Human-Induced Pluripotent Stem Cell Culture Methods Under cGMP Conditions

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The discovery of induced pluripotent stem cells (iPSCs) revolutionized the approach to cell therapy in regenerative medicine. Reprogramming of somatic cells into an embryonic-like pluripotent state provides an invaluable resource of patient-specific cells of any lineage. Implementation of procedures and protocols adapted to current good manufacturing practice (cGMP) requirements is critical to ensure robust and consistent high-quality iPSC manufacturing. The technology developed at Allele Biotechnology for iPSC generation under cGMP conditions is a powerful platform for derivation of pluripotent stem cells through a footprint-free, feeder-free, and xeno-free reprogramming method. The cGMP process established by Allele Biotechnology entails fully cGMP compliant iPSC lines where the entire manufacturing process, from tissue collection, cell reprogramming, cell expansion, cell banking and quality control testing are adopted. Previously, we described in this series of publications how to create iPSCs using mRNA only, and how to do so under cGMP conditions. In this article, we describe in detail how to culture, examine and storage cGMP-iPSCs using reagents, materials and equipment compliant with cGMP standards. © 2020 The Authors.

Basic Protocol 1: iPSC Dissociation
Support Protocol 1: Stem cell media
Support Protocol 2: ROCK inhibitor preparation
Support Protocol 3: Vitronectin coating
Basic Protocol 2: iPSC Cryopreservation
Basic Protocol 3: iPSC Thawing

Keywords: cGMP • induced pluripotent stem cells • iPSC • mRNA reprogramming • xeno-free

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How to cite this article:
Rivera, T., Zhao, Y., Ni, Y., & Wang, J. (2020). Human-induced pluripotent stem cell culture methods under cGMP conditions. *Current Protocols in Stem Cell Biology*, *54*, e117. doi: 10.1002/cpsc.117

INTRODUCTION

The arrival and development of induced pluripotent stem cells (iPSCs), thanks to Dr. Yamanaka and other stem cell researchers (Takahashi & Yamanaka, 2006), provided new possibilities to cell therapy and regenerative medicine. iPSCs are derived from somatic cells that are reprogrammed back into an embryonic-like pluripotent state by forced

expression of key transcription factors (Takahashi et al., 2007; Yu et al., 2007). iPSCs have the capacity to self-renew and the potential to differentiate into all cell lineages of the three germ layers. Streamlined cGMP cell reprogramming allows generation of patient-specific differentiated cells to replace diseased or damaged tissues. In addition to regenerative medicine, iPSCs have numerous biomedical applications in drug screening, toxicological studies, and disease modeling (Shi, Inoue, Wu, & Yamanaka, 2017).

Prior to 2007, derivation of pluripotent stem cells from pre-implantation embryos, known as embryonic stem cells (ESC), opened new avenues for the development of treatments of many degenerative diseases. However, generation of human ESC lines raises ethical and political concerns as it requires the destruction of human embryos (Aach, Lunshof, Iyer, & Church, 2017). In addition, the use of differentiated cells from ESC for clinical applications suffers from the concerns of immune rejection after allogenic cell transplantation (de Almeida, Ransohoff, Nahid, & Wu, 2013). Therefore, the use of iPSC in regenerative medicine holds great promise as a therapeutic product circumventing the ethical problems associated with the use of human embryos and immune rejection after transplantation.

The development of directed differentiation protocols using human iPSC into various cell types has allowed the initiation of several cell-therapy human trials based on iPSCs. The first reported clinical trial involving iPSC-derived retinal pigment epithelial cells was designed to treat the Age-Related Macular Degeneration (AMD) (Mandai, Kurimoto, & Takahashi, 2017). In 2018, the Kyoto University announced the first clinical trial to treat Parkinson's disease using iPSC-derived dopaminergic neurons to be transplanted into human patients (Takahashi, 2019). iPSC-derived cells are also used in clinical trials for cancer immunotherapy to treat advanced solid tumors (ClinicalTrials.gov Identifier: NCT02923375). Recently, a clinical trial using iPSC-derived cardiomyocytes has been approved for heart diseases (Cyranoski, 2018).

As any therapeutic material to be applied to human, iPSC-derived clinical products should be well defined for the safety and efficacy of the therapeutic applications, and development of clinical-grade iPSC lines suitable for various applications as a starting material for cell therapy is a priority to the whole field.

This article describes the procedures for human iPS cell culture and handling techniques compliant with cGMP regulations in order to generate high quality iPSC for use in clinical applications. This protocol has been tested and validated using multiple cGMP-grade iPSC lines generated with Allele reprogramming core technology at Allele's GMP facility in San Diego, California. To adhere to cGMP regulations for cell manufacturing, reagents, materials, and equipment compliant with cGMP standards were used and the best practices for cell culture and quality control were adopted. In addition to the implementation of this protocol for iPSC culture, a cGMP-compliant facility that meets the FDA regulations and requirements imposed by institutional review boards (IRBs) is required to successfully obtain cGMP compliant iPSC.

Basic Protocol 1 covers iPSC dissociation procedures during iPSC expansion and selection. cGMP stem cell media is described in Support Protocol 1. ROCK inhibitor reconstitution is detailed in Support Protocol 2 and coating of culture vessels in Support Protocol 3. How the cGMP iPSC lines are cryopreserved is described in Basic Protocol 2 and finally, how the iPSC frozen stocks are thawed is outlined in Basic Protocol 3.

STRATEGIC PLANNING

Manufacturing of high-quality iPSCs using a robust cGMP-compliant process is essential for iPS cell-based therapies, suitable methods for every step throughout iPS cell

manufacturing should be established to assure the desired high quality. Using cGMP-grade and xeno-free reagents is required to ensure defined and consistent culture conditions for iPSC manufacturing. Well-characterized and quality-controlled solutions and reagents must be obtained from reliable sources and the corresponding Certificate of Analysis should be acquired. Routine iPSC cell culture manipulations should be performed inside a Class II biological safety cabinet (BSC). All reagents and materials described in this protocol are sterile and should be disinfected with proper aseptic techniques before introducing into BSC. During iPSC culture under cGMP conditions there are critical factors and components that require detailed evaluation as discussed below.

## Critical Factors of iPSC Culture

Cell morphology

Evaluation of the culture process is critical to ensure manufacturing of high-quality iPSCs. It is, therefore, essential to examine iPS cell culture every day under a microscope to check for iPSC-like morphology, presence of any differentiated cells and cell culture confluency. Cell morphology of human iPSC has been reported to resemble that of human ESC (Takahashi et al., 2007), these characteristics include a high nucleus:cytoplasm ratio, prominent nucleoli, scant cytoplasm, and formation of compact colonies with clear and smooth colony edges (Yu et al., 2007) (Fig. 1).

High-quality iPS cells are attainable only when optimal cell culture conditions are achieved. In order to maintain cell growth in exponential phase it is important that iPSCs be sub-cultured every 4-5 days according to cell density described below; if the density of the culture is too high, iPSCs tend to grow as 3D-like aggregates and cell viability decreases. Representative images of iPSC cultures grown to different density are shown in Figure 2.

