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## Passaging and colony expansion of human pluripotent stem cells by enzyme-free dissociation in chemically defined culture conditions

Jeanette Beers<sup>1</sup>, Daniel R. Gulbranson<sup>2,3</sup>, Nicole George<sup>4</sup>, Lauren I. Siniscalchi<sup>1</sup>, Jeffrey Jones<sup>4,5</sup>, James A. Thomson<sup>2,3,6</sup>, and Guokai Chen<sup>1,2</sup>

Jeanette Beers: beersj@nhlbi.nih.gov; Daniel R. Gulbranson: daniel.gulbranson@colorado.edu; Nicole George: ngeorge@wicell.org; Lauren I. Siniscalchi: lsiniscalchi.22@gmail.com; Jeffrey Jones: jjones@wicell.org; James A. Thomson: jthomson@morgridgeinstitute.org

<sup>1</sup>Center for Molecular Medicine, National Heart, Lung and Blood Institute, Bethesda, Maryland; Building 10, Magnuson Clinical Center, 5N214; 10 Center Dr.; Bethesda, MD 20892, USA

<sup>2</sup>Morgridge Institute for Research, Madison, Wisconsin; 330 N. Orchard St.; Madison, WI 53715; USA

<sup>3</sup>Department of Cell and Regenerative Biology, School of Medicine and Public Health, University of Wisconsin, Madison, Wisconsin; 1300 University Ave; Madison, WI 53706; USA

<sup>4</sup>WiCell Institute, Madison, Wisconsin; P.O. Box 7365; Madison, WI 53707; USA

<sup>5</sup>Department of Obstetrics and Gynecology, University of Wisconsin School of Medicine and Public Health, Madison, WI 53706: USA

<sup>6</sup>Department of Molecular, Cellular, & Developmental Biology, University of California, Santa Barbara; Life Sciences Building, Room 3314; Santa Barbara, CA 93106; USA

### Abstract

This protocol describes an EDTA-based passaging procedure to be used with chemically defined E8 medium that serves as a tool for basic and translational research into human pluripotent stem cells (iPSCs). In this protocol, passaging one six-well or 10 cm plate of cells takes about 6–7 min. This enzyme-free protocol achieves maximum cell survival without enzyme neutralization, centrifugation, or drug treatment. It also allows for higher throughput, requires minimal material and limits contamination. Here we describe how to produce a consistent E8 medium for routine maintenance and reprogramming and how to incorporate the EDTA-based passaging procedure into human induced PSC (iPSC) derivation, colony expansion, cryopreservation and teratoma formation. This protocol has been successful in routine cell expansion, and efficient for expanding large-volume cultures or a large number of cells with preferential dissociation of PSCs. Effective for all culture stages, this procedure provides a consistent and universal approach to passaging human pluripotent stem cells in E8 medium.

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Correspondence to: Guokai Chen, guokai.chen@nih.gov; Tel: 301-594-4717.

### Contributions

G.C. and J.A.T. conceived the experiments and supervised the project; G.C. developed dissociation protocol; J.B. and G.C. performed the reprogramming experimental procedure in the paper; G.C. and D.R.G. demonstrated preferential dissociation of EDTA; N.G., J.J., D.R.G. and G.C. performed long-term culture; G.C., D.R.G. and J.B. performed teratoma formation assay; L.I.S. performed immunostaining and EDTA sequential dissociation imaging; J.B. and G.C. wrote the paper.

### Competing Financial interests

J.A.T. is a founder, stockowner, consultant, and board member of Cellular Dynamics International. He also serves as scientific advisor to and has financial interests in Tactics II Stem Cell Ventures.

## Keywords

Cell culture; human embryonic stem cells; induce pluripotent stem cells Cell survival; cell dissociation

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## Introduction

The study of human embryonic stem cells (ESCs), the first established human PSC lines<sup>1,2</sup>, led to the derivation of iPSCs from human somatic cells<sup>3-5</sup>. Human ESCs and iPSCs are both capable of generating cells from all three germ lines and have thus gathered tremendous interest for their practical and scientific values. Maintained in cell culture, both are affected by different culture conditions and cell handling techniques.

Cell culture conditions for human ESCs and iPSCs have evolved from feeder-dependent and feeder-free medium to defined medium on defined extracellular matrix (ECM)<sup>1,2,6-9</sup>. Improved understanding of self-renewal and differentiation means that growth media and ECM are becoming increasingly defined and simplified. At the same time, multiple cell handling techniques have evolved to facilitate PSC research. However, no single technique can be used at every stage of culture, from expansion through differentiation and cryopreservation.

## Dissociation of ESCs

For regular cell culture practices, cells are often individualized during passaging to achieve even distribution and uniform treatments. However, human ESCs survive poorly after individualization (i.e., being made single cell), because these cells are more sensitive to treatments and are prone to cell death, a fact that has made the development of a universal dissociation method particularly challenging. In routine experiments, dissociation methods are chosen on the basis of either cell survival or sensitivity. In regular expansion, cell survival is the priority. ESCs and iPSCs are usually passaged as aggregates with enzymatic dissociation, with collagenase used for culture on feeder cells<sup>1,2</sup> and Dispase used for culture on feeder-free cells<sup>8</sup>. Mechanical approaches, such as cell scrapers and other passaging tools, have also been developed to dissociate cells as aggregates. Furthermore, in a differentiation or transfection experiment, TryPLE and Accutase can be used to individualize ESCs, but poor survival often leads to abnormal karyotypes<sup>10,11,12</sup>; small chemicals, such as Rho-associated protein kinase (ROCK) inhibitors, must be used to boost cell survival in this process<sup>13</sup>. All these methods require specialized tools or reagents that are costly for long-term or large-scale experiments. At the same time, the consistency of enzymatic methods is usually affected by the quality of enzymes from batch to batch. Given the variability of these methods, it is highly desirable to find a universal approach for all purposes.

The quality of culture conditions is also crucial to the maintenance and expansion of the PSCs. The medium components related to feeder cells or animal products often greatly affect the consistency of the cell culture, which could be even more problematic when cells have potential applications in translational research. To improve the consistency of cell culture, we systematically analyzed the functions of individual medium components, and finally formulated a xeno-free, chemically defined stem cell medium E8, which contains eight essential factors for human ESCs<sup>14</sup>. With minimal modification of E8, human iPSCs can be directly derived from skin biopsies in chemically defined conditions. This culture system could have great applications in future PSC research. In companion with the development of cell culture medium, a consistent xeno-free dissociation method is important to fulfilling the potential of the defined culture conditions.

