TECHNICAL MANUAL

Integrated Workflow for the Isolation, Expansion, and Reprogramming of Erythroid Progenitor Cells

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1.0 Introduction

Peripheral Blood Cells for Reprogramming

Human induced pluripotent stem (iPS) cells are generated by reprogramming somatic cells to a pluripotent state, through the transient overexpression of key reprogramming factors.

Dermal fibroblasts were the first human cell type to be converted to iPS cells and are still one of the most common sources used for reprogramming experiments.[1,2] Since then, numerous primary cell sources such as keratinocytes, mesenchymal stem cells, T cells, B cells, hematopoietic progenitor cells, and urine epithelial cells have also been reprogrammed to iPS cells.[1–8] The choice of starting cell type is influenced by factors such as availability of donor tissue from normal and diseased patients, invasiveness of sample collection procedures, genomic integrity, epigenetic memory, and reprogramming efficiency.

Peripheral blood (PB) is a popular tissue source for generating iPS cells.[9] Blood collection is a well-established and minimally invasive procedure, and collected cells are naturally replaced as the tissue is self-renewing. Banked blood samples are also available for a wide variety of disease, age, gender, and geographical subtypes. Since the cells are continually replenished from stem cells in the bone marrow, it is expected that they will contain fewer environment-associated point mutations than skin, which is exposed to long-term ultraviolet radiation.

However, PB contains a heterogeneous mixture of cell types, the most prevalent of which are enucleated (e.g. mature red blood cells [RBCs] and platelets) and therefore not suitable for reprogramming. The first step in preparing whole PB samples for reprogramming is therefore to separate the PB mononuclear cell (PBMC) fraction from the RBCs and platelets. This is generally done by density gradient centrifugation.

The PBMC fraction consists of T and B lymphocytes, macrophages, monocytes, erythroid progenitor cells, and rare circulating stem cells. Many of these cell types have been successfully reprogrammed with varying efficiencies.[9] While T cells and B cells are the most abundant cell types in the PBMC fraction and have been successfully reprogrammed, they contain V(D)J genomic rearrangements of the T cell receptor or immunoglobulin loci, respectively. The ability to generate iPS cells from specific T cells has been utilized for proof-of-principle experiments in T cell therapy,[10] but little is known about how these rearrangements may affect the function of other downstream cell lineages.

Less abundant cell types, including CD34+ hematopoietic stem and progenitor cells and erythroid progenitor cells, are attractive for reprogramming due to their lack of genomic rearrangements and demonstrated reprogramming ability.[7,11] However, owing to their low frequency in whole blood, these cells need to be isolated from PB and/or expanded in vitro to obtain sufficient cell numbers for reprogramming.

A Complete Workflow

We have developed an integrated set of tools to facilitate the reprogramming of PB to human iPS cells. First, RBCs, platelets, and lineage-committed cells are depleted from blood using a RosetteSep™ cocktail and SepMate™ density gradient centrifugation tubes. Enriched cells can then be directly cultured in StemSpan™ media and supplements for erythroid cell expansion.

Once sufficient cell numbers have been generated, reprogramming can begin through transfection/transduction of reprogramming factors. ReproTeSR™ is a xeno-free medium that supports rapid and efficient feeder-free reprogramming of blood-derived cells. iPS cells generated from this workflow can transition seamlessly to our TeSR™ family of iPS cell maintenance media and the STEMdiff™ suite of products (e.g. STEMdiff™ Neural Induction Medium, Catalog #05835) for directed differentiation.

2.0 Materials, Reagents, and Equipment

2.1 Erythroid Progenitor Reprogramming Kit (Catalog #05924)

The following table lists the components of the Erythroid Progenitor Reprogramming Kit. The materials provided in the kit are sufficient to process 10 mL of fresh peripheral blood.