Cell proliferation

Maintenance of indefinite self-renewal and unlimited expansion potential are fundamental hallmarks of successful stem cell culture. ESCs and iPSCs present a short cell cycle and their doubling time is approximately 16-20 hr (Hanna et al., 2010), allowing for very fast cell division, which supports pluripotency. When pluripotent stem cells differentiate, the cell cycle duration lengthens to >24 hr, which is the typical cell cycle of a differentiated somatic cell (Becker, Stein, Lian, van Wijnen, & Stein, 2010). Several
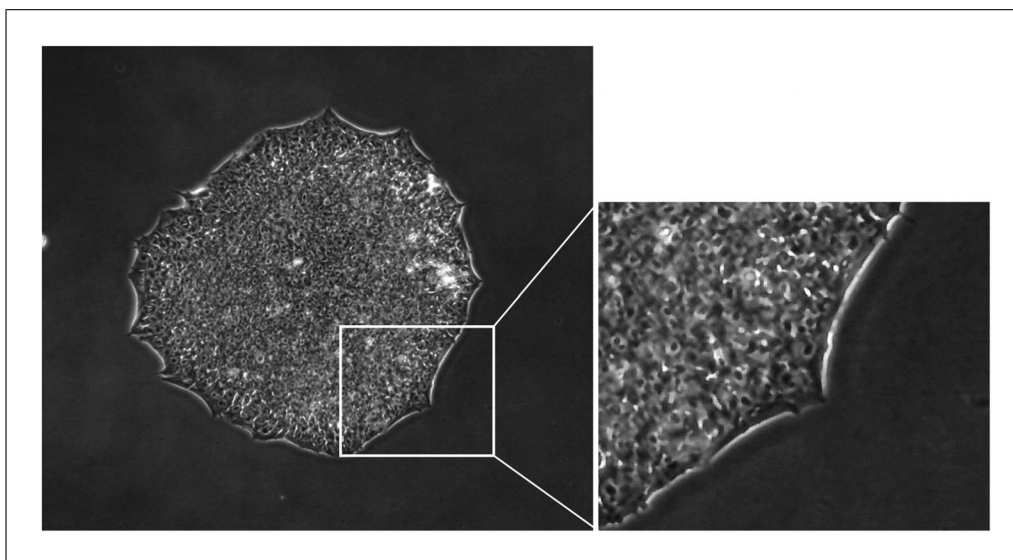
Figure 1 iPSC Morphology. iPSC colony comprised of highly packed cells and well-defined borders. Enlargement of the colony is shown on the right. 20× magnification.
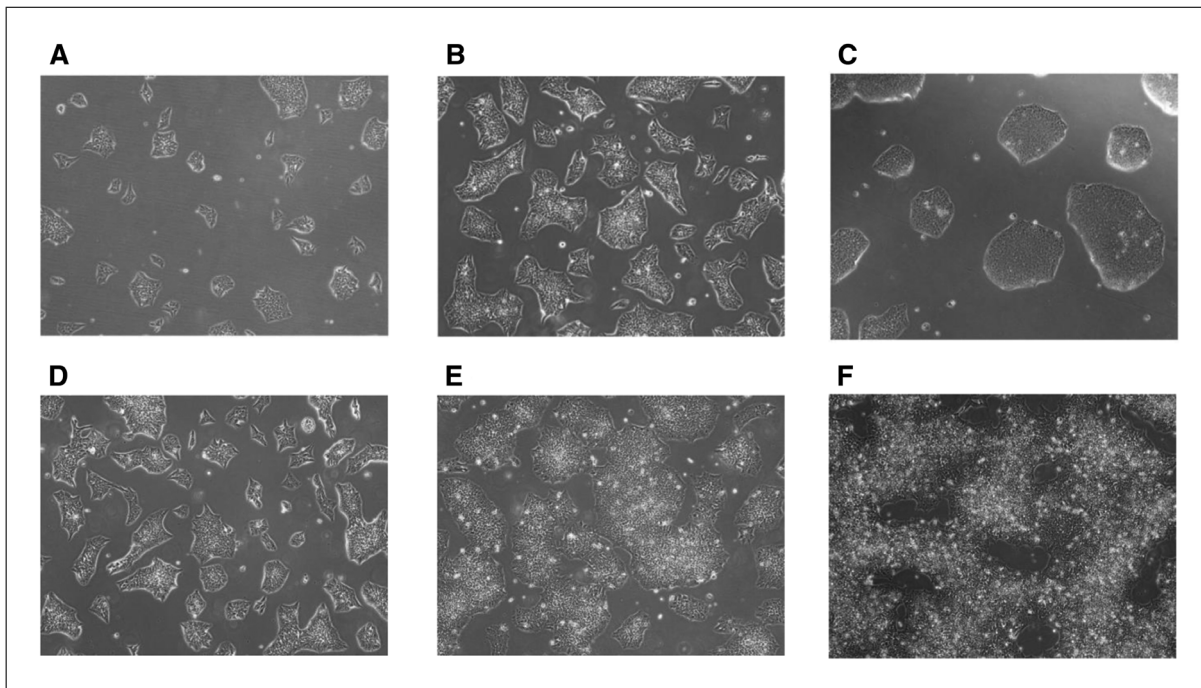
Figure 2 Confluency of iPSC culture. (**A**) Small- and medium-sized iPSC colonies. (**B** and **C**) Medium- and large-sized iPSC colonies. (**D**) iPSC culture at 40% confluence. (**E**) iPSC culture at 60%-75% confluence. (**F**) Overgrown iPSC culture. 10× magnification.

reports showed that elongated or arrested cell cycle results in spontaneous differentiation (Neganova, Zhang, Atkinson, & Lako, 2009; Ruiz et al., 2011). Accordingly, analysis of cell proliferation and stem cell renewal was used as a test method to evaluate robustness of iPS cell culture.

Cell proliferation is determined by analyzing the doubling time of the cell culture. The information required for calculation of the doubling time includes: date, starting and ending time of the culture, as well as initial and final cell number.

Doubling time is calculated as follows:

$$\text{Doubling time} = \text{duration of the culture} \times \log\left(2\right) / \left[\log\left(\text{total cells harvested}\right) - \log\left(\text{cells plated}\right)\right].$$

We have established and validated a quality control test to perform the "Cell Counting and Viability" assay of iPSC cultures. This assay is used to determine iPS cell number, viability, and proliferation. We have incorporated a cGMP-compliant automated cell counter into our iPSC culture platform that allows determination of the viability and cell concentration of aggregating cells such as iPSCs. The cell counter includes fluorescent dyes to determine the total cell number and dead cell population of a given sample using image analysis. Since it allows a cell count of aggregated cells, no single-cell dissociation of the iPSC culture is required to perform the analysis.

Critical Components of iPSC Culture

Stem cell medium

The quality and consistency of the stem cell medium is crucial to the maintenance and expansion of iPSCs. Essential 8™ (E8) Medium is a fully defined, xeno-free, and feeder-free medium formulated for the growth and expansion of human pluripotent stem cells. It was originally developed by Chen et al. (2011) and showed to support iPSC growth for >50 passages.

cGMP-compliant E8 medium (Gibco^{TM}, ThermoFisher, Waltham, MA) was evaluated to support iPSC growth during our Process Development phase. We have observed high proliferation rates (doubling time around 20 hr) and typical iPSC morphology in cultures grown in cGMP-E8 medium. Consequently, we established cGMP-E8 as a suitable medium for iPSC manufacture.

Essential 8^{TM} Medium has been extensively tested and is proven to be able to maintain pluripotency in multiple pluripotent stem cells lines. Unlike most feeder-free media, Essential 8^{TM} medium does not require the presence of BSA (bovine serum albumin) or HSA (human serum albumin) that contributes to lot-to-lot variability.

Cell culture matrix

We aimed for and have established a defined iPSC culture system that is completely xeno-free, including both the medium and vessel coating material. We previously showed that the synthetic peptide-based surface-coating Synthemax II-SC (Corning), certifiable under cGMP, could be used to support iPSC growth (Ni, Zhao, Warren, Higginbotham, & Wang, 2016). We have further explored the use of other extracellular matrices (ECM) manufactured under cGMP guidelines.

We evaluated Vitronectin (VTN) as a xeno-free ECM to culture iPSC under cGMP conditions. VTN is a recombinant human protein used as ECM substrate, which was shown to support human pluripotent stem cell sell-renewal (Braam et al., 2008). The vitronectin variant, VTN-N, lacks the N-terminal SMB functional domain of the native vitronectin and has been shown to support human pluripotent attachment and survival better than wild-type vitronectin (Chen et al., 2011).

A cell culture system based on the above-described cGMP components provides an optimal system for the in vitro maintenance of iPSC, supporting self-renewal and high proliferative rates in a way that is highly consistent and practical for cGMP operations. Pluripotent stem cells maintained on CTS^{TM}-VTN-N matrix appear to be homogeneous of typical iPSC colonies with no spontaneous differentiation observed even after long-term culturing.

Cell culture conditions/oxygen

iPSC cultures should be maintained in a multi-gas incubator to provide a stable environment of 37°C, 5% CO_2, 5% O_2 and relative humidity of about 95%. CO_2 and O_2 sensors should be calibrated regularly according to cGMP Equipment and Operation Qualification schedules to ensure accurate measurements.

These parameters are defined based on the culture requirements of iPSC. E8 Base Medium is suitable for 5% CO_2 conditions providing a pH of 7.4. Moreover, regulation of oxygen concentration towards physiological levels (2%-5%), which is natural to pluripotent stem cells, is critical for reducing spontaneous differentiation and chromosomal abnormalities while supporting stem cell renewal and increasing cell proliferation (Dakhore, Nayer, & Hasegawa, 2018). During our Process Development, low oxygen tension at 3%-5% was found to be optimal.