We previously studied cell death mechanisms after individualization and found that myosin-actin dependent contraction leads to cell death, and that cell-cell adhesions promote survival by inhibiting the contraction<sup>15</sup>. Cells survived by reforming small aggregates in the first few hours after dissociation. This observation provided the theoretical foundation for a method that generates aggregates strong enough to survive, but small enough to be accessed by growth factors or transfection reagents. In combination with the development of chemically defined cell culture, we set out to devise a universal dissociation technique for the expansion, cryopreservation, and experimentation of human PSCs in defined conditions. We found that, after a specific EDTA treatment, cells can be partially dissociated to generate small aggregates that survive as well as Dispase-treated cells, and which are more accessible to various treatments. When used with the chemically defined E8 medium, this dissociation method can be used to handle human PSCs during derivation, expansion, and preservation. In this protocol we describe how to derive iPSCs from fibroblasts as an example of how to use our EDTA dissociation method and defined chemical culture conditions in each stage of the iPSC derivation process.

## Development of the protocol

Conventionally, human PSCs are passaged as aggregates with enzymes, with individualized cells typically dying from actomyosin contraction. However, individualized cells can survive through the inhibition of ROCK-actin-myosin pathway components. Alternatively, cells can survive naturally through reaggregation of adjacent cells, indicating that higher local density could lead to reassociation efficiency independent of drug treatment (Fig. 1a). We found that EDTA treatment could partially dissociate human ESCs, and that ESCs were easily washed off with medium (Supplementary Fig. 1a). Loose adhesions between the cells generated very high local density beneficial to cell survival. Most cells were in small aggregates, which attached to a Matrigel-coated plate within minutes (5 min), spread in 2 h and survived as colonies (24 h; Fig. 1b). EDTA-dissociated ESCs survive significantly better than individualized cells by TryPLE, and the survival efficiency is similar to passaging cells as large aggregates using a Dispase protocol (Fig. 1c and Supplementary Methods). Cell survival when using this EDTA method is comparable to that observed in cells treated by ROCK inhibitor (Supplementary Fig. 1b), and it is E-cadherin dependent (Supplementary Fig. 1c). This indicates that cells harvested by EDTA survive without drug treatment, but require direct cell-cell adhesion.

Because of the survival efficiency of EDTA dissociation, we have used it extensively in long-term culture expansion of human PSCs, on more than 50 different human ESCs and iPSCs in different feeder-free media, including TeSR and E8. In each of these cases, normal karyotypes were effectively maintained in long-term culture. The longest iPSC culture conducted in our research lasted more than 51 passages and longer than 6 months, and the cells maintained normal karyotypes (Supplementary Table 1 lists some of these ESC and iPSC lines). Cells dissociated by EDTA efficiently survived not only on Matrigel and vitronectin surfaces, but also on synthetic surface independent of ROCK inhibitors (Supplementary Fig. 1d). Because of its simplicity and consistency, this protocol has been used for our routine cell culture practices<sup>14-17</sup>, and other groups have successfully used EDTA dissociation to maintain human PSCs<sup>18</sup>.

EDTA dissociation is also useful in transfection and differentiation experiments (Supplementary Fig. 2 and Supplementary Methods). In transfection experiments, EDTA-dissociated cells were transfected efficiently while maintaining high survival rate (Supplementary Fig. 2a-c). At the same time, these cells were sensitive to growth factor-induced differentiation (Supplementary Fig. 2d). We hypothesize that EDTA dissociation enables small aggregates to survive better than individualized cells, and that the use of this

method allows easier access to reagents used in differentiation experiments when compared with conventional aggregates collected with enzymes such as Dispase. The EDTA method can also be used to harvest PSCs for effective teratoma formation in immune-deficient mice. EDTA dissociation has been successfully used for all culture stages of human PSCs.

EDTA dissociation also improves the handling of PSCs mixed with differentiated cells. In reprogramming experiments iPSC colonies are mechanically isolated for colony expansion, and in the process they are often contaminated with unprogrammed somatic cells. Conventionally, colony picking is used to enrich stem cells, but this approach is prone to bacterial and fungal contamination, and is not practical when too many lines are involved. We were able to avoid contamination by combining the advantages of E8 medium and the EDTA dissociation method. First, defined E8 medium allows stem cells to grow faster than fibroblasts, needing additional factors such as hydrocortisone. Even without colony isolation, PSCs could potentially outgrow somatic cells in a few passages with high passaging efficiency. Second, we found that PSCs respond to EDTA differently from somatic cells, such as fibroblasts. EDTA treatment preferentially harvests ESC colonies, leaving most fibroblast cells on the original plate (Supplementary Fig. 3a,b). In comparison, TrypLE ubiquitously dissociates both ESCs and fibroblasts.

These observations led us to use EDTA dissociation for iPSC derivation and colony expansion, which requires both human PSCs and somatic cells. In a conventional iPSC derivation procedure, cells are often passaged as individual cells, with ROCK inhibitors boosting iPSC survival before individual colonies are picked. However, we have used EDTA differential dissociation and harvested iPSCs with high survival rates without the help of a ROCK inhibitor (Supplementary Fig. 3c); we found that most EDTA-dissociated cells expressed the stem cell marker SSEA-4 (Supplementary Fig. 3d). EDTA dissociation thus enables the enrichment of potential iPSCs in an overcrowded reprogramming culture in which a secondary passaging is needed. We have also used EDTA dissociation to expand isolated iPSC colonies. We have often left differentiated cells in the original plate and found that stem cells reached a high purity in one or two passages. In addition, in EDTA-based cell expansion, we routinely maintained established ESC and iPSC lines with high purity (i.e., >95% of the population is positive for the stem cell marker Oct4) without enrichment by manual picking. It is possible that spontaneous differentiation is suppressed by the easy access to growth factors by small aggregates created by EDTA passaging, and that differential dissociation allows potential enrichment of stem cells after each passage while leaving some differentiated cells behind.

In addition to preferential dissociation, EDTA passaging has another advantage: it is a quick procedure with minimal opportunity for contamination. The procedure can be performed in a biosafety cabinet, and it does not require enzymatic neutralization and centrifugation. We are thus able to passage 24 individual lines in 15 minutes with minimal risk of contamination. In addition, as EDTA treatment does not damage the ECM, a portion of the iPSCs can be maintained in the original wells after dissociation, providing a backup or duplicate plate for maintenance or further characterizations.

## Experimental Design

### E8 medium production

This protocol describes how to produce chemically defined stem cell medium E8 with the formulas listed in Tables 1 and 2. To maintain consistency in the cell culture, we recommend making large quantities of medium each time, storing it in the freezer and performing a batch test before applying the medium in real experiments. A base medium, which can be used for all stages of stem cell culture, is first prepared according to Table 1

(see also Supplementary Table 2). Full E8 medium can be prepared with additional self-renewal essential factors (Table 2). The medium can also be prepared on a small scale with the reagents listed in Tables 1 and 2.

### **Routine cell culture expansion with EDTA dissociation**

When the E8 medium is ready, we test it with H1 ESCs and one control fibroblast iPSC line. EDTA dissociation is used to passage the cells for at least five passages, and cells are then analyzed with flow cytometry and karyotyping. After the E8 medium passes the test, the frozen stock can be used for routine cell culture expansion and other experiments. We find that OCT4-positive cells consistently compose >95% of the whole population when we use the EDTA passaging method with E8 medium.