COMPONENT/PRODUCT NAME	QUANTITY/SIZE	COMPONENT #
SepMate™-15 (sample size)	4 x 15 mL tubes	15410
Lymphoprep™	250 mL	07801
RosetteSep™ Human Progenitor Cell Basic Pre-Enrichment Cocktail (sample size)	0.5 mL	15216
StemSpan™ SFEM II	100 mL	09605
StemSpan™ Erythroid Expansion Supplement (100X)	1 mL	02692
ReproTeSR™	500 mL Kit	05926
TeSR™-E7™/ReproTeSR™ Basal Medium	480 mL	05919
ReproTeSR™ 25X Supplement	20 mL	05928

2.2 Additional Materials and Reagents

PRODUCT	CATALOG #
Tissue culture-treated cultureware	e.g. 38016 (6-well plates)
Falcon® Conical Tubes	38009 (15 mL) AND 38010 (50 mL)
D-PBS (Without Ca++ and Mg++)	37350
Dulbecco's Phosphate Buffered Saline with 2% Fetal Bovine Serum (PBS + 2% FBS)	07905
DMEM/F-12 with 15 mM HEPES	36254
Corning® Matrigel® hESC-Qualified Matrix	Corning 354277
3% Acetic Acid with Methylene Blue	07060
Epi5™ Episomal iPSC Reprogramming Kit	Thermo Fisher A15960
P3 Primary Cell 4D-Nucleofector™ X Kit L • P3 Primary Cell Nucleofector™ Solution	Lonza V4XP-3024
iPS cell maintenance medium • mTeSR™1 OR • mTeSR™ Plus OR • TeSR™-E8™ OR • TeSR™2	 85850 OR 05825 OR 05990 OR 05860
Y-27632	72302
Passaging reagent • Gentle Cell Dissociation Reagent OR • ReLeSR™	 07174 OR 05872

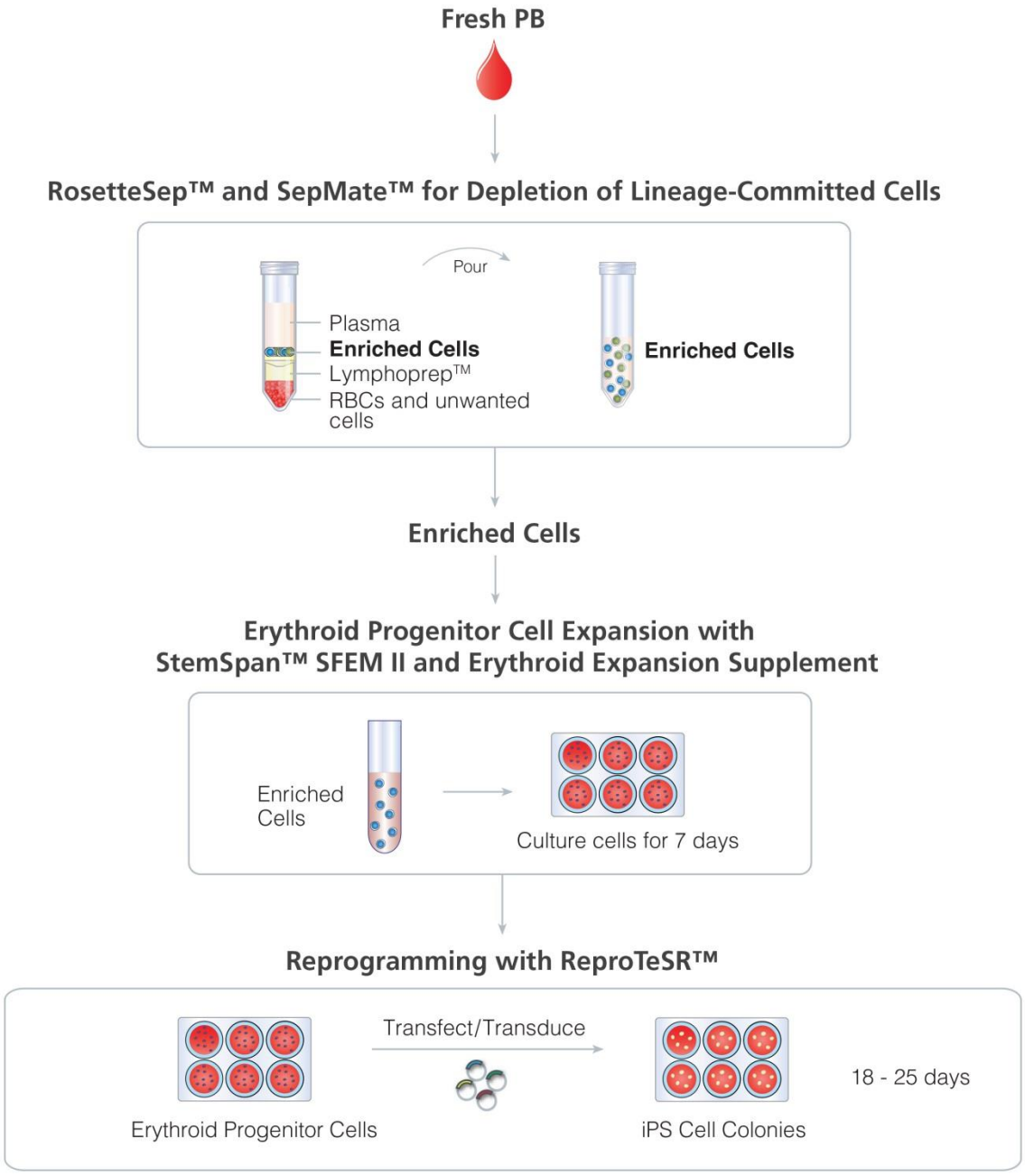
For a complete list of products available from STEMCELL Technologies Inc., visit www.stemcell.com.

