubate at 37 °C for 5 min (*see* **Note 12**).

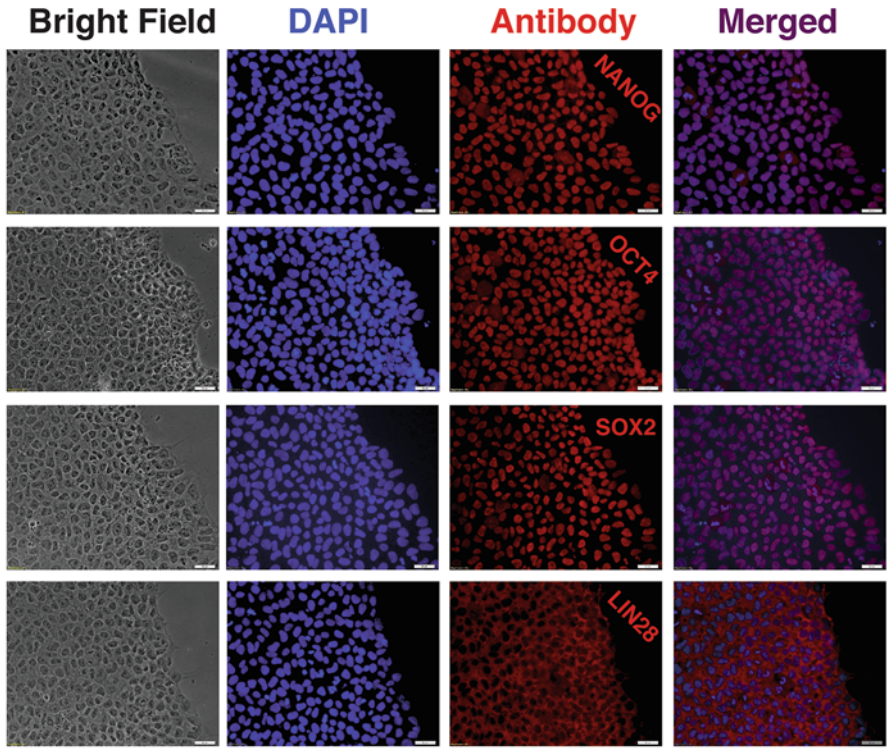

Fig. 1 Immunocytochemistry of human iPSCs. Scale, 20 μm. Images were taken at 40× magnitudes. Proteins stained are indicated. Nuclei were stained with DAPI

3. Pipette the cell suspension up and down to promote dissociation of cells and then transfer the cells to a 15-mL tube.
4. Add 5–10 mL of E8 medium into the tube and mix thoroughly.
5. To remove cell clumps, filter the cells vial a 70- or 100-μM cell strainer into a clean 15-mL tube (*see* **Note 13**).
6. Take an aliquot of 10 μL of the cell suspension and quantitate the cells using a hemacytometer.
7. Centrifuge the cells at 300 × *g* at 4 °C for 5 min.
8. Resuspend the cell pellet in at least 1.3 mL of FACS buffer in order to obtain a cell concentration of 0.5–1 × 10^6 cells/100 μL (0.5–1 × 10^4 cells/μL) (*see* **Note 14**). Keep the cells on ice as much as possible from this step on.
9. Label the flow cytometry tubes without cell strainer caps as SSEA3, SSEA4, TRA-2-49, TRA-1-60, TRA-1-81, SSEA1, Mouse IgM, Mouse IgG, Rat IgM, TRA-2-49/No-7AAD, 7-AAD only, and No Stain (*see* **Note 15**). The tubes of Mouse IgM, Mouse IgG, and Rat IgM serve as the isotype controls.

10. Transfer 100 μL of the cell suspension into each tube prepared above.
11. Add the defined amount of the PE-labeled antibodies to each tube (*see* Table 1), mix by pipetting or a quick vortex. Incubate on ice for 1 h in the dark.
12. Add 4 mL of FACS buffer to each tube and centrifuge at 300 × *g* for 5 min at 4 °C.
13. Decant the FACS buffer containing the antibodies by inverting the tubes. Add 4 mL of FACS buffer into each tube and centrifuge at 300 × *g* for 5 min at 4 °C.
14. Decant the FACS buffer by inverting the tubes and resuspend the iPSC pellet in 1 mL of the FACS buffer.
15. Label the flow cytometry tubes with cell strainer caps (mesh size, 35 μm) the same way as in **step 9** of this section.
16. In order to remove any cell clump, filter the cell suspension by transferring the cells into new flow cytometry test tubes through the cap cell strainer labeled above (*see* **Note 16**).
17. Add 7-AAD solution to a final concentration of 0.5 μg/mL and incubate for 5 min on ice before analysis with a flow cytometer.
18. Conduct cell immunophenotyping using a flow cytometer and acquire at least 10,000 cell events for each sample (*see* **Note 17**).
19. Analyze the data using FlowJo (Fig. 2).

