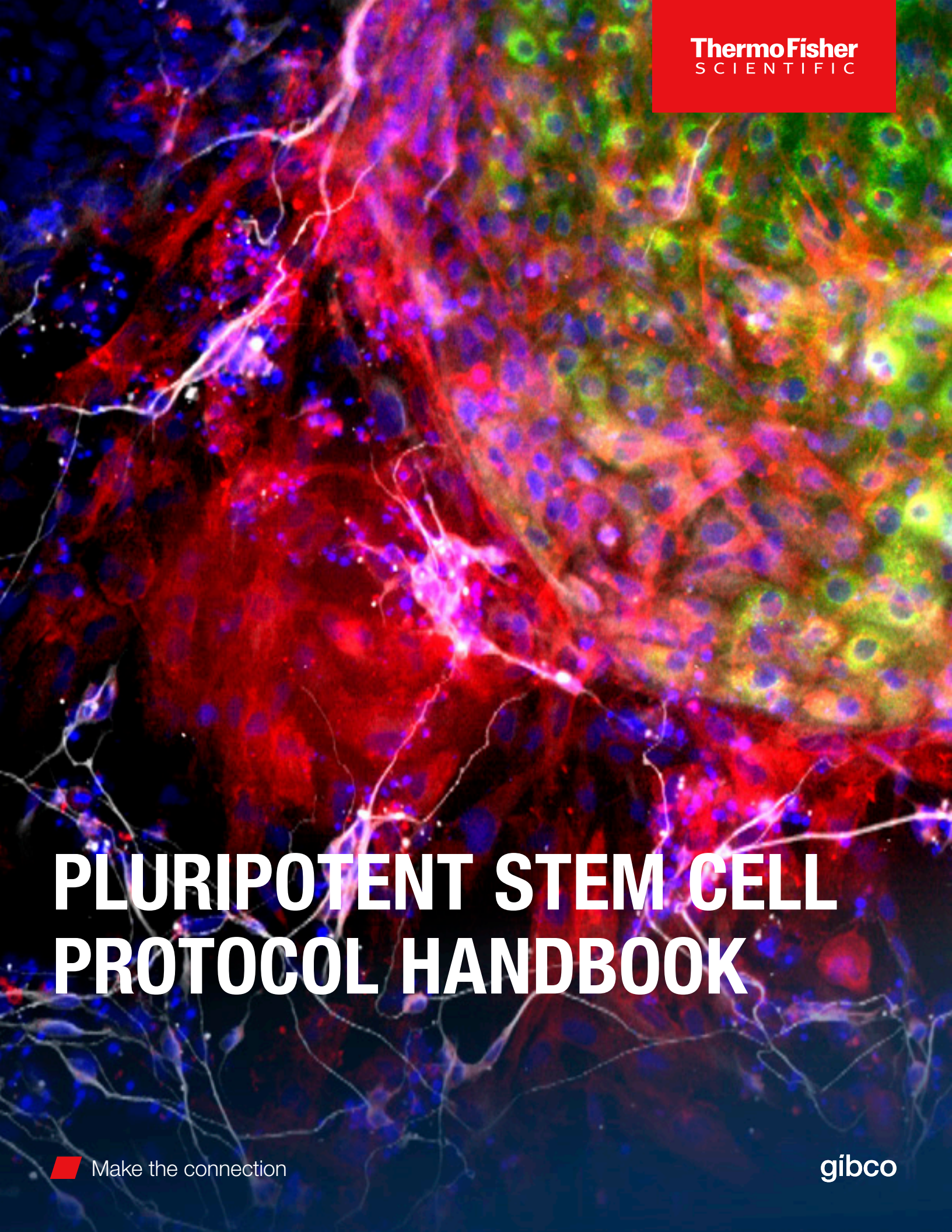

Thermo Fisher SCIENTIFIC

PLURIPOTENT STEM CELL PROTOCOL HANDBOOK

Make the connection

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Introduction

Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), sometimes collectively referred to as PSCs, are cells that have the ability to renew themselves indefinitely and differentiate into almost any cell type when exposed to the right microenvironment. Because of these unique properties, PSCs have many potential applications in regenerative medicine, disease research, drug discovery, and basic research. Across these different areas, there are several common steps in the PSC workflow: The PSCs are most frequently derived through reprogramming of somatic cells into iPSCs. They are then characterized to confirm cell type identity, pluripotency, and genetic stability. Finally, they are cultured and expanded to generate enough cells for downstream applications such as genome editing and differentiation. In this handbook, we provide a collection of the most commonly used protocols, showing where our products fit in the steps of the PSC workflow. Additional protocols and details regarding these products can be found in the respective product manuals.

Contents

1. Reprogramming

Reprogramming is the process by which somatic cells like fibroblasts are forced back into a pluripotent state through the ectopic expression of specific transcription factors. The reprogramming process, which typically lasts from 3 to 4 weeks, involves the culture of somatic cells, the delivery of the reprogramming factors, and the eventual transition into the pluripotent state. However, in order to establish new iPSC lines, clones need to be picked, expanded up to 10 passages, characterized, and banked to confirm genetic stability and pluripotency. All of these steps will be covered in this protocol handbook.

This section will describe how to reprogram fibroblasts using the nonintegrating Invitrogen™ CytoTune™-iPS 2.0 Sendai Reprogramming Kit as well has how to quickly characterize and pick emerging iPSC clones. There is an option for reprogramming under feeder-dependent conditions, which is very robust and enables high reprogramming efficiency. There are also options for feeder-free reprogramming with three media systems, depending on research needs: Gibco™ Essential 8™ or Essential 8™ Flex Medium for reprogramming under xeno-free conditions, or Gibco™ StemFlex™ Medium. Essential 8 Flex and StemFlex Media also enable an every-other-day feeding schedule, rather than the standard every-day schedule.

For additional details on the reprogramming system and for reprogramming of other cell types, refer to the detailed manual (Pub. No. MAN0009378) for the CytoTune-iPS 2.0 Sendai Reprogramming Kit. For translational research, use the Invitrogen™ CTS™ CytoTune™ 2.1 Sendai Reprogramming Kit with Gibco™ CTS™ Essential 8™ Medium, as described in the manual (Pub. No. MAN0016994) for the CytoTune-iPS 2.1 Sendai Reprogramming Kit.

1.1 Reprogramming fibroblasts with the CytoTune-iPS 2.0 Sendai Reprogramming Kit

1.1A CytoTune-iPS 2.0 Sendai Reprogramming Kit

The CytoTune-iPS 2.0 Sendai kit uses vectors based on a modified, nontransmissible form of the Sendai virus (SeV) to safely and effectively deliver and express key genetic factors necessary for reprogramming somatic cells into iPSCs. In contrast to many available protocols, which rely on viral vectors that integrate into the genome of the host cell, the CytoTune-iPS 2.0 Sendai kit uses vectors that are nonintegrating and remain in the cytoplasm (i.e., they have zero genomic footprint). In addition, the host cell can be cleared of the vectors and reprogramming factor genes by exploiting the cytoplasmic nature of SeV and the functional temperature-sensitive mutations introduced into key viral proteins.

The CytoTune-iPS 2.0 Sendai Reprogramming Kit contains three SeV-based reprogramming vectors that are optimized for generating iPSCs from human somatic cells such as fibroblasts, as described in these feeder-dependent or feeder-free protocols. Reprogramming protocols for other cell types are included in the product manual (Pub. No. MAN0009378).

Important: The Sendai reprogramming vectors of the CytoTune-iPS 2.0 kit are not compatible with the reprogramming vectors of the original Invitrogen™ CytoTune™-iPS Reprogramming Kits (Cat. No. A13780-01, A13780-02). Do not mix or substitute the reprogramming vectors of the CytoTune-iPS 2.0 kit with the reprogramming vectors of the original kits.

Important: This product must be used according to biosafety level 2 (BSL-2) containment requirements, which include a biological safety cabinet and laminar flow hood, and the appropriate personal safety equipment to prevent mucosal exposure and/or contact with splashes.

Contents and storage

Component*	Cap color	Amount**		Storage
		A16517	A16518	
Polycistronic Klf4–Oct3/4–Sox2 (KOS)	Clear	100 µL	3 x 100 µL	–80°C
cMyc	White			
Klf4	Red			

* The titer of each CytoTune-iPS 2.0 Sendai reprogramming vector is lot-dependent. For the specific titers of your vectors, refer to the Certificate of Analysis (CoA) available at **thermofisher.com/cytotune** and search by the product lot number that is printed on the vial.

** Each vial contains 100 µL of one of the CytoTune-iPS 2.0 Sendai reprogramming vectors at a concentration of ≥8 x 10^7 cell infectious units/mL (CIU/mL).

Safety information

SeV host species: The host species reported so far for SeV are mouse, rat, hamster, and guinea pig, all of which have been described as serologically positive.

Transmission of SeV: SeV is transmitted by aerosol and contact with respiratory secretions. The virus is highly contagious, but the infection does not persist in immunocompetent animals.

CytoTune-iPS 2.0 kit Sendai reprogramming vectors: The reprogramming vectors in this kit are based on a modified, nontransmissible form of SeV, which has the fusion protein deleted, rendering the virus incapable of producing infectious particles from infected cells.

Inoculating animals with transduced cells: Although the CytoTune-iPS 2.0 kit Sendai vectors are nontransmissible, cells that have been exposed to the virus should be tested with PCR or antibody staining to ensure the absence of the virus before being inoculated into animals. Animals that have already been infected with wild-type SeV may be able to make infectious viruses based on the vectors of the CytoTune-iPS 2.0 kit.

Required materials for reprogramming of feeder-dependent human fibroblasts

Cells and vectors

- CytoTune-iPS 2.0 Sendai reprogramming vectors
  Note: For successful reprogramming, you need all three tubes of reprogramming vectors.
- Human fibroblasts to reprogram
- **Optional:** Human neonatal foreskin fibroblasts—ATCC™ CRL-2522™ strain BJ—as a positive reprogramming control
- Gibco™ CF1 Mouse Embryonic Fibroblasts, irradiated (Cat. No. A34181)

Media and reagents

- Gibco™ DMEM, high glucose, GlutaMAX™ Supplement, pyruvate (Cat. No. 10569-010)
- Gibco™ KnockOut™ DMEM/F-12 (Cat. No. 12660-012)
- Gibco™ Embryonic Stem Cell–Qualified FBS, US origin (Cat. No. 16141-079)
- Gibco™ KnockOut™ Serum Replacement – Multi-Species (Cat. No. A3181501)
- Gibco™ MEM Non-Essential Amino Acids Solution (100X) (Cat. No. 11140-050)
- Gibco™ FGF-Basic (AA 1–155) Recombinant Human Protein (Cat. No. PHG0264)
- Gibco™ 2-Mercaptoethanol (Cat. No. 21985-023)
- Gibco™ Attachment Factor Protein (1X) (Cat. No. S-006-100)
- Gibco™ TrypLE™ Select Enzyme (1X) (Cat. No. 12563029) or Gibco™ Trypsin-EDTA (0.05%) (Cat. No. 25300054)
- Gibco™ DPBS, no calcium, no magnesium (Cat. No. 14190359)
- **Optional:** Gibco™ Penicillin-Streptomycin (10,000 U/mL) (Cat. No. 15140-122)

Media for reprogramming of feeder-dependent human fibroblasts

For optimal reprogramming of human neonatal foreskin fibroblasts using the CytoTune-iPS 2.0 Sendai Reprogramming Kit to generate iPSCs cultured on mouse embryo fibroblast (MEF) feeder cells, use the following media at the designated stages of the reprogramming experiment:

Fibroblast medium: Used for plating cells prior to transduction, expansion, posttransduction recovery of cells, and plating of transduced cells on MEF culture dishes

To prepare 100 mL of complete fibroblast medium, aseptically mix the following components:

DMEM, high glucose, GlutaMAX Supplement, pyruvate	89 mL
Embryonic Stem (ES) Cell–Qualified FBS, US origin	10 mL
MEM Non-Essential Amino Acids Solution (100X)	1 mL
2-Mercaptoethanol	100 µL

Complete fibroblast medium can be stored at 2–8°C for up to 4 weeks.

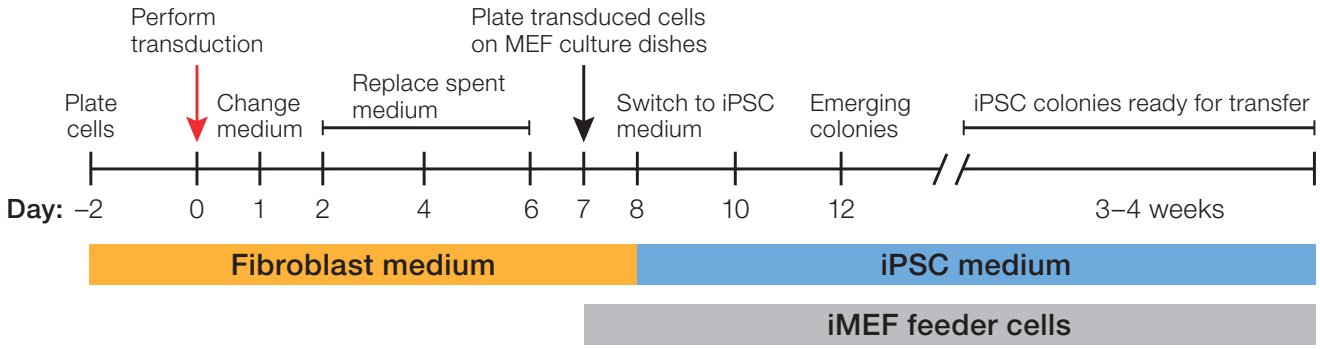
iPSC medium: Used for expansion of transduced cells on MEF culture dishes, live staining, and picking of iPSCs

To prepare 100 mL of complete iPSC medium, aseptically mix the following components:

KnockOut DMEM/F-12	78 mL
KnockOut Serum Replacement – Multi-Species	20 mL
MEM Non-Essential Amino Acids Solution (100X)	1 mL
2-Mercaptoethanol	100 µL
Penicillin-Streptomycin (10,000 U/mL) (optional)	1 mL
FGF-Basic (AA 1–155) Recombinant Human Protein*	40 µL

* Prepare the iPSC medium without Gibco FGF-Basic (AA 1–155) Recombinant Human Protein (bFGF), and then supplement with fresh bFGF when the medium is used.

Complete iPSC medium (without bFGF) can be stored at 2–8°C for up to 3 weeks.



Reprogramming workflow timeline

Day –2: Plate human fibroblasts with a passage number of 5 or lower into at least 2 wells of a 6-well plate in fibroblast medium so that they will be 30–60% confluent on the day of transduction (day 0).

Note: The kit supplies sufficient virus to transduce cells in at least 5 wells of a 6-well plate.

Day 0: Transduce the cells using the CytoTune-iPS 2.0 Sendai reprogramming vectors at the appropriate multiplicity of infection (MOI). Incubate the cells overnight.

Day 1: Replace the medium with fresh complete fibroblast medium to remove the CytoTune-iPS 2.0 vectors.

Day 2–6: Replace the spent medium every other day.

Day 5 or 6: Prepare MEF culture dishes.

Day 7: Plate transduced cells on MEF culture dishes in fibroblast medium.

Day 8: Change the medium to iPSC medium.

Day 9–28: Replace the spent medium every day, and monitor the culture vessels for the emergence of iPSC colonies. When iPSC colonies are ready for transfer, perform live staining, and pick and transfer undifferentiated iPSCs onto fresh MEF culture dishes for expansion.

Figure 1.1. Reprogramming workflow for human fibroblasts, using the CytoTune-iPS 2.0 Sendai Reprogramming Kit. The major steps of reprogramming human neonatal foreskin fibroblasts using the CytoTune-iPS 2.0 Sendai Reprogramming Kit to generate iPSCs cultured on iMEF (inactivated MEF) feeder cells are shown above. Note that the timeline is provided as a guideline for experimental planning; the actual timeline can vary based on the cell type and experimental conditions.

Reprogramming fibroblasts

The following protocol has been optimized to transduce 1 well of human neonatal foreskin fibroblasts, ATCC CRL-2522 BJ strain, as a positive control. We recommend that you optimize the protocol for your cell type, and add an appropriate number of conditions and/or wells to utilize the entire volume of virus.

Day –2: Prepare the cells for transduction

1. Two days before transduction, plate human neonatal foreskin fibroblasts onto at least 2 wells of a 6-well plate at the appropriate density to obtain between 2 x 10^5 and 3 x 10^5 cells per well on the day of transduction (day 0). One of the wells will be used to count cells for viral volume calculations.

   Note: Each CytoTune-iPS 2.0 Sendai Reprogramming Kit supplies sufficient virus to transduce cells in at least 5 wells of a 6-well plate. We recommend using the entire volume of virus.

   Note: We recommend about 30–60% confluency on the day of transduction. Because overconfluency results in decreased transduction efficiency, we recommend replating your cells to achieve 30–60% confluency if your cells have become overconfluent during culturing.

2. Culture the cells for 2 more days, ensuring the cells have fully adhered and extended.

Day 0: Perform transduction

3. On the day of transduction, in a water bath, warm 1 mL of fibroblast medium for each well to be transduced.

4. Harvest the cells from one well to perform a cell count. These cells will not be transduced, but will be used to estimate the cell number in the other well(s) plated in step 1.

5. Remove the cells from this well using 0.5 mL of TrypLE Select Enzyme (1X) or trypsin-EDTA (0.05%), following the procedure recommended by the manufacturer and incubating at room temperature. When the cells have rounded up after 1–3 minutes, add 1 mL of fibroblast medium to each well, and collect the cells from each well into a 15 mL conical centrifuge tube.

6. Count the cells using the desired method (e.g., Invitrogen™ Countess™ Automated Cell Counter, Cat. No. AMQAX1000), and calculate the volume of each virus needed to reach the target MOI, using the live cell count and the titer information on the CoA.

$$\text{Volume of virus (µL)} = \frac{\text{MOI (CIU/cell) x number of cells}}{\text{Titer of virus (CIU/mL) x } 10^{-3} \text{ (mL/µL)}}$$

Note: We recommend initially performing the transductions with MOIs of 5, 5, and 3 (i.e., KOS MOI = 5, cMyc MOI = 5, and Klf4 MOI = 3). These MOIs can be optimized for your application.

Note: The titer of each CytoTune-iPS 2.0 Sendai reprogramming vector is lot-dependent. For the specific titers of your vectors, go to **thermofisher.com/cytotune** and search for the CoA by the product lot number that is printed on the vial. Avoid refreezing and thawing of the reprogramming vectors since viral titers can decrease dramatically with each freeze/thaw cycle.

7. Remove one set of viral vector tubes from –80°C storage. Thaw each tube one at a time by first immersing the bottom of the tube in a 37°C water bath for 5–10 seconds, and then removing the tube from the water bath and allowing it to thaw at room temperature. Once thawed, briefly centrifuge the tube and place it immediately on ice.

8. Add the calculated volumes of each of the three viral vectors to 1 mL of fibroblast medium, prewarmed to 37°C. Ensure that the solution is thoroughly mixed by pipetting the mixture gently up and down. Complete the next step within 5 minutes.

9. Aspirate the fibroblast medium from the cells, and add the reprogramming virus mixture prepared in step 8 to the well containing the cells. Incubate the cells overnight in a 37°C incubator with a humidified atmosphere of 5% CO_2.

Day 1: Replace medium and culture cells

10. At 24 hours after transduction, replace the medium with fresh fibroblast medium.

    Note: Depending on your cell type, you should expect to see some cytotoxicity 24–48 hours posttransduction, which may affect >50% of your cells. This is an indication of high uptake of the virus. We recommend that you continue culturing your cells and proceed with the protocol.

11. Culture the cells for 6 more days, changing the spent medium with fresh fibroblast medium every other day.

    Note: Depending on your cell type, you may observe high cell density before day 5. We do **not** recommend passaging your cells onto MEF culture dishes before 7 days posttransduction. You may replace spent medium daily with fresh fibroblast medium if cultures become very dense.

Day 5 or 6: Prepare MEF culture dishes

12. 1–2 days before passaging the transduced fibroblasts onto MEF feeder cells, prepare 6-well MEF culture dishes. (Refer to section 2.1A for detailed instructions on preparing MEF culture dishes.)

Day 7: Plate transduced cells on MEF culture dishes

13. At 7 days after transduction, fibroblasts are ready to be harvested and plated on the MEF culture dishes. Remove the medium from the fibroblasts and wash the cells once with DPBS.

14. To remove the cells from the 6-well plate, use 0.5 mL of TrypLE Select Enzyme (1X) or trypsin-EDTA (0.05%) following the procedure recommended by the manufacturer and incubate at room temperature. When the cells have rounded up (1–3 minutes later), add 2 mL of fibroblast medium into each well, and collect the cells in a 15 mL conical centrifuge tube.

    Note: Because the cells can be very sensitive to trypsin at this point, minimize trypsin exposure time and incubate the cells at room temperature.

15. Centrifuge the cells at 200 x *g* for 4 minutes, aspirate the medium, and resuspend the cells in an appropriate amount of fibroblast medium.

16. Count the cells using the desired method (e.g., Countess Automated Cell Counter), and seed the MEF culture dishes with 2 x 10^4–1 x 10^5 cells per well and incubate overnight in a 37°C incubator with a humidified atmosphere of 5% CO_2.

    Note: Reprogramming efficiency can vary widely, so we recommend plating between 2 and 4 different densities. Depending on your cell type, you may need to plate most of your cells in the same well to ensure sufficient numbers of colonies.

    Note: We strongly recommend setting aside cells at this point for RNA extraction to be used as a positive control in the RT-PCR or quantitative PCR (qPCR) detection of the CytoTune-iPS 2.0 Sendai vectors. It is very important to include this positive control when performing detection of the CytoTune-iPS 2.0 Sendai vectors.

Day 8 to 28: Feed and monitor the cells

17. After 24 hours, change the medium to iPSC medium and replace the spent medium every day after that.

18. Starting on day 8, observe the plates every other day under a microscope for the emergence of cell clumps indicative of reprogrammed cells.

    Note: For BJ fibroblasts, we normally observe colony formation on day 12 posttransduction. However, depending on your cell type, you may need to culture for up to 4 weeks before seeing colonies.

19. At 3–4 weeks after transduction, colonies should have grown to an appropriate size for transfer. The day before transferring the colonies, prepare MEF culture plates using 6- or 12-well plates coated with Gibco Attachment Factor Protein.

    Note: We typically harvest colonies closer to 3 weeks posttransduction to avoid differentiation.

20. When colonies are ready for transfer, perform live staining as described in section 1.2 in order to identify reprogrammed colonies.

21. Manually pick colonies and transfer them to MEF plates as described in section 1.2C.

Required materials for reprogramming of feeder-free human fibroblasts

Cells and vectors

- CytoTune-iPS 2.0 Sendai reprogramming vectors
  Note: For successful reprogramming, you need all three tubes of reprogramming vectors.
- Human fibroblasts to reprogram
- **Optional:** Human neonatal foreskin fibroblasts—ATCC CRL-2522 strain BJ—as a positive reprogramming control
- Gibco CF1 Mouse Embryonic Fibroblasts, irradiated (Cat. No. A34181)

Media and reagents

- DMEM, high glucose, GlutaMAX Supplement, pyruvate (Cat. No. 10569-010)
- ES Cell–Qualified FBS, US origin (Cat. No. 16141-079)
- **Optional:** Gibco Penicillin-Streptomycin (10,000 U/mL) (Cat. No. 15140-122)
- TrypLE Select Enzyme (1X) (Cat. No. 12563029) or Gibco Trypsin-EDTA (0.05%) (Cat. No. 25300054)
- DPBS, no calcium, no magnesium (Cat. No. 14190359)
- Feeder-free PSC culture medium:
  - Essential 8 Medium (Cat. No. A1517001), or
  - Essential 8 Flex Medium (Cat. No. A2858501), or
  - StemFlex Medium (Cat. No. A3349401)
- Matrix:
  - Gibco™ Vitronectin (VTN-N) Recombinant Human Protein, Truncated (Cat. No. A14700), or
  - Gibco™ Geltrex™ LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix (Cat. No. A1413302), or
  - Gibco™ rhLaminin-521 (Cat. No. A29248)

Table 1.1. Feeder-free PSC culture media selection guide.

	Essential 8 Medium	Essential 8 Flex Medium	StemFlex Medium
Benefit during reprogramming	High reprogramming efficiency	High reprogramming efficiency	Results in large colonies that are easy to pick
Flexible feeding schedule	No	Yes	Yes
Xeno-free	Yes	Yes	No
Recommended matrix	Vitronectin	Vitronectin	Geltrex matrix
Compatible matrices	Vitronectin, Geltrex matrix, or rhLaminin-521	Vitronectin, Geltrex matrix, or rhLaminin-521	Vitronectin, Geltrex matrix, or rhLaminin-521

Media for reprogramming of feeder-free human fibroblasts

For optimal reprogramming of human neonatal foreskin fibroblasts using the CytoTune-iPS 2.0 Sendai Reprogramming Kit to generate feeder-free iPSCs, use the following media at the designated stages of the reprogramming experiment:

Fibroblast medium: Used for plating cells prior to transduction, expansion, posttransduction recovery of cells, and plating of transduced cells on matrix-coated culture dishes

To prepare 100 mL of complete fibroblast medium, aseptically mix the following components:

DMEM, high glucose, GlutaMAX Supplement, pyruvate	89 mL
ES Cell–Qualified FBS, US origin	10 mL
MEM Non-Essential Amino Acids Solution (100X)	1 mL
2-Mercaptoethanol	100 µL

Complete fibroblast medium can be stored at 2–8°C for up to 1 week.

Feeder-free PSC culture media: Used for expansion of transduced cells on matrix-coated culture dishes, live staining, and picking of iPSCs

For coating protocols, refer to section 2.2A. For preparation of feeder-free media, refer to section 2.2B.

Reprogramming fibroblasts

The following protocol has been optimized to transduce 1 well of human neonatal foreskin fibroblasts, ATCC CRL-2522 BJ strain, as a positive control. We recommend that you optimize the protocol for your cell type, and add an appropriate number of conditions and/or wells to utilize the entire volume of virus.

Day –2: Prepare the cells for transduction

1. Two days before transduction, plate human neonatal foreskin fibroblasts onto at least 2 wells of a 6-well plate at the appropriate density to obtain between 2×10^5 and 3×10^5 cells per well on the day of transduction (day 0). One of the wells will be used to count cells for viral volume calculations.

   Note: Each CytoTune-iPS 2.0 Sendai Reprogramming Kit supplies sufficient virus to transduce cells in at least 5 wells of a 6-well plate. We recommend using the entire volume of virus.

   Note: We recommend about 30–60% confluency on the day of transduction. Because overconfluency results in decreased transduction efficiency, we recommend replating your cells to achieve 30–60% confluency if your cells have become overconfluent during culturing.

2. Culture the cells for 2 more days, ensuring the cells have fully adhered and extended.

Day 0: Perform transduction

3. On the day of transduction, warm 1 mL of fibroblast medium in a water bath for each well to be transduced.

4. Harvest the cells from one well to perform a cell count. These cells will not be transduced, but will be used to estimate the cell number in the other well(s) plated in step 1.

5. Remove the cells from the 6-well plate using 0.5 mL of TrypLE Select Enzyme (1X) or trypsin-EDTA (0.05%) following the procedure recommended by the manufacturer and incubating at room temperature. When the cells have rounded up (1–3 minutes later), add 1 mL of fibroblast medium into each well, and collect the cells in a 15 mL conical centrifuge tube.

6. Count the cells using the desired method (e.g., Countess Automated Cell Counter, Cat. No. AMQAX1000), and calculate the volume of each virus needed to reach the target MOI using the live-cell count and the titer information on the CoA.

$$\text{Volume of virus (µL)} = \frac{\text{MOI (CIU/cell) x number of cells}}{\text{Titer of virus (CIU/mL) x } 10^{-3} \text{ (mL/µL)}}$$

Note: We recommend initially performing the transductions with MOIs of 5, 5, and 3 (i.e., KOS MOI = 5, cMyc MOI = 5, Klf4 MOI = 3). These MOIs can be optimized for your application.

Note: The titer of each CytoTune-iPS 2.0 Sendai reprogramming vector is lot-dependent. For the specific titers of your vectors, go to **thermofisher.com/cytotune** and search for the CoA by the product lot number that is printed on the vial. Avoid refreezing and thawing of the reprogramming vectors since viral titers can decrease dramatically with each freeze/thaw cycle.

7. Remove one set of CytoTune-iPS 2.0 Sendai viral vector tubes from the –80°C storage. Thaw each tube one at a time by first immersing the bottom of the tube in a 37°C water bath for 5–10 seconds, and then removing the tube from the water bath and allowing it to thaw at room temperature. Once thawed, briefly centrifuge the tube and place it immediately on ice.

8. Add the calculated volumes of each of the three viral vectors to 1 mL of fibroblast medium, prewarmed to 37°C. Ensure that the solution is thoroughly mixed by pipetting the mixture gently up and down. Complete the next step within 5 minutes.

9. Aspirate the fibroblast medium from the cells, and add the reprogramming virus mixture prepared in step 8 to the well containing the cells. Incubate the cells overnight in a 37°C incubator with a humidified atmosphere of 5% CO_2.

Day 1: Replace medium and culture cells

10. At 24 hours after transduction, replace the medium with fresh fibroblast medium.

    Note: Depending on your cell type, you should expect to see some cytotoxicity 24–48 hours posttransduction, which may affect >50% of your cells. This is an indication of high uptake of the virus. We recommend that you continue culturing your cells and proceed with the protocol.

11. Culture the cells for 6 more days, changing the spent medium with fresh fibroblast medium every other day.

    Note: Depending on your cell type, you may observe high cell density before day 5. We do not recommend passaging your cells before 7 days posttransduction. You may replace spent medium daily with fresh fibroblast medium if cultures become very dense.

Day 7: Plate transduced cells on matrix-coated culture dishes

12. Coat a sufficient number of 6-well tissue culture dishes with matrix (see section 2.2A for coating protocols).

    Note: We generally recommend using vitronectin with Essential 8 or Essential 8 Flex Medium and the Geltrex matrix with StemFlex Medium.

13. 7 days after transduction (step 9, above), fibroblasts are ready to be harvested and plated on matrix-coated culture dishes. Remove the medium from the fibroblasts, and wash cells once with DPBS.

14. To remove the cells from the 6-well plate, use 0.5 mL of TrypLE Select Enzyme (1X) or trypsin-EDTA (0.05%) following the procedure recommended by the manufacturer and incubate at room temperature. When the cells have rounded up (1–3 minutes later), add 2 mL of fibroblast medium into each well, and collect the cells in a 15 mL conical centrifuge tube.

    Note: Because the cells can be very sensitive to trypsin at this point, minimize trypsin exposure time and incubate the cells at room temperature.

15. Centrifuge the cells at 200 x *g* for 4 minutes, aspirate the medium, and resuspend the cells in an appropriate amount of fibroblast medium.

16. Count the cells using the desired method (e.g., Countess Automated Cell Counter), and seed the matrix-coated culture dishes with 2 x 10^4–1 x 10^5 cells per well of a 6-well plate (or the equivalent for other types of plates) and incubate overnight in a 37°C incubator with a humidified atmosphere of 5% CO_2.

    Note: Reprogramming efficiencies will typically be lower when using feeder-free conditions, so the number of cells plated should be increased accordingly. We recommend plating between 2 and 4 different densities per well. Depending on your cell type, you may need to plate most of your cells on the same plate to ensure sufficient numbers of colonies.

    Note: We strongly recommend that you set aside cells at this point for RNA extraction to be used as a positive control in the RT-PCR or qPCR detection of the CytoTune-iPS 2.0 Sendai viral vectors. It is very important to include this positive control when performing detection of the CytoTune-iPS 2.0 Sendai viral vectors.

Day 8 to 28: Feed and monitor the cells

17. After 24 hours, change the medium to a feeder-free PSC culture medium. If using Essential 8 Medium, replace the spent medium every day after that. If using Essential 8 Flex or StemFlex Medium, replace spent medium every other day after that.

18. Starting on day 8, observe the plates every other day under a microscope for the emergence of cell clumps indicative of reprogrammed cells.