Cell density

Maintaining a proper colony density is another critical aspect of growing human iPS cells in vitro. Frequent dissociation of the iPSC colonies can cause cell stress and poor cell survival; on the other hand, chromosomal abnormalities and spontaneous differentiation may arise in overgrown iPSC cultures. Therefore, it is essential to find the proper cell density in order to maintain cultures of high quality iPSCs.
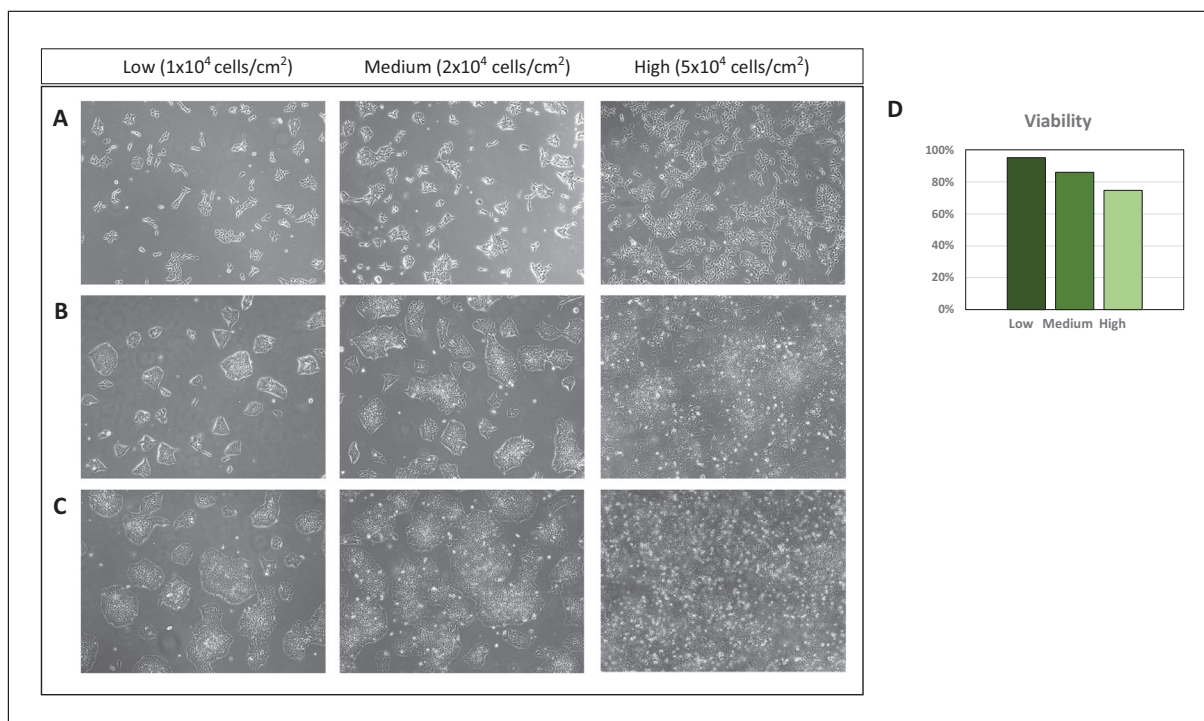
Figure 3 Cell seeding density. iPSCs were seeded at three different cell densities: low (1×10^4 cells/cm^2), medium (2×10^4 cells/cm^2), or high (5×10^4 cells/cm^2) in VTN-coated flasks and cultured at 37°C, 5% CO_2, 5% O_2 for 3 days. Representative images 24 hr (**A**), 48 hr (**B**), and 72 hr (**C**) after seeding. (**D**) Cell viability at 72 hr post-seeding from each cell density culture. 10× magnification.

The criteria used in assessing the timing for iPSC dissociation was based on colony size or overall confluency of the culture (Fig. 2A-F). We have determined that iPSCs should be passaged when the colonies have reached medium-to-large colony size (Fig. 2B and C) or when culture reaches approximately 60% to 75% confluency (Fig. 2E) to ensure healthy high proliferative cultures. Microscopic observation is therefore essential to evaluate cell culture confluency. Overgrown cultures can be easily detected as increased number of non-viable cells remain floating in the media and the center of the colonies appear to be densely packed, compromising cell viability of the culture (Fig. 2F).

To reliably and consistently achieve the accuracy of cell density, we perform three cell counts every time and calculate their average. We have evaluated different cell seeding numbers and tested cell viability under these conditions. When cell seeding was high, reduced cell viability was found and frequent cell dissociation was required. Thus, in order to avoid frequent dissociations of the colonies that can lead to poor cell survival, we recommend cell seeding numbers ranging from 1×10^4 to 2×10^4 cells/cm^2 (Fig. 3). When seeding cells near the lower limit of the optimal range, typically a 4-day dissociation/splitting interval can be established for fast dividing cell populations.

iPSC DISSOCIATION

BASIC PROTOCOL 1

Culture conditions, including cell passaging methods, affect the genetic and epigenetic stability of iPSCs; importantly, enzymatic passaging of iPSCs is associated with increased genomic instability of iPSCs (Garitaonandia et al., 2015). To circumvent this problem, enzyme-free dissociation methods, such as the EDTA-based passaging procedure, have been developed to successfully derive and expand iPSC (Beers et al., 2012).

CRITICAL STEP: During iPSC selection and expansion, cell survival is the priority. Procedures related to iPSC dissociation should be performed quickly to avoid keeping cell cultures outside of incubator for long periods of time. We recommend completing this procedure in approximately 30 min or less.

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For iPSC expansion, we recommend treatment with a dissociation reagent (Allele Biotechnology) for 2 to 5 min at 37°C to preserve iPSC clusters. Microscope observation can be performed every 2 min to evaluate the dissociation of the colonies. iPSCs are ready for dissociation when cells become rounded and separated throughout the colony, usually after 3 min of treatment (Fig. 4C), and small clusters of iPSCs are dissociated from the original tissue culture vessel. Tapping of the tissue culture vessel after treatment was found to induce dissociation of the colonies into single cells, therefore gentle rocking, instead of tapping, is recommended to preserve iPSC clusters.

Materials

E8: Essential 8™ Medium, cGMP grade (Gibco™, ThermoFisher, cat. no. A1517001)

ROCK inhibitor (ROCKi): 1 mM ROCK inhibitor solution in water (see Support Protocol 2)

DPBS with no calcium and magnesium, cGMP grade (Gibco™, ThermoFisher, cat. no. 14190144)

iPSC culture

70% USP-grade isopropanol wipes, Contec™ PROSAT™ Presaturated Knitted Polynit Wipes (Fisher Scientific, cat. no. 19-120-817)

Dissociation reagent, EDTA-containing solution, (Allele Biotechnology, cat. no. ABP-SC-DR100)

Multi-gas Incubator (Panasonic MCO, CO_2/O_2 Incubator)

Vitronectin- (VTN) coated flasks (see Support Protocol 1)

Conical tubes (ThermoFisher™, Nunc™, 15 ml and 50 ml, cat. no. 339653 and 339651)

Microscope (Live Technologies EVOS™ XL Core Imaging System, cat no. AMEX1000)

Automated Cell Counting instrument (ChemoMetec NucleoCounter® NC-200™ System)

Dissociation of iPSCs from VTN-coated flasks

1. Prior to cell dissociation, reagents including: DPBS (without calcium and magnesium), dissociation reagent, and E8 should be pre-warmed at 37°C from 30 min to 2 hr.