2.3 Equipment Required

- Biohazard safety cabinet certified for Level II handling of biological materials
- Incubator with humidity and gas control to maintain 37°C and 95% humidity in an atmosphere of 5% CO_2 in air
- Low-speed centrifuge with a swinging bucket rotor
- Pipette-aid with appropriate serological pipettes
- Pipettor with appropriate tips
- 22 gauge needle or pulled glass pipette
- Inverted microscope with a total magnification of 20X to 100X
- -20°C freezer
- Refrigerator (2 - 8°C)
- 4D-Nucleofector™ System (Lonza AAF-1001B)

3.0 Procedure Diagram

Erythroid Progenitor Cell Reprogramming



4.0 Preparation of Reagents and Materials

The following reagents and materials are required in the protocols in sections 5.0 - 7.0. Prepare as needed. For storage and stability information, refer to the Product Information Sheets (PIS's) supplied with each product.

4.1 Erythroid Expansion Medium

Use sterile techniques when preparing Erythroid Expansion Medium (StemSpan™ SFEM II + StemSpan™ Erythroid Expansion Supplement [100X]). The following example is for preparing 100 mL of Erythroid Expansion Medium. If preparing other volumes, adjust accordingly.

1. Thaw StemSpan™ Erythroid Expansion Supplement (100X) at room temperature (15 - 25°C) until just thawed. Mix thoroughly.

   Note: If necessary, centrifuge for 30 seconds to recover liquid from cap.

2. Add 1 mL of StemSpan™ Erythroid Expansion Supplement (100X) to 99 mL of StemSpan™ SFEM II. Mix thoroughly.

4.2 ReproTeSR™

Use sterile techniques when preparing complete ReproTeSR™ medium (TeSR™-E7™/ReproTeSR™ Basal Medium + 25X Supplement). The following example is for preparing 500 mL of ReproTeSR™ medium. If preparing other volumes, adjust accordingly.

1. Thaw ReproTeSR™ 25X Supplement at room temperature (15 - 25°C) or at 2 - 8°C. Mix thoroughly.
2. Add 20 mL of ReproTeSR™ 25X Supplement to 480 mL of TeSR™-E7™/ReproTeSR™ Basal Medium. Mix thoroughly.