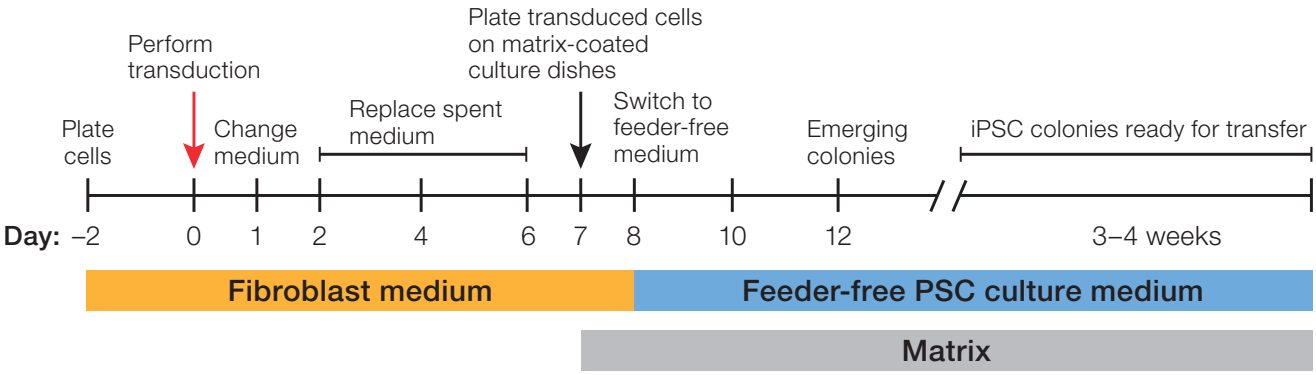
    Note: For BJ fibroblasts, we normally observe colony formation on day 12 posttransduction. However, depending on your cell type, you may need to culture for up to 4 weeks before seeing colonies.

19. 3–4 weeks after transduction, colonies should have grown to an appropriate size for transfer.

    Note: We typically harvest colonies closer to 3 weeks to avoid differentiation.

20. When colonies are ready for transfer, perform live staining as described in section 1.2 in order to identify reprogrammed colonies.

21. Manually pick undifferentiated iPSC colonies and transfer them onto 6- or 12-well, prepared, matrix-coated culture plates for further expansion or analysis, as described in section 1.2.



Reprogramming workflow timeline

Day –2: Plate human fibroblasts into at least 2 wells of a 6-well plate in fibroblast medium so that they are 30–60% confluent on the day of transduction (day 0).

Note: The kit supplies sufficient virus to transduce cells in at least 5 wells of a 6-well plate.

Day 0: Transduce the cells using the CytoTune-iPS 2.0 Sendai reprogramming vectors at the appropriate MOI. Incubate the cells overnight.

Day 1: Replace the medium with fresh complete fibroblast medium to remove the CytoTune-iPS 2.0 Sendai reprogramming vectors.

Day 2–6: Replace the spent medium every other day.

Day 7: Plate transduced cells on matrix-coated culture dishes in fibroblast medium.

Day 8: Change the medium to complete Gibco Essential 8, Essential 8 Flex, or StemFlex medium.

Day 9–28: Replace spent medium every day, and monitor the culture vessels for the emergence of iPSC colonies. When iPSC colonies are ready for transfer, perform live staining, and pick and transfer undifferentiated iPSCs onto fresh culture dishes for expansion.

Figure 1.2. Reprogramming workflow (feeder-free). The major steps required for reprogramming human neonatal foreskin fibroblasts using the CytoTune-iPS 2.0 Sendai Reprogramming Kit to generate feeder-free iPSCs are shown above. Note that the timeline is provided as a guideline for experimental planning; actual timeline can vary based on the cell type and experimental conditions.

1.2 Identifying and picking emerging iPSC clones

At 3–4 weeks posttransduction, emerging iPSC clones can be identified on a reprogramming plate as colonies by their cells and defined edges. These colonies can differ in quality, from partially to fully reprogrammed clones. A more reliable approach for identifying reprogrammed colonies is to perform live staining with PSC-specific dyes or conjugated antibodies. This section provides protocols for staining with the Invitrogen™ Alkaline Phosphatase (AP) Live Stain or Invitrogen™ live cell imaging kits. It also includes instructions for subsequently picking an emerging iPSC clone for expansion.

1.2A Staining with AP Live Stain

AP is a phenotypic marker of PSCs, including undifferentiated ESCs, iPSCs, and embryonic germ cells (EGCs) [1-4]. While AP is expressed in most cell types, its expression is highly elevated in PSCs. AP staining has therefore been used to differentially stain PSCs to easily distinguish them from MEFs used as feeders and parental fibroblasts commonly used in reprogramming experiments [5,6]. However, the AP substrates that are currently available are toxic to the cells, which prevent them from propagating once stained.

The AP Live Stain can be applied to adherent PSCs in culture without loss of proliferation or pluripotency. Staining is specific to PSCs with minimal background in MEF feeders, primary fibroblasts, and other somatic cell types commonly used for reprogramming, providing an easy-to-use, live-monitoring method to track cells during reprogramming or during routine culture of ESCs and iPSCs. After removal of dye from the media, fluorescently labeled cells lose their signal within 60–90 minutes.

Note: In general, AP is not an ideal marker for distinguishing between undifferentiated and early differentiating cells. Therefore, the AP Live Stain is not meant for distinguishing undifferentiated cells from differentiated cells [7].

Product

AP Live Stain (Cat. No. A14353), stored at –20°C

Features

- Differentially stains PSCs
- Has specificity and staining pattern similar to other AP dyes
- Unlike other AP dyes, maintains the integrity of the cells

Thawing AP Live Stain

Remove the AP Live Stain vial from the –20°C freezer and thaw at room temperature. Avoid repeated freeze/thaw cycles and aliquot the AP Live Stain into smaller volumes if necessary.

Note: It is important to completely thaw the AP Live Stain in room temperature.

Preparing the staining solution

To prepare a 1X AP Live Stain working solution, dilute the 500X stock solution in Gibco™ DMEM/F-12 with GlutaMAX™ Supplement (Cat. No. 10565-018) as described in the table below. Use the diluted dye immediately.

Note: It is important to dilute AP Live Stain stock solution in a basal media, such as DMEM/F-12 with GlutaMAX Supplement.

Culture area	AP Live Stain (500X)	DMEM/F-12 with GlutaMAX
2 cm^2	1 µL	0.5 mL
10 cm^2	3 µL	1.5 mL
20 cm^2	6 µL	3.0 mL
60 cm^2	12 µL	6.0 mL

Staining procedure

1. Remove the growth medium from the cultures to be stained with AP Live Stain.
2. Wash the culture with prewarmed DMEM/F-12 with GlutaMAX Supplement for 2–3 minutes. Aspirate and repeat.
3. Prepare a 1X AP Live Stain working solution by diluting the 500X stock solution in DMEM/F-12 with GlutaMAX Supplement (see "Preparing the staining solution"). Apply an appropriate amount of the 1X AP Live Stain solution directly on to the adherent cell culture.
4. Incubate culture for 20–30 minutes.

   Note: Incubation with media containing FBS, KnockOut SR, or feeder-free supplements designed for the growth and maintenance of ESCs and iPSCs can interfere with the AP Live Stain reaction.
5. Remove the AP Live Stain and wash twice with DMEM/F-12 with GlutaMAX solution for 5 minutes per wash.

   Note: This step removes the excess AP Live Stain and reduces the background signal. Handle the cells aseptically, and carefully add and remove the medium with minimal disruption to the adherent cells during this step to avoid damaging the cells.
6. Following the final wash, add fresh DMEM/F-12 with GlutaMAX solution prior to the visualization of fluorescently labeled colonies under fluorescence microscopy using a standard FITC filter.

   Note: Media containing FBS, KnockOut SR, or feeder-free supplements designed for the growth and maintenance of ESCs and iPSCs can interfere with fluorescence visualization.
7. Images can be captured within 30–90 minutes of staining and the most robust fluorescent colonies can be marked for selection and expansion.
8. Following visualization, replace the DMEM/F-12 with GlutaMAX Supplement with fresh growth medium. Selected colonies can be either manually picked or returned to the normal culture conditions.
9. Stained colonies may be restained as early as 24 hours after the initial staining with the AP Live Stain.

Expected results

The images below show various cells stained with the AP Live Stain. The differential staining of pluripotent cells easily distinguishes them from the feeder cells on which they have been cultured.

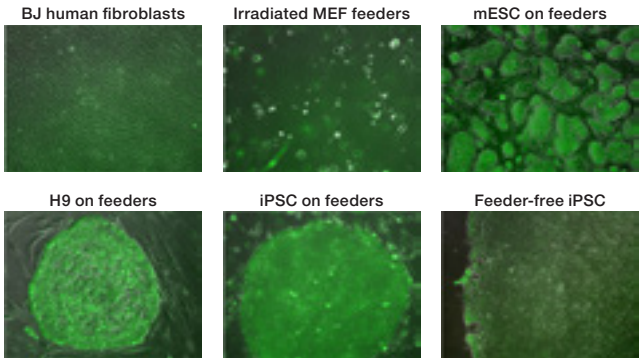


Figure 1.3. Specificity of the AP Live Stain.

Troubleshooting

No signal observed

Be sure to use the DMEM/F-12 with GlutaMAX Supplement to dilute the AP Live Stain and during wash steps.

High background staining in MEFs

Since the AP Live Stain depends on the differential expression of AP, dim staining of MEFs may be observed. This pattern is consistent with that observed with other AP dyes (Figure 1.4).

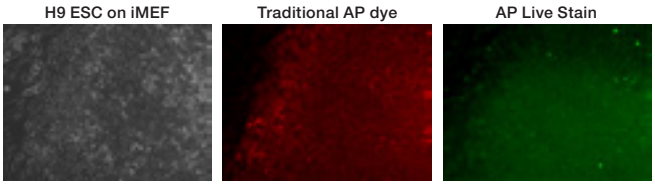


Figure 1.4. Traditional AP dye vs. the AP Live Stain.

- Wash steps are critical to removing the excess dye and to eliminating background staining. To further decrease background staining, perform 3 washes of 5 minutes each for a total of 15 minutes.
- Depending on the sensitivity of the imaging device and the background observed, AP Live Stain concentrations in the range of 0.2–1X can be used, and the time of exposure to the stain can be reduced to 10 minutes or increased to 40 minutes.

Intense staining seen in a few fibroblast-like cells

Sometimes cells that have a distinct fibroblast morphology may show robust staining with the AP Live Stain. These AP-positive fibroblast-like cells are seen only in ESC-MEF co-cultures and never in the MEF cultures alone (see Figure 1.5). Observe the entire dish to determine if the robust staining in fibroblast-like structures is in a small fraction of cells.

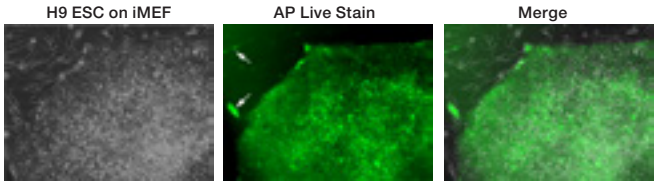


Figure 1.5. Staining of H9 ESCs and MEF feeder layers.

Survival of cells is low

It is important to handle the cells carefully during staining and subsequent washes. It has been experimentally confirmed that when cells are handled with care during staining and subsequent washing steps, the AP Live Stain does not alter cell survival.

References

1. Andrews et al. (1984) Two monoclonal antibodies recognizing determinants on human embryonal carcinoma cells react specifically with the liver isozyme of human alkaline phosphatase. *Hybridoma* 3:33–39.
2. Pera et al. (2000) Human embryonic stem cells. *J Cell Sci* 113:5–10.
3. Shamblott et al. (1988) Derivation of pluripotent stem cells from cultured human primordial germ cells. *Proc Natl Acad Sci USA* 95:13726–13731.
4. O'Connor et al. (2008) Alkaline phosphatase-positive colony formation is a sensitive, specific, and quantitative indicator of undifferentiated human embryonic stem cells. *Stem Cells* 26:1109–1116.
5. Thomson et al. (1998) Embryonic stem cell lines derived from human blastocysts. *Science* 282:1145–1147.
6. Takahashi et al. (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131:861–872.
7. Ramirez et al. (2011) Benchmarking human pluripotent stem cell markers during differentiation into the three germ layers unveils a striking heterogeneity: all markers are not equal. *Stem Cells* 29:1469–1474

1.2B Staining with live-cell imaging kits

Invitrogen™ stem cell antibody kits for live-cell imaging pair with Invitrogen™ Alexa Fluor™ dye–conjugated antibodies against important cell-surface stem cell markers in a superior imaging medium, Gibco™ FluoroBrite™ DMEM, which enhances fluorescence signals while preserving cell health during live-cell imaging workflows. These reagents are specially formulated and sterile-filtered to meet the demands of live-cell imaging applications; they contain no cytotoxic preservatives (e.g., sodium azide) and are tested to ensure that endotoxin levels are low and that they are free of common microbial contaminants. TRA-1-60 antibody is a commonly used positive cell-surface marker for PSC identification. The fibroblast marker CD44 can be used as a negative marker during reprogramming. The combination of both markers is useful for identifying fully reprogrammed iPSC colonies.

Table 1.2. Contents and storage.

Kit	Cat. No.	Kit components	Cat. No.	Concentration	Volume	Storage and handling
CD44 Alexa Fluor 488 Conjugate Kit for Live Cell Imaging	**A25528**	CD44 Rat Anti-Human/Mouse mAb, Alexa Fluor 488 Conjugate	A25527	50X	200 µL	• Use aseptic technique • Store at 2–8°C for up to 6 months • Protect from light • Do not freeze
		FluoroBrite DMEM	A1896701	1X	500 mL	
TRA-1-60 Alexa Fluor 488 Conjugate Kit for Live Cell Imaging	**A25618**	TRA-1-60 Mouse Anti-Human mAb, AlexaFluor 488 Conjugate	A25617	50X	200 µL	
		FluoroBrite DMEM	A1896701	1X	500 mL	
TRA-1-60 Alexa Fluor 555 Conjugate Kit for Live Cell Imaging	**A24879**	TRA-1-60 Mouse Anti-Human mAb, Alexa Fluor 555 Conjugate	A24874	50X	200 µL	
		FluoroBrite DMEM	A1896701	1X	500 mL	
TRA-1-60 Alexa Fluor 594 Conjugate Kit for Live Cell Imaging	**A24882**	TRA-1-60 Mouse Anti-Human mAb, Alexa Fluor 594 Conjugate	A24880	50X	200 µL	
		FluoroBrite DMEM	A1896701	1X	500 mL	

Approximate fluorescence excitation/emission wavelength maxima:
Alexa Fluor 488 conjugate: Ex/Em 495/519 nm (green, FITC filter–compatible)
Alexa Fluor 555 conjugate: Ex/Em 555/565 nm (orange, Cy®3, TRITC filter–compatible)
Alexa Fluor 594 conjugate: Ex/Em 590/617 nm (red, Texas Red filter–compatible)

Experimental protocol

See Table 1.3 for recommended volumes.

Caution: Use aseptic technique when handling live-cell imaging reagents to prevent contamination.

1. Centrifuge the dye-conjugated antibody solution (e.g., 2 minutes at 10,000 x *g*) and only use the supernatant.

   Note: This step minimizes transferring protein aggregates that may have formed during storage, thereby reducing nonspecific background staining.

2. Add the dye-conjugated antibody in a volume ratio of 1:50 directly to the cell culture medium of the cells to be stained (see Table 1.3 for guidance). Mix by gentle swirling.

3. Incubate for 30 minutes at 37°C.

4. Remove the staining solution and gently wash the cells 2–3 times with FluoroBrite DMEM.

5. For optimal results, image the cells immediately (i.e., within 30 minutes).

   Note: To continue culturing the cells, replace the FluoroBrite DMEM with fresh cell culture medium and return the cells to the 37°C incubator.

Table 1.3. Recommended volumes for the staining protocol.

Culture vessel	Number of tests*	Staining volume	Volume of 50X antibody to add
96-well plate	200	50 µL/well	1 µL
48-well plate	100	100 µL/well	2 µL
24-well plate	50	200 µL/well	4 µL
12-well plate	25	400 µL/well	8 µL
6-well plate	10	1 mL/well	20 µL
35 mm dish	10	1 mL/well	20 µL
60 mm dish	5	2 mL/well	40 µL
100 mm dish	2	5 mL/well	100 µL
4-well chamber slide	25	400 µL/well	8 µL
8-well chamber slide	50	200 µL/well	4 µL

* When the suggested staining volume is used, this kit contains sufficient reagents for the indicated number of tests.

Example data

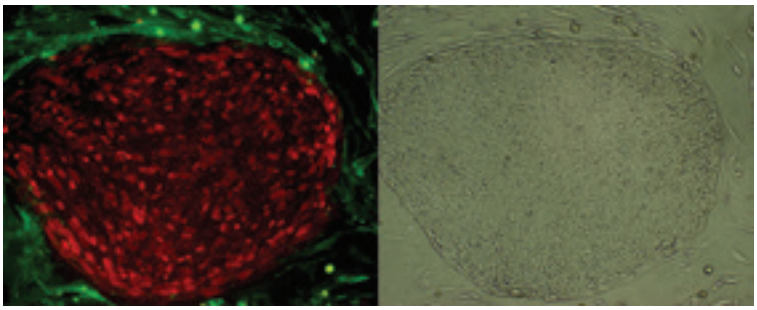


Figure 1.6. iPSCs growing on a mouse fibroblast feeder layer, stained with dye-labeled antibodies for the cell-surface markers TRA-1-60 (red, a positive pluripotency marker) and CD44 (green, a fibroblast marker that also serves as a negative pluripotency marker).

1.2C Picking emerging iPSC clones

Picking iPSC colonies (feeder-dependent)

1. Place the culture dish containing the reprogrammed cells under an inverted microscope and examine the colonies under 10x magnification.
2. Mark the colony to be picked on the bottom of the culture dish.

   Note: We recommend picking at least 10 distinct colonies by the end of each reprogramming experiment and expanding them in separate 6- or 12-well MEF culture plates (see below).
3. Transfer the culture dish to a sterile cell culture hood (i.e., biosafety cabinet) equipped with a stereomicroscope.
4. Using a 25-gauge, 1½-inch needle, cut the colony to be picked into 5–6 pieces in a grid-like pattern.
5. Using a 200 µL pipette, transfer the cut pieces to a freshly prepared 6- or 12-well MEF culture plate (see page 22) containing iPSC medium (see page 6).
6. Incubate the MEF culture plate containing the picked colonies in a 37°C incubator with a humidified atmosphere of 5% CO_2.
7. Allow the colonies to attach to the culture plate for 48 hours before replacing the spent medium with fresh iPSC medium. After that, change the medium every day.
8. When the colonies cover ~85% of the surface area of the culture dish, the culture is ready for passaging. From passages 1 to 5 (P1–P5) use manual passaging, as the colonies have not matured enough to use enzymatic harvesting methods. To perform manual passaging follow steps 1–7 above, except that all undifferentiated colonies will be passaged; hence, specific colonies do not have to be marked. From passage 5 onwards, use 1X collagenase to passage cells, as described on page 27.

Picking iPSC colonies (feeder-free)

1. Pick the iPSCs as described for the feeder-dependent colonies, up to step 4.
2. Using a 200 µL pipette, transfer the cut pieces onto a matrix-coated 6- or 12-well culture plate (section 2.2A) containing complete feeder-free PSC culture medium.
3. Incubate the matrix-coated culture plate containing the picked colonies in a 37°C incubator with a humidified atmosphere of 5% CO_2.
4. Allow the colonies to attach to the culture plate for 48 hours before replacing the spent medium with fresh complete feeder-free PSC culture medium. After that, change the medium every day.
5. When the colonies cover ~85% of the surface area of the culture dish, the culture is ready for passaging. From passages 1 to 5 (P1–P5) use manual passaging, as the colonies have not matured enough to use chemical harvesting methods. To perform manual passaging follow steps 1 and 2 above, except that all undifferentiated colonies will be passaged; hence, specific colonies do not have to be marked. From passage 5 onwards, use Gibco™ Versene™ Solution (Cat. No. 15040066) or trypsin-EDTA (0.05%) prepared in DPBS with no calcium or magnesium, as described in section 2.2B.

   Note: Enzymes such as collagenase and dispase do not work well with cells cultured in Essential 8 Medium on vitronectin-coated plates. Use of these enzymes for passaging cells results in compromised viability and attachment.
6. Continue to culture, expand, and maintain the reprogrammed colonies in complete Essential 8 Medium until you can freeze down cells from two 60 mm plates.

1.3 Establishing and banking iPSC clones

Once emerging iPSC colonies have been identified and picked, we recommend culturing the cells for 8–10 passages to help stabilize the clone and generate enough cells for a master bank and for more comprehensive characterization. Detailed protocols for the culture, expansion, and cryopreservation of iPSCs under feeder-dependent or feeder-free conditions are described in section 2 of this book. Guidance on the comprehensive characterization of PSCs is provided in section 3. Here we describe a sample scheme for generating enough cells for maintenance, banking, and characterization of the new iPSC line (Figure 1.7).

At about passage 8 or 9, split the cells such that there are at least two 60 mm dishes of cells. At the next passage, split at a ratio of 1:4, generating six to seven 60 mm dishes and one T-25 flask. These should serve as a good starting point for the establishment of the new iPSC line, with one dish for maintaining the culture, four dishes for creating the master bank, two dishes for analysis of PSC marker expression and trilineage potential, and the T-25 flask for karyotyping. (**Note:** The T-25 flask is used to ship live cells for G-banding. Alternatively, another 60 mm dish can be used for Applied Biosystems™ KaryoStat™ analysis as described in section 3.1.)

From one 60 mm dish, 3 vials can be made for cryopreservation. From four 60 mm dishes, 12 vials can be generated. To create a larger master bank or to perform more characterization on the cells, more dishes can be generated at passage 8 or 9, each split at 1:4. Once the master bank is made, vials can be thawed from the bank, cultured for a passage or two and then subjected to further experiments or further expansion. The same approach can be used for iPSCs grown on feeders or in feeder-free conditions.

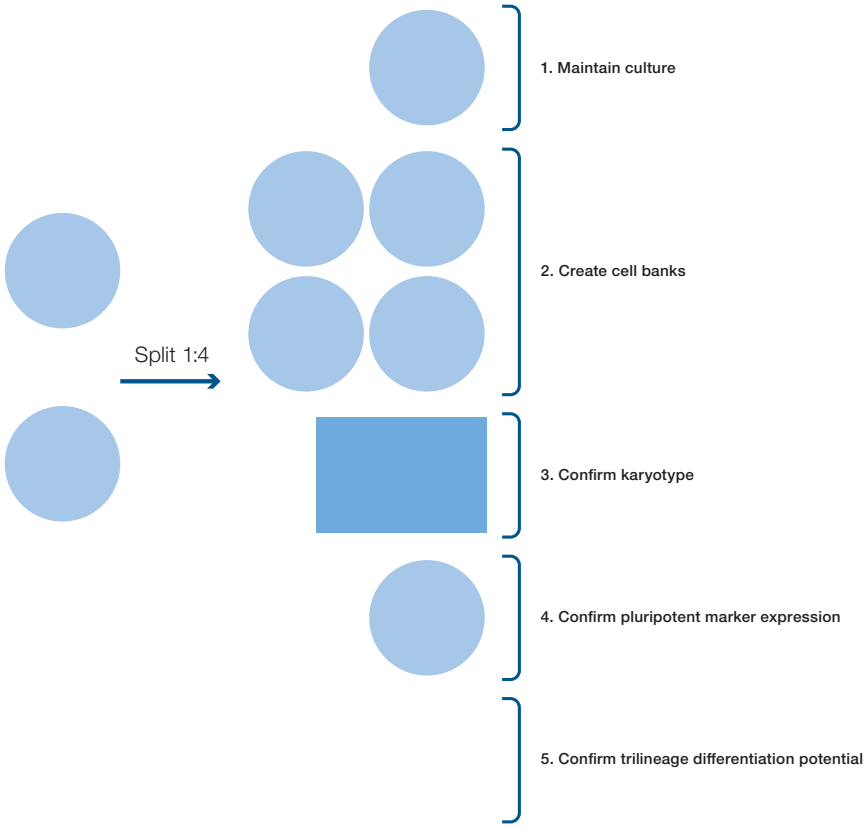


Figure 1.7. Workflow schematic for generating new iPSCs.

2. Culture and expansion of PSCs

2.1 Culturing PSCs with feeders

Culturing pluripotent stem cells (PSCs) requires a compatible combination of media, matrices, and passaging methods that support cell health and pluripotency. When human embryonic stem cells (hESCs) were first derived by Thomson et al. in 1998, they were cultured on mitotically inactivated mouse embryonic fibroblast (MEF) feeder cells using a complete medium containing basal medium and basic fibroblast growth factor (bFGF) supplemented with fetal bovine serum (FBS). To reduce variable components and animal material, FBS was eventually replaced with Gibco™ KnockOut™ Serum Replacement, a supplement for FBS-free, feeder-dependent culture. We have developed a research-use version called Gibco™ KnockOut™ Serum Replacement – Multi-Species. Eventually, media and matrices were developed to support PSCs completely independently of feeder cells, significantly simplifying the PSC culture system and workflow. Coming full circle in 2011, the Thomson lab published a fully defined, feeder-free culture system that supported PSCs without any animal components; the medium is now available as Essential 8 Medium. A newer medium developed by us based on the same formulation, Gibco™ Essential 8™ Flex Medium, enables flexible feeding schedules. Additionally, we developed a richer feeder-free medium called Gibco™ StemFlex™ Medium to optimally support flexible feeding schedules and challenging PSC applications such as single-cell passaging and gene editing.

All together, a variety of culture systems now exist to satisfy the requirements of different PSC applications, from basic research to translational applications and from simple cell culture to gene editing. Protocols for using these culture systems are described in this section, including guidance on cryopreserving and thawing cells, single-cell passaging, and adaptation from one system to another.

Get assistance with finding the right PSC culture tools at **thermofisher.com/pscculture**.

As the name implies, feeder-dependent cultures rely on feeder cells to provide many of the proteins, most often growth factors and extracellular matrix proteins that are necessary for PSCs to grow in culture. With the abundance of components to support PSC growth, feeder-dependent culture systems are considered rich and robust and are still widely used years after feeder-free systems have been introduced. This section describes the preparation of the MEF feeder layer as well as the thawing and culture of PSCs under feeder-dependent conditions.

2.1A Preparation of MEF culture plates

Inactivated MEFs are used as feeder layers for culturing PSCs in their undifferentiated state. Gibco™ MEFs are isolated from mouse embryos under sterile conditions, expanded for up to 3 passages, and then mitotically inactivated by gamma irradiation or mitomycin C treatment. The growth-arrested feeder layer supports the PSC culture by providing nutrients, growth factors, and matrix components, and it enables PSCs to survive and proliferate readily in culture. This protocol describes the preparation of MEF culture plates using the most widely used Gibco MEFs for routine human PSC culture. See a list of available Gibco MEFs for routine culture or for drug selection at **thermofisher.com/gibcomefs**.

Required materials

- Gibco™ CF1 Mouse Embryonic Fibroblasts, irradiated (Cat. No. A34181)
- Gibco™ DMEM, high glucose, GlutaMAX™ Supplement, pyruvate (Cat. No. 10569010)
- Gibco™ DPBS, no calcium, no magnesium (Cat. No. 14190144)
- Gibco™ Embryonic Stem Cell–Qualified FBS, US origin (Cat. No. 16141061)
- Gibco™ MEM Non-Essential Amino Acids Solution (100X) (Cat. No. 11140-050)
- Gibco™ 2-Mercaptoethanol (Cat. No. 21985023)
- Gibco™ Attachment Factor Protein (1X) (Cat. No. S006100)

Overview of procedural guidelines

Follow these guidelines to use inactivated MEFs as feeder layers to culture mouse and human ESCs and iPSCs:

- All solutions and equipment that come in contact with the cells must be sterile; always use proper aseptic technique and work in a laminar flow hood.
- MEFs should be plated ~24 hours in advance.
- After thawing, transfer MEFs into prewarmed medium.
- Plate MEFs on culture vessels coated with Attachment Factor (AF) Protein (1X) solution.
- For best results, use MEF dishes or plates the day after seeding and culture with ESCs or iPSCs for up to 4 more days.

Before you begin

Before starting experiments, make sure to have frozen ESC or iPSC stocks on hand.

Coat culture vessels with AF

1. Cover the whole surface of each culture vessel with AF solution.

Culture vessel	AF coating volume
96-well plate	0.1 mL
24-well plate	0.3 mL
12-well plate	0.5 mL
6-well plate	1 mL
60 mm dish	3 mL
100 mm dish	9 mL
25 cm² flask	3 mL
75 cm² flask	9 mL

2. Incubate the vessels for 1 hour at 37°C.
3. Using sterile technique in a laminar flow culture hood, completely remove the AF solution from the culture vessel by aspiration.

   Note: It is not necessary to wash the culture surface before adding cells or medium.

4. Use the coated vessels immediately or store them at room temperature for up to 24 hours.

Prepare MEF medium

Prepare 500 mL of MEF medium by mixing the following components (prewarmed in a 37°C, 5% CO_2 incubator):

DMEM, high glucose, GlutaMAX Supplement, pyruvate	450 mL
Embryonic Stem Cell–Qualified FBS, US origin	50 mL
MEM Non-Essential Amino Acids Solution, 10 mM	5 mL
2-Mercaptoethanol	500 µL

Complete MEF medium can be stored at 2–8°C for up to 4 weeks.

Thawing of MEFs

1. Remove the cryovial containing the inactivated MEFs from the liquid nitrogen storage tank.
2. Briefly roll the vial between gloved hands to remove frost, then swirl gently in a 37°C water bath.
3. When only a small ice crystal remains in the vial, remove from water bath. Spray the outside of the vial with 70% ethanol before placing it in the cell culture hood.
4. Pipette the thawed cells gently into a 50 mL conical tube.
5. Add 10 mL of prewarmed MEF medium to the cells dropwise while gently swirling the conical tube. Gently mix by pipetting up and down.

   Note: Adding the medium slowly helps the cells avoid osmotic shock.
6. Transfer the entire cell suspension to a 15 mL conical tube and centrifuge at 200 x *g* for 5 minutes.
7. Aspirate the supernatant and resuspend the cell pellet in an appropriate volume of prewarmed MEF medium.
8. Use an appropriate volume of the cell suspension to determine the viable cell number using your method of choice (e.g., Invitrogen™ Countess™ Automated Cell Counter, Cat. No. AMQAX1000).

Plating of MEFs

1. Aspirate the gelatin solution from the AF-coated culture vessels, as applicable.
2. Add the appropriate amount of MEF medium into each culture vessel.

Culture vessel	MEF medium volume
96-well plate	0.1 mL
24-well plate	0.5 mL
12-well plate	1 mL
6-well plate	2 mL
60 mm dish	5 mL
100 mm dish	10 mL
25 cm^2 flask	5 mL
75 cm^2 flask	15 mL

3. Add the appropriate amount of MEF suspension into each culture vessel.

Culture vessel	Number of MEFs
96-well plate	1.0 x 10^4 cells/well
24-well plate	6.0 x 10^4 cells/well
12-well plate	1.5 x 10^5 cells/well
6-well plate	3.0 x 10^5 cells/well
60 mm dish	6.0 x 10^5 cells
100 mm dish	1.8 x 10^6 cells
25 cm^2 flask	7.5 x 10^5 cells
75 cm^2 flask	2.3 x 10^6 cells

   Note: The appropriate cell density should be optimized for the specific application. We recommend 3.0 x 10^4 MEFs/cm^2 as a good starting point, but the typical range is 2.0 x 10^4 to 5.5 x 10^4 MEFs/cm^2.
4. Move the culture vessels in several quick back-and-forth and side-to-side motions to disperse the cells across the surface of the vessels.
5. Incubate the cells in a 37°C incubator with a humidified atmosphere of 5% CO_2.
6. Use the MEF culture vessels the day after plating.

2.1B Culturing feeder-dependent PSCs

Human PSCs such as iPSCs and ESCs can be maintained on a layer of inactivated MEF feeder cells for many passages without compromising the cells' proliferation or pluripotency and differentiation potential. This protocol describes how to thaw and culture established human PSCs on feeder cells using KnockOut Serum Replacement – Multi-Species, which uses the same trusted formulation as the original KnockOut Serum Replacement.

Required materials

- DMEM, high glucose, GlutaMAX Supplement, pyruvate (Cat. No. 10569-010)
- Embryonic Stem Cell–Qualified FBS, USDA-approved regions (Cat. No. 10439016)
- Gibco™ DMEM/F-12, GlutaMAX™ Supplement (Cat. No. 10565-018)
- KnockOut Serum Replacement (SR) – Multi-Species (Cat. No. A3181502)
- Gibco™ MEM Non-Essential Amino Acids Solution (100X) (Cat. No. 11140-050)
- Gibco™ 2-Mercaptoethanol (Cat. No. 21985023)
- Gibco™ CF1 Mouse Embryonic Fibroblasts (Cat. No. A34181)
- Gibco™ FGF-Basic (AA 1–155) Recombinant Human Protein (bFGF) (Cat. No. PHG0264)
- Gibco™ GlutaMAX™ Supplement (Cat. No. 35050-079)
- Gibco™ Collagenase, Type IV, powder (Cat. No. 17104-019) for enzymatic passaging or Gibco™ StemPro™ EZPassage™ Disposable Stem Cell Passaging Tool (Cat. No. 23181-010) for mechanical passaging
- Cell Scraper
- Gibco™ DPBS, calcium, magnesium (Cat. No. 14040-133)
- Gibco™ DPBS, no calcium, no magnesium (Cat. No. 14190144)
- Attachment Factor Protein (Cat. No. S006100)
- Gibco™ PSC Cryomedium (Cat. No. A2644401), available as part of the Gibco™ PSC Cryopreservation Kit (Cat. No. A2644601)
- 37°C water bath
- Appropriate tissue culture plates and supplies

Preparing media and materials

10 µg/mL bFGF Solution (1,000 µL)

1. To prepare 1 mL of 10 µg/mL bFGF solution, aseptically mix the following components:

bFGF	10 µg
DPBS, no calcium, no magnesium	980 µL
KnockOut SR	10 µL

2. Aliquot and store at –20°C for up to 6 months.

1 mg/mL Collagenase Type IV solution

1. Add DMEM/F-12 with GlutaMAX Supplement to Collagenase Type IV to make a 10 mg/mL stock solution. Gently vortex to suspend and filter-sterilize the solution. This solution can be aliquotted and frozen at –20°C until use.
2. Make a working solution of 1 mg/mL Collagenase Type IV in DMEM/F-12 with GlutaMAX Supplement.