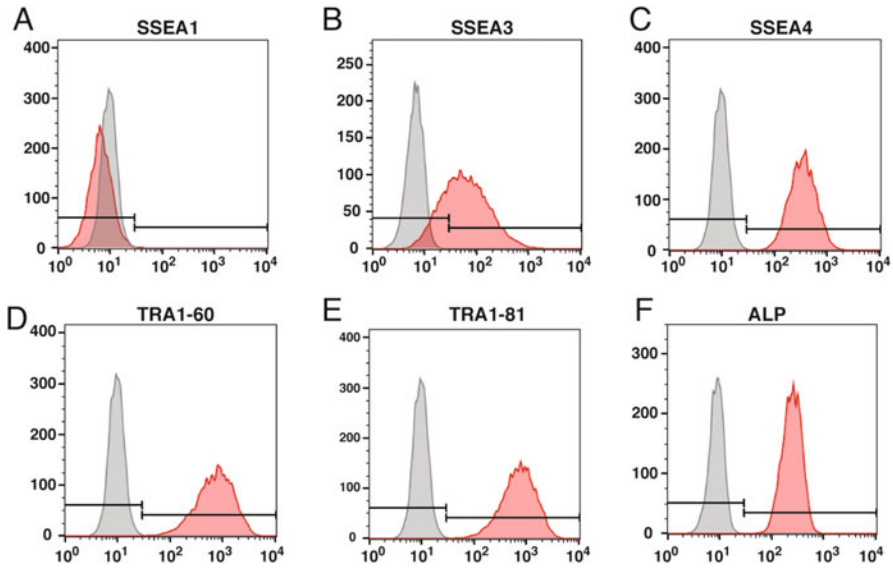

Fig. 2 Immunophenotyping of human iPSCs using the five pluripotency surface markers as indicated. SSEA1 is used as the negative control. Grey histogram is isotype control; and pink is antibodies as indicated

3.3 Evaluate Human iPSC Reprogramming Efficiency by the Number of the Colonies Positive for Alkaline Phosphatase

The efficiency of human iPSC reprogramming is commonly evaluated by the numbers of the colonies positive for alkaline phosphatase. The starting materials for this section is the reprogramming cells from day 8 to day 30 described in some of the other chapters of this volume depending on the users' motivation for this evaluation. This protocol can also be used to stain the iPSCs of the established iPSC lines.

1. Aspirate the media from the reprogramming cells or the established iPSCs and wash the cells with PBS once (1 mL for one well of a 12-well plate, 2 mL for each well of a 6-well plate).
2. Add 100% methanol pre-cooled to −20 °C into each well (1 mL for one well of a 12-well plate, 2 mL for each well of a 6-well plate) and incubate at RT for 10 min (*see* **Note 18**).
3. When fixation is going on, prepare the working BCIP/NBT solution using the stock BCIP, NBT solutions, and the ALP reaction buffer (*see* Subheading 2).
4. Aspirate the methanol and rinse the cells with PBS two times.
5. Rinse the cells with the ALP reaction buffer once.
6. Add BCIP/NBT solution (1 mL for one well of a 12-well plate, 2 mL for each well of a 6-well plate) and incubate for 15 to 25 min in the dark at RT. Check the color intensity periodically to decide when to stop the colorimetric reaction.
7. After the incubation, aspirate the BCIP/NBT solution and add PBS containing 0.05% sodium azide solution (1 mL for one well of a 12-well plate, 2 mL for each well of a 6-well plate) (*see* **Note 19**).
8. Count number of the ALP^+ colonies for each treatment of reprogramming and calculate reprogramming efficiency based on the number of the starting cells.
9. Scan the wells of the stained iPSC colonies using a photo scanner or take pictures with a microscope (*see* **Note 20**) (Fig. 3).

3.4 Evaluate Human iPSC Reprogramming Efficiency by the Number of Colonies Positive for TRA-1-60

The efficiency of human iPSC reprogramming is also evaluated by the number of the colonies positive for TRA-1-60. The starting materials for this section is the reprogramming cells from day 8 up to day 30 described in some of the other chapters of this volume depending on the purpose of this evaluation. It can be used to stain iPSCs of the established cell lines as well.