   CRITICAL STEP: *Repeated pre-warming of E8 medium may reduce the effectiveness of growth factors in cell culture media; therefore, only the amount of E8 medium required for each procedure should be pre-warmed.*

2. Prepare the amount of E8 + ROCKi required for each procedure according to Table 1.
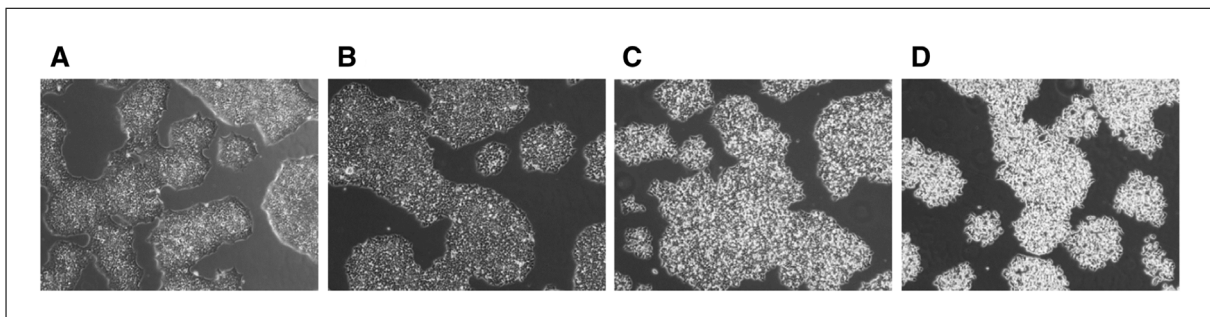
Figure 4 Dissociation of iPSCs. (**A**) iPSCs before dissociation. (**B**) Treatment of iPSCs with dissociation reagent for 1 min at 37°C; cells inside the colony become loose but colonies are not ready to dissociate. (**C**) iPSCs treated for 3 min at 37°C with dissociation reagent; cells inside the colony become rounded and uniformly separated, cells are ready for dissociation. (**D**) After treatment of iPSC for 5 min at 37°C with dissociation reagent, colonies are dissociated to small clusters and single cells. 10× magnification.

3. Retrieve the iPSC culture from the incubator, disinfect the outside with 70% iso-propanol wipes, and transfer into BSC.

4. Aspirate medium from iPSC culture that is about to be dissociated.

5. Add pre-warmed DPBS (with no calcium and magnesium) to rinse the cells. Incubate for 1 min at room temperature in the BSC.

   Recommended DPBS volumes: 3, 5, and 50 ml for 12.5-, 25-, and 225-cm^2 flasks, respectively.

6. Aspirate DPBS from the flask.

7. Add pre-warmed dissociation solution and spread gently by rocking the flask side to side. Incubate for 1 min at room temperature in BSC.

   Recommended dissociation solution volumes: 1, 3, and 15 ml for 12.5-, 25-, and 225-cm^2 flasks, respectively.

   CRITICAL STEP: *Dissociation solution should be added slowly to the wall of the flask to avoid washing the iPSC colonies off the flask.*

8. Carefully remove most of dissociation solution without drying out the cells. Incubate the flask in the incubator for 2-5 min.

   The purpose of removing most of the dissociation reagent is to eliminate the need of additional centrifugation steps to remove the dissociation reagent. We recommend removing 50%-70% of dissociation solution, which allows enough dissociation of iPSCs without drying out the cell culture. Longer incubation times will result in smaller colony size. It is important to check the cells under microscope after incubation with dissociation solution to confirm that ~90% of the cells have rounded up (see Fig. 4). If cells are sticking to the surface of the flask, gentle tapping of one side of the flask will help releasing iPSC form cell culture surface.

9. Transfer the required volume of E8+ROCKi into the flask (3 ml for 12.5-cm^2 and 25-cm^2 flasks and 15 ml for 225-cm^2 flasks), pipette cell suspension up and then gently allow it to flow back down over the cell culture surface to help release remaining iPSC colonies. Transfer the entire cell suspension to conical tube and gently pipette up and down to suspend iPSCs without breaking apart the colonies.

   If iPSC counting and viability tests are required, pipette up and down cell suspension approximately 5 times to mix and transfer a small volume (0.3 ml to 0.5 ml) to the microcentrifuge tube to perform cell counting. Follow the corresponding aggregated cell counting protocol according to manufacturer's instructions.

Plating iPSCs on VTN-coated flask

10. Disinfect with 70% isopropanol wipes and transfer a new VTN-coated flask into BSC. Aspirate the coating solution, transfer the required volume of cell suspension and E8+ROCKi into new flask according to Table 1.

    CRITICAL STEP: *iPSC seeding numbers suggested by us have been optimized for fast dividing cell populations. If the cells show slower doubling times, the seeding number*

Table 1. Required Volume of Reagents and iPSC Seeding Number According to Flask Size

Flask size	DPBS without calcium and magnesium	Dissociation reagent	E8+ROCKi	iPSC seeding number
12.5 cm^2	3 ml	1 ml	3 ml	<5E5
25 cm^2	5 ml	3 ml	5 ml	5E5~1E6
225 cm^2	50 ml	15 ml	50 ml	1E6~5E6

should be adjusted accordingly. If cell counting is not required, 1:6 to 1:10 split ratio is recommended.

11. Shake the flask back and forth and side to side to distribute the cells evenly. Place the flask in the incubator (settings: 37°C, 5% CO_2, 5% O_2).

12. On the next day, observe the cells under the microscope to confirm cell attachment and confluency.

13. Monitor the cell culture daily and evaluate readiness to cell dissociation according to cell density.

STEM CELL MEDIA

Complete Essential 8™ Medium is prepared by adding Essential 8™ Supplement (50×, provided with the medium) to Essential 8™ Basal Medium. Once E8 complete medium is prepared, it should be stored up to 2 weeks at 2°C-8°C.

To ensure adequate nutrition levels in a highly proliferative iPSC culture and avoid spontaneous differentiation, media change every day is recommended. Cell proliferation is optimal at 37°C whereas low temperatures can slow down cell growth and negatively impact the quality of cells; therefore, media used for daily change should be pre-warmed at 37°C. On the other hand, previous studies have shown that growth factors in stem cell media are sensitive to proteolysis and thermal denaturation and degradation of some of these factors are observed after 24 hr at 37°C (Levenstein et al., 2006). Thus, long-term exposure to 37°C of E8 medium should be avoided prior to use. During our process development work, we have found optimal performance of cGMP-E8 medium can be achieved by warming the medium between 30 and 120 min.

ROCK INHIBITOR PREPARATION

Human embryonic stem cells are prone to cell death after individualization during cell dissociation treatments. Rho-associated protein kinase (ROCK) inhibitor has been shown to increase cell survival of dissociated cells by preventing dissociation-induced apoptosis in human embryonic stem cells (Watanabe et al., 2007). Therefore, we recommend adding ROCK inhibitor (ROCKi) to E8 medium at concentrations of 5 or 10 µM after cell dissociation, which is not necessary during daily iPSC culture medium change without passaging.

ROCK inhibitor (Y-27632, Sigma Aldrich, cat. no. SCM075) is supplied as a solid powder in 5 mg vials. For reconstitution, add 14.8 ml sterile cell culture grade water to make 1 mM stock solution. Dividing into small volume aliquots is recommended (30- and 250-µl aliquots are used in this protocol). Aliquots are stored up to 1 year at −80°C per Allele's cGMP protocol. We have evaluated working ROCKi concentrations of 5 and 10 µM in E8 medium and demonstrated that both concentrations efficiently inhibit apoptosis of dissociated hiPSCs and promote cell survival without affecting pluripotency. We routinely add ROCKi to E8 medium at 5 µM. Whenever relevant in the following protocols, ROCKi from Sigma Aldrich will be included at such a concentration. Note that cGMP grade ROCKi is now also available from Tocris Bioscience and can be incorporated into our protocol described below once it passes the Quality Control Testing in our cGMP system.

VITRONECTIN COATING

We have tested cell therapy-grade Vitronectin (CTS™-VTN-N, Gibco™, ThermoFisher Scientific) for iPSC culture and observed consistent support of pluripotent stem cell

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renewal. VTN-N (Gibco[TM], ThermoFisher) is provided in 1-ml aliquots at 0.9 mg/ml by the manufacturer with a recommended coating concentration range of 0.1-1.0 μg/cm^2 on the culture vessel surface. We recommend using a solution concentration at 9 μg/ml with a minimum amount of 0.9 μg/cm^2.

Materials

Vitronectin: CTS[TM]-VTN-N solution at 0.9 mg/ml (Gibco[TM],ThermoFisher, cat. no. A27940)

DPBS without calcium and magnesium, cGMP grade (Gibco[TM], ThermoFisher, cat. no. 14190144)

12.5-cm^2 Non-treated cell culture flasks (Thomas Scientific, cat. no.1228K57)

25-cm^2 Non-treated cell culture flasks (Thomas Scientific, cat. no. 1194Z14)

75-cm^2 Non-treated cell culture flasks (Thomas Scientific, cat. no. 1194Z15)

225-cm^2 Non-treated cell culture flasks (Thomas Scientific, cat. no. 1194Z17)

1.5-ml Polypropylene tubes (Axygen[TM] Scientific, cat. no. SCT150SSCS)

15- and 50-ml Conical tubes (ThermoFisher[TM], Nunc[TM,] cat. no. 339653 and 339651)

Coating flasks with vitronectin

1. Prior to use, thaw the stock vial of VTN at room temperature and prepare small aliquots (12.5-, 25-, 75-, and 225-μl aliquots for 12.5-, 25-, 75-, and 225-cm^2 flasks, respectively) in polypropylene tubes. Freeze aliquots up to one year at $-80°C$.