PSC culture medium

1. To prepare 100 mL of complete PSC culture medium, aseptically mix the following components:

DMEM/F-12 with GlutaMAX Supplement	79 mL
KnockOut SR	20 mL
MEM Non-Essential Amino Acids Solution (100X)	1 mL
bFGF (10 µg/mL)*	40 mL
2-Mercaptoethanol	100 µL

* Add bFGF at the time of medium change (final concentration 4 ng/mL).

2. Complete PSC culture medium can be stored at 2–8°C for up to 4 weeks.

Preparing MEF dishes

Refer to section 2.1A.

Recovery of cryopreserved PSCs on feeders

1. Aspirate the MEF medium from a dish containing inactivated MEFs and add prewarmed PSC culture medium to the dish, 3–4 hours before plating hESCs.
2. Label the dish containing the inactivated MEF cells with the passage number from the vial, the date, and the user's initials.
3. Remove the vial of hESCs from liquid nitrogen storage using metal forceps. Add the appropriate amount of MEF medium into each culture vessel.

   Note: If the vial is exposed to ambient temperatures for more than 15 seconds between removal and thawing, transfer the vial into a container containing a small amount of liquid nitrogen.
4. Roll the vial between your gloved hands until the outside is free of frost. This should take ~10–15 seconds.
5. Immerse the vial in a 37°C water bath without submerging the cap. Swirl the vial gently.
6. When only an ice crystal remains, remove the vial from the water bath.
7. Spray the outside of the vial with 70% ethanol and place it in the hood.
8. Pipet cells gently into a sterile 50 mL conical tube using a 5 mL sterile pipette.
9. Slowly add 10 mL of PSC culture medium dropwise to cells in the 50 mL conical tube. While adding the medium, gently move the tube back and forth to mix the hESCs. This reduces osmotic shock to the cells.
10. Rinse the vial with 1 mL of PSC culture medium and add to the 50 mL conical with cells.
11. Transfer cell suspension to a 15 mL conical tube and centrifuge the cells at 1,000 rpm for 2 minutes.
12. Aspirate and discard the supernatant.
13. Resuspend the cell pellet in sufficient volume of PSC culture medium according to the table on page 27 by gently pipetting the cells up and down in the tube a few times.
14. Aspirate the spent PSC culture medium from the MEF dish and slowly add the thawed colonies onto the dish. Place the dish gently into the 37°C, 5% CO_2 incubator and move the dish in several quick back-and-forth and side-to-side motions to disperse cells across the surface of the dishes.
15. Incubate the cells overnight.
16. The next day, remove the spent medium with debris using a sterile serological pipette and transfer it into a prepared MEF dish. You can use this dish as a backup in case there is a problem with the main dish.

17. Add fresh PSC culture medium to each dish according to the volumes in the table below. Place both plates gently into a 37°C, 5% CO_2 incubator overnight.

Culture vessel	Surface area	Volume
6-well plate	10 cm^2/well	2.0 mL per well
12-well plate	4 cm^2/well	1.0 mL per well
24-well plate	2 cm^2/well	0.5 mL per well
35 mm dish	10 cm^2	2.0 mL
60 mm dish	20 cm^2	4.0 mL
100 mm dish	60 cm^2	10.0 mL

18. Examine cells under the microscope, and replace spent medium daily from both plates. If feeding more than one plate, use a different pipette for each well to reduce risk of contamination. Colonies may not be visible for up to a week.

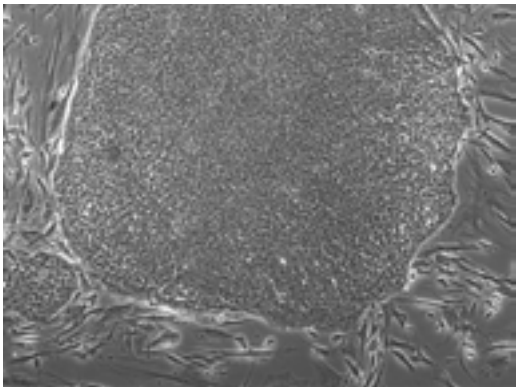


Figure 2.1. H9 hESCs cultured on mitotically inactivated MEF feeder layer in PSC culture medium containing KnockOut SR.

Passaging of feeder-dependent PSCs

When to split cells

In general, split cells when one of the following occurs:

- The MEF feeder layer is 2 weeks old
- hESC colonies are becoming too dense or too large
- Increased differentiation occurs

Split ratio

The split ratio can vary, though it is generally between 1:2 and 1:4. Occasionally, cells will grow at a different rate and the split ratio will need to be adjusted. A general rule is to observe the last split ratio and adjust the ratio according to the appearance of the hESC colonies.

If the cells look healthy and colonies have enough space, split using the same ratio. If they are overly dense and crowding, increase the ratio. If the cells are sparse, decrease the ratio. Cells will need to be split every 4–10 days based upon appearance.

hESCs do well in MEF plates that have been conditioned with PSC culture medium. It is common practice to condition new feeder plates before passaging hESCs into them.

Enzymatic passaging using collagenase

You may passage cells via the enzymatic method as described below, or mechanically as described in the next section.

1. Aspirate the MEF medium from a dish containing inactivated MEFs and add prewarmed PSC culture medium to the dish, 3–4 hours before plating hESCs.
2. Label the new MEF dish with the cell line name, the new passage number, the date, the split ratio, and the user's initials. Return the plate to the incubator.
3. Under a dissecting microscope, remove differentiated colonies from the dish to be passaged.
4. Aspirate the spent medium from the dish with a Pasteur pipette.
5. Add Collagenase Type IV (1 mg/mL) solution to the dish containing hESCs. Adjust the volume of Collagenase Type IV for various dish sizes (e.g., 35 mm dishes require 1 mL of Collagenase Type IV).

6. Incubate the dish(es) for 30–60 minutes in a 37°C, 5% CO_2 incubator. Note that the incubation times may vary among different batches of collagenase; therefore, examination of the colonies is needed to determine the appropriate incubation time.

   Note: As an alternative to Collagenase Type IV, you may use dispase at a concentration of 2 mg/mL and incubate the dish(es) for 2–3 minutes in a 37°C, 5% CO_2 incubator.

7. Stop the incubation when the edges of the colonies are starting to pull away from the plate (see Figure 2.2).

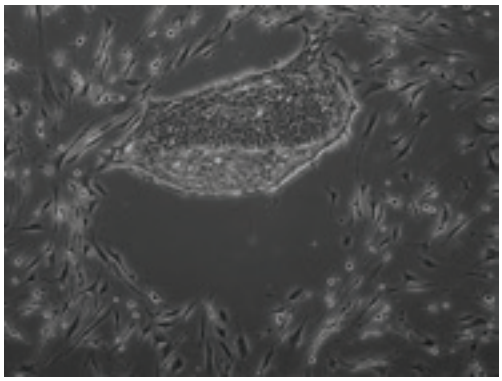


Figure 2.2. Colony pulling away from MEF layer after treatment with enzyme.

8. Aspirate the Collagenase Type IV solution with a Pasteur pipette. Remove the collagenase carefully without disturbing the attached cell layer.

9. Add PSC culture medium to each dish. Use a 5 mL pipette to gently blow the cells off the surface of the dish while pipetting up and down. Make sure to pipet gently to minimize the formation of bubbles.

10. After the hESCs have been removed from the surface of the well, pool the contents of the wells into a 15 mL conical tube.

11. Using a 5 mL pipette, add PSC culture medium to the dish to wash and collect any residual cells. Pipet up the medium and cells, and then add the collected cells to the 15 mL tube.

12. Pipet cells up and down gently a few times in the 15 mL tube to further break up cell colonies. Pipet carefully to reduce foaming.

    Note: Avoid making a single cell suspension.

13. Centrifuge at 200 x *g* for 2 minutes, and then aspirate the supernatant from the hESC pellet.

14. Resuspend the pellet with an appropriate amount of PSC culture medium (refer to the table on page 27). This is dependent on the split ratio and the number of dishes used.

15. Mix the cell suspension well with a 10 mL pipette. Be careful not to break up the colonies too much or cause bubbles in the medium.

16. Add the appropriate volume of cell suspension to each dish. Return the dish to the incubator.

17. Move the dish(es) in several quick back-and-forth and side-to-side motions to disperse cells across the surface of the dishes.

18. Incubate cells overnight to allow colonies to attach. Replace spent medium daily.

    Note: While cells are attaching, be careful when opening and closing the incubator doors to avoid disturbing the even distribution of cells across the surface of the wells.

Mechanical passaging using the StemPro EZPassage Disposable Cell Passaging Tool

19. Replace the medium in the dish containing the cells with fresh PSC culture medium.

20. Under a laminar flow hood, open the package containing the StemPro EZPassage tool and remove the tool.

21. Hold the culture vessel in one hand and pull (roll) the StemPro EZPassage tool across the entire dish in one direction. Apply gentle but firm pressure so that the entire roller blade touches the dish and maintains uniform pressure during the rolling action.

22. Keep rolling the StemPro EZPassage tool parallel to the first pass until the entire dish has been covered.

23. Rotate the culture dish 90°, and then repeat rolling the cell layer as described above.

24. When you are finished, discard the StemPro EZPassage tool and do not reuse. Use a cell scraper to lift cell clusters off the plate, if necessary.

25. Using a serological pipette, rinse the dish with medium so that the cut colonies are suspended in the medium.

26. Transfer the medium containing the colonies to a 15 mL sterile tube.

27. Seed the cell colonies on dishes plated with mitotically inactivated MEFs at an appropriate density.

28. Place the plates into a 37°C, 5% CO_2 incubator. Shake the plates gently to evenly spread out cells.

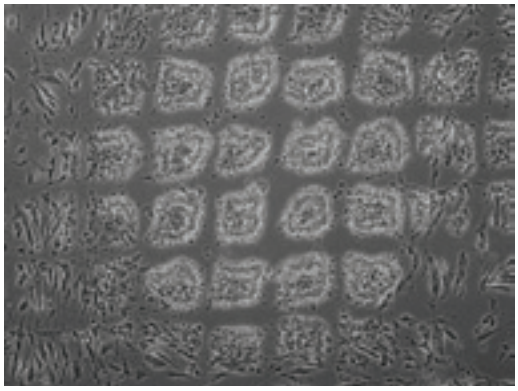


Figure 2.3. Colony after being cut with the StemPro EZPassage Disposable Cell Passaging Tool.

Cryopreservation of PSCs

1. Thaw and prechill PSC Cryomedium at 2–8ºC.

2. Harvest PSCs according to standard passaging protocol.

3. Centrifuge the cell suspension at 200 x *g* for 4 minutes.

4. Aspirate the medium, being careful not to disturb the cell pellet.

5. Add prechilled PSC Cryomedium dropwise to the cells while gently rocking the tube back and forth followed by gentle resuspension of cell pellet.

   Note: In general, from a 100 mm dish, 8–12 vials containing 1 x 10^6 viable cells/mL can be generated.

6. Dispense aliquots of the suspension into cryogenic vials according to manufacturer's specifications (i.e., 1.5 mL in a 2 mL cryovial).

   Note: Mix the cell suspension in PSC Cryomedium frequently to maintain a homogeneous suspension. Do this through gentle inversion to prevent breaking cells into smaller clumps.

7. Cryopreserve cells in an automated or manual controlled-rate freezing apparatus following standard procedures (approximately 1ºC decrease per minute).

8. Transfer frozen cell vials to liquid nitrogen (vapor phase); we recommend storage from –200°C to –125°C.

2.2 Culturing PSCs without feeders

While PSCs have traditionally been maintained on feeder layers, feeder-free culture systems using a medium such as Gibco™ Essential 8™, Essential 8 Flex, or StemFlex Medium are simpler, eliminating the need to prepare MEFs prior to culture or remove MEFs prior to downstream applications. In addition, Essential 8 and Essential 8 Flex media offer fully defined, xeno-free formulations, while StemFlex Medium supports robust PSC culture whether using clump passaging or more challenging applications such as gene editing or single-cell passaging. With Gibco™ Flex™ technology that stabilizes wild-type bFGF activity, both StemFlex and Essential 8 Flex media also enable a flexible feeding schedule rather than the traditional every-day feeding schedule. This section provides protocols that enable you to use the Gibco feeder-free culture system that best fits your needs.

2.2A Preparation of matrix-coated dishes

A variety of matrices can be used to support feeder-free culture of PSCs, including Gibco™ Geltrex™ matrix, vitronectin, and rhLaminin-521. The Geltrex matrix consists of basement membrane proteins derived from Engelbreth-Holm-Swarm tumors in mice. Unlike the Geltrex matrix, vitronectin and rhLaminin-521 are defined, xeno-free, recombinant human matrix proteins, which are more appropriate for translational work. In terms of performance, the vitronectin substrate is based on the VTN-N variant of the protein, which supports hPSC attachment and survival better than the wild-type variant. On the other hand, Gibco™ rhLaminin-521 is the most robust matrix. It can be used for routine culture but is particularly useful in stressful applications such as adaptation of PSCs from richer to leaner media, and single-cell passaging or clonal outgrowth following fluorescence-activated cell sorting (FACS), even in the absence of Rho-associated protein kinase (ROCK) inhibitors.

When performing clump passaging, Essential 8 or Essential 8 Flex media are typically recommended for use with vitronectin, while StemFlex Medium is typically recommended with a Geltrex matrix. However, Essential 8, Essential 8 Flex, and StemFlex media can be used with any of the three matrices, depending on preference or experimental need. This section describes how to coat dishes or plates using each of the three matrices.

Preparation of Geltrex matrix–coated culture plates

Gibco™ Geltrex™ LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix (Cat. No. A1413302) is used routinely for attachment and maintenance of hESCs. Each lot of Geltrex matrix has been function tested to provide the unique microenvironmental requirements for the growth and maintenance of pluripotency of hESCs, therefore eliminating the need for customers to test multiple lots. Geltrex matrices can also be used for promoting and maintaining a differentiated phenotype in a variety of cell cultures, including primary epithelial cells, endothelial cells, smooth muscle cells, and human iPSCs.

Important information

- Source: Murine Engelbreth-Holm-Swarm (EHS) tumor, protein concentration ranges from 12–18 mg/mL; refer to Certificate of Analysis for specific lot information
- Thaw Geltrex matrix in a refrigerator at 2–8°C overnight.
- When working with smaller volumes of Geltrex matrix, dispense appropriate required working volumes and store between –80°C and –20°C.
- Avoid multiple freeze/thaw cycles.
- Geltrex matrix gels in 5–10 minutes above 15°C. When working from a full 5 mL vial, it is unnecessary to keep it on ice if used within 5 minutes and the environmental temperature does not exceed 25°C; however, since smaller volumes warm more quickly, partial tubes and aliquots should be kept on ice to prevent premature gelling.

Coating procedures

Geltrex matrix is tested for hESC applications. A protein concentration of ≥9 mg/mL is used for differentiation studies of hESCs. An extract diluted to less than 9 mg/mL does not form a gel and will only support the propagation and maintenance of pluripotency of hESCs when grown with media designed for feeder-free propagation of hESCs.

Important: We recommend that the following procedures be performed in an aseptic environment using aseptic techniques to prevent contamination.

Product	Storage	Shelf life*	Amount	Cat. No.
Geltrex LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix	–80°C to –20°C	18 months	1 mL	A1413301
			5 mL	A1413302

* Shelf life duration is determined from date of manufacture.

Thin gel method (non-gelling) for propagation of hESCs

1. Thaw the Geltrex matrix solution (see “Important information” on previous page).
2. Mix the Geltrex matrix solution by slowly pipetting up and down, being careful not to introduce air bubbles.
3. Dilute 1 mL Geltrex matrix solution into 99 mL prechilled (4°C) DMEM/F-12 with GlutaMAX Supplement (or equivalent). Determine optimal coating concentration for your application empirically. Adjust volumes accordingly.
4. Add sufficient diluted Geltrex matrix solution to cover the entire growth surface area (e.g., 1.5 mL for 35 mm dish, 3 mL for 60 mm dish). The coated dish is stable for 2 weeks when wrapped with Parafilm™ sealing film and stored at 4°C.

   Important: Do not allow the coated surface to dry out. It is critical to maintain a storage temperature of 4°C to avoid premature gelling.
5. Incubate coated plates at 37°C for a minimum of 60 minutes.
6. At the time of use, carefully aspirate off the supernatant above the Geltrex coating and immediately plate cells in pre-equilibrated cell culture medium.

Preparation of vitronectin-coated culture plates

Truncated recombinant human vitronectin (VTN-N), corresponding to the amino acid fragment 62–478 of human vitronectin expressed in *E. coli*, is purified from inclusion bodies and refolded for use as a substrate for the feeder-free culture of human PSCs in Essential 8 Medium. When used with Essential 8 Medium, VTN-N has been proven to maintain pluripotency and normal growth characteristics in multiple PSC lines.

Working concentration

The optimal working concentration of vitronectin is cell line–dependent and must be determined empirically. We recommend using a final coating concentration of 0.5 µg/cm^2 on the culture surface. Prior to coating culture vessels, calculate the working concentration of vitronectin using the formula below and dilute the stock appropriately. Refer to the table on the next page for volume required by culture surface area.

$$\text{Working conc.} = \text{Coating conc.} \times \frac{\text{Culture surface area of cells}}{\text{Vol. required for surface area}}$$

$$\text{Dilution factor} = \frac{\text{Stock concentration (0.5 mg/mL)}}{\text{Working concentration}}$$

For example, to coat a 6-well plate at a coating concentration of 0.5 µg/cm^2, you will need to prepare 6 mL of diluted vitronectin solution (e.g., 10 cm^2/well surface area and 1 mL of diluted vitronectin/well) at the following working concentration:

$$\text{Working conc.} = 0.5\ \mu g/cm^2 \times \frac{10\ cm^2}{1\ mL} = 0.5\ \mu g/mL$$

$$\text{Dilution factor} = \frac{0.5\ mg/mL}{5\ \mu g/mL} = 100X \text{ (i.e., 1:100 dilution)}$$

Coat culture vessels with vitronectin

Instructions for coating a 6-well culture plate with vitronectin at a coating concentration of 0.5 µg/cm^2 are provided below. For volumes used in other culture vessels, refer to the table on this page. To calculate the working concentration of vitronectin used with other coating concentrations and to determine the appropriate dilution factor, use the equations in "Working concentration".

1. Upon receipt, thaw the vial of vitronectin at room temperature and prepare 60 µL aliquots of vitronectin in polypropylene tubes. Freeze the aliquots at –80°C or use immediately.
2. To coat the wells of a 6-well plate, remove a 60 µL aliquot of vitronectin from –80°C storage and thaw at room temperature. You will need one 60 µL aliquot per 6-well plate.
3. Add 60 µL of thawed vitronectin into a 15 mL conical tube containing 6 mL of sterile DPBS without calcium and magnesium (Cat. No. 14190144), at room temperature. Gently resuspend by pipetting the vitronectin dilution up and down.

   Note: This results in a working concentration of 5 µg/mL (i.e., a 1:100 dilution).
4. Add 1 mL of the diluted vitronectin solution to each well of a 6-well plate (refer to the table on the right for the recommended volumes for other culture vessels). When used to coat a 6-well plate (10 cm^2/well) at 1 mL/well, the final concentration will be 0.5 µg/cm^2.
5. Incubate the coated plates at room temperature for 1 hour. **Note:** The culture vessel can now be used or stored at 2–8°C wrapped in laboratory film for up to a week. Do not allow the vessel to dry. Prior to use in the next step, prewarm the culture vessel to room temperature for at least 1 hour.
6. Aspirate and discard the vitronectin solution. It is not necessary to rinse off the culture vessel after the removal of vitronectin. Cells can be passaged directly onto the vitronectin-coated culture vessels.

Culture vessel	Approx. surface area	Diluted vitronectin solution volume
6-well plate	10 cm^2/well	1.0 mL per well
12-well plate	4 cm^2/well	0.4 mL per well
24-well plate	2 cm^2/well	0.2 mL per well
35 mm dish	10 cm^2	1.0 mL
60 mm dish	20 cm^2	2.0 mL
100 mm dish	60 cm^2	6.0 mL
T-25 flask	25 cm^2	2.5 mL
T-75 flask	75 cm^2	7.5 mL

Product	Storage	Shelf life*	Amount	Cat. No.
Vitronectin (VTN-N) Recombinant Human Protein, Truncated	Store at –80°C	24 months	1 mL (0.5 mg/mL)	A14700

* Shelf life duration is determined from date of manufacture.

Preparation of rhLaminin-521–coated culture plates

rhLaminin-521 is a recombinant human protein that provides a defined surface for feeder-free culture of PSCs. Laminin-521 is a natural component of the stem cell niche *in vivo*. rhLaminin-521 recapitulates a natural environment for maintenance of self-renewal, normal morphology, pluripotency, and karyotype of PSCs cultured in chemically defined, feeder-free, and xeno-free stem cell culture media such as Essential 8 Medium. Furthermore, rhLaminin-521 supports cell health across the stem cell workflow, enabling improved reprogramming efficiency, efficient passaging of PSCs as a single cell suspension in the absence of inhibitors of apoptosis as well as efficient transfer of existing feeder-dependent PSC cultures to feeder-free conditions.

Important information

- Thaw rhLaminin-521 slowly at 2–8°C and avoid extended exposure of protein to ambient temperatures; for long coating procedures, the laminin stock solution should be kept on ice.
- Once thawed, the rhLaminin-521 stock is stable for up to 3 months when stored at 2–8°C.
- Divide the thawed rhLaminin-521 into usage-size aliquots and store in a frost-free freezer at –30°C to –10°C. Avoid repeated freeze-thaw cycles.
- Plates can be coated in advance of experiments, sealed with Parafilm sealing film, and stored at 2–8°C under aseptic conditions for up to 2 weeks; do not allow the culture surface to dry.

Working concentration

The optimal working concentration of rhLaminin-521 is cell line–dependent and must be determined empirically. We recommend using an initial coating concentration of 0.5 µg/cm^2 on the culture surface. Prior to coating culture vessels, calculate the working concentration according to the formula below and dilute the stock appropriately. Refer to the table below for culture surface area and required coating volumes.

Culture vessel	Surface area	Volume
6-well plate	10 cm^2/well	2.0 mL per well
12-well plate	4 cm^2/well	0.8 mL per well
24-well plate	2 cm^2/well	0.4 mL per well
35 mm dish	10 cm^2	2.0 mL
60 mm dish	20 cm^2	4.0 mL
100 mm dish	60 cm^2	12.0 mL

$$\text{Working conc.} = \text{Coating conc.} \times \frac{\text{Culture surface area of cells}}{\text{Vol. required for surface area}}$$

$$\text{Dilution factor} = \frac{\text{Stock concentration (100 µg/mL)}}{\text{Working concentration}}$$

For example, to coat a 6-well plate at a coating concentration of 0.5 µg/cm^2, you will need to prepare 12 mL of diluted rhLaminin-521 solution (2 mL per well) at the following working concentration:

$$\text{Working conc.} = 0.5\ \mu g/cm^2 \times \frac{10\ cm^2}{2\ mL} = 2.5\ \mu g/mL$$

$$\text{Dilution factor} = \frac{100\ \mu g/mL}{2.5\ \mu g/mL} = 40X \text{ (i.e., 1:40 dilution)}$$

Product	Storage	Shelf life*	Amount	Cat. No.
rhLaminin-521	–30°C to –10°C	2 years from date of receipt	100 µg**	A29248
			1 mg = 100 µg x 10	A29249

* Shelf-life duration is determined from date of receipt when stored at recommended storage conditions.

** Also available as a kit with Essential 8 Medium: Gibco™ Essential 8™ Adaptation Kit (Cat. No. A25935).

Coat culture vessels with rhLaminin-521
Instructions for coating a 6-well culture plate with rhLaminin-521 at a coating concentration of 0.5 µg/cm^2 are provided below. For volumes used in other culture vessels, refer to the table on page 34. To calculate the working concentration of rhLaminin-521 used with other coating concentrations and to determine the appropriate dilution factor, use the equations in "Working concentration".

1. Upon receipt, thaw the vial of rhLaminin-521 slowly at 2–8°C, mix by gentle trituration, and prepare usage size aliquots in polypropylene tubes. Freeze aliquots at –30°C to –10°C or store aliquots at 2–8°C for up to 3 months.
2. To coat the wells of a 6-well plate, add a 300 µL aliquot of rhLaminin-521 into a 15 mL conical tube containing 12 mL of sterile DPBS with calcium and magnesium (Cat. No. 14040-133). Gently resuspend by pipetting the rhLaminin-521 dilution up and down.

   Note: This results in a working concentration of 2.5 µg/mL (i.e., a 1:40 dilution).
3. Add 2 mL of the diluted rhLaminin-521 solution to each well of a 6-well plate (see table on page 34 for the recommended volumes for other culture vessels). When used to coat a 6-well plate (10 cm^2/well) at 2 mL/well, the final coating concentration will be 0.5 µg/cm^2.
4. Incubate the plates in a 37°C, 5% CO_2 for 2 hours, for efficient coating.

   Note: Alternatively, the plate can be coated at 2–8°C overnight. Do not allow the culture vessel to dry. Prior to use, prewarm the culture vessel to room temperature.
5. Aspirate the rhLaminin-521 solution and discard. It is not necessary to rinse off the culture vessel after the removal of rhLaminin-521. Cells can be passaged directly onto the rhLaminin-521–coated culture vessels.

2.2B Feeder-free culture of PSCs using Essential 8 or Essential 8 Flex Media

Essential 8 Medium is a fully defined, feeder-free medium formulated for the growth and expansion of human PSCs. Unlike most feeder-free media, Essential 8 Medium does not require the presence of bovine serum albumin (BSA) or human serum albumin (HSA) that contributes to lot-to-lot variability. In addition, most serum-free media consist of more than 20 components, adding complexity, time, and cost, while Essential 8 Medium consists of only eight components. Thus, this uncomplicated, xeno-free medium minimizes batch variability. For an animal and human origin–free version of Essential 8 Medium that is more appropriate for cell therapy applications, use Gibco™ CTS™ Essential 8™ Medium.

Based on the original Essential 8 Medium formulation, the Essential 8 Flex Medium is simple, defined, and does not include BSA or HSA, thereby minimizing batch variability. However, unlike Essential 8 Medium, Essential 8 Flex Medium is formulated to stabilize bFGF activity. As such, this medium supports the culture and expansion of PSCs without the need for daily feeding. This protocol describes how to thaw, culture, and cryopreserve PSCs in either Essential 8 or Essential 8 Flex Medium. It also describes how to adapt PSCs from other culture systems into Essential 8 or Essential 8 Flex Medium.

Required materials

- Essential 8 Medium (Cat. No. A1517001) or Essential 8 Flex Medium (Cat. No. A28585-01)
- Matrix:
  - Vitronectin (VTN-N) Recombinant Human Protein, Truncated (Cat. No. A14700)
  - Geltrex LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix (Cat. No. A1413301)
  - rhLaminin-52 (Cat. No. A29248)
- DPBS, no calcium, no magnesium (Cat. No. 14190-144)
- Gibco™ Versene™ Solution (Cat. No. 15040-066) or Invitrogen™ UltraPure™ 0.5M EDTA, pH 8.0 (Cat. No. 15575-020)
- PSC Cryopreservation Kit (Cat. No. A2644601), which consists of PSC Cryomedium (Cat. No. A2644401) and Gibco™ RevitaCell™ Supplement (100X) (Cat. No. A2644501)
- 37°C water bath
- Appropriate tissue culture plates and supplies

Preparation of media and reagents

Essential 8 or Essential 8 Flex Medium (500 mL)

1. Thaw the frozen Essential 8 or Essential 8 Flex Supplement at room temperature for ~1 hour. Supplement may also be thawed at 2–8°C overnight; small amounts of precipitate or presence of some red coloring may be observed, but this will not affect product performance.

   Important: Do not thaw the frozen supplement at 37°C.

2. Mix the thawed supplement by gently inverting the vial a couple of times. Remove 10 mL from the bottle of Essential 8 or Essential 8 Flex basal medium, and then aseptically transfer the entire contents of the supplement to the bottle of basal medium. Swirl the bottle to mix and to obtain 500 mL of homogeneous complete medium.
3. Complete Essential 8 or Essential 8 Flex Medium can be stored at 2–8°C for up to 2 weeks. Before use, warm complete medium required for that day at room temperature until it is no longer cool to the touch. Do not warm the medium at 37°C.

0.5 mM EDTA in DPBS (50 mL)

4. To prepare 50 mL of 0.5 mM EDTA in DPBS, aseptically mix the following components in a 50 mL conical tube in a biological safety cabinet:

DPBS, no calcium, no magnesium	50 mL
UltraPure 0.5M EDTA, pH 8.0	50 µL

5. Filter-sterilize the solution. The solution can be stored at room temperature for up to 6 months.

Matrix-coated dishes

We generally recommend using Essential 8 and Essential 8 Flex media with VTN-N as the matrix. However, these media can also be used with a Geltrex matrix or rhLaminin-521. Refer to section 2.2A for detailed instructions on how to coat dishes with these matrices.

Recovery of cryopreserved PSCs in Essential 8 or Essential 8 Flex Medium

1. Prewarm complete Essential 8 or Essential 8 Flex Medium and VTN-N–coated 6-well plates to room temperature.

2. Remove the vial of PSCs from liquid nitrogen storage and transfer it on dry ice to the tissue culture room.

3. Immerse the vial in a 37°C water bath without submerging the cap. Swirl the vial gently. When only an ice crystal remains, remove the vial from the water bath, spray the outside with 70% ethanol, and place it in the hood.

4. Transfer the thawed cells to a 15 mL conical tube and slowly add 10 mL of complete Essential 8 or Essential 8 Flex Medium dropwise to the cells. This reduces osmotic shock to the cells. While adding the medium, gently move the tube back and forth to mix the PSCs. Rinse the vial with 1 mL of complete Essential 8 or Essential 8 Flex Medium and add to the 15 mL tube with cells.

5. Centrifuge the cells at 200 x *g* for 5 minutes, aspirate and discard the supernatant, and resuspend the cell pellet in 2 mL of complete Essential 8 or Essential 8 Flex Medium by gently pipetting the cells up and down a few times.

6. Slowly add the PSC suspension into a prewarmed, VTN-N–coated 6-well plate, plating 1 vial of ~1 x 10^6 viable thawed cells per well.

7. **Optional:** To improve cell survival, you can use RevitaCell Supplement (100X) (Cat. No. A2644501) at 1X final concentration in the cell culture (i.e., 20 µL per 2 mL of cell suspension) for the first 24 hours postthaw to minimize apoptosis and necrosis. Using this supplement for the recovery of PSCs requires a lower seeding density; therefore, seed 1 vial containing ~1 x 10^6 viable cells across 2 wells of a 6-well plate (i.e., half the seeding density than for recovery in Essential 8 or Essential 8 Flex Medium alone).

   Note: Do not add any additional ROCK inhibitors to the growth medium.

7. Move the plate in several quick side-to-side motions to disperse the cells across the surface of the wells, and place the plate gently into the 37°C, 5% CO_2 incubator.

8. The next day, replace the spent medium with fresh complete Essential 8 or Essential 8 Flex Medium.

9. If using Essential 8 Medium, replace the medium daily until the cells are ~85% confluent. If using Essential 8 Flex Medium, additional feeds are not required. If the cells are to be left without feeding for longer than 48 hours (for example, during a weekend), double the feed volume (Figure 2.4).

Passaging PSCs cultured in Essential 8 or Essential 8 Flex Medium

General guidelines

Split cultures when the first of the following occurs:

- PSC colonies are becoming too dense or too large
- PSC colonies are showing increased differentiation
- The colonies cover ~85% of the surface area of the culture vessel, usually every 3 to 4 days

The split ratio can vary, though it is generally between 1:2 and 1:4 for early passages and between 1:3 and 1:12 for established cultures. Occasionally, cells will grow at a different rate and the split ratio will need to be adjusted.

As a general rule, observe the last split ratio and adjust the ratio according to the appearance of the PSC colonies. If the cells look healthy and the colonies have enough space, split using the same ratio. If the colonies are overly dense and crowding, increase the ratio; if they are sparse, decrease the ratio.