### **Derivation of human iPSCs from fibroblast cells**

After E8 components pass the quality test, a set of E8-based media are used to reprogram human skin fibroblast cells. After transduction, cells are first cultured in E8-based reprogramming medium 1, and then in reprogramming medium 2 (Table 2). We usually perform the reprogramming experiment in a six-well plate so that multiple treatments can be applied to the cells in the same experiment (Fig. 2). Sodium butyrate treatment is usually used to improve reprogramming efficiency.

Reprogramming cells usually show up about 3–5 d after transduction, and the colonies mature 20–30 d after transduction. During this period, cells usually need to be passaged to avoid overconfluence, which can inhibit the emergence of true iPSC colonies. The EDTA method is applied to dissociate cells after the emergence of reprogramming cells, which preferentially enrich potential iPSCs.

The procedure has been successfully used in lentiviral-, sendai virus- and episomal DNA-based reprogramming. For simplicity, we used commercially available STEMCCA polycistron lentivirus in this protocol. This procedure could also be modified to reprogram human umbilical vein endothelial cells (HUVECs) and adipocyte cells.

### **Colony expansion and preservation of human iPSCs**

When iPSCs are mature, individual colonies are mechanically isolated into a 24-well plate for colony expansion. Cells are subsequently expanded with the EDTA method, and can be cryopreserved in 3–5 d. The cells can be expanded and should be characterized by alkaline phosphatase staining (APS) and other appropriate characterizations. After further confirmation by karyotyping and immunostaining for pluripotency markers, validated iPSC lines can be used for teratoma formation with the EDTA method.

## **MATERIALS**

### **REAGENTS**

#### **Cells**

Human ESCs: H1 cells, WA01 (US National Institutes of Health (NIH), human ESC registry no. 0043)

Human iPSCs from fibroblasts: ND1 (NIH control iPSC line)

Human foreskin fibroblasts: CCD-1079Sk (ATCC cat. no. CRL-2097)

SCID-Beige mice (optional for teratoma only, step 28C; Taconic cat. no. CBSCBG-F)

Critical: Multiple mice are usually needed for each injection, and female mice are often used.

Caution: Experiments involving live rodents must conform to Local and National regulations.

Human STEMCCA Cre-Excisable Constitutive Polycistronic (OKSM) Lentivirus reprogramming kit (Millipore cat. no. SCR545)

Sodium Chloride (Sigma S5886 or equivalent)

Sodium Chloride (NaCl, Sigma, cat. no. S5886 or equivalent)

DMSO (Sigma, cat. no. D4540 or equivalent)

Matrigel, growth factor reduced (BD Biosciences, cat. no. 354230)

Critical: Always store in a non-antifreeze freezer.

Rock Inhibitor Y-27623 (Tocris, cat. no. 1254)

Holo-transferrin (Sigma, cat. no. T0665 or equivalent)

Recombinant human fibroblast growth factor (FGF, Peprotech, cat. no.100-18B or equivalent)

Recombinant human transforming growth factor (TGF- $\beta$ 1, R&D Systems, cat. no. 240-B/CF)

Insulin, human, 10 mg/mL solution (Sigma, cat. no. I9278-5ML)

Hydrocortisone (Sigma, cat. no. H-0396)

Sodium butyrate (Sigma, cat. no. 303410)

Dulbecco's PBS, calcium and magnesium Free (Invitrogen, cat. no. 14190-250 or equivalent)

E8 media components (Tables 1–2)

Reprogramming media components (Tables 3–4)

Polybrene (Sigma, cat. no. H9268)

TrypLE (Invitrogen, cat. no. 12563)

BCIP/NBT alkaline phosphatase substrate kit IV (Vector Laboratories, cat. no. SK-5400)

Tris-HCl, 100 mM, pH 9.5

EDTA, 0.5M, pH8.0 stock solution (K&D Medical cat. no. RGF-3130 or equivalent)

Human Serum Albumin(HSA, 0.05% (wt/vol); Sigma, cat. no. A6608; see Reagent Setup)

HSA in HCl in PBS (0.05% (wt/vol); Sigma, cat. no. A6608; see Reagent Setup)

Liquid Nitrogen

Ethanol

Dulbecco's modified Eagle medium (DMEM)-F1

Sodium Selenite

L-ascorbic acid

Sodium Hydroxide

## EQUIPMENT

Laminar Flow Hood with Vacuum

Centrifuge (Beckman Coulter Allegra X15-R with SX4750 swinging bucket rotor or equivalent)

Cell Culture Incubator (37°C, 95% humidity, 5% CO<sub>2</sub> and 5% O<sub>2</sub> atmosphere, Heracell, Thermo Scientific)

Inverted phase contrast microscope (x4 and x10 objectives, Zeiss or equivalent)

Freezer –80°C (Thermo Scientific)

Waterbath, 37°C Isopropanol Cell-Freezing Container

Cryovials, 1.2 ml (USA Scientific, cat. no. 1412-9100 or equivalent)

Six-well Nunclon Delta Surface tissue culture dishes (Thermo Scientific cat. no. 140675 or equivalent)

P1000 pipetman and sterile tips with filter

Pipet-aid and sterile 5- and 10-ml plastic disposable pipettes

Sterile filter units, 500 ml (Millipore, Stericup, cat. no. SCGPU05RE)

Conical tubes, 15 and 50 ml (Falcon, cat. nos. 352097 and 352098 or equivalent)

Eppendorf tube

Gauge needle (20.5)

## Reagent Setup

**EDTA dissociation solution**—Add 500 µl 0.5 M EDTA (pH 8.0) stock into 500 ml calcium/magnesium-free PBS. Add 0.9g of NaCl and adjust the osmolarity to 340 mOsm. Sterilize the solution by filtration, and store it at 4°C for up to 6 months.

Critical: To achieve the least disturbance of cells during dissociation, the osmolarity of EDTA solution is designed to be the same as that of E8 medium.

**Matrigel**—See Box 1 for Matrigel Preparation

**ROCK inhibitor**—Y-27632 is the most commonly used ROCK inhibitor, and it can be dissolved in H<sub>2</sub>O or DMSO. Dissolve the Y-27632 in sterile H<sub>2</sub>O or sterile DMSO with a final concentration of 10 mM (1000x), then aliquot and store it at –80°C. The solution is stable for at least 1 year.

Critical: With the EDTA protocol, ROCK inhibitor is not as essential as it is in enzymatic protocols. In routine maintenance of iPSC lines, ROCK inhibitor treatment is optional. However, if the stem cell cultures are too confluent, or have not been passaged for 4 d or more, ROCK inhibitor can greatly increase cell survival. If most of the cells are individualized, ROCK inhibitor treatment is recommended.

**Holo-transferrin (1000x)**—Dissolve 500 mg of Holo-transferrin in 50 mL of Dulbecco's PBS and filter to sterilize. This makes a 1000x (10 mg/ml) stock that can be divided into 500-µl aliquots and frozen at –80°C, at which temperature it is stable for at least 1 year.