2. Prior to coating, thaw one aliquot of VTN at room temperature and dilute VTN in DPBS (with no calcium and magnesium) at 1:100 and gently resuspend by pipetting up and down.

3. Add diluted VTN solution to each flask at 0.1 ml/cm^2, close the flask tightly and tap the flask to spread and cover the entire cell culture surface with the coating solution.

4. Place the flask into BSC for 1 min and then make sure that the solution covers the whole surface of the flask.

   The purpose of this short incubation time in the BSC is to ensure homogeneous distribution of the coating solution over the surface of the vessel before placing it into incubator.

5. If the coating solution does not cover the entire surface, gently tap the flask again to help homogeneous distribution of the coating reagent. Once an even coating is achieved, incubate the coated flask for 1 to 3 hr at 37°C.

   CRITICAL STEP: *Uneven coating coverage will result in poor iPSC attachment after seeding. Make sure that the entire surface is covered both before and after incubation at 37°C. Incubation can be performed at room temperature; however, better coating is achieved at 37°C.*

6. Prior to use, aspirate the VTN solution and discard.

   VTN-coated flasks can be stored for up to 3 days at 4°C and wrapped to avoid drying. However, optimal results are achieved when coated flasks are used the same day. If a coated flask is stored at 4°C, pre-equilibration of the flask in the incubator is recommended prior to use.

iPSC CRYOPRESERVATION

The quality of iPSC can be severely influenced by culture conditions and culture manipulation techniques, with cryopreservation being a major potential obstacle to maintaining iPSC quality. Human embryonic stem cells typically suffer from very low post-thaw recoveries. Similarly, cryopreservation is one of the most critical steps after iPSC expansion. Suboptimal cryopreservation methods lead to poor recovery of iPSCs after thawing, resulting in low survival rate of their population. The low cryopreservation efficiency of

human pluripotent stem cells often leads to the arising of abnormal cells as well. To counter this, we (as described below) and others (Liu & Chen, 2014) have extensively tested and achieved technical improvements in cryopreservation methods.

Cryopreservation parameters, including components of cryopreservation medium and methods for processing samples during cryopreservation, were thoroughly evaluated in our Process Development:

-Methods: Optimization of cryopreservation procedure begins with good training of operators to ensure robustness and consistency to provide uniform cell population for downstream clinical applications. Critical steps during cryopreservation should be performed in a timely and efficient manner to preserve cell viability, especially when generating Master Cell Banks (MCB). The protocol shown here for cryopreservation of iPSC has been validated for large cGMP banking and, importantly, provides a robust method and easily performed by different operators.

-Cell freezing containers: The CoolCell® freezing containers available from BioCision (San Rafael, California) have already been adopted for use in cGMP cleanrooms. These containers provide highly reproducible, alcohol-free $-1°C$/min controlled rate freezing when transferred to a $-80°C$ freezer. The $-80°C$ freezer must follow the cGMP requirements for temperature-controlled storage equipment.

-Cryopreservation medium: A critical component of the cryopreservation medium is the cryopreservant dimethyl sulfoxide (DMSO), we established that DMSO concentration at 10% in E8 medium provides high cryopreservation efficiency.

Protein-rich medium has been shown to increase cryopreservation efficiency; thus, serum is commonly added to cryomedium in most research labs. However, medium containing serum is not recommended to manufacture cGMP-grade iPSC. Human serum albumin (HSA) had been widely used as replacement of serum for human pluripotent stem cell cultivation (Ludwig et al., 2006). Along this line, we investigated cryopreservation efficiencies using cryomedium containing different concentrations of HSA and found high cryopreservation efficiency in medium containing 1% or 2.5% HSA. Post-thaw cell viability of iPSC lines cryopreserved under these conditions was generally higher than 90% (Fig. 5).

Therefore, to ensure high cryopreservation efficiencies and good cell survival (>90% viability) we recommend cryopreserving iPSCs in E8 medium containing 10% DMSO and HSA at concentrations ranging from 1% to 2.5%. We routinely use cryomedium containing 1% HSA but this concentration can be adapted according to each cell line's growth conditions.

The following procedure describes cryopreservation of iPSC at a concentration of 1×10^6 cells /ml in 1 ml of cryopreservation medium.

Volume of cryopreservation medium and number of cryogenic vials to prepare are determined by the results of live cell number of iPSCs obtained during cell count of the flask being cryopreserved.

If only a fraction of the iPSCs are to be cryopreserved, calculate the volume of cryopreservation medium accordingly, but maintain concentration of 1×10^6 cells/ ml to preserve high survival rate.

Materials

70% USP-grade isopropanol wipes, Contec[TM] PROSAT[TM] Presaturated Knitted Polynit Wipes (Fisher Scientific, cat. no. 19-120-817)
DMSO: Dimethyl Sulfoxide, USP grade (Sigma Aldrich, cat. no. D2438)
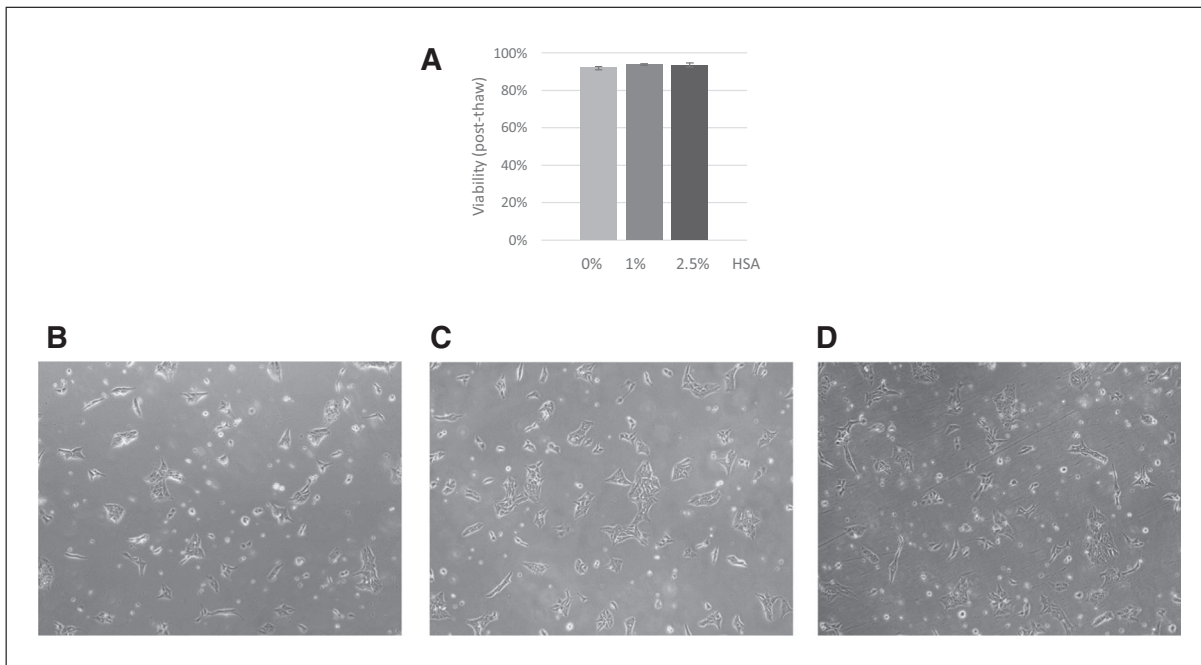
Figure 5 HSA concentration in cryopreservation medium. Comparison between cryopreservation medium containing different concentrations of HSA for cell recovery after cryopreservation. (**A**) Cells were thawed in E8 medium with ROCKi and cell viability was determined after thawing. Error bars indicate standard deviation ($n = 3$). (**B-D**) Representative images of iPSC cultures 24 hr after thawing, iPSCs were cryopreserved in E8 medium with 10% DMSO and 0% (**B**), 1% (**C**) or 2.5% (**D**) HSA. $10\times$ magnification.