Newly derived PSC lines may contain a fair amount of differentiated cells through passage 4. It is not necessary to remove differentiated material prior to passaging. By propagating/splitting the cells, the overall culture health should improve throughout early passages.

Enzymes such as collagenase and dispase do not work well with cells cultured in Essential 8 or Essential 8 Flex Medium and on vitronectin. Use of these enzymes for passaging cells results in compromised viability and attachment.

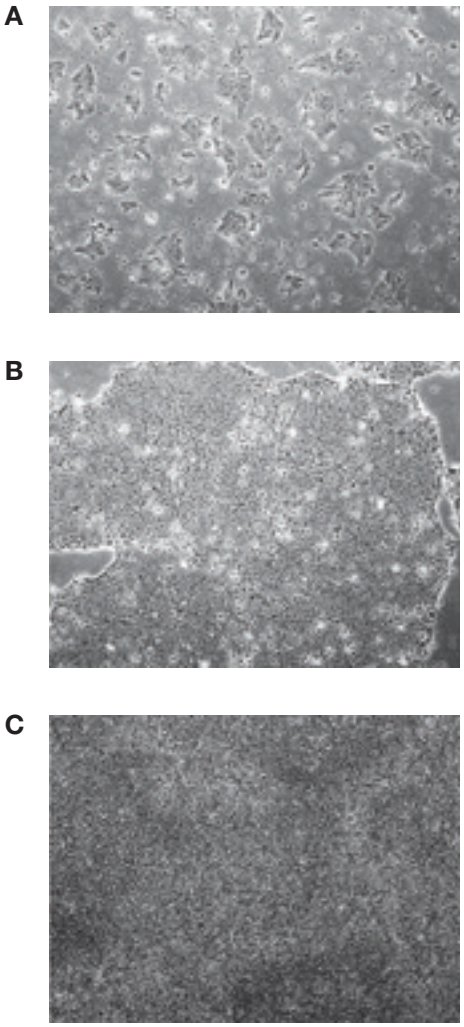


Figure 2.4. PSCs growing in Essential 8 Medium at varying levels of confluency. (A) PSCs growing in Essential 8 Medium on vitronectin 24 hours after a passage, prior to changing the medium. **(B).** PSCs growing in Essential 8 Medium on vitronectin that are ready for passage. **(C)** PSCs growing in Essential 8 Medium on vitronectin that are overconfluent.

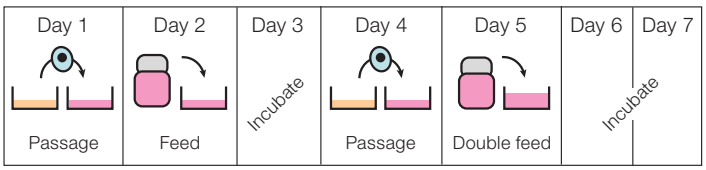


Figure 2.5. Typical weekly PSC culture workflow using the Essential 8 Flex Medium.

Passaging PSCs using Versene solution

1. Prewarm complete Essential 8 or Essential 8 Flex Medium, VTN-N–coated 6-well culture plate, and the Versene solution to room temperature.

2. Aspirate the spent medium from each well containing PSCs, and rinse each well twice with DPBS without calcium and magnesium (see Table 2.1 for recommended volume).

3. Add the Versene solution to each well containing PSCs (see Table 2.1). Swirl the vessel to coat the entire cell surface.

4. Incubate the plate at room temperature for 5–8 minutes or at 37°C for 4–5 minutes. When the cells start to separate and round up, and the colonies appear to have holes in them when viewed under a microscope, they are ready to be removed from the wells.

5. Aspirate the Versene solution, and add prewarmed complete Essential 8 or Essential 8 Flex Medium to each well (see Table 2.1).

6. Remove the cells from the well(s) by gently squirting medium over the surface of the well a few times and pipetting up the colonies. Avoid creating bubbles. Collect the cells in a 15 mL conical tube. There may be obvious patches of cells that were not dislodged and left behind. Do not scrape the cells from the plate in an attempt to recover them. Do not over-triturate the cell suspension.

   Note: Depending upon the cell line, work with no more than 1–3 wells at a time, and work quickly to remove the cells after adding Essential 8 or Essential 8 Flex Medium to the well(s), which quickly neutralizes the initial effect of the Versene solution. Some lines re-adhere rapidly after addition of medium, and must be removed 1 well at a time. Others are slower to reattach, and may be removed 3 wells at a time.

7. Add an appropriate volume of prewarmed complete Essential 8 or Essential 8 Flex Medium to each well of a VTN-N–coated 6-well plate so that each well contains 2 mL of medium after the cell suspension has been added. Refer to Table 2.1 for the recommended volumes for other culture vessels.

8. Mix the cell suspensions from step 6 by gently inverting a few times and transfer the appropriate volume of cell suspension into each well containing prewarmed complete Essential 8 or Essential 8 Flex Medium.

9. Move the plate in several quick side-to-side motions to disperse the cells across the surface of the wells. Incubate the cells in the 37°C, 5% CO_2 incubator overnight.

10. Feed the PSCs the day after splitting. If using Essential 8 Medium, replace the medium daily after that until the cells are ready for passaging. If using Essential 8 Flex Medium, additional feeds are not required (Figure 2.5). If the cells are to be left without feeding for longer than 48 hours (for example, during a weekend), double the feed volume.

11. **Optional:** To improve cell survival, you can add RevitaCell Supplement (Cat. No. A2644501) to 1X final concentration (i.e., 20 µL per 2 mL of cell suspension) for the first 24 hours post-passage.

    Note: It is normal to see cell debris and small colonies after passaging.

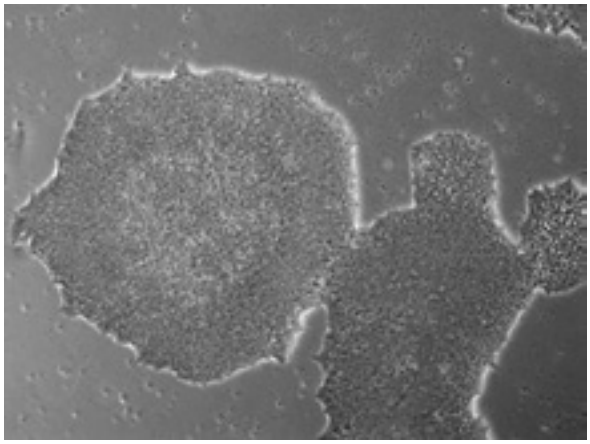


Figure 2.6. Normal pluripotent stem cell morphology. PSC colonies are expected to be tightly packed with defined borders and a high nucleus-to-cytoplasm ratio. The image above shows PSCs at passage 6.

Table 2.1. Reagent volumes (in mL per well or per dish).

Culture vessel (approx. surface area)	Vitronectin solution*	DPBS	Versene solution	Complete medium
6-well plate (10 cm^2/well)	1 mL	2 mL	1 mL	2 mL
12-well plate (4 cm^2/well)	0.4 mL	1 mL	0.4 mL	1 mL
24-well plate (2 cm^2/well)	0.2 mL	0.5 mL	0.2 mL	0.5 mL
35 mm dish (10 cm^2)	1 mL	2 mL	1 mL	2 mL
60 mm dish (20 cm^2)	2 mL	4 mL	2 mL	4 mL
100 mm dish (60 cm^2)	6 mL	12 mL	6 mL	12 mL
T-25 flask (25 cm^2)	2.5 mL	4–5 mL	2–3 mL	4–5 mL
T-75 flask (75 cm^2)	7.5 mL	12–15 mL	5–8 mL	12–15 mL

* The optimal working concentration of vitronectin solution is cell line–dependent. We recommend using a final coating concentration of 0.1–1.0 µg/cm^2 on the culture surface, depending on your cell line.

Cryopreservation of PSCs

1. Thaw and prechill PSC Cryomedium at 2–8°C.
2. Harvest PSCs according to standard single- or clumped cell passaging protocols.

   Note: Recommended passaging reagents for use with Essential 8 and Essential 8 Flex media include EDTA (Versene Solution) for clumped cell passaging, or Gibco™ TrypLE™ Select Enzyme (1X) or Gibco™ StemPro™ Accutase™ Cell Dissociation Reagent for single-cell passaging.
3. Centrifuge the cell suspension at 200 x *g* for 4 minutes.
4. Aspirate the medium, being careful not to disturb the cell pellet.
5. Add prechilled PSC Cryomedium dropwise to the cells while gently rocking the tube back and forth followed by gently resuspending the cell pellet.

   Note: In general, from a 100 mm dish, 8–12 vials containing 1 x 10^6 viable cells/mL can be generated.
6. Dispense aliquots of the suspension into cryogenic vials according to manufacturer's specifications (e.g., 1.5 mL in a 2 mL cryovial).

   Note: Mix the cell suspension in PSC Cryomedium frequently to maintain a homogeneous suspension. If utilizing clumped passaging methods at cell harvest, then mix cell suspension by gentle inversion to prevent breaking cells into smaller clumps.
7. Cryopreserve cells in an automated or manual controlled-rate freezing apparatus following standard procedures (approximately 1ºC decrease per minute).
8. Transfer frozen cell vials to liquid nitrogen (vapor phase); we recommend storage at –200°C to –125°C.

Adaptation of hPSCs from different culture systems to Essential 8 or Essential 8 Flex Medium

Required materials

- Essential 8 Medium (Cat. No. A1517001) or Essential 8 Flex Medium (Cat. No. A2858501)
- DPBS, no calcium, no magnesium (Cat. No. 14190250)
- Collagenase Type IV (Cat. No. 17104019) or Gibco™ Dispase II, powder (Cat. No. 17105041)
- Versene Solution (Cat. No. 15040066) or UltraPure 0.5 M EDTA, 8.0 pH (Cat. No. 15575020)
- DMEM/F-12, GlutaMAX Supplement (Cat. No. 10565018)
- KnockOut Serum Replacement – Multi-Species (Cat. No. A3181502)
- FGF-basic (AA 1–155) Recombinant Human (Cat. No. PHG0264)
- MEM Non-Essential Amino Acids Solution (100X) (Cat. No. 11140050)
- 2-Mercaptoethanol (Cat. No. 21985023)
- Matrix:
  - Geltrex LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix (Cat. No. A1413302) for general applications
  - Vitronectin (VTN-N) Recombinant Human Protein, Truncated (Cat. No. A14700) for xeno-free applications
  - rhLaminin-521 (Cat. No. A29248) for best results and for xeno-free applications
- Sterile cell culture hood (i.e., biosafety cabinet)
- Inverted microscope
- Incubator set at 37°C, 5% CO_2
- Water bath set at 37°C
- Sterile serological pipettes (5 mL, 10 mL)
- Centrifuge
- 15 mL centrifuge tubes
- Appropriate tissue culture plates and supplies

Preparation of media and reagents

Refer to section 2.1 for instructions on how to prepare media and reagents used for feeder-dependent culture. Refer to the first parts of this section for instructions on how to prepare media and reagents for feeder-free culture with Essential 8 or Essential 8 Flex Medium.

Adaptation of feeder-dependent cultures into Essential 8 or Essential 8 Flex Medium

Feeder-dependent cultures can be adapted into Essential 8 or Essential 8 Flex Medium using the modified collagenase- or dispase-based passaging method below (Figure 2.7). Cells can be transferred directly into Essential 8 or Essential 8 Flex Medium with vitronectin or a Geltrex matrix. Alternatively, for best results, cells can be transferred to rhLaminin-521 or the complete Essential 8 Adaptation Kit (Cat. No. A25935) as described in the product insert (Pub. No. MAN0014536) before transitioning to vitronectin.

Note: The volumes given in the following adaptation procedure are for 60 mm culture dishes. For culture vessels with different sizes, adjust the volumes appropriately.

1. Prepare a 1:100 Geltrex matrix solution (for general applications) in DMEM/F-12 or a 1:50 vitronectin solution (for xeno-free applications) in DPBS without calcium and magnesium. Coat culture dishes with your matrix of choice and incubate for 1 hour at 37°C (see section 2.2A for the recommended coating procedure).
2. Aspirate the spent PSC medium from the dish containing PSCs on feeder cells and wash once with DPBS without calcium and magnesium.
3. Aspirate the DPBS, and add 2 mL of 1X Collagenase Type IV (1 mg/mL), prewarmed to 37°C.

   Note: Alternatively, you can use a Dispase II solution (2 mg/mL) in place of the Collagenase Type IV solution.
4. Incubate the cells for 30–45 minutes in the 37°C, 5% CO_2 incubator.

   Note: If using Dispase II solution (2 mg/mL), incubation time is 15–25 minutes.
5. Stop the incubation when the edges of the colonies begin to curl from the plate.
6. Add 2 mL of complete PSC medium and gently dislodge the colonies from the plate by washing off with a 5 mL serological pipette.

   Tip the plate at a 45-degree angle and rotate the plate as you begin to triturate the clusters of colonies into smaller fragments. Repeat until the desired fragment size is achieved.

   Note: Optimal fragment size is critical for successful adaptation. Colony fragments that are too large will form embryoid body (EB)–like clusters when reseeded, and fragments that are too small will differentiate.
7. Transfer the suspended colony clusters into a 15 mL conical tube.
8. Add an additional 2 mL of complete PSC medium to dislodge the remaining colonies and transfer them to the 15 mL tube.

   Note: If desired, you can triturate the PSC colonies by pipetting them up and down 5–7 times in the 15 mL conical tube rather in the tissue culture dish. This can reduce the chances of contamination.
9. Let the colony fragments sediment at the bottom of the 15 mL tube for 2–5 minutes by gravity.
10. Discard the supernatant, add 4 mL of complete PSC medium, and gently resuspend the sedimented colony fragments by pipetting up and down 2 times.
11. Gravity sediment the clusters again for 2–5 minutes.

12. While colony fragments are sedimenting, aspirate the matrix solution from the freshly prepared dish and add 4 mL of complete Essential 8 or Essential 8 Flex Medium.

13. Aspirate the supernatant from the sedimented clusters and add 4 mL of complete Essential 8 or Essential 8 Flex Medium.

14. Resuspend the PSC clusters by gently pipetting them up and down 2 times, taking care not to further break down the clusters; the goal is to just resuspend the PSC clusters for seeding (split ratio of 1:4).

15. Distribute 1 mL of the suspended PSC clusters into each matrix-coated dish containing 4 mL of complete Essential 8 or Essential 8 Flex Medium. Move the dish in several quick figure-8 motions to disperse the cells across the surface of the dish.

16. Place the culture dishes containing the PSC clusters into the 37°C, 5% CO_2 incubator.

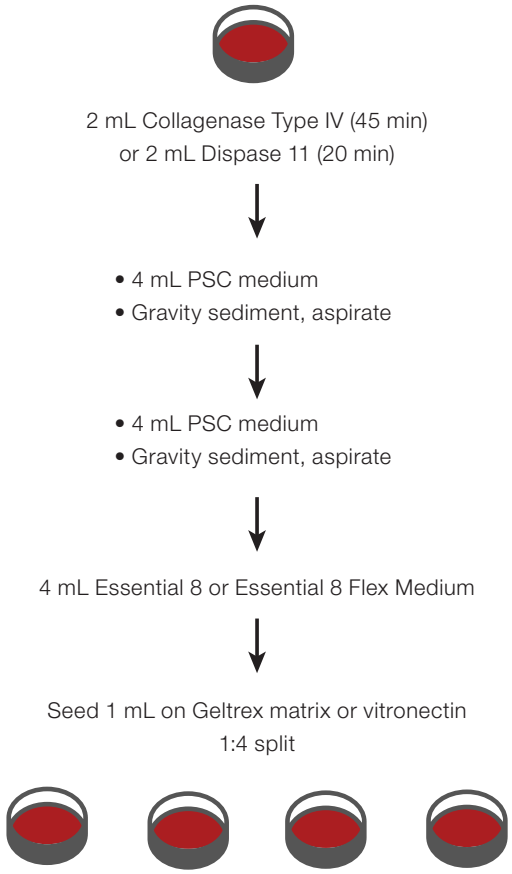


Figure 2.7. Procedure for adaptation of PSCs into Essential 8 or Essential 8 Flex Medium. Performing the gravity sedimentation twice is essential for the attachment and adaptation of the colonies on plates coated with vitronectin or Geltrex matrix.

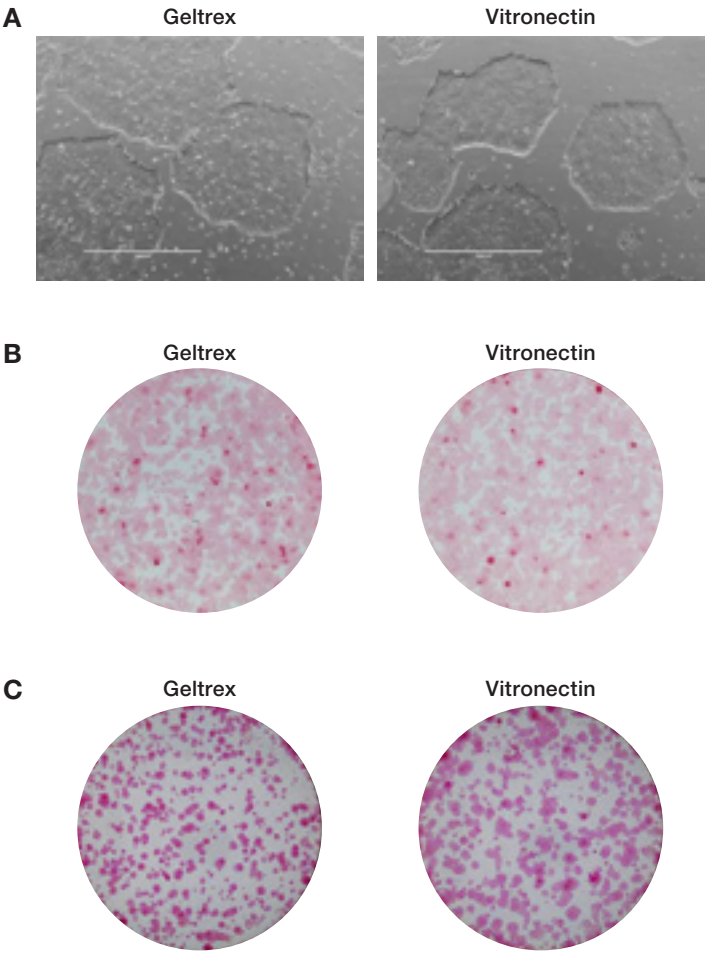


Figure 2.8. PSCs adapted from feeder-dependent cultures into Essential 8 Medium. (A) 50x phase contrast images, **(B)** whole-well alkaline phosphatase staining of PSCs adapted from feeder-dependent cultures into Essential 8 Medium on Geltrex matrix– or vitronectin-coated dishes using Collagenase Type IV, and **(C)** without Collagenase Type IV.

Adaptation of other feeder-free cultures into Essential 8 or Essential 8 Flex Medium

PSCs cultured in other feeder-free media like mTeSR™1 medium or Gibco™ StemPro™ hESC Serum-Free Medium (SFM) can be adapted into Essential 8 or Essential 8 Flex Medium using the same EDTA or Versene solution–based passaging method that will later be used for routine passaging of the newly adapted cultures. When transitioning from a feeder-free culture grown on Matrigel™ or Geltrex matrix, we recommend maintaining the same matrix with Essential 8 or Essential 8 Flex Medium for at least one passage. The matrix can then be changed to vitronectin on the next passage. Use Figure 2.9 as a reference for the appropriate adaptation scheme.

Alternatively, for best results, cells can be transferred to rhLaminin-521 or the complete Essential 8 Adaptation Kit as described in the product insert (Pub. No. MAN0014536) before transitioning to vitronectin.

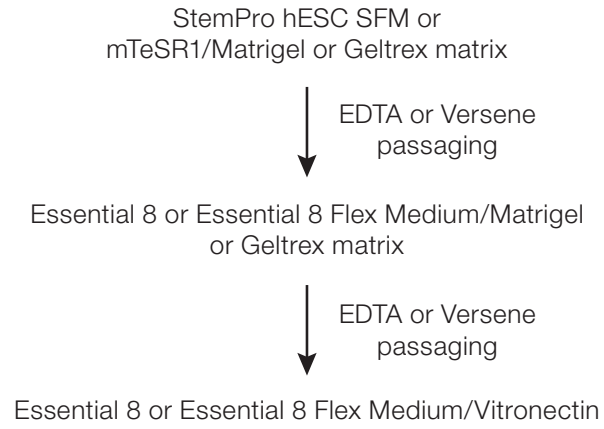


Figure 2.9. Scheme for adapting PSCs from other feeder-free systems into Essential 8 or Essential 8 Flex Medium with vitronectin.

2.2C Feeder-free culture of PSCs using StemFlex Medium

StemFlex Medium supports the robust expansion of feeder-free PSCs and is optimized to support novel applications including single-cell passaging, gene editing, and reprogramming. The unique formulation enables a flexible feeding schedule and the ability to choose the matrix and passaging reagent that best suit a specific application. This protocol describes how to thaw, culture, and cryopreserve PSCs in StemFlex Medium. It also describes how to adapt PSCs from other culture systems into StemFlex Medium and how to perform single-cell passaging with PSCs with the robust support of StemFlex Medium.

Materials needed

- StemFlex Medium (Cat. No. A3349401)
- Matrix:
  - Geltrex LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix (Cat. No. A1413301)
  - rhLaminin-521 (Cat. No. A29248)
  - Vitronectin (VTN-N) Recombinant Human Protein, Truncated (Cat. No. A14700)
- DPBS, no calcium, no magnesium (Cat. No. 14190144)
- Versene Solution (Cat. No. 15040066) or UltraPure 0.5 M EDTA, pH 8.0 (Cat. No. 15575020)
- PSC Cryopreservation Kit (Cat. No. A2644601), which consists of PSC Cryomedium (Cat. No. A2644401) and RevitaCell Supplement (Cat. No. A2644501)
- TrypLE Select Enzyme (1X), no phenol red (Cat. No. 12563029)
- DMEM/F-12, GlutaMAX Supplement (Cat. No. 10565018)
- Collagenase, Type IV, powder (Cat. No. 17104019)
- Water bath set at 37°C
- Appropriate tissue culture plates and supplies

Preparation of media and reagents

StemFlex Medium (500 mL)

1. Thaw the frozen StemFlex Medium at room temperature for ~2 hours or overnight at 2–8°C.

   Important: Do not thaw the frozen supplement at 37°C.

2. Mix the thawed supplement by gently inverting 3–5 times.

3. Aseptically transfer 50 mL of StemFlex supplement to the bottle of StemFlex basal medium (450 mL fill). Gently invert the bottle several times to obtain 500 mL of homogeneous complete medium.

4. Store complete StemFlex Medium at 2–8°C for up to 2 weeks.

5. Before use, warm complete medium required for the day at room temperature and until it is no longer cool to the touch.

   Alternatively, an aliquot for use that day may be prewarmed in a 37°C water bath until no longer cool to the touch. Avoid extended dwell times at 37°C. Following reconstitution, complete media can be aliquotted and stored at –5°C to –20°C for up to 6 months.

   Alternatively, usage-size aliquots of the supplement can be made and frozen at –5 to –20°C for up to 6 months. Avoid multiple freeze/thaw cycles.

0.5 mM EDTA in DPBS (50 mL)

1. To prepare 50 mL of 0.5 mM EDTA in DPBS, aseptically mix the following components in a 50 mL conical tube in a biological safety cabinet:

DPBS, no calcium, no magnesium	50 mL
0.5M EDTA	50 µL

2. Filter-sterilize the solution. The solution can be stored at room temperature for up to 6 months.

Matrix-coated dishes

We generally recommend using StemFlex Medium with a Geltrex matrix as the substrate for routine culture with clump passaging. However, StemFlex Medium can also be used with vitronectin or rhLaminin-521. Refer to section 2.2A for detailed instructions on how to coat dishes with these matrices.

Procedural guidelines

Guidelines for culturing human PSCs in StemFlex Medium

Split cultures when the first of the following occurs:

- PSC colonies become too dense or too large
- PSC colonies show increased differentiation
- Colonies cover ~85% of the surface area of the culture vessel (usually every 3 to 5 days)

The split ratio can vary, though it is generally between 1:2 and 1:4 for newly derived PSCs, and between 1:3 and 1:12 for established cultures. Occasionally, cells may recover at a different rate and the split ratio will need to be adjusted.

As a general rule, observe the last split ratio and adjust the ratio according to the appearance of the PSC colonies. If the cells look healthy and the colonies have enough space, split using the same ratio. If the colonies are overly dense and crowding, increase the ratio; if they are sparse, decrease the ratio.

Newly derived PSC lines may contain a fair amount of differentiation through passage 4. It is not necessary to remove differentiated material prior to passaging. By propagating/splitting the cells, the overall culture health should improve throughout the early passages.

Do not scrape the cells from the culture vessel during clump cell passaging using Versene or 500 µM EDTA solution.

For complete transition to the StemFlex Medium system from other culture systems, a minimum of a 2-passage adaptation is recommended.

Recover frozen PSCs in complete StemFlex Medium
If using precoated plates stored at 2–8°C, prewarm Geltrex matrix–coated plates to room temperature. Prewarm complete StemFlex Medium to room temperature.

1. Precoat plates with Geltrex matrix as described in section 2.2A. See the table below for recommended volumes. The optimal working concentration of Geltrex matrix is cell line–dependent.

Culture vessel	Surface area	Volume
6-well plate	10 cm^2/well	1.5 mL per well
12-well plate	4 cm^2/well	0.6 mL per well
24-well plate	2 cm^2/well	0.3 mL per well
35 mm dish	10 cm^2	1.5 mL
60 mm dish	20 cm^2	3.0 mL
100 mm dish	60 cm^2	9.0 mL

Refer to section 2.2A for procedural guidelines.

2. Remove a vial of PSCs from liquid nitrogen storage and transfer it on dry ice to the tissue culture room.
3. Immerse the vial in a 37°C water bath without submerging the cap; swirl the vial gently.
4. When only an ice crystal remains, remove the vial from the water bath, spray the outside with 70% ethanol, and place it in the hood.
5. Transfer the thawed cells to a 15 mL or 50 mL conical tube and add 3 mL of complete StemFlex Medium to the cells dropwise to reduce osmotic shock. While adding the medium, gently move the tube back and forth to mix the PSCs.
6. Rinse the vial with 1 mL of complete StemFlex Medium and add to the conical tube containing the cells.
7. Centrifuge the cells at 200 x *g* for 4 minutes, aspirate and discard the supernatant, and resuspend the cell pellet in 1 mL of complete StemFlex Medium by gently pipetting the cells up and down a few times.
8. Immediately prior to plating of cells and following coating of culture vessel for >1 hour at 37°C, 5% CO_2, aspirate Geltrex matrix from the wells and discard. Be certain to not allow the culture surface to dry out.
9. Slowly add the PSC suspension into the Geltrex matrix–coated plate, plating ~100,000 viable cells per cm^2 of plate for conditions seeded in the absence of ROCK inhibitor.

   Note: This amount may need to be adjusted based upon the solution used for cryopreservation.

   Optional: To improve efficiency of cell survival 24 hours postthaw, inclusion of RevitaCell Supplement (Cat. No. A2644501) may be used at 1X final concentration (i.e., 10 µL per 1 mL of cell suspension) for the first 24 hours postthaw to minimize apoptosis and necrosis. When using this supplement for recovery of your PSCs, lower cell seeding densities are required; plating at a viable cell density of ~20,000–40,000 viable cells/cm^2 will allow for recovery in 3–4 days postthaw. **Important:** Do not include additional ROCK inhibitors such as Y27632 or thiazovivin when using RevitaCell Supplement.

Culture vessel	Surface area	Volume
6-well plate	10 cm^2/well	2.0 mL per well
12-well plate	4 cm^2/well	1.0 mL per well
24-well plate	2 cm^2/well	0.5 mL per well
35 mm dish	10 cm^2	2.0 mL
60 mm dish	20 cm^2	4.0 mL
100 mm dish	60 cm^2	12.0 mL

10. Move the plate in several quick side-to-side motions to disperse the cells across the surface of the wells, and place the plate gently into the 37°C, 5% CO_2 incubator.

11. Feed the PSCs the day after seeding followed by an every-other-day feeding schedule.

    If the cells are to be left without feeding for 2 days (for example, over a weekend), double the feed volume (e.g., 4 mL added per well of 6-well plate). Refer to Figure 2.10 for a flexible feeding schedule.

    Note: Cells should be passaged upon reaching ~85% confluency to maintain culture health.

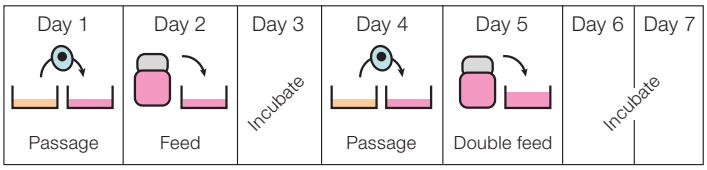


Figure 2.10. Typical weekly PSC culture workflow using StemFlex Medium.

Clump cell passage PSCs using Versene or 500 µM EDTA solution for routine culture

If using precoated plates stored at 2–8°C, prewarm Geltrex matrix–coated plates to room temperature. Prewarm StemFlex Medium and Versene or 500 µM EDTA solution to room temperature.

1. Precoat plates with Geltrex matrix as described in section 2.2A. See the table below for recommended coating conditions. The optimal working concentration of Geltrex matrix is cell line–dependent.

Culture vessel	Surface area	Volume
6-well plate	10 cm^2/well	1.5 mL per well
12-well plate	4 cm^2/well	0.6 mL per well
24-well plate	2 cm^2/well	0.3 mL per well
35 mm dish	10 cm^2	1.5 mL
60 mm dish	20 cm^2	3.0 mL
100 mm dish	60 cm^2	9.0 mL

Refer to section 2.2A for procedural guidelines.

2. Aspirate the spent medium from the vessel containing PSCs and rinse the vessel once with DPBS without calcium and magnesium. See the table below for recommended volumes.

Culture vessel	Surface area	Volume
6-well plate	10 cm^2/well	2.0 mL per well
12-well plate	4 cm^2/well	1.0 mL per well
24-well plate	2 cm^2/well	0.5 mL per well
35 mm dish	10 cm^2	2.0 mL
60 mm dish	20 cm^2	4.0 mL
100 mm dish	60 cm^2	12.0 mL

3. Add Versene or 500 µM EDTA solution to the side of the vessel containing PSCs (see table below for recommended volume), then swirl the vessel to coat the entire well surface.

Culture vessel	Surface area	Volume
6-well plate	10 cm^2/well	1.0 mL per well
12-well plate	4 cm^2/well	0.4 mL per well
24-well plate	2 cm^2/well	0.2 mL per well
35 mm dish	10 cm^2	1.0 mL
60 mm dish	20 cm^2	2.0 mL
100 mm dish	60 cm^2	6.0 mL

4. Incubate the vessel at room temperature for 5–8 minutes or at 37°C for 4–5 minutes.

   When the cells start to separate and round up, and the colonies appear to have holes in them when viewed under a microscope, they are ready to be removed from the vessel. Remove the cells from the well(s) by gently flushing medium over the surface of the well a few times.

   Note: Cells should not be incubated to the extent that the colonies float off the surface of the culture vessel.

5. Aspirate the Versene or 500 µM EDTA solution, and add prewarmed complete StemFlex Medium to the vessel. See the table below for recommended volumes.

   Note: Step 5 can be completed immediately prior to passaging the cells and cells can be directly passaged onto the new plate rather than transferred to a conical tube.

Culture vessel	Surface area	Volume
6-well plate	10 cm^2/well	2.0 mL per well
12-well plate	4 cm^2/well	1.0 mL per well
24-well plate	2 cm^2/well	0.5 mL per well
35 mm dish	10 cm^2	2.0 mL
60 mm dish	20 cm^2	4.0 mL
100 mm dish	60 cm^2	12.0 mL

6. Remove the cells from the well(s) by gently flushing medium over the surface of the well a few times.

7. Collect cells in a 15 mL or 50 mL conical tube.

   There may be obvious patches of cells that were not dislodged and left behind. Do not scrape the cells from the dish in an attempt to recover them.

   Note: Depending upon the cell line, work with no more than 1 to 3 wells at a time, and work quickly to remove cells after adding StemFlex Medium to the well(s), as it quickly neutralizes the initial effect of the Versene or 500 µM EDTA solution. Some lines re-adhere very rapidly after medium addition, and must be removed 1 well at a time. Others are slower to reattach, and may be removed 3 wells at a time.

8. Following coating of the culture vessel for >1 hour at 37°C and 5% CO_2, aspirate the Geltrex matrix from the culture vessel and discard.

   Important: Do not allow the culture surface to dry out.

9. Immediately add an appropriate volume of prewarmed complete StemFlex Medium to each well of a Geltrex matrix–coated plate so that each well contains the recommended volume of complete medium after the cell suspension has been added. See the table below for recommended volumes.

   Note: The split ratio can vary, though it is generally between 1:2 and 1:4 for newly derived PSCs, and between 1:3 and 1:12 for established cultures. Occasionally, cells may recover at a different rate and the split ratio will need to be adjusted.