**FGF (1000x)**—One milligram of FGF should be dissolved in 10 mL of sterile 0.05% HSA in PBS. This 1000x FGF (0.1 mg/ml) should be divided into 500- $\mu$ l aliquots and frozen at  $-80^{\circ}\text{C}$ , at which temperature it is stable for at least 1 year.

**TGF- $\beta$ 1 (1000x)**—TGF $\beta$ 1 should be resuspended to a concentration of 1.74 $\mu\text{g}/\text{ml}$  (1000x) in sterile 0.05% HSA in 4 mM HCl in PBS. This is then aliquotted into 500- $\mu$ l aliquots and frozen at  $-80^{\circ}\text{C}$ ; it is stable at this temperature for at least 1 year.

**Hydrocortisone (10,000x)**—A measure of 3.625 mg of hydrocortisone is dissolved in 1 ml of sterile water (10mM stock). This solution is then divided into 50- $\mu$ l aliquots and frozen at  $-80^{\circ}\text{C}$ ; it is stable at this temperature for at least 1 year.

**Sodium butyrate (1,000x)**—A measure of 110 mg of sodium butyrate is dissolved in 10 ml of sterile water and filtered (100 mM stock). This is then divided into 500- $\mu$ l aliquots and frozen at  $-80^{\circ}\text{C}$ ; it is stable at this temperature for at least 1 year.

**Cryopreservation media (2x)**—Add 2 ml of sterile DMSO and 20  $\mu$ l 10 mM ROCK inhibitor stock into 8 ml E8 medium to make 2x cryopreservation medium for iPSCs in colony expansion. Medium can be stored at  $4^{\circ}\text{C}$  for up to 1 week.

**Polybrene Stock (1000x)**—Dissolve the polybrene in PBS to 1000x (6  $\mu\text{g}/\text{ml}$ ) and filter to sterilize. Freeze the solution in small aliquots at  $-80^{\circ}\text{C}$  and store them for up to 1 year before use. The final concentration on cells during infection will be 6 ng/ml.

**E8 base medium**—Pour 50 liters of DMEM-F12 medium into a suitable container, and stir it on a stir plate. Save the empty bottles. Add 3,200 mg L-ascorbic acid and 970 $\mu$ l sodium selenite (0.7 mg/ml stock) into medium and mix well. Adjust the pH to 7.4 with 10 N NaOH. Add NaCl to adjust the osmolarity to 340 mOsm. Aliquot the medium back into the original bottles. The final composition of the medium should contain 64 mg/l L-ascorbic acid and 13.6 $\mu\text{g}/\text{l}$  sodium selenite. The details of how to make this medium are also given in Table 1. This base E8 medium should be frozen and stored at  $-20^{\circ}\text{C}$ , it is stable at this temperature for at least 1 year.

**Full E8 (TGF $\beta$ 1) medium**—Thaw one bottle (500 ml) of E8 base medium. Add 500 $\mu$ l of 1000x holo-transferrin, 500 $\mu$ l of 1000x FGF, 500 $\mu$ l of 1000x TGF $\beta$ 1 and 1 ml 10 mg/ml insulin. Filter the E8 (TGF $\beta$ 1) medium through a 500-ml Millipore Stericup filter. Store the medium at  $4^{\circ}\text{C}$  before use. The medium is stable for 2–3 weeks at  $4^{\circ}\text{C}$ . Do not warm this medium in a  $37^{\circ}\text{C}$  water bath before use, as it will shorten the life of the growth factors. Note that full E8 medium can now be purchased as Essential 8 media from Life Technologies (cat. no. A14666SA) or from Stem Cell Technologies (cat. no. 05840) as TeSR-E8. The details of how to make this medium are also given in Table 2.

Caution: These purchased media are only for stem cell maintenance. They contain TGF- $\beta$ 1, which can interfere with reprogramming.

**Reprogramming media 1 and 2**—Prepare 500 ml of reprogramming medium with the same formula as Full E8 medium, but leave out the TGF- $\beta$ 1. In place of the TGF- $\beta$ 1, either add 50 $\mu$ l 10,000s hydrocortisone (reprogramming Medium 1) or 500 $\mu$ l of 1000x sodium butyrate (reprogramming media 2) before filtering.

The details of how to make these media are also given in Table 2. Media can be stored at  $4^{\circ}\text{C}$  for up to 2 weeks.



**HSA in PBS**—Add 25 mg HAS to 50 ml of PBS and filter to sterilize. Store it for 1–2 months at 4°C or make aliquots and store them at –20°C for 1 year.

**HAS in HCl-PBS**—Dissolve 25 mg of HAS in 50 ml of 4 mM HCl in PBS and filter to sterilize. Store it for 1–2 months at 4°C or make aliquots and store them at –20°C for 1 year.

## Procedure

### Steps 1–9: Human PSC expansion with EDTA in full E8 Medium (TGF- $\beta$ 1 medium)

#### Timing, 6–7 minutes per plate

- 1| Place a new six-well plate that has been coated with Matrigel (Box 1) and warmed to room temperature (RT, 20–25°C) in the tissue culture hood. In addition, keep the EDTA solution ready, which has been warmed to RT.
- 2| Label the new Matrigel-coated plate and aspirate the Matrigel from the well. Replace with 1.5–2 ml full E8 medium (or E8 medium + ROCK Inhibitor if desired) per well. **Critical Step:** This step can also be performed during the EDTA incubation, but if a large number of cells are being passed, it is better to prepare plates ahead, as time is limited.
- 3| Aspirate media from the ESCs or iPSCs that are about to split. Rinse the wells with 1 ml per well of EDTA solution to remove the magnesium and calcium from the medium. **Critical Step:** When rinsing, add the EDTA solution to the wells and then immediately aspirate the EDTA off again without incubating. **Critical Step:** When washing with EDTA, add the EDTA to the wall of the well slowly to avoid washing the colonies off the plate too early.
- 4| Repeat the EDTA wash (as described in Step 3) a second time to ensure that all magnesium and calcium is removed. Add another 1 ml per well of the EDTA solution and let it sit for 2–5 min at RT within the hood. **Critical Step:** The longer the cells sit in the EDTA, the smaller the colonies that will result. In addition, it is important to keep the movement of the plate to a minimum to avoid lifting the colonies completely off the plate during this step.
- 5| After 2–5 min, carefully aspirate the EDTA solution from the cells. Use 1–4 ml of E8 medium (Or E8 medium + ROCK inhibitor) and add it rapidly to wash the colonies off the plate and disperse the colonies as quickly as possible. The calcium and magnesium in the medium will neutralize any remaining EDTA. **Critical Step:** Do not break apart the colonies too much by excess pipetting. **Critical Step:** This and the next step should be performed quickly, as the colonies start to reattach to the plate very rapidly after the addition of calcium and magnesium.
- 6| Ensure that the cell suspension is well mixed and then plate the desired cell amount per well in the newly prepared plate.
- 7| Shake the plate back and forth and side to side to distribute the cells. Place the plate in an incubator. Let it sit overnight for maximum attachment.
- 8| On the next day, take E8 medium out from a 4°C refrigerator, let it sit at RT to warm, if desired. Remove the medium from cells, and add 2 ml of E8 medium to each well in the six-well plate.
- 9| On each following day, repeat Step 8, monitor cells daily, and when confluence reaches ~80% repeat Steps 1–7 (usually between 3 and 5 d after seeding).