1. Aspirate the culture medium from the 6-well plate (*see* **Note 21**) and rinse the wells twice with 2 mL of 1× PBS for each well. After the last rinse, add 2 mL of 100% methanol pre-cooled to −20 °C and fix at room temperature for 10 min (*see* **Note 22**).

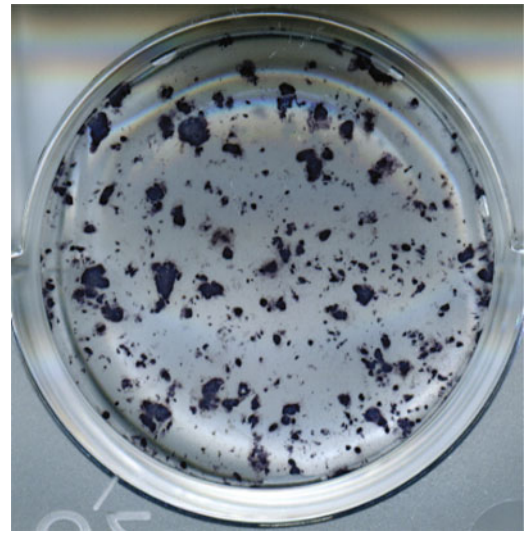

Fig. 3 A representative scanned image of reprogramming cells stained for the ALP activity

2. Remove the fixative from the wells and rinse the cells twice with 2 mL of 1× PBS each time.
3. To reduce the background, block the cells with 1% BSA in PBS at room temperature for 30 min. After blocking, rinse the cells twice with 2 mL of 1× PBS each time.
4. Dilute the biotin-conjugated TRA-1-60 antibody (1:200) in 1.5 mL of the staining buffer and add the diluted antibody into one well of a 6-well plate. Incubate overnight at 4 °C.
5. Aspirate the primary antibody and wash the cells three times with 2 mL of 1× PBS for 2 min each time with very gentle agitation.
6. Dilute streptavidin-HRP-conjugated secondary antibody (1:500) in the staining buffer. Aspirate PBS and add 1.5 mL of the diluted secondary antibody into each well. Incubate at room temperature in the dark for 2 h.
7. Remove the diluted secondary antibody reagent and wash the wells five times with 2 mL of 1× PBS for 2 min each time with very gentle agitation.
8. Prepare the substrate working solution from the DAB HRP kit: to 5 mL of water, add two drops of reagent 1, four drops of reagent 2, two drops of reagent 3, and two drops of the nickel solution. Add 1.5 mL of the substrate working solution into each well and incubate at room temperature for 10–15 min.

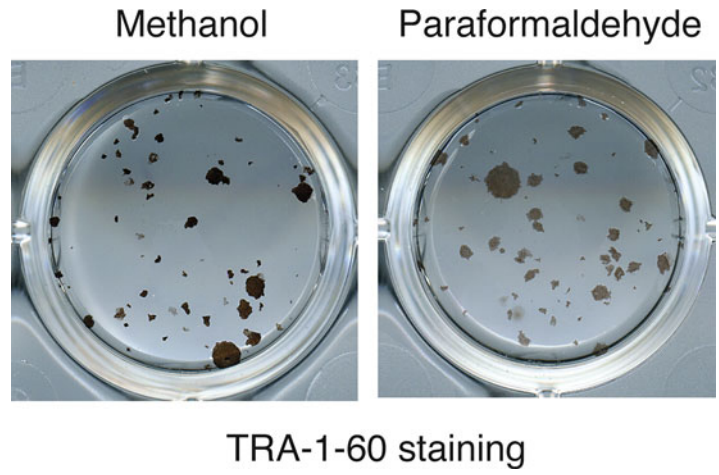

Fig. 4 Representative scanned images of the reprogramming cells stained for TRA-1-60. Please note the more intense staining for colonies fixed with methanol than that with paraformaldehyde

9. Remove the substrate and rinse the cells three times with 2 mL of 1× PBS each time. Add 2 mL of preservation buffer into each well and store the cells at 4 °C if analysis cannot be conducted right away (*see* **Note 19**).
10. Count the TRA-1-60^{+} colonies. Calculate the reprogramming efficiency.
11. Take images using photo scanner and/or microscope (Fig. 4) (*see* **Note 20**).