4.3 Coating Cultureware with Corning® Matrigel®

Corning® Matrigel® hESC-Qualified Matrix is recommended for reprogramming. It should be previously aliquoted and frozen. Consult the Certificate of Analysis supplied with Corning® Matrigel® for the recommended aliquot size ("Dilution Factor") to prepare 25 mL of diluted matrix. Ensure to keep Matrigel® on ice when thawing and handling to prevent it from gelling.

Note: ***Use tissue culture-treated cultureware****.*

1. Thaw one aliquot of Corning® Matrigel® on ice.
2. Dispense 25 mL of cold DMEM/F-12 into a 50 mL conical tube and keep on ice.
3. Add thawed Matrigel® to the cold DMEM/F-12 and mix well. The vial may be washed with cold medium if desired.
4. Immediately use the diluted Matrigel® solution to coat tissue culture-treated cultureware. See Table 1 below for recommended coating volumes.

Table 1. Recommended Volumes for Coating Cultureware

CULTUREWARE	VOLUME OF DILUTED MATRIGEL®
6-well plate	1 mL/well
100 mm dish	6 mL
T-25 cm^2 flask	3 mL
T-75 cm^2 flask	8 mL

5. Swirl the cultureware to spread the Matrigel® solution evenly across the surface.

   Note: If the cultureware surface is not fully coated by the Matrigel® solution, it should not be used for human ES or iPS cell culture.

6. Incubate at room temperature (15 - 25°C) for at least 1 hour before use. Do not let the Matrigel® solution evaporate.

   Note: If not used immediately, the cultureware must be sealed to prevent evaporation of the Matrigel® solution (e.g. with Parafilm®) and can be stored at 2 - 8°C for up to 1 week after coating. Allow stored coated cultureware to warm to room temperature (15 - 25°C) for 30 minutes before proceeding to the next step.

7. Gently tilt the cultureware onto one side and allow the excess Matrigel® solution to collect at the edge. Remove the excess solution using a serological pipette or by aspiration. Ensure that the coated surface is not scratched.

8. Immediately add the appropriate medium (e.g. 2 mL/well if using a 6-well plate).

5.0 Erythroid Progenitor Cell Pre-Enrichment and Expansion

5.1 Pre-Enrichment of Hematopoietic Progenitor Cells from Whole Blood

RosetteSep™ + SepMate™ Pre-Enrichment

Ensure that the blood sample, PBS + 2% FBS, Erythroid Expansion Medium, Lymphoprep™, and centrifuge are all at room temperature (15 - 25°C).

1. Add RosetteSep™ Human Progenitor Cell Basic Pre-Enrichment Cocktail to the blood sample at **5 µL/mL** of blood (e.g. for 10 mL of blood, add 50 µL of cocktail).
2. Incubate at room temperature (15 - 25°C) for **10 minutes**.
3. Dilute sample with an equal volume of PBS + 2% FBS. Mix gently.

   For example, dilute 10 mL of sample with 10 mL of PBS + 2% FBS.
4. Add Lymphoprep™ to the SepMate™-15 tube by carefully pipetting it through the central hole of the SepMate™ insert.

   Note: For 10 mL of sample, it is recommended to add 3.5 mL of Lymphoprep™ to each of two SepMate™-15 tubes. For other sample volumes, refer to the PIS for SepMate™.
5. Keeping the SepMate™-15 tube vertical, add the diluted sample by pipetting it down the side of the tube. The sample will mix with the density gradient medium above the insert.

   Note: For 10 mL of initial sample, add 10 mL of diluted sample to each of two SepMate™-15 tubes.

   Note: The sample can be poured down the side of the tube. Take care not to pour the diluted sample directly through the central hole.
6. Centrifuge at **1200 x *g*** for **10 minutes** at room temperature (15 - 25°C), with the **brake on**.