Passage the cells for the desired number of passages. After five passages we recommend collecting some cells for FACS to measure pluripotency markers and for karyotyping. If the FACS and karyotyping results are satisfactory, the media components are suitable for iPSC maintenance and reprogramming. This enzyme-free passaging protocol can be used during the derivation of iPSC cells from other cell types. When you are ready to begin reprogramming, continue to the next section.

### Steps 10–11: Transduction of human fibroblasts

#### Timing: 3 d

- 10| Take a low-passage-number fibroblast culture and plate it in one well of a six-well dish so that cells will be about 80% confluent the next day (100,000 or 150,000 cells per well) in fibroblast medium (usually 10% (vol/vol) FBS and Penicillin-streptomycin, L-glutamine and non-essential amino acids in DMEM; however, if your fibroblasts are growing in another medium, use that medium). Incubate the cells overnight.
- 11| Late on the next day, mix together 1.5 ml of fibroblast medium with polybrene (to obtain 6 ng/ml final concentration). Add Millipore STEM-CCA virus at the desired titer, add to mixture to the cells, and leave on the cells overnight.. Our current protocol utilizes a multiplicity of infection (MOI) of ~5–10. On the next day, if cells are nearly confluent, change medium to fresh fibroblast medium to remove polybrene. Coat two six-well plates with Matrigel, as described in Box 1.

### Steps 12–18: Culture of transduced fibroblasts on Matrigel

#### Timing: 3–4 weeks

- 12| Still on the same day, passage the well of reprogramming cells using TrypLE. TrypLE dissociation preserves cells better than regular trypsin-EDTA, and it does not require serum-based neutralization. To do this, remove the medium from the cells and add 0.5 ml of TrypLE to the wells. Incubate for 5 min in an incubator, and then wash the cells off the plate with fibroblast medium into a centrifuge tube and dilute them fivefold with fibroblast medium to inactivate TrypLE. **Critical Step:** Some cell lines do not come off the plate well with TrypLE. With these lines, you may want to wash the plate twice with EDTA before adding TrypLE.
- 13| Spin cells down for 5 min at RT at 200g in a swinging bucket rotor and resuspend them in 24 ml of fibroblast medium. Remove the Matrigel/DMEM-F12 from the two six-well plates and add 2 ml of cells per well. This gives a final passage of one well of infected cells into two six-well plates coated with Matrigel in fibroblast medium or directly into reprogramming medium 1.
- 14| Maintain the cells in reprogramming medium 1, replacing medium every other day for 3–5d.
- 15| Remove the medium and replace it with reprogramming medium 2. To improve reprogramming efficiency, add 100  $\mu$ M sodium butyrate into medium 2 on one plate. **Critical Step:** The time at which to replace reprogramming media 1 with 2 is based on cell confluence. Medium 1 acts to promote fibroblast growth, and should be switched to medium 2 when the confluence reaches ~20–30%. If cells are still very sparse you may want to keep them in reprogramming medium 1 for a few more days.

- 16| Continue replacing the medium every other day, using reprogramming medium 2 with or without sodium butyrate.
- 17| Monitor the cells daily, if cells become too confluent while you are waiting for iPSC colonies to mature, cells may need to be passaged with EDTA at some point during the 2 weeks. Choose three or four wells to passage with EDTA onto two new Matrigel-coated plates (1:3 ratio). Use the EDTA passaging method as described in Steps 1–7 above, but using reprogramming medium 2 instead of full E8 (TGF- $\beta$ 1) medium.
- 18| Twenty to twenty-five days after transduction, colonies should be ready for picking. At this point, proceed to Step 19. Around this time, begin feeding the original reprogramming plates full E8 (TGF- $\beta$ 1) medium daily.

### Steps 19–27: Mechanical isolation of human iPSC colonies

#### Timing: 2–3 d

- 19| For picking, prepare a 24-well plate by coating with Matrigel (as described in Box 1, using 250  $\mu$ l per well of the Matrigel stock for a total of 1 mg of Matrigel per 24-well plate).
- 20| After the 30-min Matrigel incubation, replace Matrigel/DMEM-F12 with full E8 (TGF- $\beta$ 1) medium containing 1x Rock inhibitor. **Critical Step:** Although Rock inhibitor is described as optional in the EDTA passaging method, ROCK inhibitor will greatly increase the survival chances of newly picked colonies.
- 21| Spray 70% (vol/vol) ethanol on a microscope and on the surrounding area, as well as on a pipet and box of tips. **Critical Step:** We pick the colonies on a benchtop in the laboratory using a normal inverted light microscope. If space allows, a microscope can be placed inside a laminar flow hood or a bench top PCR clean hood to allow for a sterile field while picking colonies. If preferred, a dissection microscope can be used to find and manually passage the colonies.
- 22| While wearing a facemask, find iPSC colonies under the microscope with the x4 objective. By using a P20 pipette with a tip, circle around the colony until it is loosened from surrounding cells.
- 23| While still using a pipette tip, cross-hatch the colony so that it will come off the plate in smaller pieces.
- 24| Next, use the pipette to push the colony off the plate and suck it into a pipette tip. Transfer the colony pieces into one well of the 24-well plate.
- 25| Repeat Steps 22–24 with other colonies, placing one colony in each well of the 24-well plate. Incubate the cells overnight in the tissue culture incubator.
- 26| On the day after picking, remove the medium and replace it with full E8 (TGF- $\beta$ 1) medium without rock inhibitor.
- 27| Replace the E8 medium daily until the attached colony is big enough to passage. The colony is usually ready to passage after 2–3 d.

### Step 28: Ongoing passage and analysis of colonies

#### Timing, 5–7 days

- 28| When the colony is ready for passaging, use EDTA to passage as described in Steps 1–7. However, leave some of the cells in the original well of the 24-well plate while transferring most or them to a new Matrigel-coated well on a 12 - or

6-well plate (again in 1x Rock inhibitor). Continue to incubate the cells, replacing the medium daily and passing as described in Steps 1–7 when cells become confluent. Once you have one well in a six-well plate ready to passage, freeze two vials of cells (option A) and leave some cells (~10–20%) in the well to continue growing. Cells can now be further expanded for further cryopreservation (option A), APS staining (option B), analysis of teratoma formation (option C) and other characterizations such as FACS staining.

**Critical Step:** When expanding each colony, keep track of passage number (manually picking and transferring into the 24-well plate, as described in Steps 19–24, is passage 1).