HSA: Human Serum Albumin (100 mg/ml), USP grade (Irvine Scientific, cat. no. 9988)

E8: Essential 8™ Medium, cGMP grade (Gibco™, ThermoFisher, cat. no. A1517001)

ROCK inhibitor (ROCKi): 1 mM ROCK inhibitor solution in water (see Support Protocol 2)

1.2-ml Cryogenic vials (Corning® External Thread Cryogenic Vials, cat. no. 430658)

60-ml Reagent bottle (Thermo Scientific, cat. no. 3420200060)

Cell freezing container, CoolCell® BioCision

Automated cell counting instrument, ChemoMetec NucleoCounter® NC-200™ System

Ultra-low freezer, Panasonic MDF-U76VC-PA

Collecting iPSCs and preparing cryopreservation medium

1. Perform iPSC dissociation and cell count as described in the Basic Protocol 1, steps 1 to 9.

2. Calculate volume of cryopreservation medium according to Table 2.

3. Prepare cryopreservation medium as follows:

    a. Disinfect with 70% isopropanol wipes and transfer into BSC: cryogenic vials, one reagent bottle, consumables and reagents including, E8 medium aliquot, ROCKi aliquot(s), DMSO vial(s), HSA aliquot(s).

    b. Add corresponding volume of E8 and ROCKi (according to Table 2) to the reagent bottle.

    c. Transfer the required volume of DMSO (according to Table 2) to the reagent bottle. Swirl the bottle gently while adding DMSO to help mix evenly.

    d. Transfer the required volume of HSA (according to Table 2) to the reagent bottle.

Table 2. Cryopreservation Medium Formulation

Required volume	Abbreviation	Formula
Cryopreservation medium (ml)	Vol. cryo	Vol. cryo = Total live cell number[a]/1 × 10^6
E8 (ml)	Vol. E8	Vol. E8 = (Vol. cryo × 0.8) – Total volume of cell suspension[b] (ml)
ROCK inhibitor	Vol. ROCKi	Vol. ROCKi = Vol. E8 (ml)/200
DMSO	Vol. DMSO	Vol. DMSO = Vol. cryo (ml) × 0.1
HSA	Vol. HSA	Vol. HSA = Vol. cryo (ml) × 0.1

[a]Total live cell number: obtained from cell count and viability test.
[b]Total volume of cell suspension: volume of cell suspension in E8+ROCKi in which cells were collected from flask after iPSC dissociation.

    e. Mix the reagents by gently pipetting up and down at least 5 times. Label the reagent bottle as Cryopreservation Medium (CM).

Cryopreserving iPSCs

4. Mix dissociated cells by gently pipetting and transfer the required cell suspension into the reagent bottle containing CM. Carefully mix the cell suspension in CM.

    CRITICAL STEP: *CM should be added slowly to avoid osmotic damage to the cells. Alternatively, if slow speed is difficult to achieve through continuous pipetting, drop-wise addition of CM should be performed. Be careful not to over-pipet the cell suspension to avoid breaking apart iPSC colonies. Once cell suspension is added to CM, next steps should be performed quickly as speedy processing is critical to preserve cell viability. We recommend completing this procedure in approximately 15 min or less.*

5. Transfer 1 ml of cells in CM into each cryotube.

    CRITICAL STEP: *If many aliquots are going to be prepared in cryovials, frequent mixing of cell suspension is required to ensure homogeneous cell suspension in CM.*

6. Upon completion of vialing, immediately place cryogenic vials into cell freezing container and transfer into ultra-low temperature freezer.

7. Transfer cryogenic vials into liquid nitrogen for long-term storage after 24 hr of cryopreservation.

    CRITICAL STEP: *Do not use cryogenic vials for storage in the liquid phase of liquid nitrogen, store cryogenic vials only in the vapor phase.*

iPSC THAWING AND SEEDING

The following steps describe the thawing procedure of one cryogenic vial of iPSC and cell seeding into 75- cm^2 VTN-coated flask.

In our protocols, thawing of cells in automated cell thawing instruments replaces common methods of cell thawing such as water baths to ensure consistency, as rapid and reproducible thawing reduces operation variability and the risk of contamination. Cryovials are retrieved from the liquid nitrogen tank or −80°C freezer and thawed in BioCision ThawSTAR® (BioCision LLC, Larkspur, CA) automated cell thawing instrument according to manufacturer's instructions.

Materials

Cryopreserved iPSC (see Basic Protocol 2)
70% USP-grade isopropanol wipes, Contec™ PROSAT™ Presaturated Knitted Polynit Wipes (Fisher Scientific, cat. no. 19-120-817)

E8: Essential 8^{TM} Medium, cGMP grade (Gibco^{TM}, ThermoFisher, cat. no. A1517001)

ROCK inhibitor (ROCKi): 1 mM ROCKi solution in water (see Support Protocol 2)

Automated Cell Thawing instrument, BioCision^{TM} ThawSTAR$^{®}$

Automated Cell Counting instrument, ChemoMetec NucleoCounter$^{®}$ NC-200^{TM} System

1.5-ml Microcentrifuge tubes (Axygen^{TM} Scientific cat. no. SCT150SSCS)

15- and 50-ml Conical tubes (ThermoFisher^{TM}, Nunc^{TM,} cat. no. 339653 and 339651)

Centrifuge, Eppendorf $^{®}$Centrifuge 5810/5810R

VTN-coated flask: one 75-cm^2 VTN-flask per line (see Support Protocol 3)

Multi-gas incubator, Panasonic MCO, CO_2/O_2 Incubator

Microscope, Live Technologies EVOS^{TM} XL Core Imaging System (cat. no. AMEX1000)

Thawing cryopreserved iPSCs

1. Retrieve cryovials with cryopreserved iPSC from liquid nitrogen.

2. Thaw the cells in an automated cell thawing instrument according to manufacturer's instructions.

   CRITICAL STEP: *Vials should be placed quickly into the cell thawing instruments once retrieved from liquid nitrogen.*

3. Once the cell thawing program is completed, hold the vial and turn the vial upside down to check for the disappearance of ice.

4. Disinfect the vial with 70% isopropanol wipes and transfer into BSC.

5. Transfer a small volume of cell suspension (100 μl) into a microcentrifuge tube to perform cell count and viability test according to procedure determined by the automated cell counter.

6. Disinfect with 70% isopropanol wipes and transfer of E8 medium and ROCKi aliquot of appropriate quantity into BSC.

7. Combine pre-warmed E8 medium and ROCKi in a 50-ml conical tube (recommended volume is 30 ml of E8 medium and 150 μl ROCKi) and mix by pipetting.

8. Transfer approximately 9 ml of E8+ROCKi into a new 15-ml conical tube and add cell suspension from the cryogenic vial; gently mix by pipetting up and down 5 times.

9. Place the conical tube in the centrifuge and pellet the cells for 2 min at $300 \times g$, room temperature.

Seeding iPSCs in VTN-coated flask

10. Disinfect with 70% isopropanol wipes the conical tube and VTN-coated 75-cm^2 flask with 70% isopropanol wipes and transfer into BSC.

11. Discard VTN coating solution from flask.

12. Carefully remove the supernatant from the conical tube in step 9 without disturbing the cell pellet.

13. Transfer approximately 5 ml of E8+ROCKi from step 7 into conical tube and gently pipette up and down to resuspend cell pellet.

14. Transfer the entire cell suspension from step 13 into VTN-coated flask.

15. Add approximately an additional 10 ml of E8+ROCKi from step 7 into VTN-coated flask. Close the flask and move the flask back and forth and side to side to distribute cells across the entire cell culture surface.

16. Perform microscope observation to ensure that cell suspension is evenly distributed.

17. Place the flask into the incubator (settings: 37°C, 5% CO_2, 5% O_2).

   iPSC attachment and iPSC morphology are evaluated 24 hr after thawing. Small typical iPSC colonies should be observed.

UNDERSTANDING RESULTS

When establishing an iPSC culture from a cryopreserved cell population, a 24-48 hr "recovery period" is normal since cell proliferation could be slower than expected, but exponential cell growth should resume after this period. This recovery stage should be taken into consideration when calculating the first post-thawing doubling time. However, when high levels of apoptosis and cellular debris are observed 24 hr post-thaw, it might be indicative of suboptimal cryopreservation storage or thawing procedures. An extended post-thaw recovery time can be considered in this situation and media change can be skipped the next day to allow more cells to attach. Alternatively, cells in suspension can be re-seeded 24 hr post-thaw into a new coated vessel to rescue some of the cell population that would otherwise be lost.