   Note: For samples older than 24 hours, a centrifugation time of 20 minutes is recommended.
7. Pour off the top layer, which contains the pre-enriched cells, into a new tube. Do not hold the SepMate™ tube in the inverted position for longer than 2 seconds. Pool samples from the same donor into 1 tube.

   Note: Some RBCs may be present on the surface of the SepMate™ insert after centrifugation. This will not affect performance.
8. Wash pre-enriched cells by topping up the tube with PBS + 2% FBS and centrifuge at **300 x *g*** for **8 minutes** with the **brake on**.
9. Resuspend cell pellet in approximately 50 µL of Erythroid Expansion Medium (section 4.1) per 10 mL of blood sample.

5.2 Expansion of Erythroid Progenitor Cells

1. **Day 0**: Count pre-enriched cells (obtained in section 5.1 step 9) using 3% Acetic Acid with Methylene Blue, and resuspend to a cell density of 1.5 - 2.5 x 10^5 cells/mL in Erythroid Expansion Medium.

   Note: For instructions on cell counting, refer to the PIS for 3% Acetic Acid with Methylene Blue, available at www.stemcell.com or contact us to request a copy.

2. Plate 3 - 5 x 10^5 PBMCs (2 mL) per well of a 6-well plate.

   Note: The Erythroid Progenitor Reprogramming Kit contains enough Erythroid Expansion Medium to expand 8 wells (in 6-well plates) for 7 days.

3. Incubate at 37°C and 5% CO_2 overnight.

4. **Day 1**: Transfer each 2 mL cell suspension to a new well.

   Note: During the first 24 hours of culture, some blood cell types may adhere to the tissue culture dish. Transferring the non-adherent cells to a new dish helps remove these unwanted cells and does not affect the expansion of erythroid progenitor cells.

5. **Days 2, 4, and 6**:

   a) Transfer cells to a 15 mL conical tube.

   b) Centrifuge cells at 300 x *g* for 5 minutes.

   c) Remove supernatant and resuspend cells in 2 mL of fresh Erythroid Expansion Medium.

   d) Plate 2 mL per well of a 6-well plate. Incubate at 37°C and 5% CO_2.

6. **Day 7**: Centrifuge cells at 300 x *g* for 5 minutes. Remove supernatant and resuspend cells in 2 mL of fresh Erythroid Expansion Medium. Perform a cell count using 3% Acetic Acid with Methylene Blue. Cells are now ready for transfection/transduction (section 6.0).

   Note: Enrichment of erythroid progenitor cells is typically seen by day 7, but can be further enriched by extending culturing time to days 8 or 9. To examine enrichment of erythroid progenitor cells, flow cytometry analyses for erythroid cell surface markers CD71 and Glycophorin A (GlyA) can be performed (Figure 1). For further information, refer to the Brochure: An Integrated Workflow for Reprogramming Blood Cells (Document #28722), available at www.stemcell.com or contact us to request a copy.