## A) Cryopreservation and recovery of human iPSC colonies

### Timing, 6–10 min per line

- i. Split cells with 1:4 ratio 2–3 d before cryopreservation as described in Steps 1–7.
- ii. When cells reach to ~70–80% confluence, dissociate the cells with the EDTA method (as described in Steps 3 and 4).
- iii. After 2–5 min, carefully aspirate the EDTA solution from the cells and quickly add 1 ml of full E8 medium per well of a six-well plate.
- iv. In a dropwise manner, add an equal volume (1 ml) of 2x cryopreservation medium (20% (vol/vol) DMSO in full E8 medium) into the EDTA-harvested cell suspension to yield a final concentration of 10% (vol/vol) DMSO.
- v. Aliquot 250–500  $\mu$ l of cells into each labeled cryotube. For one well from a six-well plate with 70–80% confluence, it is possible to freeze four to eight vials. During early reprogramming, if iPSCs are more sparse, it is more common to freeze two vials from one well.
- vi. Freeze cells at  $-80^{\circ}\text{C}$  in an isopropanol freezing container for at least 2 h.
- vii. Transfer the cells to liquid nitrogen for long-term storage.
 

**Pause Point:** Cells can be stored in liquid nitrogen for more than 5 years.
- viii. To thaw cells, take the cryotube out of the liquid nitrogen tank and place it directly in a  $37^{\circ}\text{C}$  water bath.
- ix. Gently stir the water with the tube, and check closely for the disappearance of ice.
- x. When only a small ice particle is floating, spray the vial thoroughly with 70% (vol/vol) ethanol and transfer the tube into a biosafety cabinet.
- xi. Transfer the cells into a 15-ml conical tube
- xii. Add 10 ml of E8 medium dropwise while continuously mixing the solution in the tube.
- xiii. Centrifuge cells at 200g for 5 minutes at  $4^{\circ}\text{C}$
- xiv. Carefully aspirate the supernatant and use E8 medium to resuspend the cells. Plate cells into one to three wells of a Matrigel coated six-well plate. ROCK inhibitors can be added into E8 medium when plating, if desired.

**B) APS staining****Timing 1–2 h**

- i. When passaging colonies by the EDTA method (Steps 1–7), take a small portion of the cells and seed a 12-well Matrigel-coated plate.
- ii. When cells have grown and are ready to passage, they are ready for APS staining. By using the BCIP/NBT alkaline phosphatase substrate kit IV, stain the cells for APS. First mix enough reagents for the wells you are staining (0.5 ml per well).
- iii. To 5 ml of 100 mM Tris-HCl, pH 9.5, add two drops of Reagent A and mix well. Add two drops of Reagent B and mix, then add two drops of reagent C and mix (note that Reagents A, B and C are part of the kit).
- iv. Remove the medium from the cells and add 0.5 ml per well of the APS staining reagent mix. Colonies are usually visible after 20 min. When the colonies are visible, remove the liquid; otherwise, wait until the colonies are stained with distinctive signal in contrast to surrounding cells.

**C) Teratoma formation assay with the EDTA method****Timing 6–9 weeks**

- i. After iPSCs are confirmed by different characterization, select a cell line for teratoma formation assay. Split selected cells in a 1:4 ratio (as described in Steps 1–7). This should be done 2–3 d before you plan to inject.
- ii. When cells reach ~70–80% confluence, dissociate the cells with EDTA (as described in Steps 3–4). Meanwhile prethaw 200  $\mu$ l Matrigel on ice and dilute it with 400  $\mu$ l ice-cold E8 medium.
- iii. Collect the cells after the EDTA incubation with 1 ml of E8 medium per well (six-well plate).
- iv. Spin cells in a 1.5-ml Eppendorf tube at 100g for 5 minutes at RT.
- v. Remove the supernatant and resuspend the pellet with 600  $\mu$ l of the E8 medium/Matrigel mixture.
- vi. Take up the cell suspension into a 1000- $\mu$ l syringe, and keep it on ice before injection.
- vii. By using a 20.5 gauge needle, inject 100–300  $\mu$ l of suspension per mouse as soon as possible. For intramuscular injection, the cell suspension should be injected into the muscle center in the hind leg quadriceps along its long axis.
- viii. House mice normally and assess them regularly for the formation of teratomas. These should be seen at ~4 weeks after injection.
- ix. Around 2 weeks after teratomas are first detected, remove the teratoma for histological analysis. **Caution:** Ensure that the method used for removal follows all relevant animal handling and experimentation guidelines.

**Troubleshooting**

Troubleshooting advice can be found in Table 3.

**Timing**

Reagent Preparation, Large-scale medium preparation: 2 d

Steps 1–7, medium certification with EDTA passaging: 6–7 min per plate

Steps 8 and 9, feeding and iPSC maintenance passaging every 3–4 d: (optional) continue for at least 20 d to certify a batch of medium

Steps 10 and 11, fibroblast culture and viral transduction: 3 d

Steps 12–18, reprogramming in chemically defined medium: 3–4 weeks

Steps 19–28, mechanical isolation and colony expansion: 1–2 weeks

Step 28A, cryopreservation, 6–10 min per line

Step 28B, APS staining: 1–2 h

Step 28C, teratoma formation: 6–9 weeks.

## ANTICIPATED RESULTS

Chemically defined E8 medium provides ideal cell culture conditions for human PSC research. By modifying a few growth factors, we are able to create cell culture conditions for human iPSC derivation and expansion (Tables 1 and 2). EDTA dissociation also provides a simple and efficient way to handle stem cells in different circumstances (Table 4). This combination of medium and handling method enables effective culture expansion and other treatments. All these experiments can be performed without drug treatment, but ROCK inhibitor is included in this protocol for those who prefer its use.

When human fibroblasts are reprogrammed in the E8-based feeder-free system, transformed cells are visible 5–6 d after transduction. It takes 20–30 d before true iPSC colonies mature enough for colony expansion (Fig. 2c). Overcrowded fibroblast cells often suppress the emergence of iPSC colonies, and thus EDTA dissociation could be used to differentially enrich iPSCs.

After mechanical isolation of iPSC colonies, EDTA dissociation enables effective expansion and cryopreservation in 1–2 weeks, markedly decreasing the time needed to preserve the cells. Owing to its simplicity, we are able to passage 24 individual lines from a 24-well plate in 15 minutes.

Expanded iPSCs should be first confirmed by APS staining and by pluripotency marker staining using flow cytometry or immunostaining (Fig. 2d,e and Supplementary Fig. 4b). Teratoma formation assays should be performed on selected iPSC lines by injecting cells into SCID mice. Teratomas usually emerge after 4 weeks, and are ready to be analyzed 6–7 weeks after injection.