Culture vessel	Surface area	Volume
6-well plate	10 cm^2/well	2.0 mL per well
12-well plate	4 cm^2/well	1.0 mL per well
24-well plate	2 cm^2/well	0.5 mL per well
35 mm dish	10 cm^2	2.0 mL
60 mm dish	20 cm^2	4.0 mL
100 mm dish	60 cm^2	12.0 mL

10. Move the vessel in several quick side-to-side motions to disperse the cells across the surface of the vessels.

11. Place the vessel gently into the 37°C, 5% CO_2 incubator and incubate the cells overnight.

12. Feed the PSCs the day after passaging followed by an every-other-day feeding schedule after that until the cells are ~85% confluent.

    If the cells are to be left without feeding for longer than 48 hours (for example, during a weekend), double the feed volume (e.g., 4 mL added per well of 6-well plate).

    Note: It is normal to see cell debris and small colonies after passage. Cells should be passaged once reaching ~85% confluency to maintain culture health.

Cryopreservation of PSCs

1. Thaw and prechill PSC Cryomedium at 2–8ºC.

2. Harvest PSCs according to standard single-cell or clumped cell passaging protocols.

3. Centrifuge the cell suspension at 200 x *g* for 4 minutes.

4. Aspirate the medium, being careful not to disturb the cell pellet.

5. Add prechilled PSC Cryomedium to the cells dropwise while gently rocking the tube back and forth followed by gentle resuspension of the cell pellet.

   Note: In general, from a 100 mm dish, 8–12 vials containing 1 x 10^6 viable cells/mL can be generated.

6. Dispense aliquots of the suspension into cryogenic vials according to manufacturer's specifications (i.e., 1.5 mL in a 2 mL cryovial).

   Note: Mix the cell suspension in PSC Cryomedium frequently to maintain a homogeneous suspension. If utilizing clumped passaging methods at cell harvest, then mix cell suspension by gentle inversion to prevent breaking cells into smaller clumps.

7. Cryopreserve cells in an automated or manual controlled-rate freezing apparatus following standard procedures (approximately 1ºC decrease per minute).

8. Transfer frozen cell vials to liquid nitrogen (vapor phase); we recommend storage at –200°C to –125°C.

Adaptation of mTeSR1 medium–cultured PSCs to StemFlex Medium on Geltrex matrix–coated vessels

When mTeSR1 medium–based cultures achieve passaging confluency (i.e., 60–85% confluency), the cells are ready for adaptation to the StemFlex Medium.

Passage PSCs as described in "Clump cell passage PSCs using Versene or 500 µM EDTA solution for routine culture".

Note: For complete transition to the StemFlex Medium system from mTeSR1 medium, a minimum of a 2-passage adaptation is recommended.

Adaptation of feeder-dependent PSCs to StemFlex Medium on Geltrex matrix–coated vessels

When the feeder-dependent cultures reach passaging confluency (i.e., 60–85% confluent with round colonies that are not overcrowded), the cells are ready for adaptation to feeder-free culture conditions.

Note: The following instructions are for use of Geltrex matrix in transition. For cell lines that are difficult to transition, rhLaminin-521 can be implemented for one passage followed by Versene solution passaging onto Geltrex matrix–coated plates.

If using precoated plates stored at 2–8°C, prewarm the Geltrex matrix–coated plates to room temperature.

1. Precoat plates with Geltrex matrix. See the following table for recommended coating conditions.

   The optimal working concentration of Geltrex matrix is cell line–dependent. See section 2.2A for procedural guidelines.

Culture vessel	Surface area	Volume
6-well plate	10 cm^2/well	1.5 mL per well
12-well plate	4 cm^2/well	0.6 mL per well
24-well plate	2 cm^2/well	0.3 mL per well
35 mm dish	10 cm^2	1.5 mL
60 mm dish	20 cm^2	3.0 mL
100 mm dish	60 cm^2	9.0 mL

2. Prepare a fresh 1 mg/mL Collagenase Type IV solution in DMEM/F-12 with GlutaMAX Supplement, filter-sterilize using a 0.2 µm filter unit, and prewarm in a 37°C water bath.

3. Perform manual cleanup of feeder-dependent cultures to remove areas of aberrant differentiation ahead of passaging of cultures.

4. Aspirate the spent medium from the vessel containing PSCs and rinse the vessel once with DPBS without calcium and magnesium. See the table below for recommended volumes.

Culture vessel	Surface area	Volume
6-well plate	10 cm^2/well	2.0 mL per well
12-well plate	4 cm^2/well	1.0 mL per well
24-well plate	2 cm^2/well	0.5 mL per well
35 mm dish	10 cm^2	2.0 mL
60 mm dish	20 cm^2	4.0 mL
100 mm dish	60 cm^2	12.0 mL

5. Aspirate the DPBS and add prewarmed 1 mg/mL Collagenase Type IV solution.

Culture vessel	Surface area	Volume
6-well plate	10 cm^2/well	1.0 mL per well
12-well plate	4 cm^2/well	0.5 mL per well
24-well plate	2 cm^2/well	0.25 mL per well
35 mm dish	10 cm^2	1.0 mL
60 mm dish	20 cm^2	2.0 mL
100 mm dish	60 cm^2	6.0 mL

6. Incubate the dish for ~45 minutes in a 37°C, 5% CO_2 incubator.

   Note: Stop the incubation when the edges of the colonies begin to curl from the plate. Do not overincubate.

7. Add complete StemFlex Medium and gently dislodge the colonies from the plate by washing off colonies with a 5 mL serological pipette or 1 mL pipettor.

Culture vessel	Surface area	Volume
6-well plate	10 cm^2/well	1.0 mL per well
12-well plate	4 cm^2/well	0.5 mL per well
24-well plate	2 cm^2/well	0.25 mL per well
35 mm dish	10 cm^2	1.0 mL
60 mm dish	20 cm^2	2.0 mL
100 mm dish	60 cm^2	6.0 mL

8. Transfer the suspended colony clusters into a 15 mL conical tube.

9. Add complete StemFlex Medium to the vessel to dislodge the remaining colonies, and transfer them to the 15 mL conical tube. Repeat trituration of contents transferred to the 15 mL conical tube until the desired cluster size is achieved.

Culture vessel	Surface area	Volume
6-well plate	10 cm^2/well	1.0 mL per well
12-well plate	4 cm^2/well	0.5 mL per well
24-well plate	2 cm^2/well	0.25 mL per well
35 mm dish	10 cm^2	1.0 mL
60 mm dish	20 cm^2	2.0 mL
100 mm dish	60 cm^2	6.0 mL

10. Let the colony fragments sediment to the bottom of the 15 mL conical tube for 5 minutes by gravity.

11. Discard the supernatant, add complete StemFlex Medium, and gently resuspend the sedimented colony fragments by pipetting up and down 2 times.

12. Gravity-sediment the clusters for 2–5 minutes.

13. While the colony fragments are sedimenting, aspirate the matrix solution from the Geltrex matrix–coated vessel and add complete StemFlex Medium.

Culture vessel	Surface area	Volume
6-well plate	10 cm^2/well	2.0 mL per well
12-well plate	4 cm^2/well	1.0 mL per well
24-well plate	2 cm^2/well	0.5 mL per well
35 mm dish	10 cm^2	2.0 mL
60 mm dish	20 cm^2	4.0 mL
100 mm dish	60 cm^2	12.0 mL

14. Aspirate the supernatant and resuspend the sedimented PSC clusters by gently pipetting them up and down 2 times, taking care not to break them down further.

Culture vessel	Surface area	Volume
6-well plate	10 cm^2/well	2.0 mL per well
12-well plate	4 cm^2/well	1.0 mL per well
24-well plate	2 cm^2/well	0.5 mL per well
35 mm dish	10 cm^2	2.0 mL
60 mm dish	20 cm^2	4.0 mL
100 mm dish	60 cm^2	12.0 mL

15. Distribute the resuspended PSC clusters into the Geltrex matrix–precoated vessel, then move the vessel in several quick side-to-side motions to disperse the cells across the surface of the vessel.

Culture vessel	Surface area	Volume
6-well plate	10 cm^2/well	0.5 mL per well
12-well plate	4 cm^2/well	0.25 mL per well
24-well plate	2 cm^2/well	0.125 mL per well
35 mm dish	10 cm^2	0.5 mL
60 mm dish	20 cm^2	1.0 mL
100 mm dish	60 cm^2	3.0 mL

16. Place the vessel gently into a 37°C, 5% CO_2 incubator and incubate the cells overnight.

17. For the first passage posttransition, feed cells daily.

    Note: It is normal to see cell debris and small colonies after passaging.

18. Passage cells per instructions for "Clump cell passage PSCs using Versene or 500 µM EDTA solution for routine culture" on page 48 upon attaining ~85% confluency to maintain culture health.

    Following passaging with Versene solution, cells can be fed using the optional flexible feed schedules.

Single-cell passaging of PSCs using TrypLE Select Enzyme for high-throughput screening (HTS) or gene editing applications

If using precoated plates stored at 2–8°C, prewarm rhLaminin-521–coated plates to room temperature. Prewarm StemFlex Medium and TrypLE Select solution to room temperature.

1. Precoat plates with rhLaminin-521 as described in section 2.2A. See the table below for recommended volumes. The optimal working concentration of rhLaminin-521 is cell line–dependent.

Culture vessel	Surface area	Volume
6-well plate	10 cm^2/well	2.0 mL per well
12-well plate	4 cm^2/well	0.8 mL per well
24-well plate	2 cm^2/well	0.4 mL per well
35 mm dish	10 cm^2	2.0 mL
60 mm dish	20 cm^2	4.0 mL
100 mm dish	60 cm^2	12.0 mL

2. Aspirate the spent medium from the culture vessel.

3. Rinse the vessel once with recommended volume of DPBS without calcium and magnesium. See the table below for recommended volumes.

Culture vessel	Surface area	Volume
6-well plate	10 cm^2/well	2.0 mL per well
12-well plate	4 cm^2/well	1.0 mL per well
24-well plate	2 cm^2/well	0.5 mL per well
35 mm dish	10 cm^2	2.0 mL
60 mm dish	20 cm^2	4.0 mL
100 mm dish	60 cm^2	12.0 mL

4. Add the recommended volume of prewarmed TrypLE Select enzyme. See below for recommended volumes.

Culture vessel	Surface area	Volume
6-well plate	10 cm^2/well	1.0 mL per well
12-well plate	4 cm^2/well	0.4 mL per well
24-well plate	2 cm^2/well	0.2 mL per well
35 mm dish	10 cm^2	1.0 mL
60 mm dish	20 cm^2	2.0 mL
100 mm dish	60 cm^2	6.0 mL

5. Incubate the vessel at 37°C, 5% CO_2 for 5 minutes.

   Note: Avoid extended incubation of PSCs with dissociation reagent to minimize cellular toxicity.

6. Gently pipet the cells up and down 5–10 times with a 1 mL pipette to generate a single-cell suspension.

7. Transfer the cell suspension to a conical tube containing the recommended neutralization volume of StemFlex Medium to dilute the dissociation reagent. See table for recommended volumes.

Culture vessel	Surface area	Volume
6-well plate	10 cm^2/well	3.0 mL per well
12-well plate	4 cm^2/well	1.2 mL per well
24-well plate	2 cm^2/well	0.6 mL per well
35 mm dish	10 cm^2	3.0 mL
60 mm dish	20 cm^2	6.0 mL
100 mm dish	60 cm^2	18.0 mL

8. Centrifuge the PSCs at 200 x *g* for 4 minutes.

9. Aspirate and discard the supernatant, flick the tube 3–5 times to loosen the pellet, and resuspend the cells by pipetting them up and down 5–10 times in the recommended resuspension volume of StemFlex Medium. See the table for recommended volumes.

Culture vessel	Surface area	Volume
6-well plate	10 cm^2/well	2.0 mL per well
12-well plate	4 cm^2/well	1.0 mL per well
24-well plate	2 cm^2/well	0.5 mL per well
35 mm dish	10 cm^2	2.0 mL
60 mm dish	20 cm^2	4.0 mL
100 mm dish	60 cm^2	12.0 mL

10. Determine the viable cell density and percent viability using an Invitrogen™ Countess™ II Automated Cell Counter or similar automated or manual method.

11. Adjust the concentration of the cell suspension using StemFlex Medium to achieve the cell seeding density recommended for your culture vessel. See Table 2.2 for seeding densities.

12. Immediately prior to plating of cells and following coating of culture vessel for >2 hours at 37°C, 5% CO_2, aspirate rhLaminin-521 from the wells and discard.

    Important: Do not allow the culture surface to dry out.

13. Transfer the cell suspension to the precoated culture vessel.

    Note: If using alternative substrates such as Geltrex matrix or vitronectin, we recommend including RevitaCell Supplement for the first 24 hours post-passage, whereas rhLaminin-521 does not require the use of RevitaCell Supplement.

14. Move the vessel in several quick side-to-side motions to disperse the cells across the surface of the vessel. Place the vessel gently into the 37°C, 5% CO_2 incubator and incubate the cells overnight.

Table 2.2. Recommended cell seeding densities.

Culture vessel (surface area)	Number of viable cells added*		Plating volume of StemFlex Medium
	12,500 cells/cm²	25,000 cells/cm²	
6-well plate (10 cm²/well)	125,000	250,000	2.0 mL per well
12-well plate (4 cm²/well)	50,000	100,000	1.0 mL per well
24-well plate (2 cm²/well)	25,000	50,000	0.5 mL per well
35 mm dish (10 cm²)	125,000	250,000	2.0 mL
60 mm dish (20 cm²)	250,000	500,000	4.0 mL
100 mm dish (60 cm²)	750,000	1,500,000	12.0 mL

* Time to confluency is 4–5 days for a seeding density of 12,500 cells/cm² and 3–4 days for a seeding density of 25,000 cells/cm².
Note: Cell seeding densities are cell line–dependent and thus may need to be optimized for your cell line.

15. Feed the PSCs the day after passaging followed by an every-other-day feeding schedule after that.

    If the cells are to be left without feeding for longer than 48 hours (for example, during a weekend), double the feed volume (e.g., 4 mL added per well of 6-well plate).

    Note: It is normal to see cell debris and small colonies after passaging.

16. Cells should be passaged once reaching ~85% confluency to maintain culture health.

3. Characterization of PSCs

3.1 Genetic analysis of PSCs using the KaryoStat Assay

With recent technological advances, induced pluripotent stem cells (iPSCs) can now be derived from various somatic cells using different reprogramming methods and can be cultured with a variety of media and matrices. As diverse PSC lines are derived, modified, and cultured under different conditions, and furthermore as PSC lines are subjected to prolonged culture or genome editing, there is a need for reliable characterization methods to confirm the quality of the PSCs.

Current PSC characterization practices use a panel of assays that primarily test functional pluripotency and detect abnormalities that can affect cell behavior and safety. Therefore, some of these assays can be performed even on emerging iPSC colonies, whereas most of the other assays are performed after clones have been expanded. Such assays would include a variety of molecular analyses as well as fixed-cell staining of additional PSC markers.

This section contains guidance for three molecular assays: the Applied Biosystems™ KaryoStat™ Assay is an array-based alternative to G-band karyotyping; the Applied Biosystems™ TaqMan® hPSC Scorecard™ Panel is used for RT-qPCR–based analysis of self-renewal and trilineage differentiation potential; and the Applied Biosystems™ PrimeView™ Human Gene Expression Array is for use with PluriTest online analysis tool for pluripotency confirmation based on global gene expression profiles.

The KaryoStat Assay (Cat. No. 905403) is an alternative to G-banding karyotyping, offering accurate genotyping (sample ID) plus whole-genome coverage for accurate detection of chromosomal abnormalities with results in 2.5 days. The assay enables the detection of aneuploidies, submicroscopic aberrations, and mosaic events for research use. Because the assay provides genotype information, it can help in the identification of triploidies and copy neutral events such as absence of heterozygosity. Our proprietary manufacturing technology produces arrays that are highly reproducible between each batch with no risk of probe dropout inherent in bead manufacturing techniques. The assay includes arrays, reagents, and easy-to-use data analysis software for a cost-effective and streamlined analysis of PSC lines.

The KaryoStat Assay is optimized for processing 8–24 samples at a time. It is recommended that at least one of these samples be a negative control. This quick reference guide outlines the 3.5-day protocol. Alternatively, the assay can be completed in 2.5 days. For more details, refer to the KaryoStat Assay user guide.

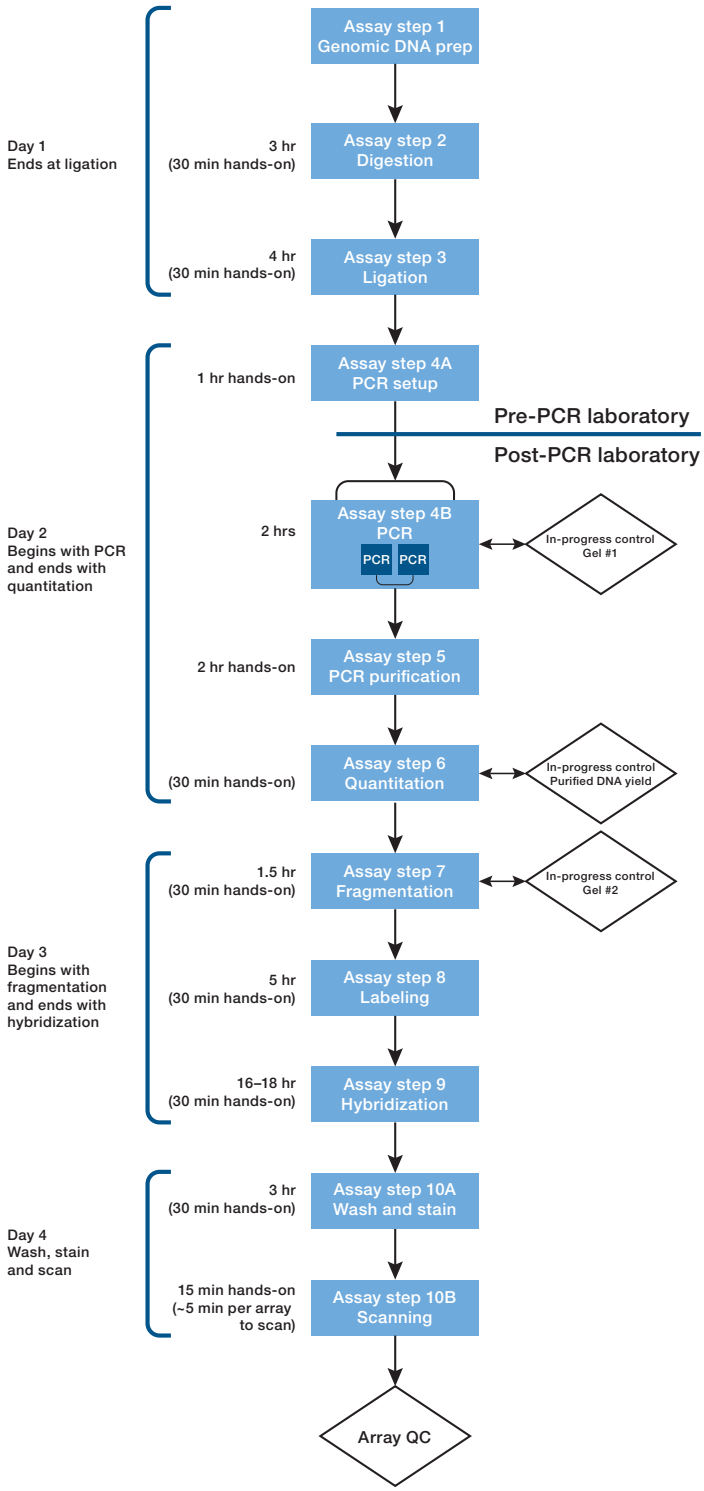


Figure 3.1. KaryoStat Assay workflow overview.

For a list of required materials, see **Appendix C: Required equipment, consumables, and reagents** in the KaryoStat Assay user guide (Pub. No. MAN0017068).

Preparation of genomic DNA (gDNA) plate

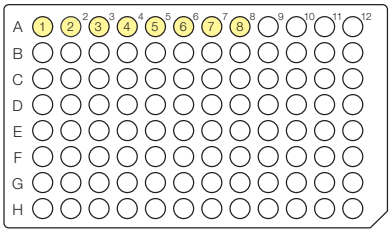
Dilute stock gDNA to working concentration

1. Place the gDNA plate in the upper half of a cooling block on ice.
2. Place the gDNA at room temperature (15–30°C) until thawed (≤30 minutes); place in a cooling block on ice. Use within 1 hour.
3. Vortex gDNA samples for 3 seconds.
4. Centrifuge at 650 x *g* for 1 minute; place in cooling block.
5. Use a double-strand–specific quantitation method to determine sample concentration.
6. Dilute each sample to 50 ng/µL with low-EDTA Tris-EDTA (TE) buffer in separate wells of a 96-well plate.
7. Tightly seal the plate.
8. Centrifuge at 650 x *g* for 1 minute.
9. Place on cooling block.

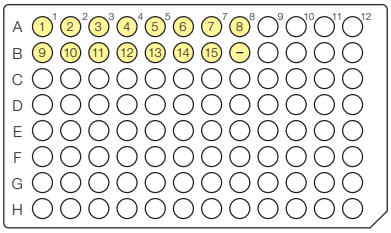
Aliquot prepared gDNA into assay plate

1. Number a 96-well plate (see diagram).
2. Place plate on lower half of cooling block (Chemglass Life Sciences LLC, Cat. No. CLS3621).
3. Transfer 5 µL of first sample from gDNA stock plate to well A1 of DIG-LIG plate to perform digestion (DIG) and ligation (LIG); transfer all samples in same way.

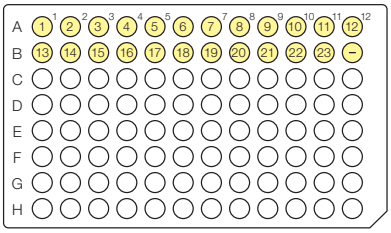
8-sample assay plate



16-sample assay plate



24-sample assay plate



4. Add gDNA to wells marked 1–8. For negative control, add 5 µL of low-EDTA TE buffer instead of gDNA to a well and mark well clearly with "–".
5. Tightly seal DIG-LIG plate with new seal; centrifuge at 650 x *g* for 1 minute and place on cooling block on ice.

Stage 1: Digestion

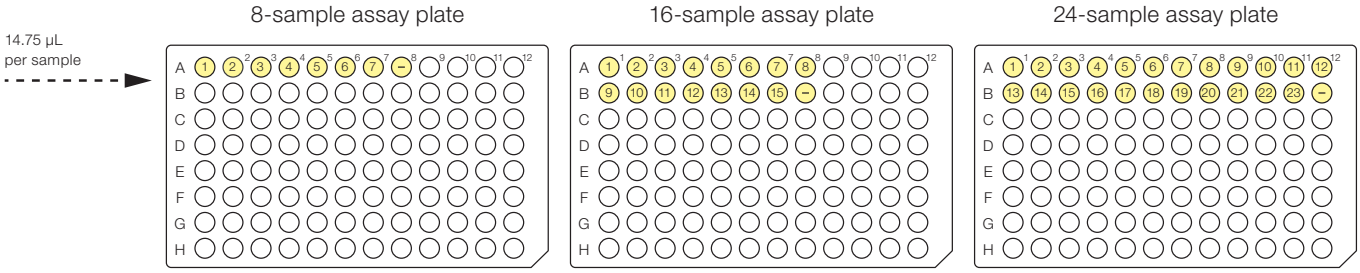
1. Thaw Thermo Scientific™ NspI buffer (10X Tango™ buffer) and 100X BSA ≤30 minutes at room temperature. Vortex and spin down; place on ice. Use within 1 hour.
2. Leave NspI at –15 to –20°C until ready to use.
3. Prepare a digestion master mix (MM) according to the table below. Be sure to vortex before adding the enzyme.

Reagent	Per sample	MM for 8 samples (with 20% overage)	MM for 16 samples (with 20% overage)	MM for 24 samples (with 20% overage)
Water, nuclease-free	11.55 µL	110.9 µL	221.8 µL	332.6 µL
NspI buffer	2.00 µL	19.2 µL	38.4 µL	57.6 µL
100X BSA	0.20 µL	1.9 µL	3.8 µL	5.8 µL
NspI	1.00 µL	9.6 µL	19.2 µL	28.8 µL
Total volume	**14.75 µL**	**141.6 µL**	**283.2 µL**	**424.8 µL**

4. Vortex the digestion master mix at high speed 3 times, 1 second each time; spin down.

5. Add the digestion master mix to samples within 10 minutes.

Samples	Volume/sample
gDNA (50 ng/µL)	5.00 µL (250 ng)
Digestion MM	14.75 µL
Total volume	**19.75 µL**



6. Ensure lid of thermal cycler is preheated.

7. Seal plate with adhesive film.

8. Vortex plate at high speed in 5-sector format, 1 second per sector.

9. Spin down at 650 x *g* for 1 minute.

10. Load plate onto thermal cycler and run the following program:

Temperature	Time
16°C	3 hours
70°C	20 minutes
4°C	5 minutes
4°C	∞

Note: You can hold plate in thermal cycler at 4°C for up to 16–20 hours.

11. Proceed to “Stage 2: Ligation”.

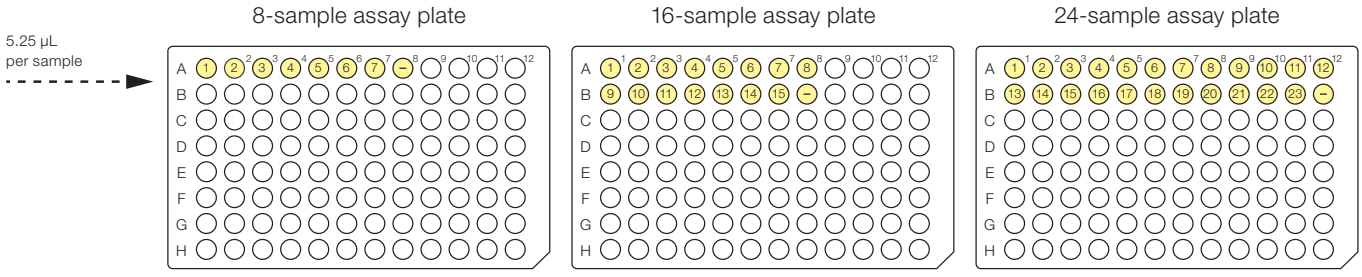
Stage 2: Ligation

1. Thaw DNA ligase buffer and NspI adaptor at room temperature (≤30 minutes). Vortex to ensure any precipitate is resuspended and DNA ligase buffer is clear. Place on ice. Use within 1 hour.
2. Leave DNA ligase at –15 to –20°C until ready to use.
3. Prepare a ligation master mix (MM) according to the table. Be sure to vortex before adding the enzyme.

Reagent	Per sample	MM for 8 samples (with 25% overage)	MM for 16 samples (with 25% overage)	MM for 24 samples (with 25% overage)
DNA ligase buffer	2.50 µL	25.0 µL	50.0 µL	75.0 µL
Adaptor, NspI	0.75 µL	7.5 µL	15.0 µL	22.5 µL
DNA ligase	2.00 µL	20.0 µL	40.0 µL	60.0 µL
Total volume	**5.25 µL**	**52.5 µL**	**105.0 µL**	**157.5 µL**

4. Vortex the ligation master mix at high speed 3 times, 1 second each time; spin down.
5. Add the ligation master mix to samples within 10 minutes.

Samples	Volume/sample
NspI-digested sample	19.75 µL
Ligation MM	5.25 µL
Total volume	**25.00 µL**



6. Ensure lid of thermal cycler is preheated.
7. Seal plate with adhesive film.
8. Vortex plate at high speed in 5-sector format, 1 second per sector.
9. Spin down at 650 x *g* for 1 minute.
10. Load plate onto thermal cycler and run the following program:

Temperature	Time
16°C	3 hours
70°C	20 minutes
4°C	5 minutes
4°C	∞

Note: You can hold plate in thermal cycler at 4°C for up to 16–20 hours.

11. Proceed to "3A: PCR".

Optional stopping point: You can freeze the plate at –15 to –25°C for up to 1 week.

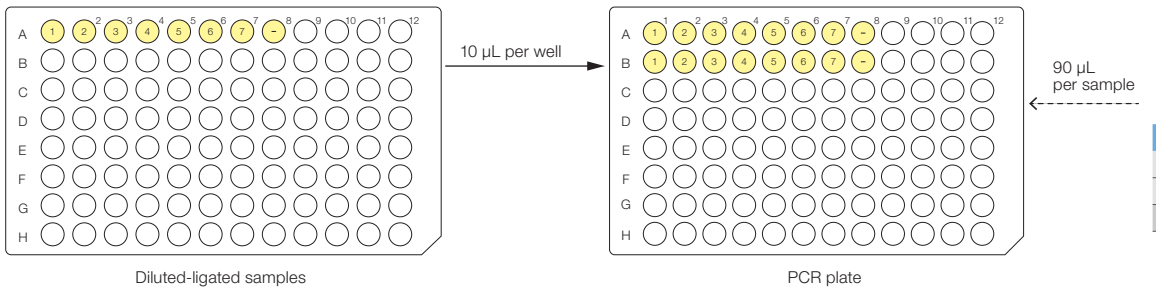
Stage 3A: PCR

1. Thaw the Applied Biosystems™ CytoScan™ *Taq* Buffer, dNTPs, and PCR primer at room temperature (≤30 minutes). Place on ice and use within 1 hour.
2. Ensure the ligation plate is sealed properly.
3. Spin down at 650 x *g* for 1 minute.
4. Thaw the ligated samples at room temperature (≤30 minutes). Immediately place on ice and dilute samples according to the table below. Process within 1 hour.

Samples	Volume/sample
Ligated sample	25 µL
Water, nuclease-free (chilled)	75 µL
Total volume	**100 µL**

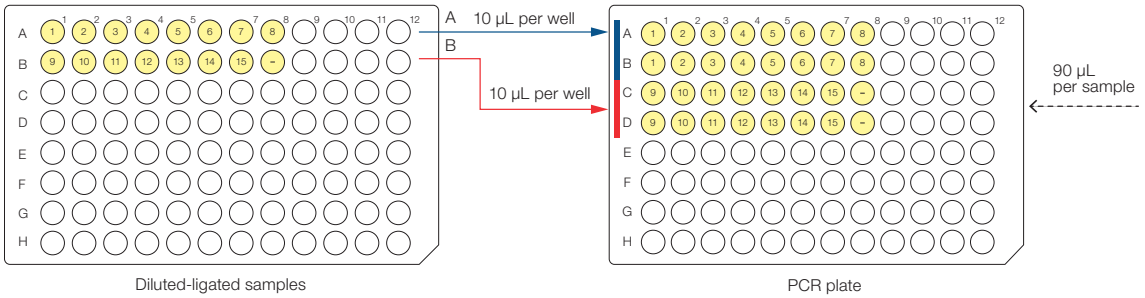
5. Seal plate with adhesive film.
6. Vortex at high speed in 5-sector format, 1 second per sector. Spin down at 650 x *g* for 1 minute.
7. Transfer two 10 µL aliquots of each sample to PCR plate.

8-sample assay plate

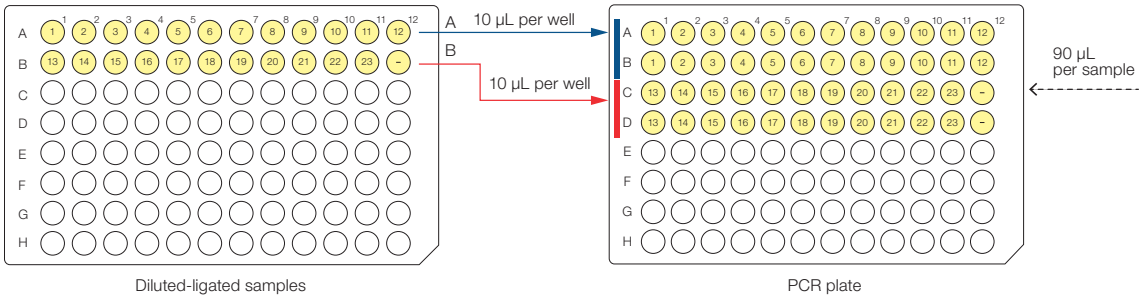


Samples	Volume/sample
Ligated sample	10 µL
PCR MM	90 µL
Total volume	100 µL

16-sample assay plate



24-sample assay plate



8. Vortex and spin down all reagents.

9. Keep 5M betaine and nuclease-free water (NFW) on ice. If 5M betaine is frozen, thaw and vortex until fully dissolved.

10. Leave the Applied Biosystems™ CytoScan™ *Taq* DNA Polymerase at –15 to –20°C until ready to use.

11. Prepare a PCR master mix (MM) in a 15 mL centrifuge tube. Assemble the master mix on ice. Be sure to vortex before adding the enzyme. Dispense within 20 minutes.