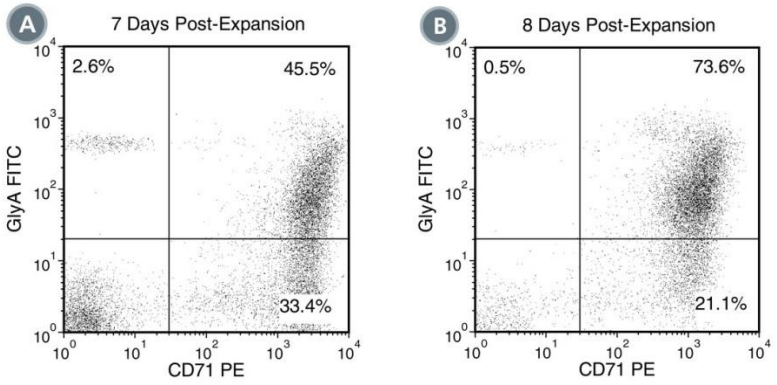


Figure 1. Extending Culture Time Increases Purity of Erythroid Progenitor Cells
Flow cytometry analyses for erythroid markers CD71 and GlyA are shown. **(A)** On day 7 of culture, ~80% of cells express erythroid cell markers CD71 and **(B)** Extending the culture time to day 8 increased the erythroid cell purity to ~95% in this example. The bulk of the cell population consists of CD71+GlyA+ erythroblasts. More immature CD71+GlyA- progenitor cells and proerythroblasts, as well as more differentiated CD71-/low GlyA+ normoblasts, were also present at lower frequencies.

6.0 Transfection/Transduction of Erythroid Progenitor Cells

1. Transfer 1 x 10^6 culture-expanded erythroid cells (from section 5.2 step 6) into a 15 mL conical tube.
2. Centrifuge at 300 *x g* for 5 minutes. Remove supernatant and resuspend cells in 100 µL of P3 Primary Cell Nucleofector™ Solution.
3. Add 1 µg of each episomal vector to the cell suspension and mix well.

   Note: It is recommended to use the set of 5 episomal vectors provided in the Epi5™ Episomal iPSC Reprogramming Kit . The amount of episomal vectors used may require optimization depending on the transfection efficiency and cell source.
4. Electroporate cells with reprogramming vectors according to the manufacturer's instructions.

   Note: For transfection of episomal vectors using the Lonza Nucleofector™, use the electroporation setting indicated for human CD34+ cells.
5. Transfer cells to a 15 mL conical tube containing 6 mL of Erythroid Expansion Medium (section 4.1).

7.0 Reprogramming of Erythroid Progenitor Cells

Note: Warm ReproTeSR™ medium (section 4.2) to room temperature (15 - 25°C) before use.

1. **Day 0**: Plate 3.3 x 10^5 cells (i.e. 2 mL of cell suspension from section 6.0) in each well of a 6-well plate coated with Corning® Matrigel® (section 4.3). Incubate at 37°C.

   Note: The suggested plating density is optimized for the transfection of erythroid cells with an episomal system. Plating density may need further optimization depending on the vector system used and growth kinetics of the cells being reprogrammed.
2. **Day 2:** Add 1 mL of Erythroid Expansion Medium (section 4.1) to the transfected/transduced cells (without removing any medium from the well). Incubate at 37°C.
3. **Day 3:** Add 1 mL of ReproTeSR™ medium (without removing medium from the well). Incubate at 37°C.
4. **Day 5:** Add 1 mL of ReproTeSR™ medium (without removing medium from the well). Incubate at 37°C.
5. **Day 7**: Aspirate medium from each well and replace with 2 mL of fresh ReproTeSR™ medium. Incubate at 37°C.
6. **Day 8 - 25**: Perform daily medium changes (2 mL/well) using ReproTeSR™ medium. Monitor the cells until iPS cell colonies appear.

   Note: iPS cell colonies typically arise between days 18 - 25, but may vary depending on cell type and vector system used. To achieve optimal reprogramming efficiency, use blood-derived cells at low passage. For a representative example of an iPS cell colony, see section 7.1.

7.1 Identification and Isolation of iPS Cell Colonies

7.1.1 Identification of iPS Cell Colonies

iPS cells have characteristic cellular and colony morphology that can be used to distinguish them from non-reprogrammed cells in the same culture. Putative iPS cell colonies are recognizable by their relatively large size, tight cell packing, and well-defined borders. Identification is facilitated by the low degree of colony overgrowth from differentiated cells in feeder-free reprogramming media such as ReproTeSR™. Within iPS cell colonies, the cells are small, with high nuclear to cytoplasmic ratio. These characteristics can be used to manually select putative iPS cell colonies. Confirmation of pluripotency is recommended for all newly generated iPS cell clones, once a cell line is established.