Lines with slow proliferative rates will require adaptation to the culture method described here, in which case cell seeding densities should be adjusted accordingly in order to gain healthy cell cultures. Daily evaluation of the culture under a microscope is essential to determine the readiness for cell dissociation and to detect appearance of spontaneously differentiated cells. For morphology evaluation, it is important to know that 24-48 hr after cell dissociation, iPSCs might initially exhibit elongated cell morphology and loose colony appearance. However, they should become more compact and uniform of typical pluripotent stem cell shapes once the colonies grow bigger, normally in 1 or 2 more days.

iPSC lines generated and expanded using Allele's system under cGMP standards have exhibited: cell doubling times similar to those of human ES cells (approximately 18-20 hr) (Hanna et al., 2010), homogeneous pluripotent gene expression profile, high and broad differentiation potential, and genomic stability over 100 passages in culture. Some iPSC lines are already available and have been adopted for future clinical applications. This set of protocols has been tested with multiple iPSC lines derived from a variety of cell types from donors across different ages, genders, and disease conditions. When implemented properly, our system's consistency will benefit any cGMP iPSC operation.

Time Considerations

The method for iPSC culture shown in this protocol has been optimized to minimize operation time when working under cGMP conditions. Improvements at each step of the process, such as iPSC expansion, cryopreservation, and thawing, allows processing of multiple iPSC lines in a more confined time frame while preserving cell viability and quality.

iPSCs cultures established after thawing one cryovial of cells containing 1×10^6 cells should give enough cells to seed a 75-cm^2 flask, which will reach 60%-70% confluency within 3-4 days. At such cell confluence, iPSC yield will be approximately 5×10^6 cells. At this point, they can be further expanded into 225-cm^2 flask and grown for another 3-4 days to obtain yields between 20×10^6 and 30×10^6 cells. At this stage, cells can be used for differentiation into a desired cell type or to generate an iPS cell bank. If large batches of iPSCs are required, cells collected form 225-cm^2 flasks can be expanded

for another 3-4 days. Following our method of dissociation regimen and a cell seeding density ranging from 1×10^4–2×10^4 cells/cm^2, approximately 8×10^7–18×10^7 iPSCs can be obtained within 10 to 15 days of culture from 1×10^6 cells of a starting population.

This cell culture procedure enables the production of a working cell bank (WCB) of 100 vials of 1×10^6 iPSCs each within 10-12 days from a cryopreserved vial of iPSC of a master cell bank (MCB). It is optimized for manufacturing large numbers of cells at reduced cost by allowing industrial scale production of iPSCs in a time-efficient manner.

COMMENTARY

Background Information

Generation of iPSC for clinical applications

Genetic reprogramming of human somatic cells to a pluripotent state was first achieved in 2007 by overexpression of four pluripotent factors (Takahashi et al., 2007; Yu et al., 2007) using viral vectors that integrate into the genome. Since then, numerous methods have been developed to generate iPSCs and substantial efforts have been made to improve reprogramming efficiencies and to optimize iPSC culture conditions to obtain high-quality iPSCs that are comparable to human ES cells with respect to colony morphology, cell behavior, gene expression, epigenetic status, and differentiation potential both in vitro and in vivo (Malik & Rao, 2013).

Genomic alterations represent an obstacle for therapeutic applications, as random integration of exogenous DNA can lead to insertional mutagenesis with unpredictable effects on the quality of the cells and the potential safety after transplantation (Hacein-Bey-Abina et al., 2003). Therefore, integration-free methods for iPSC derivation are desired for manufacturing clinical-grade cells. Several non-integrating methods, including recombinant proteins, DNA plasmids, and Sendai virus, have been developed to transiently express transcription factors to induce reprogramming (Okita et al., 2011; Seki et al., 2010; Zhou et al., 2009). However, these methods show low efficiency, lack of consistency, and require the removal of vector-positive cells in DNA plasmid and Sendai virus-based cell reprogramming methods. Overcoming these obstacles, the mRNA-based reprogramming technology provides a powerful platform for generating "footprint-free" iPSC. This system allows the ectopic expression of transcription factors with no risk of genomic integration, no requirement of cleanup phase to purge residual traces of vectors, and allows dose-titratable expression of any desired factor (Warren, Ni, Wang, & Guo, 2012).

mRNA-based cell reprogramming has also been shown to be the most efficient technology (Ni et al., 2016), and, more importantly, the most suitable method to ensure genome integrity of the iPSCs. A detailed comparison of the genetic integrity of iPSCs derived by the three different integration-free methods showed extremely low rates of aneuploidy and very low karyotype abnormalities detected in RNA-derived iPSCs compared to Sendai virus or episomal-derived iPSCs (Schlaeger et al., 2015). Therefore, mRNA technology represents a very powerful tool and is justifiably the method of choice to generate high-quality cGMP iPSCs for clinical applications.

Initial studies showed that the reprogramming process itself compromises genomic integrity of iPSCs (Laurent et al., 2011). However, increasing evidence lately suggested that most genetic variations observed in iPSC clones were observed in the parental cell population they were reprogrammed from (Letourneau et al., 2014). Interestingly, reprogramming has been shown to actually rectify chromosomal abnormalities present in somatic cells (Bershteyn et al., 2014). Optimization of iPSCs derivation and expansion to promote genetic stability is a priority during iPSC manufacturing to avoid spontaneous chromosomal abnormalities.

The mRNA reprogramming technology developed by Allele Biotechnology has demonstrated to be extremely efficient, consistent, and reproducible. It allows generation of iPSCs from multiple cell types with a faster kinetics than other methods based on our comparisons. Moreover, mRNA-derived iPSCs have been shown to better recapitulate the molecular signatures of human ESCs than virally derived iPSCs (Warren et al., 2010). Differentiation potential of iPSC lines generated by Allele Biotechnology has been tested on more than a dozen different cell types successfully by us and by several teams in the industry with minimal line-to-line variations.

Critical Parameters

cGMP-iPSC

Derivation of iPSC for clinical use has been proposed to be either manufactured under cGMP requirements or converted from research-grade conditions to cGMP quality standards. However, rigorous tests need to be performed on the converted lines to verify that the cells are free of contamination since they were not under full custody of a cGMP operation all the time. Allele's platform for iPSC derivation and expansion relies on the first method where every procedure from tissue collection from the donor to iPSC generation and banking is performed under cGMP requirements.

In order to ensure consistency and uniformity of the cGMP-iPSCs, strict standards are required to develop and validate manufacturing processes. Several principles of cGMP guidelines need to be established regarding quality control and assurance, design of the facility and the equipment, adequate and precise documentation, personnel training and certification, and environmental monitoring.

Allele Biotechnology has followed well-documented quality control principles and established a quality assurance system so that the entire manufacturing operation fulfills expected requirements from the initial informed consent to the final iPSC bank. All procedures are documented in the form of standard operating procedures (SOPs), procedure descriptions, registries, records, training documents, manuals, lists, and others to assure the good management, functionality, and traceability of the cell bank.

Allele cGMP-facility is built with ISO 7/Class cleanrooms, ISO 5/Class biosafety cabinets, and ISO 8/Class Facility Support Areas. Every procedure is performed in dedicated cleanrooms equipped with qualified biosafety cabinets, incubators, freezers, refrigerators, and centrifuges for iPSC generation. All the equipment is calibrated periodically by certified vendors to ensure accuracy. To acquire quality assurance to the maximum level, we have incorporated certified materials, reliable suppliers, qualified equipment, detailed SOPs, and rigid process validations at each step. Environmental monitoring program and procedures for the manufacturing suite are conducted to assure compliance of the manufacturing environments according to the 21 Code of Federal Regulation (CFR). The GMP Manufacturing suite is monitored for non-viable airborne particulate, viable airborne particulate, and viable surface according to ISO 14644-1:2015. Personnel dedicated to processing iPSCs have been thoroughly trained and manufacturing operations are monitored and recorded daily. In addition, the Quality Control-Designated Laboratory supports all product testing.