## Supplementary Material

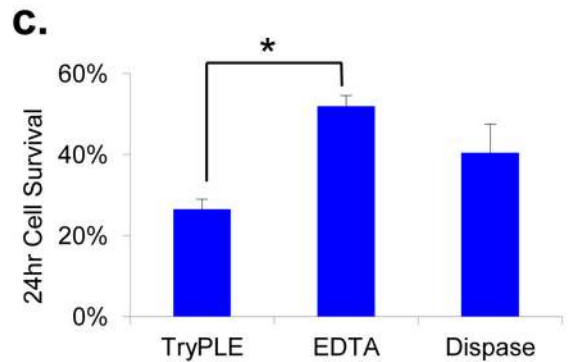
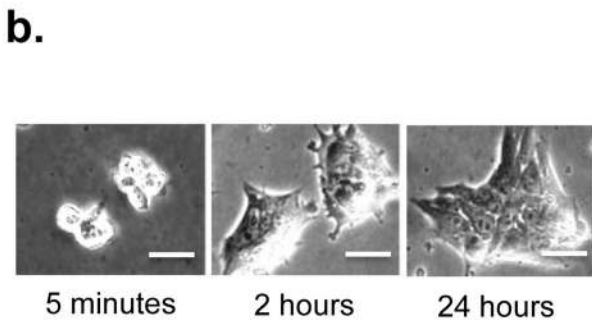
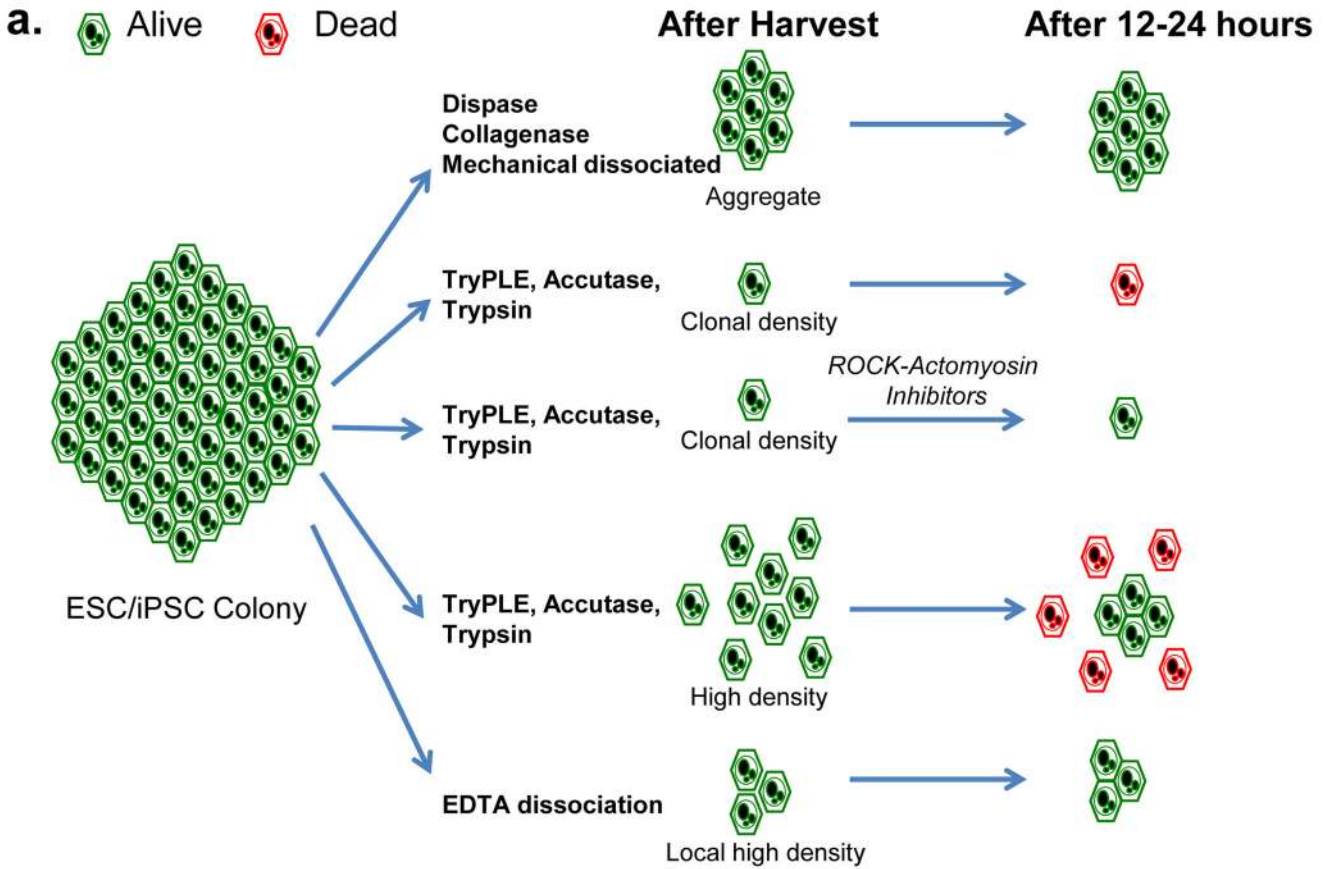
Refer to Web version on PubMed Central for supplementary material.

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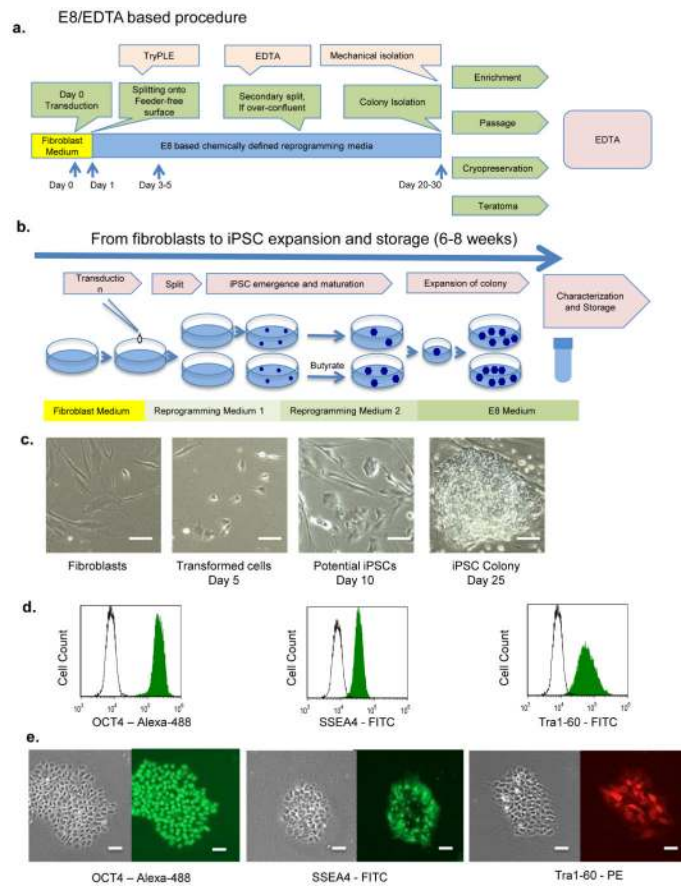
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**Figure 1. EDTA dissociation is an effective method for pluripotent stem cell passaging**  
 a) Summary of cell survival efficiency by different methods. After dissociation, enzyme/mechanic-generated large aggregates survive efficiently; individualized cells die at clonal density, but survive in the presence of inhibitors or high loading density; EDTA-generated small aggregates survive efficiently by high local density without the help of drug treatment.  
 b) Human ESC colony morphology after plating. H1 cells were partially dissociated with EDTA, and they attached to the plate in a few minutes, spread in 2 h, and survived as colonies (24 h). Scale bars, 50 μm. c) Comparison of cell survival of different passaging methods. Cells were dissociated by TryPLE, EDTA or Dispase and added onto Matrigel-



coated plates; next, live cells were counted after 24 h. Asterisk (\*) signifies that the survival by EDTA differed significantly ( $P < 0.05$ ,  $n=3$ ) from TrypLE at 24 h.