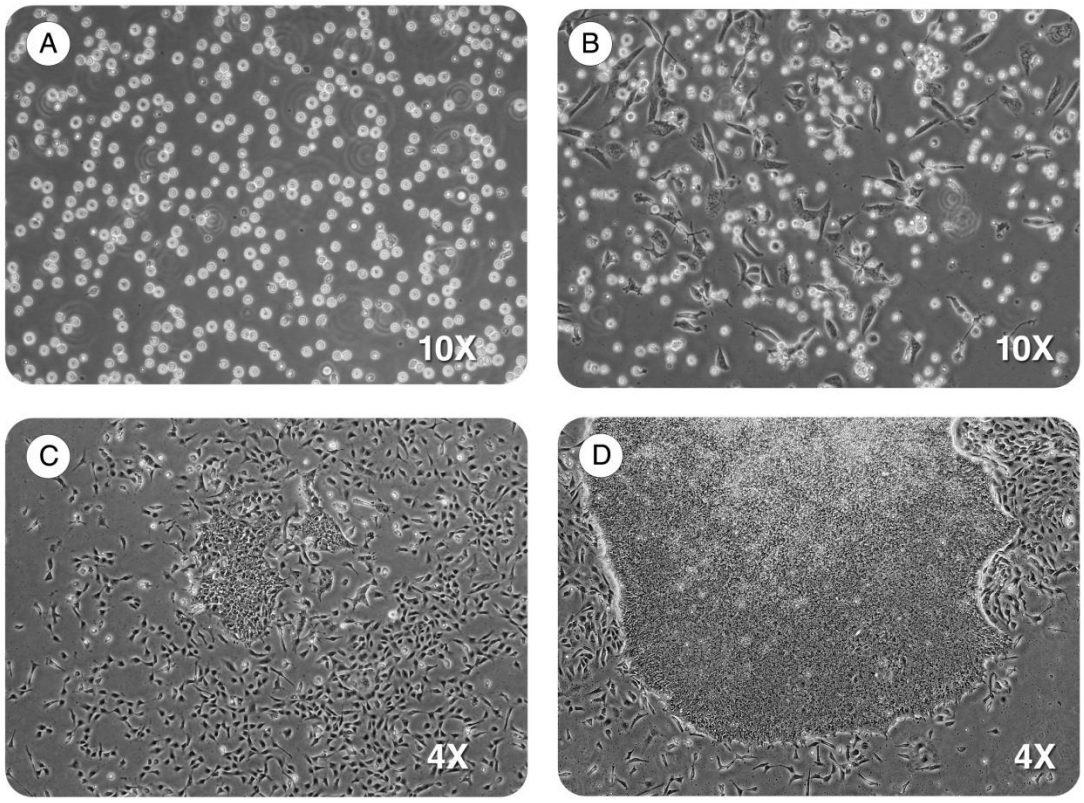


Figure 2. Morphological Changes Observed During the Induction Phase of Reprogramming Blood-Derived Cells
(A) Prior to transfection (or transduction) of reprogramming factors, blood-derived cells expand as suspension cultures.
(B) On day 7 (post-transfection of reprogramming factors), blood-derived cells are shown attached to the plate.
(C) After 2 weeks, pre-iPS cell colonies form, exhibiting epithelial-like morphology.
(D) By 3 weeks, putative iPS cell colonies arise.

7.1.2 Isolation of iPS Cell Colonies

The procedures below should be performed under a stereomicroscope using sterile conditions. Warm media and coated plates to room temperature (15 - 25°C) before use.

1. Cut the putative iPS cell colony into small fragments using either a 22 gauge needle or a pulled glass pipette (Figure 3).

   Note: If there are many untransfected, partially reprogrammed, and/or differentiated cells surrounding the putative iPS cell colony, these may need to be scraped away prior to isolating the iPS cell colony.