Quality control of iPSC

The critical quality attributes in respect to clinical-grade iPSCs are still under development by Allele Biotechnology and the entire field. The International Stem Cell Banking Initiative is currently providing general guidance regarding standards to qualify clinical grade iPSC lines. There is a general agreement in the field, as manifested through workshop discussions and publications, of the critical quality attributes that include cell identity, microbiological sterility, genetic fidelity and stability, viability, characterization, and differentiation potency. The combination of these data should provide enough quality control information at the clinical stage to determine the quality and safety of the iPSC line. However, the specific parameters, assays, and standards to validate iPSC lines still need to be defined to acquire such data. Allele Biotechnology is taking a lead in this development.

As will be described elsewhere, strict standards and methods for quality control have been adopted by Allele Biotechnology to qualify cGMP-iPSC lines. Sterility testing is performed routinely following FDA guidance for biological products, including mycoplasma and endotoxin tests specified under title 21 of the CFR. Purity of cell population is determined by expression of pluripotent markers using flow cytometry analysis and RNA sequencing. Genome integrity is evaluated by high-throughput sequencing of the donor cells and reprogrammed cells, genetic identity matching with starting material by short tandem repeat (STR) analysis, and karyotypic analysis is routinely performed to evaluate genome stability. Finally, careful analysis of differentiation potential is performed to confirm potency.

iPSC Culture under cGMP conditions

Material

Selecting GMP-grade or animal-origin-free reagents is extremely important to provide defined and consistent iPSC manufacturing. In addition, quality control methods need to be performed to demonstrate that the generated iPSCs meet quality standards. For Allele's cGMP-iPSC culture, cGMP-grade reagents

and materials were used whenever possible; otherwise, strict quality control tests have been developed and implemented to obtain quality assurance.

In the protocol published in 2016 (Ni et al., 2016), we provided a detailed method to generate iPSC under cGMP conditions using the mRNA technology; here we present a protocol to culture and expand cGMP-iPSC. We provide information regarding where to acquire reagents, vessels, and other materials that are compliant with cGMP standards and regulations.

Extensive optimization of cell culture media has been performed to develop a completely defined medium that efficiently supports derivation and culturing of human iPSCs (Chen et al., 2011). Multiple matrix proteins and synthetic surfaces have been shown to support human ES cell growth, but extensive evaluation is required to find the matrix that ensures efficient cell attachment and survival of pluripotent stem cell in defined medium (Chen et al., 2011). To find optimal cell culture conditions for iPSC under cGMP standards, we have evaluated parameters related to stem cell media, cell culture matrix, and subculture reagents in the Process Development phase. Reagents, materials, and qualified vendors are detailed in each section of the protocol.

Cell culture methods

iPSC adherent cultures have been considered labor-intensive and time-consuming; however, the protocol presented here provides a set of methods to culture iPSC in a time-efficient manner, which allows the establishment of a reliable platform for cGMP manufacturing. Our optimized cell culture techniques enable more efficient iPSC culturing by reducing the time of manufacturing operations and increasing reproducibility and quality.

Several changes in iPSC culture conditions and adoption of optimal manipulation of iPS cells have been proposed to reduce genetic aberrations during reprogramming and subsequent iPSC expansion (Lee, Tang, Rao, Weissman, & Wu, 2013). Culture conditions at physiologic oxygen levels are among the most important factors that could prevent genomic instability of iPSC lines. Several lines of evidence support this notion. For instance, reprogrammed cells have been shown to present accelerated cell growth and division when changing from oxidative respiration to oxidative glycolysis, which increases the po-

tential for leakage of reactive oxygen species (ROS) into the iPSC cytoplasm. ROS may oxidize nucleotides, leading to the mutagenic 7,8 dihydro-8-oxoguanine, capable of generating single- and double-strand DNA breaks (Turinetto, Orlando, & Giachino, 2017). Hence, finding optimal culture conditions for reprogrammed cells is critical to reduce genetic alterations associated to adaptation in culture and also to avoid lengthy culture time, particularly when large master or working cell banks are being generated. For this purpose, low oxygen environment has been incorporated into our cGMP platform for both generating and expanding iPSCs in order to ensure genomic stability throughout the whole manufacturing process.

During iPSC expansion, harvesting of iPSC clusters using non-enzymatic passaging method affords high controllability of cluster size while preserving cell viability. Moreover, EDTA-based cell dissociation does not require pretreatment of the culture with ROCK inhibitor or enzyme neutralization as posttreatment, which are recommended for enzymatic methods (Beers et al., 2012). In addition, EDTA treatment preferentially dissociates iPSCs over any differentiated cells, thereby at the same time providing a suitable method for iPSCs selection from heterogeneous populations when spontaneous differentiation occurs, avoiding the need of manual colony picking (Beers et al., 2012). The protocol we provide in this article for iPSC dissociation integrates a time-efficient procedure and cGMP grade reagents. Importantly, our optimized method eliminates the need of centrifugation of the cells prior to seeding to a new culture vessel; thus, reduces cellular stress.

This method for iPS cell culture provides high viability after dissociation as well as a consistent cell stability after cryopreservation, resulting in reduced cost and time to meet iPS cell banking requirements at various expansion stages. Cell stability of our cell bank has been validated using cell recovery and viability assays after thawing, reaching post-thaw recovery rates of over 90%.

Overall, this fully tested protocol for iPSC culture has been optimized for efficient iPSC proliferation and stemness. It allows scalable manufacturing operations that are fully compliant with cGMP standards; it can be adopted by any cGMP facility, providing a valuable platform to generate iPSCs for clinical applications. Moreover, this platform is designed to allow an easy transition to closed cell culture systems towards automation, which

facilitates scalability and controllability and reduces operator-introduced variability, a direction of development Allele is actively engaged in.

Troubleshooting

There are a number of key parameters that could impact the quality of iPSC cultures. Establishment of vigorous cGMP level protocols provides the best route to quality control of iPSC through the entire manufacturing process. During our Process Development stage, we addressed the factors that could potentially affect the quality of the iPSC culture.

Cell culture handling can be a source of severe disruption of iPSC clusters or damages to cells can be caused by excessive pipetting of cell suspension, causing low cell viability after dissociation. In addition, overconfluence of iPSC cultures should be avoided to enhance cell viability. Cell dissociation schedule should be carefully determined and consistently executed. Through early experimentation, we determined that 4 days was the optimal dissociation regimen when following our criteria for cell seeding density.

iPSC could undergo spontaneous differentiation in vitro, which is commonly characterized by the presence of colonies without distinct borders, flattened cells, or colonies with less compacted areas. Major causes for differentiation are (1) *overconfluent cultures*: Therefore, it is essential that colonies are passaged before reaching full confluence; (2) *poor quality of ECM coating*: This can be prevented by QC testing of each lot of coated vessels prior to transferring into manufacturing; alternatively, increasing the volume of coating solution could reduce the risk of coating failure; (3) *high oxygen levels* We suggest adjustment to low oxygen tension of 3%-5% O_2 for all pluripotent stem cell culturing; (4) *cell culture exposed to high fluctuations of temperature*: This can occur when cell culture is kept for extended periods of time outside of the incubator; thus, the execution of the protocol should be studied and evaluated by the managers to reduce operation time. Moreover, addition of pre-warmed reagents into cultures is recommended but prolonged exposure of stock media to 37°C should be limited to keep the growth factors from losing activities. Removal of differentiated cells can be achieved during the dissociation step by performing short incubation times with EDTA-based dissociation reagent since iPSCs will be preferentially harvested and differentiated cells will remain attached to the current culture surface.

If poor cell recovery rates or low cell attachment after cryopreservation is detected, it is advisable that the thawing procedure should be carried out more quickly and proper concentration of ROCKi added into the media at the time of thawing.

To avoid spontaneous chromosomal abnormalities in cultured iPSCs, several precautionary steps may be implemented: (1) make sure that oxygen tension is maintained at pluripotent stem cell-appropriate physiological levels, (2) careful selection of extracellular matrices that best maintain the normal karyotypes of pluripotent stem cells, such as human vitronectin or laminin-521 (Braam et al., 2008; Rodin et al., 2010), (3) use only enzyme-free methods for cell dissociation to prevent passage-induced mutations during prolonged culturing (Beers et al., 2012).

Author Contribution and Acknowledgments

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Internet Resources

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Reference of clinical trial using iPSC-derived cells for cancer immunotherapy.

Key References

Ni et al. (2016). See above.
Describes a protocol to generate iPSCs through an established cGMP process.