Reagent	Per sample	MM for 8 samples (with 15% overage)	MM for 16 samples (with 15% overage)	MM for 24 samples (with 15% overage)
Water, nuclease-free (chilled)	50.3 µL	965.8 µL	1,931.5 µL	2,897.3 µL
CytoScan *Taq* Buffer	10.0 µL	192.0 µL	384.0 µL	576.0 µL
5M betaine	20.0 µL	384.0 µL	768.0 µL	1,152.0 µL
dNTPs	3.5 µL	67.2 µL	134.4 µL	201.6 µL
PCR primer	4.2 µL	80.6 µL	161.3 µL	241.9 µL
CytoScan *Taq* DNA Polymerase	2.0 µL	38.4 µL	76.8 µL	115.2 µL
Total volume	**90 µL**	**1,728 µL**	**3,456 µL**	**5,184 µL**

12. Vortex the PCR master mix before adding the enzyme.

13. Vortex the PCR master mix at high speed 3 times, 1 second each.

14. Add the PCR master mix to the samples according to the table below.

Samples	Volume/sample
Ligated sample	10 µL
PCR MM	90 µL
Total volume	**100 µL**

15. Seal the PCR plate, vortex twice at high speed in 5-sector format, 1 second per sector. Spin down at 650 x *g* for 1 minute.

16. Keep plate on ice and bring to thermal cycler.

17. Ensure thermal cycler lid is preheated.

18. Load plate onto thermal cycler and run the following PCR program:

Temperature	Time	Cycles
95°C	1 minute	1
95°C	30 seconds	30
60°C	45 seconds	
68°C	60 seconds	
68°C	7 minutes	1
4°C	5 minutes	—
4°C	Hold (can be held overnight)	

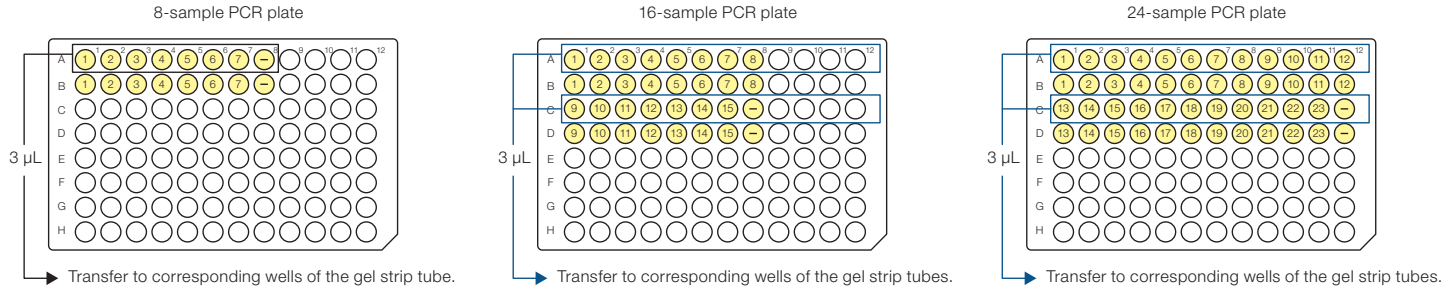
Note: Specify "Maximum mode" for 100 µL volume.

19. Proceed to "Stage 4: PCR product purification".

Optional stopping point: If not processing immediately, the plate may be stored at –20°C for up to 1 week.

Stage 3B: PCR product check

1. Transfer 3 µL of each PCR product from one row to corresponding wells of gel strip tube.
2. Add 17 µL of diluted loading buffer.
3. Seal gel strip tubes.



4. Vortex and spin down.
5. Load entire sample onto a 2% agarose gel.
6. Follow manufacturer's instructions to run gel that meets requirements (Figure 3.2).

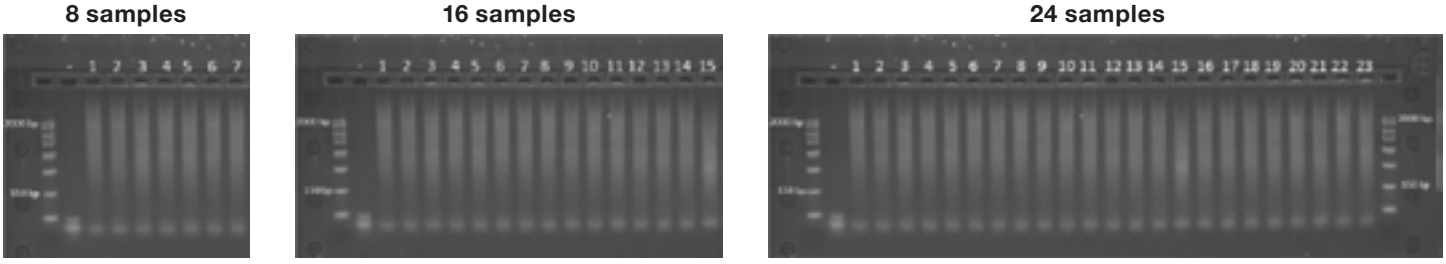
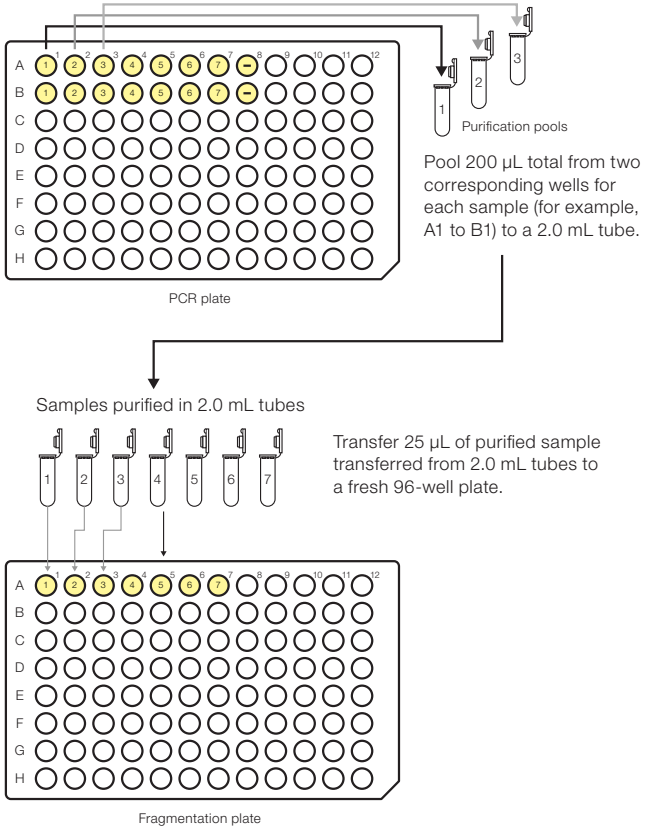


Figure 3.2. PCR products run on a 2% agarose gel. The majority of the PCR product is between 150 and 2,200 bp.

Stage 4: PCR product purification

1. Add 45 mL of absolute ethanol to Invitrogen™ PureLink™ PCR Purification Wash Buffer prior to use. Cap bottle tightly and mix by inverting Purification Wash Buffer bottle 10 times.
2. Pool both PCR products for each sample by transferring all PCR reactions to appropriately marked 2.0 mL tube.
3. Examine PCR plate to ensure that all volume from each well is transferred.
4. Thoroughly mix Purification Beads stock by inverting bottle up and down 10 times until mixture is homogeneous. Examine bottom of bottle to ensure that solution appears homogeneous.
5. Add 360 µL of Purification Beads to each pooled sample using a single-channel P1000 pipette (if >8 samples, use a multichannel pipette).
6. Securely cap each tube and mix well by inverting 10 times.
7. Incubate at room temperature for 10 minutes.
8. Centrifuge tubes (with hinges facing out) for 3 minutes at 16,000 x *g*.
9. Place tubes on magnetic stand so that cap hinge is over magnet.
10. Leaving tubes in stand, pipet off supernatant without disturbing bead pellet. Discard supernatant.
11. With P1000 pipette, add 800 µL Purification Wash Buffer to each tube.
12. Cap tubes, load into foam adapter, vortex at maximum setting for 2 minutes.
13. Centrifuge tubes (with hinges facing out) for 3 minutes at 16,000 x *g*.
14. Place tubes back on magnetic stand.
15. Leaving tubes in stand, pipet off supernatant without disturbing bead pellet. Discard supernatant.
16. Spin tubes (with hinges facing out) for 30 seconds at 16,000 x *g* with hinges facing out; place them back on magnetic stand.
17. With a P20 pipette, remove any drops of Purification Wash Buffer from bottom of each tube.
18. Allow any remaining Purification Wash Buffer to evaporate by taking tubes off the magnetic stand and leaving them uncapped at room temperature for 7 minutes.
19. With a P100 pipette, add 27 µL of Elution Buffer to each tube and dispense directly on beads.
20. Cap tubes, load into foam adapter, vortex at maximum speed for 10 minutes to resuspend beads.
21. If beads are not fully resuspended, flick tubes to dislodge pellet and vortex for an additional 2 minutes.
22. Centrifuge tubes (with hinges facing out) for 3 minutes at 16,000 x *g*.
23. Place tubes on magnetic stand for 10 minutes until all beads are pulled to side.
24. Transfer 25 µL of eluted sample to appropriate well of fresh 96-well plate.
25. Proceed to “Stage 5: Quantitation”.

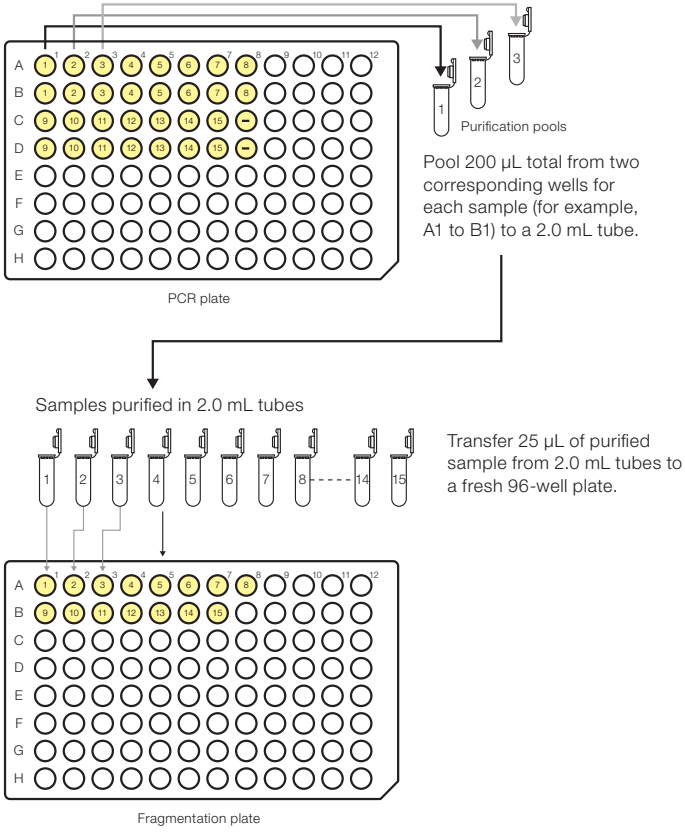
8 samples



Pool 200 µL total from two corresponding wells for each sample (for example, A1 to B1) to a 2.0 mL tube.

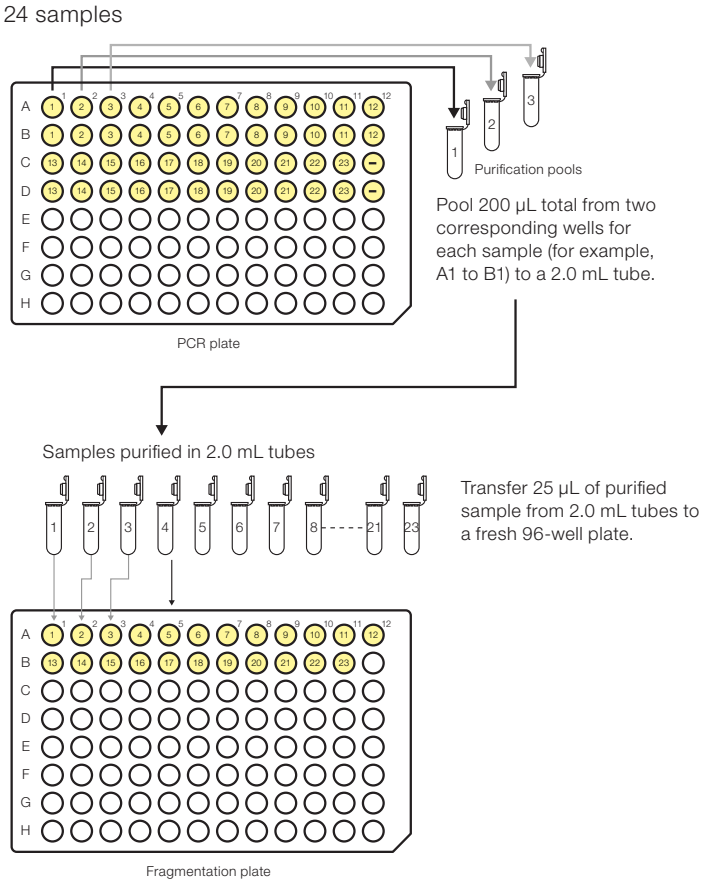
Transfer 25 µL of purified sample transferred from 2.0 mL tubes to a fresh 96-well plate.

16 samples



Pool 200 µL total from two corresponding wells for each sample (for example, A1 to B1) to a 2.0 mL tube.

Transfer 25 µL of purified sample from 2.0 mL tubes to a fresh 96-well plate.



Stage 5: Quantitation

Prepare the quantitation plate

1. Add 198 µL NFW into an ultraviolet (UV) plate.
2. Add 200 µL NFW into each well of empty row to use as blank.
3. Add 2 µL of each purified sample to corresponding well of UV plate.
4. Seal plate, vortex, and spin down.

Plate spectrophotometer

1. Use OD_{280} and OD_{320} as controls.
2. Measure the OD of each PCR product at 260, 280, and 320 nm.

3. Determine the OD_{260} measurement for the water blank and calculate average.

4. Calculate one OD_{260} reading for every sample:

   OD_{260} = (sample OD_{260}) – (average water blank OD_{260})

5. Calculate the undiluted concentration for each sample in µg/µL:

   OD_{260} x 0.05 µg/µL x 100

Assess OD readings

- Average purification yield for ≥7 samples must be ≥3.0 µg/µL; do not further process samples with yields <2.5 µg/µL.
- The OD_{260}/OD_{280} ratio should be between 1.7 and 2.1.
- The OD_{320} measurement must be very close to 0 (≤0.1).

Optional stopping point: The plate may be stored at –15 to –25°C for up to 1 week.

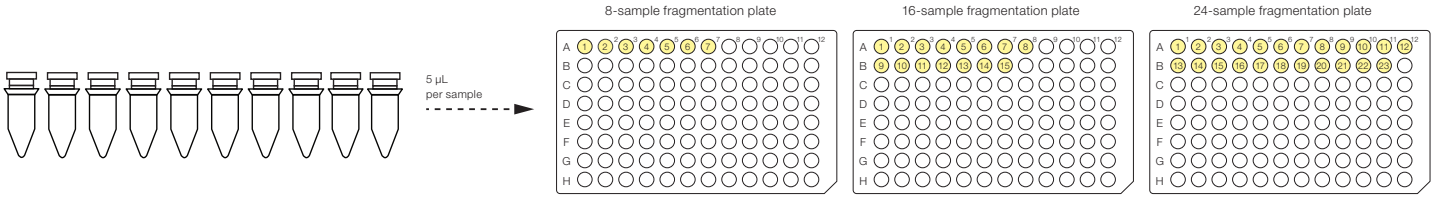
Stage 6A: Fragmentation

1. Chill the plate centrifuge to 4°C before proceeding.
2. Turn on the thermal cycler to preheat the lid.
3. Remove the plate of purified, quantitated samples from –15 to –20°C storage and thaw at room temperature (≤30 minutes). Seal tightly and spin down plate at 650 x *g* for 1 minute. Place the plate on lower half of cooling block on ice and chill for 10 minutes before use. Process within 1 hour.
4. Thaw the Fragmentation Buffer at room temperature (≤30 minutes). Vortex and spin down; place on ice. Use within 1 hour.
5. Leave the Fragmentation Reagent at –15 to –20°C until ready to use.
6. Keep all reagents, including water, on ice. Perform all additions on ice.
7. Prepare a fragmentation master mix (MM) according to the table below. Be sure to vortex before adding the enzyme. Dispense within 10 minutes.

Reagent	2.5 U/µL
Water, nuclease-free	215 µL
Fragmentation Buffer	275 µL
Fragmentation Reagent	10 µL
Total volume	**500 µL**

8. Vortex the fragmentation master mix before adding enzyme.
9. Vortex the fragmentation master mix at high speed 3 times, 1 second each time, pulse spin 3 seconds, place in cooling block.
10. Add the fragmentation master mix equally to strip tubes.

11. With a multichannel pipette, add 5 µL of fragmentation master mix to each sample:



Samples	Volume/sample
Purified PCR product	23 µL
Fragmentation MM	5 µL
Total volume	**28 µL**

12. Seal the sample plate with an adhesive film.
13. Vortex at high speed in 5-sector format, 1 second per sector.
14. Spin down at 650 x *g* for 1 minute in a prechilled centrifuge.
15. Ensure that the thermal cycler block is preheated.
16. Load the plate onto the thermal cycler and run the following program:

Temperature	Time
37°C	35 minutes
95°C	15 minutes
4°C	5 minutes
4°C	∞ (process plate within 2.5 hours)

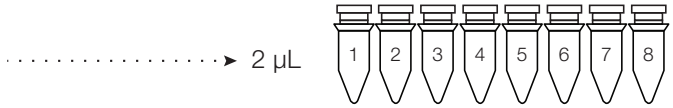
17. Proceed immediately to “Stage 6B: Fragmentation QC gel”.

Optional stopping point: If not proceeding to the next step, the fragmented DNA plate may be held at –15 to –20°C for up to 60 hours.

Stage 6B: Fragmentation QC gel

Prepare the loading buffer dilution

1. Add 14 µL NFW into strip tubes labeled “Dil”.
2. Transfer 2 µL of each fragmented sample to the corresponding Dil strip tube. Seal strip, vortex, and spin down.



Dil strip tube

3. Label the strip tubes “Gel analysis”.
4. Add 8 µL diluted fragmented product to 12 µL diluted loading buffer.
5. Load 20 µL samples onto 4% agarose gel.
6. Fill empty wells with 20 µL NFW.
7. If quality control results pass, proceed to “Stage 7: Labeling”.

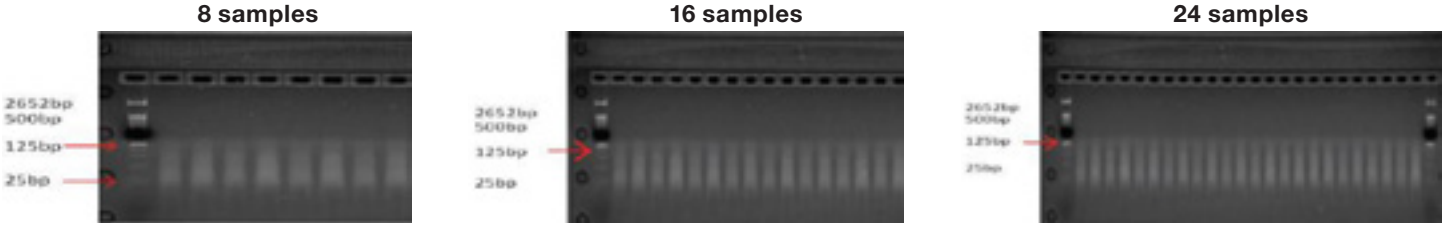


Figure 3.3. Fragmented samples run on a 4% agarose gel. Average fragment distribution must be between 25 and 125 bp.

Stage 7: Labeling

1. Thaw Thermo Scientific™ TdT buffer and DNA labeling reagent at room temperature (≤30 minutes); place on ice. Use within 1 hour.

2. Leave Thermo Scientific™ TdT enzyme at –15 to –20°C until ready to use.

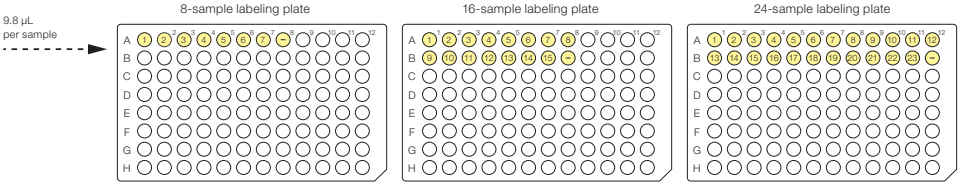
3. Prepare labeling master mix (MM). Be sure to vortex before adding the enzyme. Dispense within 10 minutes.

Reagent	Per sample	MM for 8 samples (with 20% overage)	MM for 16 samples (with 20% overage)	MM for 24 samples (with 20% overage)
TdT buffer	7.0 µL	67.2 µL	134.4 µL	201.6 µL
DNA labeling reagent	1.0 µL	9.6 µL	19.2 µL	28.8 µL
TdT enzyme	1.8 µL	16.8 µL	33.6 µL	50.4 µL
Total volume	**9.8 µL**	**93.6 µL**	**187.2 µL**	**280.8 µL**

4. Vortex labeling master mix before adding enzyme.

5. Vortex labeling master mix and spin down. Add 9.8 µL of labeling master mix to each sample.

Samples	Volume/sample
Fragmented DNA (less 2.0 µL for gel analysis)	26.0 µL
Labeling MM	9.8 µL
Total volume	**35.8 µL**



6. Tightly seal plate, vortex at high speed in 5-sector format for 1 second per sector.

7. Spin down at 650 x *g* for 1 minute.

8. Load plate onto thermal cycler and run the following program:

Temperature	Time
37°C	4 hours
95°C	15 minutes
4°C	5 minutes
4°C	∞

9. Proceed to “Stage 8: Hybridization”.

Optional stopping point: The plate may be held at –15 to –25°C for up to a week.

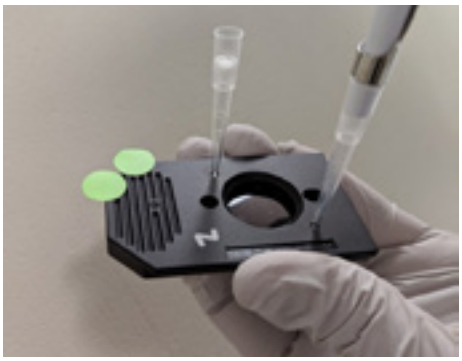
Stage 8: Hybridization

Create test request and register array

1. Unpack the arrays and allow to equilibrate to room temperature prior to use.
2. Preheat the hybridization oven for at least 1 hour at 50°C with the rotation turned on.
3. Create a batch registration file using Applied Biosystems™ GeneChip™ Command Console Software.
4. Prepare a hybridization master mix (MM) according to the table below in a 15 mL conical tube on ice.

Reagent	Per sample	MM for 8 samples (with 20% overage)	MM for 16 samples (with 20% overage)	MM for 24 samples (with 20% overage)
Hyb Buffer Part 1	82.5 µL	792.0 µL	1,584.0 µL	2,376.0 µL
Hyb Buffer Part 2	7.5 µL	72.0 µL	144.0 µL	216.0 µL
Hyb Buffer Part 3	3.5 µL	33.6 µL	67.2 µL	100.8 µL
Hyb Buffer Part 4	0.5 µL	4.8 µL	9.6 µL	14.4 µL
Oligo Control Reagent	1.0 µL	9.6 µL	19.2 µL	28.8 µL
Total volume	**95 µL**	**912 µL**	**1,824 µL**	**2,736 µL**

5. Mix well by vortexing the master mix at high speed 3 times for 3 seconds each, then pour it into a reservoir on the cooling block.



6. Add 95 µL of the hybridization master mix to each sample.
7. Tightly seal the plate, vortex twice at high speed in 5-sector format and spin down at 2,000 rpm for 1 minute.

Optional stopping point: The plate can be held at –15 to –25°C for up to a week.

8. Load the plate onto the thermal cycler and run the following program:

Temperature	Time
95°C	10 minutes
49°C	3 minutes
49°C	∞

9. Allow the samples to incubate at 49°C for at least 3 minutes before loading.

10. Leaving the samples on the thermal cycler, load 100 µL of sample onto each array using a single-channel P200 pipette. Only hybridize up to 6 arrays at a time.

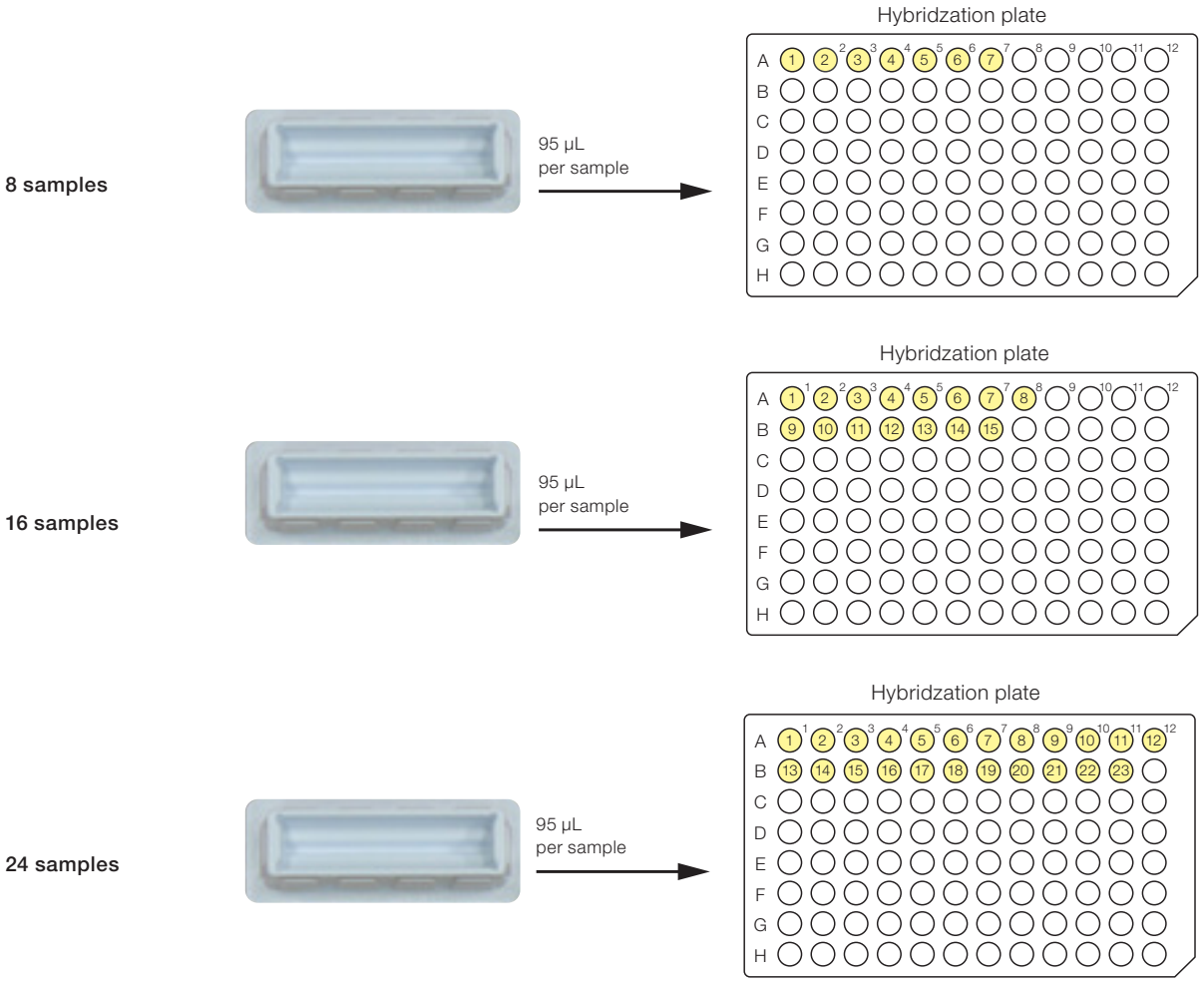
    Note: If bubbles adhere to the array surface, tap the array lightly on the edge of a countertop, then gently shake the array a few times to ensure bubbles are not visible through the window.

11. Clean any excess fluid from around the septa.

12. Apply Tough-Spots™ to the septa and press firmly.

13. Immediately load the arrays into the hybridization oven, 4–6 at a time.

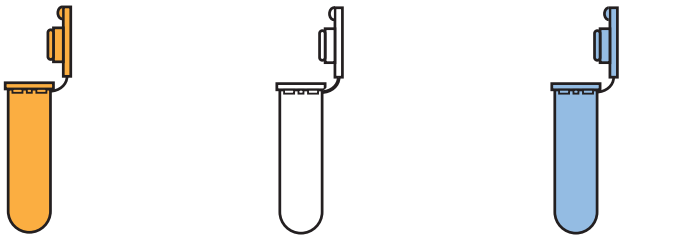
14. Hybridize the arrays for 16–18 hours at 50°C at 60 rpm.



Stage 9: Wash, stain, and scan

Aliquot the following reagents into separate 1.5 mL microfuge tubes for each array:

1. Add 500 µL Stain Buffer 1 solution into the amber tubes.
2. Add 500 µL Stain Buffer 2 into the clear tubes.
3. Add 800 µL Array Holding Buffer into the blue tubes.



500 µL Stain Buffer 1 | 500 µL Stain Buffer 2 | 800 µL Array Holding Buffer

Washing and staining arrays

1. Prime fluidics station with Wash A and Wash B. Load stain solutions and select correct fluidics protocol.
2. Start fluidics protocol and leave cartridge lever down in "eject" position.
3. Remove adhesive label dots from each array.
4. Load arrays onto fluidics station.
5. Refer to the Applied Biosystems™ KaryoStat™ Assay user guide (Pub. No. 905403) for detailed instructions on priming the fluidics stations and washing and staining the CytoScan Optima Arrays in the GeneChip Command Console.

Before scanning:

1. Ensure no bubbles are visible through window. Clean array surface.
2. Cover septa with thin, ¾-inch adhesive label dots; load onto scanner. Thicker dots can get caught in autoloader.
3. Scan arrays as described in the KaryoStat Assay User Guide (Pub. No. 905403).

Important information:

- Stain Buffer 1 and Array Holding Buffer are light-sensitive.
- If there is a delay after adding reagents to tubes, store tubes at 4°C, protected from light.
- Remove bubbles from arrays on the fluidics station, or remove bubbles manually (see Applied Biosystems™ GeneChip™ Fluidics Station 450 User Guide for AGCC, Pub. No. 08-0295).

3.2 Analyzing differentiation potential of PSCs using the TaqMan hPSC Scorecard Panel

The fast 96-well and 384-well TaqMan hPSC Scorecard Panels are standard or fast Applied Biosystems™ MicroAmp™ Optical 96- and 384-well reaction plates that contain dried-down Applied Biosystems™ TaqMan® Gene Expression Assays specifically formulated for evaluating human ESCs and PSCs to confirm their pluripotency and to predict their differentiation potential and outcome. The gene expression assays contain a collection of predesigned, gene-specific primer and probe sets for performing quantitative gene expression studies on the cDNA samples prepared from undifferentiated or differentiated human embryonic stem cells (ESCs) and iPSCs. This section provides a protocol for sample preparation and usage of the most common panel format: standard 384 wells. For details on using other formats, refer to the product manual (Pub. No. MAN0008384).

Kit contents and storage

Types of kits

The manual (Pub. No. MAN0008384) has the information on the products listed below.

Ordering information

Product	Cat. No.
TaqMan hPSC Scorecard Panel, 384-well	A15870
TaqMan hPSC Scorecard Kit, 384-well	A15872
TaqMan hPSC Scorecard Panel, Fast 96-well	A15876
TaqMan hPSC Scorecard Kit, Fast 96-well	A15871

384-well TaqMan hPSC Scorecard Panel

The 384-well TaqMan hPSC Scorecard Panel (Cat. No. A15870) and TaqMan hPSC Scorecard Kit (Cat. No. A15872) contain the components listed below. Each 384-well plate can be used to analyze 4 cDNA samples.

Component	Composition	Amount	Cat. No.	
			A15870	A15872
TaqMan hPSC Scorecard Panel, 384-well	TaqMan probes in a 384-well plate	1 plate	•	•
MicroAmp Optical Adhesive Film	Optical adhesive covers	1 each	•	•
TaqMan Gene Expression Master Mix*	Solution containing AmpliTaq Gold DNA Polymerase UP (Ultra Pure), uracil-DNA glycosylase, dNTPs with dUTP, Passive Reference 1, and optimized mix components	5 mL		•
TaqMan hPSC Scorecard Panel QRC	TaqMan hPSC Scorecard Panel Quick Reference Card	1 each	•	•

* Applied Biosystems™ TaqMan® Gene Expression Master Mix (Cat. No. 4369016) is also available separately.