4 Notes

1. The E8 medium for the maintenance of human iPSCs and embryonic stem cells are available commercially if the users of the protocol prefer not to make it by themselves.
2. We use plastic slide chambers because iPSCs attach much better to plastic slides than to glass slides.
3. Genetically modified vitronectin supporting growth of human pluripotent stem cells are available commercially [11]. Follow the protocol provided by the manufacturer if the users choose to use hESC-qualified vitronectin for coating.
4. When growing iPSC culture is available, directly start from **step 12** of Subheading 3.1.1 to transfer iPSC culture from the conventional culture vessels into slide chambers.

5. Instead of the iPSC dissociation buffer prepared in-house, we found that the commercial enzyme-free Gentle Dissociation Reagent (Stemcell Technologies, Cat#, 07174) works well.
6. Immunocytochemistry can be conducted for cells grown on conventional cell culture vessels. Go to Subheading 3.1.2 directly if you prefer to conduct immunocytochemistry on 6-well or 12-well plates. Please note that you have to modify Subheading 3.1.2 slightly because it is written specifically for immunocytochemistry of cells on a slide. You will have high-quality images of stained cells when slides are used, especially for images of high magnification (20× and 40×, or higher).
7. Coating the slides with the concentrated Matrigel promotes attachment of iPSCs to slides (dilution factor of 100 vs around 1,000 in the conventional passaging of iPSCs.).
8. Unlike the conventional passaging of iPSCs with E8 system, we do not use ROCK inhibitors when passaging iPSCs onto slide chambers. ROCK inhibitors trigger differentiation of iPSCs. Omission of ROCK inhibitors will ensure high quality of iPSCs for the subsequent marker staining. iPSCs attach well even without the use of ROCK inhibitors when we use concentrated Matrigel to coat the chamber (dilution factor of 100 vs around 1,000 in the conventional passaging of iPSCs.).
9. Keep cultures below 90% confluence to avoid spontaneous differentiation.
10. Cold wash media and fixation solution loosen the attachment of iPSC colonies to the slides, and the quality of the subsequent imaging is affected because of difficulty in focusing the entire colony. We found that warming the solutions and media to 37 °C removes this issue, and high-quality images can be achieved.
11. Add the PBS gently during washes and avoid the cells and antibodies from drying to preserve antibody integrity from this step on.
12. Using Accutase instead of trypsin to dissociate iPSCs into individual cells reduces the possible enzymatic digestion of your cell surface proteins and renders higher viability of the treated cells after cell dissociation.
13. This filtration step is optional since **step 16** will filter the cells again immediately before analysis with flow cytometry.
14. Adjust the volume appropriately considering that you will need at least 12 tubes per cell line. It is expected that you will get around 8–10 $\times$ 10^6 cells from two T25-flask cultures at 90% of confluence, which is enough to have more than 5 $\times$ 10^5 cells per tube.

15. The expression of ALP is generally high and uniform in pluripotent stem cells, giving strong signals and a narrow bell-shaped histogram. Therefore, ALP/TRA-2-49 is the chosen marker for compensation in the flow cytometry analysis. The TRA-2-49/No-7AAD tube is for compensation to set up the flow cytometer.
16. Cells do not flow through the strainer efficiently by gravity. Pipette up and down the cell suspension in the reservoir of the cell strainer to facilitate the filtration using a 1-mL pipette tip.
17. It is encouraged to conduct flow cytometry immediately after the staining. Cells should be stored on ice to prevent the cells from death if flow cytometry cannot be conducted immediately. Cells can stay on ice for 4 h before analysis. Prolonged storage of the cells after staining will result in significant cell death.
18. Do not fix the cells with paraformaldehyde because it will compromise the enzyme activity of alkaline phosphatase. To preserve the ALP activity, it is critical to fix the iPS cells with methanol, which is much milder. In our experience, ALP staining becomes very weak after fixation with paraformaldehyde, but methanol fixation preserves the ALP activity and gives intense signals.
19. At this stage, the stained plates can be stored at 4 °C for later analysis. Inclusion of sodium azide prevents growth of microbes.
20. During scanning of the stained cells, the preservation buffer should be removed to improve quality of the images. The reflective liquid in the wells will result in glare in the images.
21. This protocol is written for procedures with a well of a 6-well plate. The amount of reagents can be adjusted depending on the size of your reprogramming vessels.
22. Some published methods used 4% paraformaldehyde to fix the cells for TRA-1-60 staining [13]. We found that like staining for ALP activity, paraformaldehyde significantly damages the TRA-1-60 epitope and methanol fixation gives much stronger staining (Fig. 4).

Acknowledgments

The research in Hu laboratory is supported by an NIH 1R01GM127411-01A1 and the American Heart Association (17GRNT33670780).

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