2. Scrape and aspirate colony fragments using a 200 µL pipettor with a filtered tip.
3. Immediately plate iPS cell colony fragments on cultureware coated with desired matrix (e.g. Corning® Matrigel®, section 4.3) and containing iPS cell maintenance medium (e.g. mTeSR™1, mTeSR™ Plus, or TeSR™-E8™).

   Note: To facilitate the initial attachment of iPS cell colony fragments, add Y-27632 to the maintenance medium at a final concentration of 10 µM. After 24 hours, replace the maintenance medium (without Y-27632).

4. Incubate at 37°C and perform iPS cell maintenance medium changes accordingly.

   Note: For the first few passages perform manual (i.e. chemical- and enzyme-free) passaging of newly isolated iPS cell lines. This can help to reduce the presence of contaminating cells such as fibroblasts, partially reprogrammed cells, or differentiated cells. Once iPS cell lines are established, chemical or enzymatic passaging methods may be used (e.g. Gentle Cell Dissociation Reagent or ReLeSR™). For complete instructions on how to maintain iPS cells using mTeSR™1, mTeSR™ Plus, TeSR™-E8™, or TeSR™2, refer to the Technical Manuals available at www.stemcell.com or contact us to request a copy.

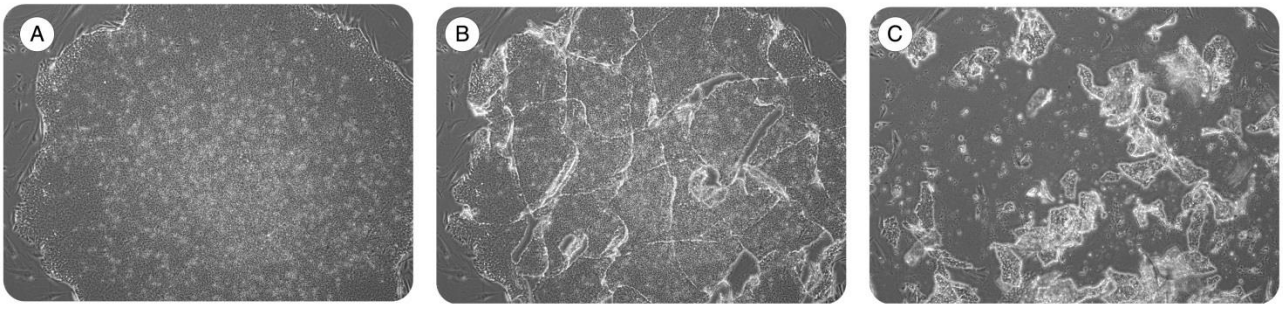


Figure 3. Manual Isolation of Putative iPS Cell Colonies
(A) Putative human iPS cell colonies exhibiting morphological characteristics similar to human ES cells with compact morphology, high nuclear-to-cytoplasmic ratio, and defined borders. **(B)** Using a 22 gauge needle or pulled glass pipette, the putative iPS colony is cut into smaller fragments. **(C)** These smaller fragments are then scraped off the plate with a filtered 200 µL pipette tip.

8.0 Troubleshooting

PROBLEM	SOLUTION
Poor expansion of erythroid cells from PBMCs	• Ensure that complete Erythroid Expansion Medium is used within 2 weeks of preparation. • Whole peripheral blood less than 24 hours old is recommended as the primary cell source.
Low reprogramming efficiency	• Depending on the vector system being used, ensure that the transfection or transduction efficiency has been optimized with erythroid progenitor cells. • Ensure that the vector being used has not been compromised by checking whether the reprogramming factors are being expressed.
Differentiation of erythroid progenitor cells	• The optimal culture time is 7 - 8 days; extended culture of erythroid progenitor cells can lead to increased spontaneous differentiation of erythroid progenitor cells to more mature erythroblasts. Differentiation results in hemoglobinization and is observed as an increased redness of the culture, which is particularly pronounced when the cells are pelleted.
Low cell viability after transfection or transduction	• Ensure transfection or transduction is optimized for erythroid progenitor cells.

9.0 References

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