Fast 96-well TaqMan hPSC Scorecard Panel

The Fast 96-well TaqMan hPSC Scorecard Panel (Cat. No. A15876) and TaqMan hPSC Scorecard Kit (Cat. No. A15871) contain the components listed below. Each 96-well plate can be used to analyze 1 cDNA sample.

Component	Composition	Amount	Cat. No.	
			A15876	A15871
TaqMan hPSC Scorecard Panel, Fast 96-well	TaqMan probes in a 96-well plate	2 plates	•	•
MicroAmp Optical Adhesive Film	Optical adhesive covers	2 each	•	•
TaqMan Fast Advanced Master Mix*	Solution containing AmpliTaq Fast DNA Polymerase, uracil-N glycosylase (UNG), dNTPs with dUTP, ROX dye (passive reference), and optimized buffer components	2 x 1 mL		•
TaqMan hPSC Scorecard Panel QRC	TaqMan hPSC Scorecard Panel Quick Reference Card	1 each	•	•

* Applied Biosystems™ TaqMan® Fast Advanced Master Mix (Cat. No. 4444556) is also available separately.

Shipping and storage

Component	Storage and handling
TaqMan hPSC Scorecard Panel, 96- and 384-well	Store at 4°C to 30°C. Maintain in foil bag until ready to for use. Briefly spin plates at 400 x *g* for 2 minutes prior to use.
MicroAmp Optical Adhesive Film	Store at 4°C to 30°C. **Protect from dust.**
TaqMan Gene Expression Master Mix	Store at 2°C to 8°C. **Do not freeze.**
TaqMan Fast Advanced Master Mix	Store at 2°C to 8°C. **Do not freeze.**

How a TaqMan hPSC Scorecard Panel works

After isolating total RNA from human ESC or iPSC cultures and using it to generate cDNA in a reverse transcription (RT) reaction, TaqMan hPSC Scorecard Panels and associated reagents are used to quantitate RNA expression levels of genetic markers for pluripotency and differentiation potential, as well as endogenous controls. The gene expression data are then analyzed using the web-based Applied Biosystems™ TaqMan® hPSC Scorecard Analysis Software for the pluripotency and differentiation potential of the cells from which the total RNA is isolated.

To do this:

- Isolate total RNA from human ESCs or iPSCs by organic phase extraction or other preferred method.
- Prepare each cDNA sample by performing 8 RT reactions per total RNA sample.
- Combine the appropriate TaqMan master mix with your cDNA sample and RNase-free water, and reconstitute each well of the TaqMan hPSC Scorecard Panel by adding 10 µL of the reaction mixture per well.
- Load and run the plates on a compatible real-time PCR instrument using either standard or fast thermal cycling conditions.
- Analyze the gene expression data using the web-based TaqMan hPSC Scorecard Analysis Software to confirm the pluripotency of the samples and predict their differentiation potential and outcome; the hPSC Scorecard Analysis Software is available at **thermofisher.com/scorecard**.

Types of TaqMan hPSC Scorecard Panels

TaqMan hPSC Scorecard Panels are available as 384-well plates (Cat. No. A15870, A15872) or as 96-well plates (Cat. No. A15876, A15871) for use under fast thermal cycling conditions.

- The 384-well TaqMan hPSC Scorecard Panel contains 384-well MicroAmp optical assay plates, which allow the analysis of 4 separate cDNA samples under standard thermal cycling conditions.
- The 96-well TaqMan hPSC Scorecard Panel contains 96-well MicroAmp optical thermal cycling plates, which reduce quantitative PCR (qPCR) run times to less than 40 minutes when used under fast thermal cycling conditions in a compatible RT-PCR system; each 96-well plate allows the analysis of one cDNA sample.

Compatible TaqMan master mixes

Each well in a TaqMan hPSC Scorecard Panel contains a pair of unlabeled PCR primers specific to a pluripotency or differentiation marker or endogenous control, and a TaqMan probe with a fluorescent dye-label on the 5′ end (e.g., FAM or VIC™ dye) and a minor groove binder (MGB) and nonfluorescent quencher (NFQ) on the 3′ end.

The assays in each well are reconstituted to a 1X formulation using the compatible TaqMan master mix as described here, and are designed to run under standard or fast cycling conditions for two-step RT-PCR.

The table below lists the TaqMan hPSC Scorecard Panel and corresponding TaqMan master mix compatible with it. Note that TaqMan hPSC Scorecard kits contain the appropriate compatible TaqMan master mix, which are not supplied with the individually packaged TaqMan hPSC Scorecard Panels and need to be purchased separately.

TaqMan hPSC Scorecard Panel	Compatible TaqMan master mix
TaqMan hPSC Scorecard Panel, 384-well	TaqMan Gene Expression Master Mix (for standard cycling)
TaqMan hPSC Scorecard Panel, Fast 96-well	TaqMan Fast Advanced Master Mix (for fast cycling)

Compatible qPCR instruments

TaqMan hPSC Scorecard Panels can be used with the Applied Biosystems™ qPCR systems listed below. Note that fast 96-well TaqMan hPSC Scorecard Panels must be run on qPCR systems that contain fast thermal cycling blocks. Alternately, the 384-well TaqMan hPSC Scorecard Panel must be run on qPCR systems with standard thermal cycling blocks.

TaqMan hPSC Scorecard Panel	Compatible Applied Biosystems qPCR systems
TaqMan hPSC Scorecard Panel, 384-well	• QuantStudio 12K Flex System with 384-well block • ViiA 7 Real-Time PCR System with 384-well block
TaqMan hPSC Scorecard Panel, Fast 96-well	• StepOnePlus Real-Time PCR System • ViiA 7 Real-Time PCR System with Fast 96-well block

3.2A Sample generation

The most common scheme for characterizing a PSC line using a TaqMan hPSC Scorecard Panel involves analyzing undifferentiated PSCs as well as samples differentiated from the same line, typically embryoid bodies (EBs) that have been allowed to differentiate spontaneously. For each sample, you will need to harvest at least 5 x 10^5 cells to isolate sufficient total RNA for the subsequent steps of the workflow. Here we describe how to generate the undifferentiated and differentiated samples.

For instructions on how to culture undifferentiated ESCs or iPSCs under feeder-free or feeder-dependent conditions, see section 2.1. Feeder-dependent ESCs or iPSCs should be cultured feeder-free on Gibco™ Geltrex™ matrix–coated culture vessels for one passage in mouse embryonic fibroblasts (MEFs)–conditioned medium before the cells are harvested and total RNA is isolated. For more detailed information on culturing PSCs in MEF-conditioned medium, refer to the TaqMan hPSC Scorecard Panel manual (Pub. No. MAN0008384).

There are several methods for creating EBs for random differentiation. One protocol is available in section 5.1, and it includes instructions for suspension EB culture, which is recommended for a maximum of 7 days. If extended EB culture is desired, we recommend seeding the EBs on a Geltrex matrix–coated culture vessel.

At the point where cultures are ready for harvest:

1. Aspirate off the medium and wash the cells once with 5 mL of DPBS for 2–3 minutes.
2. Aspirate off the DPBS wash.
3. Add 1 mL of Invitrogen™ TRIzol™ Reagent and incubate for 2–3 minutes.
4. Scrape the plate with a sterile cell scraper and collect the slurry into an RNase-free microcentrifuge tube.
5. Store at –80°C until ready for RNA isolation.

3.2B Sample preparation

Isolation of total RNA using TRIzol reagent

This section provides instructions on extracting total RNA from the ESCs, iPSCs, or EBs by TRIzol reagent-based organic phase extraction to use as a template for synthesis of single-stranded cDNA. You will need at least 5 x 10^5 cells harvested per sample to isolate sufficient total RNA for the reverse transcription reaction.

Note: For optimal performance, we recommend isolating total RNA by organic phase extraction using the TRIzol reagent. Note that column-based purification methods may also yield high-quality RNA.

Materials needed

- TRIzol reagent (Cat. No. 15596-026)
- Invitrogen™ UltraPure™ DNase/RNase-Free Distilled Water (Cat. No. 10977)
- Chloroform (Sigma, Cat. No. C-2432)
- Isopropanol (Sigma, Cat. No. I9516)
- Ethanol (Sigma, Cat. No. E7023)

Caution: TRIzol reagent contains phenol (toxic and corrosive) and guanidine isothiocyanate (an irritant), and may be a health hazard if not handled properly. Always work with TRIzol reagent in a fume hood, and always wear a lab coat, gloves and safety glasses. For more information, refer to the TRIzol reagent Safety Data Sheet (SDS), available from our website at **thermofisher.com/trizolsafety**.

Isolation of total RNA by TRIzol reagent–based organic phase extraction

1. Incubate the lysate from the last step of the harvesting procedure with TRIzol reagent at room temperature for 5 minutes to allow complete dissociation of nucleoproteins.
2. To the cells in TRIzol reagent, add 0.2 mL of chloroform per 1 mL of TRIzol reagent, and shake the tube vigorously by hand for 15 seconds.
3. Incubate the sample at room temperature for 2–3 minutes and centrifuge at 12,000 x *g* for 15 minutes at 4°C.

   Note: The mixture separates into a lower red phenol–chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The upper aqueous phase is ~50% of the total volume.
4. Carefully remove the upper aqueous phase and transfer to a new tube. Avoid drawing any of the interphase or organic layer into the pipette when removing the aqueous phase.
5. Add 0.5 mL of 100% isopropanol to the aqueous phase per 1 mL of TRIzol reagent, and incubate at room temperature for 10 minutes.
6. Centrifuge at 12,000 x *g* for 10 minutes at 4°C.
7. Carefully remove the supernatant, and wash the RNA pellet with 1 mL of 75% ethanol.

   Note: The RNA is often invisible prior to centrifugation and forms a gel-like pellet on the side and at the bottom of the tube upon centrifugation.
8. Centrifuge the tube at 7,500 x *g* for 5 minutes at 4°C.
9. Discard the supernatant and air-dry the RNA pellet for 5–10 minutes.
10. Resuspend the RNA pellet in 20–50 µL of RNase-free water.

DNase treatment

One key variable to the success of any RT-PCR experiment is the quality of the template RNA. DNA removal is critical for ensuring high-quality RNA, because DNA can serve as a template during the PCR portion of the experiment, resulting in false positives, background, etc. Ideally, the total RNA sample should have less than 0.005% of genomic DNA by weight. We recommend treating the isolated total RNA with the Invitrogen™ DNA-*free*™ DNA Removal Kit, which digests the contaminating DNA to levels below the limit of detection by routine PCR.

Materials needed

- DNA-*free* DNA Removal Kit (Cat. No. AM1906; contains rDNase I, 10X DNase I Buffer, DNase Inactivation Reagent, and nuclease-free water)

Guidelines for using the DNA-*free* kit

- We recommend conducting the reactions in 0.5 mL tubes to facilitate removal of the supernatant after treatment with the DNase Inactivation Reagent.
- DNA-*free* kit reactions can be conducted in 96-well plates; we recommend using V-bottom plates because their shape makes it easier to remove the RNA from the pelleted DNase Inactivation Reagent at the end of the procedure.
- The recommended reaction size is 10–100 µL and a typical reaction size is 50 µL.
- Routine DNase treatment removes 2 µg of genomic DNA from a 50 µL reaction with ≤200 µg/mL nucleic acid; refer to the product insert if more rigorous DNase treatment is needed.

DNA-*free* kit procedure

1. For a 50 µL reaction, combine the following reagents in a clean, DNase- and RNase-free 0.5 mL microcentrifuge tube, and mix gently.

Component	Amount
RNA sample	1–10 µg
10X DNase I Reaction Buffer	5 µL
rDNase I (2 units)	1 µL
DEPC-treated water to bring reaction to 50 µL	
Total	50 µL

2. Incubate the tube at 37°C for 20–30 minutes.
3. Resuspend the DNase Inactivation Reagent by flicking or vortexing the tube, add 5 µL (0.1 volume) of the resuspended inactivation reagent to the reaction mix, and mix well.
4. Incubate for 2 minutes at room temperature, mixing the reaction occasionally.
5. Centrifuge at 10,000 x *g* for 1.5 minutes and transfer the RNA to a fresh tube.
6. RNA is now ready for reverse transcription.

RNA quantification and quality

Introduction

We recommend using total RNA that is:

- Between 0.002 and 0.2 µg/µL
- Less than 0.005% genomic DNA by weight
- Dissolved in a PCR-compatible buffer
- Free of RNase activity
- Free of inhibitors of reverse transcription and PCR
- Not denatured

Important: Denaturation of the RNA is not necessary and may reduce the yield of cDNA for some gene targets.

Assess total RNA amount and quality

Use a Thermo Scientific™ NanoDrop™ instrument to quantify the extracted RNA sample. RNA quality is best assessed using A_{260}/A_{280}, with the recommended value close to 2.0.

RNA integrity can be further assessed by running the samples on a 1% agarose gel and assessing the 2:1 ratio of the 28S and 18S RNA bands and the absence of degraded RNA that appears as small molecular weight smear.

If using the Agilent™ Bioanalyzer, an RNA integrity number (RIN) value of higher than 5 may be sufficient, but higher than 8 is ideal for downstream applications.

cDNA preparation

Reverse transcription of total RNA

This section provides instructions on generating single-stranded cDNA from the total RNA by reverse transcription using the Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor.

Materials needed

- High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Cat. No. 4374966)

Perform RT reaction

1. Allow the components of the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor to thaw on ice.
2. Prepare a 2X RT master mix by mixing the following components:

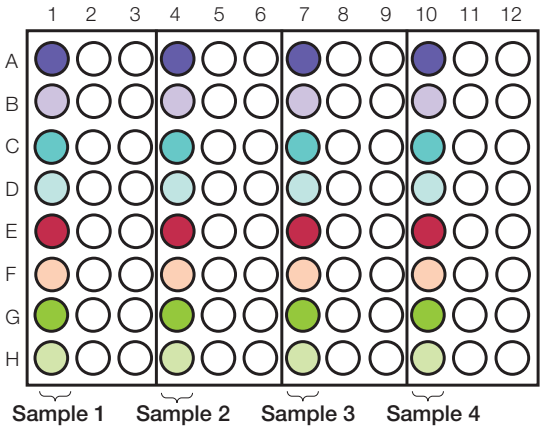
Component	Per well	1 sample (8 wells)	4 samples (8 wells/sample)
10X TaqMan RT Buffer	5 µL	50 µL	190 µL
25X dNTP mix	2 µL	20 µL	76 µL
10X random primers	5 µL	50 µL	190 µL
MultiScribe Reverse Transcriptase (50 U/µL)	2.5 µL	25 µL	95 µL
RNase Inhibitor (20 U/µL)	2.5 µL	25 µL	95 µL
RNase-free water	8 µL	80 µL	304 µL
Total	**25 µL**	**250 µL**	**950 µL**

3. Place the 2X RT master mix on ice and mix gently.

4. Prepare RNA samples by diluting 1 µg total RNA in a total of 225 µL of RNase-free water.

5. Add 225 µL of 2X RT master mix to the diluted RNA and mix well.

6. Aliquot 50 µL of the above RNA plus RT master mix in 8 vertical wells of a 96-well plate or an 8-strip PCR tube (see image below).



7. Run the RT reaction in a thermal cycler using conditions as listed below.

Step	Temperature	Time
1	25°C	10 minutes
2	37°C	120 minutes
3	85°C	5 minutes
4	4°C	∞

8. Proceed to section 3.2C. If you do not proceed immediately, store all cDNA samples at –15°C to –25°C. To minimize freeze-thaw cycles, store the cDNA in smaller aliquots.

3.2C RT-qPCR using the TaqMan hPSC Scorecard Panel

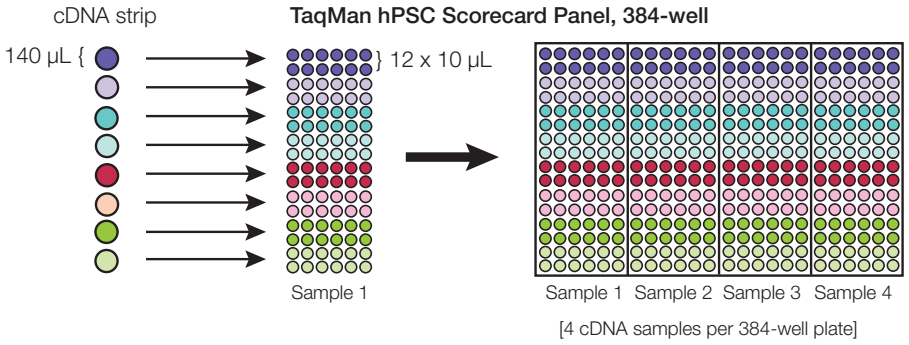
This section provides instructions for analyzing your cDNA samples by RT-qPCR using the TaqMan hPSC Scorecard Panel.

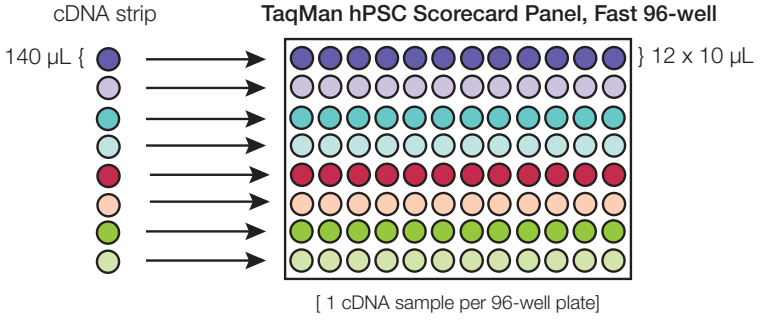
Materials needed

- TaqMan hPSC Scorecard Panel, Fast 96-well or 384-well
- TaqMan Fast Advanced Master Mix (96-well format for running in fast mode using the Fast 96-well TaqMan hPSC Scorecard Panel)
- TaqMan Gene Expression Master Mix (384-well format using the 384-well TaqMan hPSC Scorecard Panel)
- Applied Biosystems™ MicroAmp™ Optical Adhesive Film

Run RT-qPCR

1. Dilute each well containing 50 µL cDNA with 20 µL of PCR water for a final volume of 70 µL.
2. Add 70 µL of 2X TaqMan Gene Expression Master Mix (if using the 384-well TaqMan hPSC Scorecard Panel) or 70 µL 2X TaqMan Fast Advanced Master Mix (if using the Fast 96-well TaqMan hPSC Scorecard Panel).
3. Load 10 µL per well using a multichannel pipette onto the 384-well or the 96-well plate using fresh tips each time as shown. For 96-well plates, one well is sufficient to load one row of the plate.
4. Seal the plate with the MicroAmp Optical Adhesive Film, and centrifuge at 600 x *g* for 2 minutes.





5. Place the plate in a compatible RT-PCR instrument equipped with the appropriate thermal block.

6. Open the experiment template file and save a separate copy with your experimental details. Run the experiment using standard method for 384-well plates with the TaqMan Gene Expression Master Mix and fast mode for 96-well plates with the TaqMan Fast Advanced Master Mix, using the cycling parameters listed below.

   Note: The experiment template files (.edt) are available at **thermofisher.com/scorecardinstrument**.

   Refer to the appropriate instrument user guide for information on how to set up the plate document/ experiment or create a template from the setup file.

384-well panel run mode (ramp rate): standard

Step	Temperature	Time	Cycles
Hold	50°C	2 minutes	—
Hold	95°C	10 minutes	—
Hold	95°C	15 seconds	40
Anneal/extend	60°C	1 minute	40

Fast 96-well panel run mode (ramp rate): fast

Step	Temperature	Time	Cycles
Hold	50°C	20 seconds	—
Melt	95°C	1 second	40
Anneal/extend	60°C	20 seconds	40

Important: Be sure to run your RT-qPCR experiment using the standard curve method. Do not use $\Delta\Delta C_t$ comparative PCR.

Analyze the results

Analyze the gene expression data from the TaqMan hPSC Scorecard Panels using the web-based TaqMan hPSC Scorecard Analysis Software, available at **thermofisher.com/scorecard**.

The TaqMan hPSC Scorecard Analysis Software summarizes all key experimental results, including pluripotency and differentiation potential, on a single dashboard. It also allows you to tag and filter experiments, view expression, correlation, and box plots, and export experimental results and data as a PDF or as a spreadsheet.

3.3 Global gene expression analysis of PSCs using the PrimeView Human Gene Expression Array and PluriTest Online Analysis Tool

Gene expression analysis is a common method for verifying PSC identity and quality, whether it is through immunocytochemistry, RT-PCR panels, microarrays, or other methods. While analysis of a small, select set of PSC and differentiation markers can indicate pluripotency, analysis of more markers and a comparison to a large number of controls or references can provide much more certainty. The PrimeView Human Gene Expression Array is a single array comprising more than 530,000 probes covering more than 20,000 genes. Data from this array can be analyzed with the PluriTest™ Online Analysis Tool, which is a bioinformatics tool that compares the transcriptional profile of a sample to an extensive reference set of >450 cell and tissue types, including PSCs and somatic cells. Together, these tools provide a powerful method for ascertaining the pluripotency of PSCs for research. This section provides a brief overview of the global gene expression analysis with the PrimeView Human Gene Expression Array, followed by guidance on the assessment with the PluriTest Online Analysis Tool. The detailed protocol on target preparation for Applied Biosystems™ GeneChip™ 3′ Expression Arrays can be found in the user manual for Applied Biosystems™ GeneChip™ 3′ IVT PLUS Reagent Kit, while the detailed protocol for using the array is in the product sheet for PrimeView Human Gene Expression Array.

3.3A GeneChip 3′ IVT PLUS Reagent Kit

The GeneChip 3′ IVT PLUS Reagent Kit enables you to prepare RNA samples for gene expression profiling analysis with Applied Biosystems™ GeneChip™ 3′ Expression Arrays. The kit generates amplified and biotinylated complementary RNA (cRNA) from poly(A) RNA in a total RNA sample. cRNA is also known as amplified RNA or aRNA. The kit does not need an up-front removal of ribosomal RNA (rRNA) and is optimized for use with GeneChip 3′ Expression Arrays.

The GeneChip 3′ IVT PLUS Reagent Kit uses a reverse transcription priming method that primes the poly(A) tail junction of RNA to provide gene expression profiles from mRNA. RNA amplification is based upon linear amplification and employs T7 *in vitro* transcription (IVT) technology. The kit comprises reagents and a protocol for preparing hybridization-ready targets from 50 to 500 ng of total RNA (Figure 3.4). The GeneChip 3′ IVT PLUS Reagent is optimized to work with total RNA from a wide range of samples, including tissues, cells, and cell lines.

This section provides a brief overview of the global gene expression analysis with the PrimeView Human Gene Expression Array. See our product website for a list of supporting manuals and for procedures regarding target preparation, target hybridization, washing, staining, and array scanning.

Assay workflow

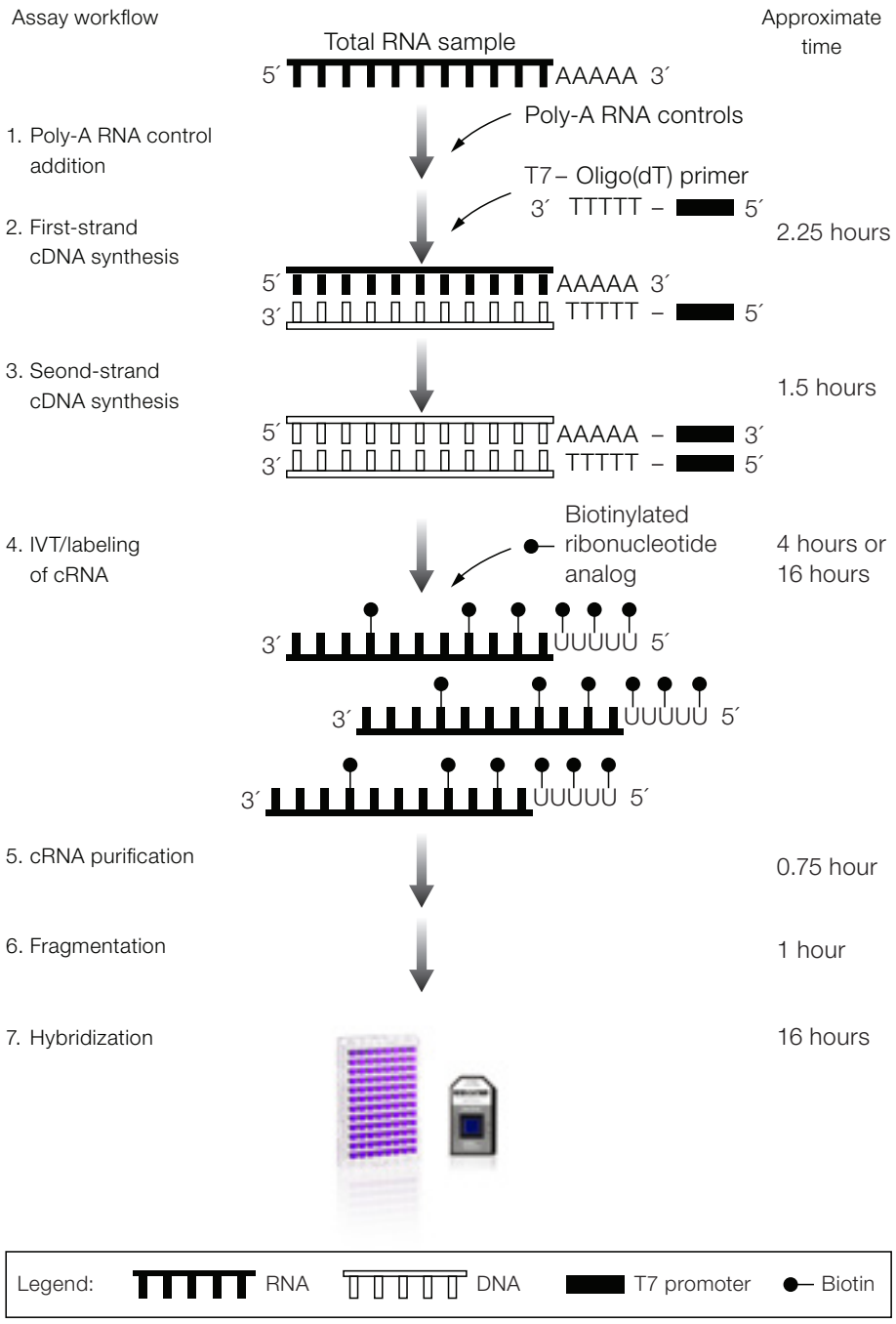


Figure 3.4. Amplification and labeling process for the GeneChip 3′ IVT PLUS kit.

3.3B Assessment with the PluriTest Online Analysis Tool

The PluriTest Online Analysis Tool is a bioinformatics tool for pluripotency assessment that provides an alternative to the teratoma assay based on global gene expression data. The PluriTest™ Assay compares the transcriptional profile of a sample to an extensive reference set of >450 cell and tissue types, including 223 hESC lines, 41 iPSC lines, somatic cells, and tissues. This free online analysis tool confirms pluripotency marker expression using two separate scores: pluripotency and novelty. The pluripotency score indicates how strongly a model-based pluripotency signature is expressed in the samples analyzed. The novelty score indicates the general model fit for a given sample.

The PluriTest Online Analysis Tool supports analysis of a number of arrays, including these gene expression arrays:

- Applied Biosystems™ GeneChip™ PrimeView™ Global Gene Expression Profile Assay (Cat. No. 905400)
- Applied Biosystems™ PrimeView™ 16 Global Gene Expression Profile Assay (Cat. No. 905402)

The source .cel and .idat files are analyzed by the PluriTest Online Analysis Tool in real time, providing instant pluripotency analysis results.

For more information about the PluriTest Online Analysis Tool, consult *Nat Methods* 8, 315-7 (2011).

Before you begin

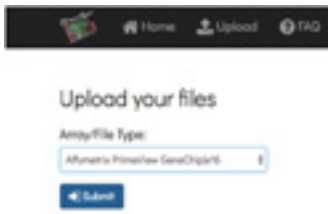
Observe these important rules and guidelines before you begin using the PluriTest Online Analysis Tool.

- You must first register to set up an account with a user name and password.
- You must use a compatible browser, specifically, the Mozilla™ Firefox™ or Google™ Chrome™ browsers.
- You must upload a minimum of 3 files at a time.
- You cannot upload more than 24 files at a time.
- An upload must contain files all of the same file type, either all .cel files or all .idat files.
- When uploading .cel files, do not upload chip-type and plate-type files in the same upload. Applied Biosystems gene expression arrays can be either by chip (GeneChip PrimeView Global Gene Expression Profile Assay) or by plate (PrimeView 16 Global Gene Expression Profile Assay).

Uploading assay files to the PluriTest Online Analysis Tool

Use PluriTest Online Analysis Tool to upload and analyze your assay files. You can upload and analyze a minimum of 3 files, and up to 24 files at one time. An upload must contain files of the same file type, either .cel or .idat, but not both types in the same upload.

1. Go to **http://pluritest.org** to open the PluriTest Online Analysis Tool web application.
2. If you are a new user, click “Register” to set up an account with a user name and password.
3. Otherwise, click “Log in” and enter your user name and password.
4. When logged in, on the welcome screen, click “Get started”.
5. On the menu bar, click “Upload”.



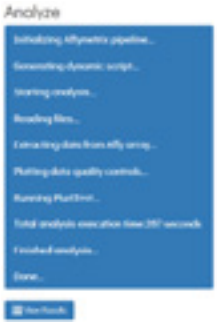
6. Select the array or file type to upload.

   Note: You cannot upload files from PrimeView GeneChip and Applied Biosystems™ PrimeView™ GeneTitan™ arrays together. They must be run in separate batches.

7. Click “Submit”.
8. In the Upload files window, click “Add files” to open a navigation window to locate the files to upload.
9. Navigate to the file folder where the files reside, select the files to upload, and then click “Open”. The files are added to the Upload files window.
10. Confirm the file list, and then click “Start upload”. You can also upload individual files by clicking “Start” by the file name.

    If you inadvertently upload files from the PrimeView GeneChip and PrimeView GeneTitan arrays together, the following message will open. Click “Cancel”, “Back”, or “Upload” to try again.

11. When the upload is complete, click “Continue”, and then in the “pending analysis” window, click “Analyze” to analyze the uploaded file set. The files are analyzed one at a time and go through multiple analysis steps, as illustrated below. The results are merged into one analysis.



    Note: Depending on the number of files being processed, internet speed, and computer speed, the analysis may take several minutes. Do not close the browser during analysis or processing will stop.

12. When the analysis is complete, click “View results” to see a summary of the analysis, including file names, file type, and date of analysis. From there, you can access detailed information.

Getting analysis results

1. When an analysis is complete, click “Results” in the menu bar.
2. In the Analysis summary window, click “Details” to access the details of the analysis.
3. In the Analysis details window, click the buttons to open windows to view specific details about the analysis (see “Analysis details window” below).

   You can print or download the analysis information from these windows.
4. After viewing the analysis details, click “Back to completed analyses” to return to the Analyses summary window.
5. **Optional:** In the Analyses summary window, click “Delete” to remove the results of the analysis, or to log off.

Analysis details window

Use the analysis details window to review the results of the analysis. This window shows the individual file names and types that were analyzed, the analysis results, and the pluripotency and novelty metrics for each file.

- “Pass” shows clear pluripotency signature
- “Fail” means the samples are not pluripotent
- “Further analyze” means the results were borderline, which can happen if the quality of the cultures is not great; test the samples again

Although the files are analyzed one at a time, the results are merged into a combined analysis for the run.

Use the buttons to view more details about the combined analysis. In most cases, the pluripotency plot will be the most useful. A representational pluripotency plot is shown in Pluripotency plot window.

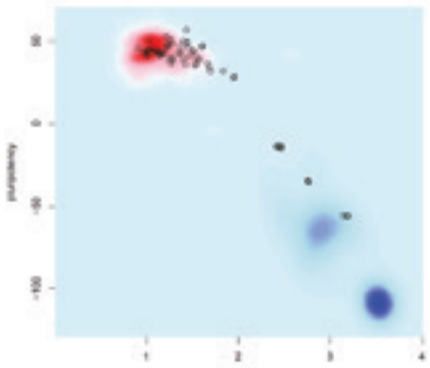
Button	
Relative Log Expression	• Opens a window where you can view the relative log expression (RLE) values of the analysis. • These values are computed by calculating for each probe set the ratio between the expression of a probe set and the median expression of this probe set across all arrays analyzed. • Most probe sets are not changed across the arrays, therefore these ratios are around 0 on a log scale. The box plots presenting the distribution of these log ratios should be centered near 0 and have similar spread. Other results would indicate low quality.
Normalized Unscaled Standard Errors	• Opens a window where you can view normalized unscaled standard errors occurring in the analysis. • The normalized unscaled standard error is the individual probe error fitting the probe-level model (the PLM expression measures using a M-estimator robust regression). The NUSE values are standardized at the probe set level across the arrays: median values for each probe set are set to 1. • Use the box plots to check for low quality. Examples of low quality are if all distributions are centered near 1 (typically an array with a box plot centered around 1.1 shows bad quality), or if one array has a globally higher spread of normalized unscaled standard error distribution than others.
Box Plots	• Opens a window where you can view box plot representations of the statistics of the analysis. • A box plot is generated after the samples are transformed with a variance stabilizing transformation (VST) and before robust spline normalization (RSN). Use this quality control plot to spot outlier arrays with too much technical variation, for example, if they show a different probe intensity distribution pattern in the box plots when compared to probes on the other arrays on the same chip or when compared to arrays on other chips.
Clustering	• Opens a window where you can view hierarchical clustering information of the statistics of the analysis. • Hierarchical clustering is a quality control plot generated after the samples were transformed with a VST and before RSN. Outlier arrays with too much technical variation might be spotted if they do not cluster with their respective technical or biological replicates from the same sample or sample type.
Pluripotency Plot	• Opens the Pluripotency plot window with a visual representation of the pluripotent samples in the analysis. • The pluripotency plot and novelty x/y scatter plot combines the pluripotency score on the y axis with the novelty score on the x axis. The red and blue background show the empirical distribution of the pluripotent (red) and nonpluripotent samples (blue) in the reference data set. • The plot image can be saved as a PDF file or printed.
Back to Completed Analyses	• Returns to the Analyses summary window.