**Figure 2. iPSC derivation from human fibroblast cells in chemically defined medium**

a) In the EDTA/E8-based iPSC derivation procedure, EDTA is the major handling method necessary for most cell culture stages. In comparison, the traditional procedure uses multiple dissociation methods (Supplementary Fig. 4a). b) A brief summary of the steps described in this protocol. c) Cell morphology changes in iPSC derivation process. Scale bars, 40  $\mu\text{m}$ . d) FACS analysis of pluripotency markers on iPSCs derived from fibroblasts. Cells were derived in defined medium, and then expanded by the EDTA method. IgG control: unfilled peak; FITC-conjugated antibody: green peak. Antibodies: OCT4-Alexa-488 (Millipore, cat. no. FCMAB113A4); SSEA4-FITC (Biolegend, cat. no. 330410); Tra1-60-FITC (Millipore, cat. no. FCMAB115F); msIgG-FITC and nonimmune control (Millipore, cat. no. MABC006F). e) Immunostaining of pluripotency markers on iPSCs derived from fibroblasts. Left, phase-contrast images; right, fluorescence images. Scale bars, 20  $\mu\text{m}$ . Antibodies: OCT4-Alexa-488 (Millipore, cat. no. FCMAB113A4); SSEA4-FITC (Biolegend, cat. no. 330410); Tra1-60-PE (Biolegend, cat. no. 330610).

Table 1

E8 Base Medium for 5% CO<sub>2</sub>.

Reagent	Stock	Amount required for 1 liter of medium	Amount required for 50 liters of medium	Final concentration	Source
DMEM-F12		1 liter	50 liters		Gibco, cat. no. 11330-032
L-ascorbic acid		64 mg	3200 mg	64 mg per liter	Sigma, cat. no. A8960
Sodium Selenite		0.7 mg per ml 19.4 $\mu$ l	970 $\mu$ l	13.6 $\mu$ g per liter	Sigma, cat. no. S5261
HCl or NaOH		Adjust pH to 7.4			
Sodium Chloride		1 g	50 g		Sigma, cat. no. S5886

Freeze at  $-20^{\circ}\text{C}$  for storageFormula suitable for 10% CO<sub>2</sub> is listed in Supplementary Table 2.

**Table 2**

E8-related media for reprogramming and maintenance

<b>Reagent</b>	<b>E8(TGF-<math>\beta</math>1)</b>	<b>Reprogramming 1</b>	<b>Reprogramming 2</b>	<b>Final Concentration</b>
E8 base medium	500 ml	500 ml	500 ml	
Holo-transferrin	500 $\mu$ l	500 $\mu$ l	500 $\mu$ l	10 $\mu$ g per ml
bFGF	500 $\mu$ l	500 $\mu$ l	500 $\mu$ l	100 ng per ml
TGF- $\beta$ 1	500 $\mu$ l	---	---	1.74 ng per ml
Insulin	1 ml	1 ml	1 ml	20 $\mu$ g per ml
Hydrocortisone	---	50 $\mu$ l	---	1 $\mu$ M
Sodium butyrate	---		500 $\mu$ l	100 $\mu$ M

**Table 3**

## Troubleshooting table

Step	Problem	Possible Causes	Solutions
5	Colonies not coming off	Pipetting too gently	Use a strong motion to rinse most cells off in one shot.
		Insufficient treatment	Extend treatment time
	Too many colonies come off during EDTA treatment	Treating for too long	Shorten treatment time or skip one washing step.
		Plate is disturbed too much during buffer change	Don't shake or swirl the plate during the treatment.
			Cells can be neutralized and collected with E8 medium. Either spin down and plate, or directly plate the suspension and change medium again after 1 h
8	Poor cell survival	Cells have not been passaged frequently enough	Cells need to be passaged around every 4 d. If the culture is beyond 5 d, add ROCK inhibitor
		Cells are overconfluent	Passage the cells when the cells reach to 80% confluence. ROCK inhibitors could be added to help the survival.
		Too much pipetting	Don't repetitively pipet the cells. After sufficient treatment, cells are easily washed off the plate.
		Overtreatment leads to too many single cells	Shorten the treatment time or skip one washing step.
		EDTA concentration is too high	Decrease the EDTA concentration from 5 to 0.5–2 mM.
9	Poor pluripotency marker expression	Reagent quality	Batch test for reagents listed in Table 2.
17	Putative iPSCs do not come off the plate	Confluent plates need more EDTA to remove calcium	Increase EDTA treatment time or add more washing steps.
			If iPSC colonies are rare, aim at potential iPSC colonies to specifically remove iPSCs.
	Poor cell survival	Cells are overconfluent	Add ROCK inhibitor.
26	No attached colony	Colony is too big	Cut the colony to small pieces before harvest.
		Non-iPSC morphology	Colonies picked may not have been iPSC colonies, improve iPSC identification
27	Poor survival	Cells are overconfluent	Add ROCK inhibitor
			Move the culture to hypoxia condition (5% O <sub>2</sub> )

**Table 4**

Comparison of human PSC dissociation protocols.

Method	Dispase	TrypLE/Accutase	EDTA
Cell Survival	Good	Poor	Good
ROCK inhibitor treatment	No	Essential	Not necessary
Rinsing or neutralization of the enzymes	Yes	Yes	No
Centrifugation	No	Yes	No
Time needed for one six-well plate	7–10 min	10–15 min	5–6 min
Damage to extracellular matrix or membrane proteins	Yes	Yes	No
Dependency on different plate coating materials <sup>14</sup>	Not Applicable	High	Low
Accessible to transfection	Less Efficient	Efficient	Efficient
Differentiation	Less sensitive	Sensitive	Sensitive
Cryopreservation	Good <sup>19</sup>	Good with ROCK inhibitors <sup>20</sup>	Good, ROCK inhibitor optional
Stem cell enrichment	No	No	Yes
Batch Consistency	Poor	Good	Good
Teratoma formation	Good	Poor	Good
Source	Commercial	Commercial	Homemade
Cost	High	High	Low