Pluripotency plot window

The pluripotency plot window provides a visual representation of the pluripotent samples in the analysis.

The pluripotency and novelty x/y scatter plot combines the pluripotency score on the y axis with the novelty score on the x axis. The red and blue background show the empirical distribution of the pluripotent (red) and nonpluripotent samples (blue) in the reference data set. The plot image can be saved as a PDF file or printed.

A representative pluripotency plot is shown below.



Pluripotency score: A score that is based on all samples (pluripotent cells, somatic cells, and tissues) in the stem cell model matrix. Samples with positive values are more similar to the pluripotent samples in the model matrix than to all other classes of samples in the matrix. The pluripotency score indicates whether a sample contains a pluripotent signature, but not necessarily if the cell preparation is a normal, bona-fide hESC or iPSC. Partially differentiated pluripotent cells, teratocarcinoma cells, or karyotypically abnormal ESCs may also have a high pluripotency score.

Novelty score: A score that is based on well-characterized pluripotent samples in the stem cell model matrix. A low novelty score indicates that the test sample can be well reconstructed based on existing data from other well-characterized iPSC and ESC lines. A high novelty score indicates that there are patterns in the tested sample that cannot be explained by the currently existing data from well-characterized, karyotypically normal PSCs. Partially differentiated pluripotent cells, teratocarcinoma cells, or karyotypically abnormal ESCs may have a high pluripotency score but cannot be reconstructed well with data from well-characterized, normal PSCs, and thus are expected have a high novelty score.

4. Genome editing of PSCs

4.1 CRISPR-Cas9 genome editing for research on hPSCs cultured in StemFlex Medium via electroporation

The advent of reprogramming technologies have allowed researchers to generate limitless pools of induced pluripotent stem cells (iPSCs) and iPSC-derived cells retaining the genetic makeup of the parental somatic cells. Combined with novel tools for gene editing, this allows researchers to generate (1) knockouts to study the impact of genes on cellular processes, (2) knock-ins to assess the impact of reversing point mutations on diseased states, or (3) reporter cell lines. To successfully perform genome editing of iPSCs, many factors need to be considered, such as choice of genome editing tools, culture media, and delivery methods. This section contains our most highly recommended genome editing protocol, combining the high efficiency of Invitrogen™ CRISPR-Cas9 editing tools and the robust support of the Gibco™ StemFlex™ culture system with electroporation-based delivery. It also provides an alternative lipid-based delivery method using the Invitrogen™ Lipofectamine™ Stem Transfection Reagent with StemFlex Medium.

Electroporation currently provides the highest efficiencies in delivering guide RNA (gRNA) complexes to PSCs, while Invitrogen™ TrueCut™ Cas9 Protein v2 enables maximum editing efficiency, and StemFlex Medium offers robust support for optimal survival of PSCs despite the stresses involved in genome editing. This protocol describes the delivery of Cas9–gRNA complexes via electroporation to PSCs cultured in StemFlex Medium, expansion post-editing, and best practices for flow sorting of cultures for clonal expansion in research applications. Refer to the user guide (Pub. No. MAN0016431) for detailed instructions on culturing human PSCs (hPSCs) under feeder-free conditions in StemFlex Medium.

Required materials

- TrueCut Cas9 Protein v2 (Cat. No. A36497)
- Invitrogen™ GeneArt™ Precision gRNA Synthesis Kit (Cat. No. A36498)
- Invitrogen™ Neon Transfection System 10 µL Kit (Cat. No. MPK1025, MPK1096)
- Invitrogen™ Qubit™ 3.0 Fluorometer (Cat. No. Q33216)
- Invitrogen™ Qubit™ RNA BR Assay Kit (Cat. No. Q10210) StemFlex Medium (Cat. No. A3349401)
- Gibco™ Geltrex LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix (Cat. No. A1413302)
- Gibco™ rhLaminin-521* (Cat. No. A29248, A29249)
- Gibco™ DMEM/F-12, GlutaMAX Supplement (Cat. No. 10565)
- Gibco™ TrypLE™ Select Enzyme (1X), no phenol red (Cat. No. 12563011)
- Gibco™ StemFlex™ Medium (Cat. No. A3349401)
- Gibco™ TrypLE Express Enzyme (1X), no phenol red (Cat. No. 12604013)

- Gibco™ DPBS, no calcium, no magnesium (Cat. No. 14190)
- Gibco™ DPBS, calcium, magnesium (Cat. No. 14040)
- Gibco™ Versene™ Solution (Cat. No. 15040)
- **Optional:** Invitrogen™ TRA-1-60 Alexa Fluor™ 488 Conjugate Kit for Live Cell Imaging (Cat. No. A25618)
- **Optional:** Gibco™ Human Episomal iPSC Line (Cat. No. A18945)
- **Optional:** Gibco™ RevitaCell™ Supplement (100X) (Cat. No. A2644501)

* Use Geltrex LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix or rhLaminin-521.

Design and generation of gRNAs by *in vitro* transcription

1. Use the Invitrogen™ GeneArt™ CRISPR Search and Design Tool, available at **thermofisher.com/crisprdesign** to search our database of >600,000 predesigned gRNA sequences specific to every gene in the human genome. Predesigned Invitrogen™ GeneArt™ gRNAs are optimized for gene knockout and typically target the first 3 transcribed exons per gene.
2. Generate your DNA template containing the T7 promoter and the gRNA sequence with the GeneArt Precision gRNA Synthesis Kit.
3. Determine gRNA concentration with the Qubit 3.0 Fluorometer coupled with the Qubit RNA BR Assay Kit.

   Note: Genome editing of hiPSC is facilitated when growing cells on rhLaminin-521 in StemFlex Medium. Before delivery of gene editing tools to the cells, we recommend adapting your cells to rhLaminin-521 and StemFlex Medium for at least two passages.

   Note: Maintaining cells on rhLaminin-521 and StemFlex Medium is generally done on 6-well plates and would be the same as for any other extracellular matrix and medium. Because cells survive better on rhLaminin-521 and StemFlex Medium, you may need to adjust the splitting dilution.

Preparation of Cas9–gRNA complex

1. Add 1.5 µL of TrueCut Cas9 Protein v2 and 300 ng of gRNA to 5 µL of Invitrogen™ Resuspension Buffer R and mix gently.

   – **Note:** The volume of gRNA should be 0.5 µL or less.
2. Determine gRNA concentration using the Qubit 3.0 Fluorometer.
3. Incubate the complex at room temperature for 10 minutes.

Procedural guidelines

Coat 24-well plates with Geltrex matrix

1. Dilute the Geltrex matrix 1:100 in cold Gibco™ DMEM/F-12, GlutaMAX™ Supplement.
2. Add 300 µL to each well.
3. Incubate plate(s) at 37°C, 5% CO_2 for >1 hour ahead of PSC seeding.

Coat 24-well plates with rhLaminin-521

The optimal working concentration of rhLaminin-521 is dependent on the cell line and ranges from 0.5 to 2.0 µg/cm^2.

1. To coat plates with 0.5 µg/cm^2, dilute 300 µL of rhLaminin-521 in 12 mL of DPBS (calcium, magnesium) or DMEM/F-12 with GlutaMAX Supplement.
2. Add 400 µL of diluted rhLaminin-521 per well.
3. Incubate plates at 37°C with 5% CO_2 ahead of PSC seeding.

Preparation of PSCs for electroporation

See "Procedural guidelines" on the previous page for plate-coating information. If using precoated plates stored at 2–8°C, prewarm rhLaminin-521– or Geltrex matrix–coated plates to room temperature. Prewarm StemFlex Medium and TrypLE Select Enzyme to room temperature.

1. Upon PSCs reaching 40–85% confluency, aspirate spent medium from the culture vessel.

2. Rinse the vessel once with the recommended volume of DPBS with no calcium or magnesium (DPBS –/–).

Culture vessel (surface area)	6-well (10 cm^2)	12-well (4 cm^2)	24-well (2 cm^2)	35 mm (10 cm^2)	60 mm (20 cm^2)	100 mm (60 cm^2)
DPBS (–/–)	2 mL/well	1 mL/well	0.5 mL/well	2 mL/dish	4 mL/dish	12 mL/dish

3. Aspirate the DPBS (–/–).

4. Add TrypLE Select Enzyme to the vessel containing PSCs, then swirl the vessel to coat the entire well surface.

Culture vessel (surface area)	6-well (10 cm^2)	12-well (4 cm^2)	24-well (2 cm^2)	35 mm (10 cm^2)	60 mm (20 cm^2)	100 mm (60 cm^2)
TrypLE Select Enzyme	1 mL/well	0.4 mL/well	0.2 mL/well	1 mL/dish	2 mL/dish	6 mL/dish

5. Incubate the vessel at 37°C with 5% CO_2 for 3–5 minutes.

6. Gently pipet the cells up and down 5–10 times with a 1,000 µL pipette to generate a single-cell suspension.

7. Transfer the cell suspension to a conical tube containing the recommended neutralization volume of StemFlex Medium to dilute the dissociation reagent.

Culture vessel (surface area)	6-well (10 cm^2)	12-well (4 cm^2)	24-well (2 cm^2)	35 mm (10 cm^2)	60 mm (20 cm^2)	100 mm (60 cm^2)
Neutralization volume, StemFlex Medium	3 mL/well	1.2 mL/well	0.6 mL/well	3 mL/dish	6 mL/dish	18 mL/dish

Centrifuge the PSCs at 200 x *g* for 4 minutes, then aspirate and discard the supernatant.

8. Flick the tube 3–5 times to loosen the pellet, then resuspend the cells by pipetting them up and down 5–10 times in a resuspension volume of StemFlex Medium.

Culture vessel (surface area)	6-well (10 cm^2)	12-well (4 cm^2)	24-well (2 cm^2)	35 mm (10 cm^2)	60 mm (20 cm^2)	100 mm (60 cm^2)
Resuspension volume, StemFlex Medium	2 mL/well	1 mL/well	0.5 mL/well	2 mL/dish	4 mL/dish	12 mL/dish

9. Determine the viable cell density and percent viability using an Invitrogen™ Countess™ II Automated Cell Counter or similar automated or manual method.

Electroporation of Cas9–gRNA complexes using the Neon Transfection System

1. Transfer 1 x 10^6 viable cells to a sterile microcentrifuge tube, and centrifuge at 200 x *g* for 4 minutes.
2. Carefully and completely aspirate the growth medium. Do not disturb the cell pellet.
3. Carefully resuspend the cell pellet in 50 µL of Resuspension Buffer R.
4. Transfer 5 µL of resuspended cells to 6 µL of the Cas9–gRNA complexes that were prepared in "Prepare Cas9–gRNA complex".
   - **Note:** If using a ssDonor for HDR-mediated editing, add 10 pmol of ssDonor in this step, maintaining the final volume at 11 µL.
   - Mix gently.
5. Pipet 10 µL of the cell suspension into the Neon Tip and electroporate with protocol 7 (1,200 V, 30 ms, 1 pulse) or protocol 14 (1,200 V, 20 ms, 2 pulses).
   - Be careful not to introduce bubbles.
   - We recommend that users optimize electroporation conditions for the Neon Transfection System for their specific cell line; the *HPRT* gRNA control is available for purchase as a custom gRNA for transfection optimization—contact us at **GEMServices@thermofisher.com** to order.
6. Immediately transfer the electroporated cells into a 24-well plate containing 0.5 mL of StemFlex Medium containing 1X RevitaCell Supplement.
7. Move the vessel in several quick side-to-side motions to disperse the cells across the surface of the vessel.
8. Carefully transfer the vessel to a 37°C incubator with 5% CO_2 and incubate the cells overnight.
9. Feed the PSCs the day after electroporation.
10. Analyze the cells 48–72 hours after electroporation.
11. Harvest cells and save a portion for continued propagation; and with the other portion, measure cleavage efficiency using the GeneArt Genome Cleavage Detection Kit.
    - With the Neon Transfection System, we have obtained up to 80% cleavage efficiency with the *HPRT* gRNA control in the Gibco Human Episomal iPSC Line expanded on a Geltrex matrix.

Expansion of PSCs following genome editing

See “Procedural guidelines” on page 93 for plate-coating information. If using precoated plates stored at 2–8°C, prewarm rhLaminin-521–coated plates to room temperature. Incubate plate(s) at 37°C with 5% CO_2 for >2 hours ahead of PSC seeding. Prewarm StemFlex Medium and Versene solution or 500 µM EDTA solution to room temperature.

1. Aspirate spent medium from the culture vessel.

2. Rinse the vessel once with recommended volume of DPBS (–/–).

Culture vessel (surface area)	6-well (10 cm^2)	12-well (4 cm^2)	24-well (2 cm^2)	35 mm (10 cm^2)	60 mm (20 cm^2)	100 mm (60 cm^2)
DPBS (–/–) wash	2 mL/well	1 mL/well	0.5 mL/well	2 mL/dish	4 mL/dish	12 mL/dish

3. Add Versene solution or 500 µM EDTA to the side of the vessel containing PSCs, then swirl the vessel to coat the entire well surface. Alternatively, TrypLE enzyme can be used as described on page 94.

Culture vessel (surface area)	6-well (10 cm^2)	12-well (4 cm^2)	24-well (2 cm^2)	35 mm (10 cm^2)	60 mm (20 cm^2)	100 mm (60 cm^2)
Versene solution	1 mL/well	0.4 mL/well	0.2 mL/well	1 mL/dish	2 mL/dish	6 mL/dish
500 µM EDTA						

4. Incubate the vessel at room temperature for 5–8 minutes or at 37°C for 4–5 minutes.

   – When the cells start to separate and round up, and the colonies appear to have holes in them when viewed under a microscope, they are ready to be removed from the vessel.

   – **Note:** Do not incubate the cells to the extent that the colonies float off the surface of the culture vessel.

5. Aspirate the Versene solution or 500 µM EDTA and add prewarm complete StemFlex Medium to the vessel. Remove the cells from the well(s) by gently flushing medium over the surface of the well a few times.

Culture vessel (surface area)	6-well (10 cm^2)	12-well (4 cm^2)	24-well (2 cm^2)	35 mm (10 cm^2)	60 mm (20 cm^2)	100 mm (60 cm^2)
Complete StemFlex Medium	2 mL/well	1 mL/well	0.5 mL/well	2 mL/dish	4 mL/dish	12 mL/dish

6. Collect cells in a 15 mL or 50 mL conical tube.

   – There may be obvious patches of cells that were not dislodged and left behind; do not scrape the cells from the dish in an attempt to recover them.

   – **Note:** Depending upon the cell line, work with no more than 1–3 wells at a time and work quickly to remove cells after adding StemFlex Medium to the well(s), which quickly neutralizes the initial effect of the Versene solution or 500 µM EDTA; some lines re-adhere very rapidly after medium addition and must be removed 1 well at a time, and others are slower to reattach and may be removed 3 wells at a time.

7. Aspirate rhLaminin-521 from the culture vessel and discard. Do not allow the culture surface to dry out.

8. Immediately add an appropriate volume of prewarmed complete StemFlex Medium to each well of an rhLaminin-521–coated plate so that each well contains the recommended volume of complete medium after the cell suspension has been added. See the table under step 5 for recommended volumes.

   – **Note:** The split ratio can vary, though it is generally between 1:6 and 1:18 for established cultures on an rhLaminin-521 matrix. Occasionally, cells may recover at a different rate and the split ratio will need to be adjusted.

4.2 CRISPR-Cas9 genome editing for research on hPSCs cultured in StemFlex Medium via lipid-based transfection

Lipid-based delivery of Cas9-gRNA complexes allows for higher-throughput gene editing of PSCs compared to electroporation-based delivery. The Lipofectamine Stem Transfection Reagent (Cat. No. STEM00001) is an efficient lipid-based reagent that provides robust delivery of gene-editing tools such as ribonucleoprotein (RNP) complexes to PSCs. In this section, we provide an optimized protocol using the Lipofectamine Stem reagent for the delivery of Cas9–gRNA complexes to cells maintained in StemFlex Medium.

To transfect PSCs cultured in StemFlex Medium, replace the medium with Gibco™ Opti-MEM™ I Reduced Serum Medium (Cat. No. 31985062) and RevitaCell Supplement. The transfection complex containing Cas9 and the gRNA is then delivered to the PSCs with the Lipofectamine Stem reagent, and the cells are incubated for 1–4 hours. Following incubation, transfection complexes are overlaid with StemFlex Medium without ROCK inhibitor or RevitaCell Supplement. The medium is fully exchanged 24 hours posttransfection, and cleavage analysis and downstream expansion for clonal analysis can be initiated 48–72 hours posttransfection.

For information on lipid-based delivery of alternative formats (e.g., protein, DNA, and/or mRNA) refer to **thermofisher.com/stemflexlipofectaminestem**.

Required materials

- StemFlex Medium (Cat. No. A3349401)
- Lipofectamine Stem Transfection Reagent (Cat. No. STEM00008)
- Geltrex LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix (Cat. No. A1413302)
- rhLaminin-521* (Cat. No. A29248)
- Gibco™ DPBS, no calcium, no magnesium (Cat. No. 14190144)
- Versene Solution (Cat. No. 15040066)
- TrypLE Express Enzyme (1X), no phenol red (Cat. No. 12604013)
- RevitaCell Supplement (Cat. No. A2644501)
- Opti-MEM I Reduced Serum Medium (Cat. No. 31985062)

* Use Geltrex LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix or rhLaminin-521.

Starting with undifferentiated human pluripotent stem cells (PSCs), expanded in a feeder-free culture system such as StemFlex Medium on a Geltrex matrix, or on a defined substrate such as rhLaminin-521, is ideal for efficient transfection.

Passaging

Maintain PSCs in the format of your choice, such as 6-well plates, 60 cm dishes, or T-75 flasks coated with Geltrex matrix or rhLaminin-521, in StemFlex Medium. Propagating PSCs in 6-well plates and transfecting in 24-well plates are convenient formats used in this protocol.

Passage PSCs every 3–5 days, before they reach ~85% confluency.

Tip: For routine passaging of PSCs with Versene Solution for expansion, the replating of large clumps of 5–10 cells promotes reattachment and survival in StemFlex Medium without the need to add RevitaCell Supplement. PSCs can be expanded in StemFlex Medium for subsequent transfection on a Geltrex matrix or rhLaminin-521.

Precoating 24-well plates with Geltrex matrix or rhLaminin-521

Coating with Geltrex matrix

1. Prepare a 1:100 dilution of Geltrex matrix in cold DMEM/F-12 with GlutaMAX Supplement.
2. Add 300 µL of diluted Geltrex matrix to each well of a 24-well plate and incubate at 37°C for ≥1 hour, before use.

Coating with rhLaminin-521

1. Prepare a 1:40 dilution of rhLaminin-521 by adding 300 µL of rhLaminin-521 stock solution (0.5 mg/mL) to 12 mL of DPBS (+/+), for a final concentration of 2.5 µg/mL.
2. Add 400 µL of diluted rhLaminin-521 to each well of a 24-well plate, and incubate at 37°C for ≥2 hours to coat the wells with 0.5 µg/cm^2 of rhLaminin-521.

- **Important:** The optimal coating concentration of rhLaminin-521 can depend on the PSC line and ranges from 0.5 to 2 µg/cm^2; increase the concentration if you observe areas of incomplete cellular attachment.
- **Tip:** Plates coated with Geltrex matrix or rhLaminin-521 can be prepared ahead of time and stored for up to 2 weeks at 4°C; equilibrate at room temperature for 1 hour before plating cells.

Seeding cells for transfection

1. To maximize transfection efficiency, seeding a single-cell suspension of PSCs prepared with TrypLE Select Enzyme is recommended.

- **Important:** As the plating efficiency of PSCs dissociated into single cells is lower than the plating efficiency of clumped cells, we recommend adding RevitaCell Supplement for overnight replating in StemFlex Medium onto a Geltrex matrix or rhLaminin-521 for transfecting the following day.

2. When feeder-free PSC cultures are less than 85% confluent, remove the StemFlex Medium and gently wash the cells twice with 2 mL of DPBS per well in a 6-well plate.
3. Add 1 mL of TrypLE Select Enzyme to each well, swirl to evenly coat the PSCs, and incubate at 37°C for 3–5 minutes.
4. Using a 1 mL pipette, gently triturate the cell suspension 5–10 times to dissociate into single cells.
5. Transfer the cell suspension into a 15 mL conical tube containing 3 mL of StemFlex Medium to inactivate the TrypLE Select Enzyme.
6. Centrifuge the cell suspension at 200 x *g* for 4 minutes.
7. Aspirate the supernatant and resuspend the pellet to a single-cell suspension in 3 mL of StemFlex Medium with RevitaCell Supplement.
8. Perform a total viable cell count with the Countess II Automated Cell Counter or another method.
9. Dilute with additional StemFlex Medium with RevitaCell Supplement to a final concentration of 100,000 cells/mL.
10. Aspirate the Geltrex matrix or rhLaminin-521 from the wells of a precoated 24-well plate.

- **Important:** Proliferating PSC cultures need room to expand during transfection, so plate the recommended starting number of cells (step 11) to achieve 30% confluency on the day of transfection.

11. Add 0.5 mL of the PSC suspension in StemFlex Medium with RevitaCell Supplement to plate 50,000 cells/well in the precoated 24-well plate.
12. Return the plate to the incubator and culture the cells at 37°C with 5% CO_2, overnight.

Changing medium on the day of transfection

Prepare a solution of Opti-MEM I medium with RevitaCell Supplement. Aspirate the StemFlex Medium and add 0.5 mL of the supplemented Opti-MEM I medium to each well just before transfection.

- **Important:** Transfect in Opti-MEM I medium with RevitaCell Supplement, not in StemFlex Medium, which can inhibit transfection.

RNP transfection protocol

RNP complex components

- TrueCut Cas9 Protein v2
- gRNA, for more information, go to **thermofisher.com/crisprgrna**

On the day of transfection (1 day after plating PSCs in a single well of a 24-well plate), perform the following steps, which have been optimized for using the Lipofectamine Stem reagent in StemFlex Medium.

Refer to pages 96–97 for more information on the expansion of the pool.

Step	Tube	Complexation component	Amount per well (24-well plate)
1	Tube 1	Opti-MEM I medium	25 µL
		Lipofectamine Stem reagent	2 µL
2	Tube 2	Opti-MEM I medium	25 µL
		Cas9 nuclease	1.5 µg
		gRNA (0.1–0.5 µg/µL)	375 ng
3	Add tube 2 solution to tube 1 and mix well. If using ssDonor for HDR editing, add 10 pmol of ssDonor during this step.		
4	Incubate mixture from step 3 for 10 minutes at room temperature.		
5	Aspirate the StemFlex Medium and add 0.5 mL of Opti-MEM I medium with RevitaCell Supplement per well just before transfection.		
6	Add 50 µL of complex from step 4 to each well; gently swirl plate to ensure even distribution of the complex across the entire well.		
7	Return culture dish to incubator and culture the cells at 37°C with 5% CO_2 for 4 hours. **Important:** After 4 hours of transfection, add 0.5 mL of StemFlex Medium warmed to room temperature to each well, return plate to incubator, and culture the cells at 37°C with 5% CO_2 overnight.		
8	The following day, aspirate the StemFlex Medium and transfection complexes and add 0.5 mL of fresh StemFlex Medium per well. If PSCs are going to be transfected for 48 hours, passage before they reach 85% confluency.		

4.3 Clonal isolation of edited pools

Deciding to move forward with clonal isolation depends on the percentage of preferred edits found in the edited pool (generated in sections 4.1–4.2) after performing quality control (QC) for indel-induced knockout (KO) or single-nucleotide polymorphism (SNP) knock-in (KI) frequency. In general, having 5% or more of the desired edits warrants continuation of the process.

For an indel-induced KO:
Process the samples using the GeneArt Genome Cleavage Detection Kit (Cat. No. A24372) to estimate cleavage efficiency, run a TIDE (Tracking of Indels by DEcomposition, **tide.nki.nl/**) analysis on the PCR product by Sanger sequencing, of the edited region or run an Ion Personal Genome Machine™ (PGM™) or S5™ sequencing run of a PCR-amplified 200 bp region of the edited gDNA to estimate indel frequency using the Integrated Genomics Viewer (IGV; The Broad Institute).

For a SNP KI:
Perform sequencing run of a PCR-amplified 200 bp region of the edited gDNA on the Ion PGM or S5 sequencer to estimate SNP change incidence using the Integrated Genomics Viewer (IGV; The Broad Institute).

Recovering the cell pool, isolating clones, and screening

If the preferred edit is detected in the cell pool at a frequency of 5% or higher, then clonal isolation is needed to isolate a homogeneous cell line. We recommend using a stringent sorting protocol to ensure seeding of a single, viable, pluripotent hiPSC. Ensure that a cell sorter with 96-well plating capability is available before you begin preparing your samples.

Note: The number of 96-well plates needed to generate single-cell clones can be estimated using the edit incidence and the need for heterozygous or homozygous clones. For example, if you need a homozygous cell line and QC results show a 5% edit, the chance of finding a homozygous clone is 1 in 40, meaning that you theoretically would need to screen at least 40 colonies to find it. To estimate the number of plates needed when using StemFlex Medium, expect approximately 15–30 colonies, but be sure to generate a few extra plates to ensure you can isolate enough clones of interest.

Required materials

- StemFlex Medium (Cat. No. A3349401)
- rhLaminin-521 (Cat. No. A29248)
- RevitaCell Supplement (Cat. No. A2644501)
- Nunc tissue culture plastics—6-well, 12-well, and 96-well plates
- DPBS, no calcium, no magnesium (DPBS –/–) (Cat. No. 14190094)
- DPBS, calcium, magnesium (DPBS +/+) (Cat. No. 14040117)
- TrypLE Express Enzyme (Cat. No. 12605093)
- Gentamicin (Cat. No. 15750078)
- Propidium Iodide (PI) (Cat. No. P3566)
- Invitrogen™ Alexa Fluor™ 488 Mouse Anti–Human TRA-1-60 (Cat. No. A25618)

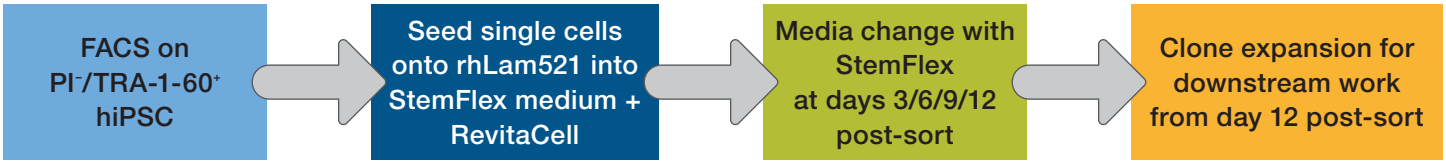


Figure 4.1. Single-cell cloning isolation workflow using a cell sorter.

Recovering the cell pool

1. Thaw the preferred cell pool (generated in step 4.1–4.2) on rhLaminin-521 in StemFlex Medium and perform one passage before cell sorting.

Preparing plates for sorting

2. Prepare coated 96-well plates by adding 50 µL rhLaminin-521 solution per well (1:40 dilution in PBS+/+).
3. Incubate rhLaminin-521–coated plates at 37°C, 5% CO_2 for 2 hours.
4. Add 150 µL of StemFlex Medium containing RevitaCell Supplement and gentamicin (100 µg/mL final concentration).

Preparing cell samples for clonal sorting

5. For single-cell sorting, aspirate the medium from 2 wells of a 6-well plate of a 70% confluent culture and wash with DPBS –/–.
6. Add 1 mL of TrypLE Express Enzyme and place into the 37°C, 5% CO_2 incubator for 3 minutes.
7. Aspirate the TrypLE Express Enzyme and resuspend the cells in StemFlex Medium plus RevitaCell Supplement, using 2 mL per well.
8. Distribute the 4 mL into four 15 mL sterile tubes: pipet 0.5 mL of dissociated cells into three tubes (for unstained, TRA-1-60–only, and PI-only sort controls) and 2.5 mL into one tube (for sorting single cells).
9. Add 2.5 µL propidium iodide (PI) to the PI control tube, add 5 µL Alexa Fluor 488 Mouse Anti-Human TRA-1-60 to the TRA-1-60 control tube, and add 12.5 µL PI and 25 µL Alexa Fluor 488 Mouse Anti-Human TRA-1-60 to the 2.5 mL tube.
10. Stain on ice for 30 minutes.
11. Wash 3 times with DPBS –/–.
12. Resuspend each sample in 2 mL of DPBS –/– plus RevitaCell Supplement and transfer to a round bottom tube with a cell strainer cap for sorting.
13. Incubate the cells on ice while setting up the controls on the sorter.
14. Sort single cells into the 96-well plates prepared above, through gating out doublets (forward and side scatter–based), dead cells (PI-positive), and differentiated cells (TRA-1-60–negative cells).

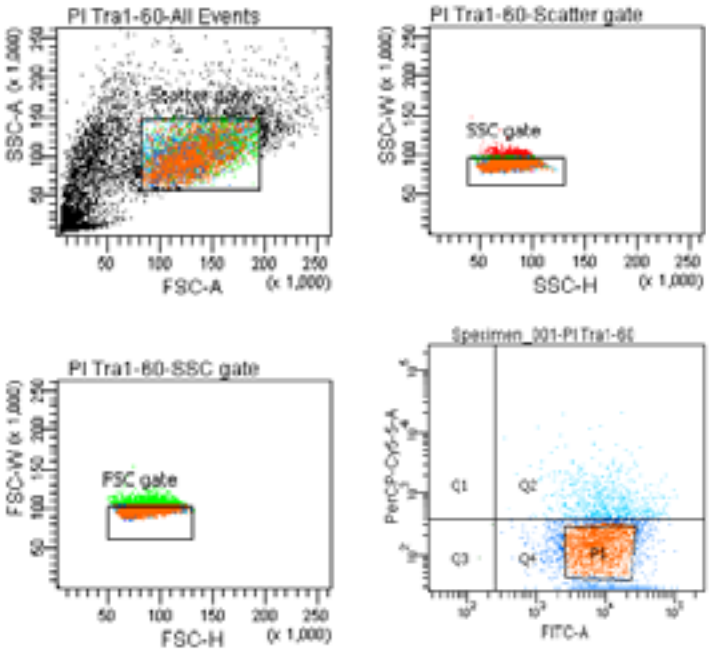


Figure 4.2. Cell-sorter gating strategy to reliably isolate single viable pluripotent cells.

Expansion of single cells post sorting

15. Right after sorting, incubate plates at 37°C, 5% CO_2 for 72 hours.
16. Perform a medium change with 175 µL medium plus gentamicin.
17. Perform media changes every 3 days until colonies are observed, maintaining gentamicin addition for the first week of culture after sorting. After 1 week, StemFlex Medium alone should be used.

18. Check on the colonies, which should begin emerging on day 8 and become clearly visible at day 14.

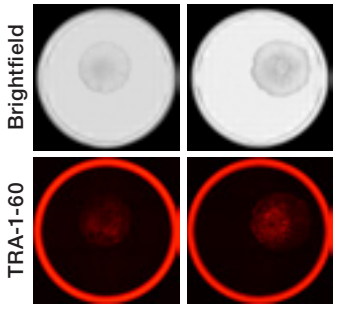


Figure 4.3. Whole-well images of single-cell clones isolated by single-cell sorting. Both brightfield and TRA-1-60 (red) images are shown.

19. On day 14, scan plates using whole-well imaging and confluency analysis to identify clones. Wells with confluency greater than 5% will typically contain a colony.

Consolidation of single-cell clones post sorting

After sorting single cells into 96-well plates, clones will emerge in random wells across the plates. To facilitate downstream processing, consolidate identified clones into a new rhLaminin-521–coated 96-well plate using the steps below.

20. From the plates containing the single cell clones, aspirate the media, wash with DPBS –/–, add 50 µL TrypLE™ Select Enzyme, and incubate at 37°C, 5% CO_2 for 3 minutes.

21. Aspirate the TrypLE™ Express Enzyme, resuspend in 150 µL medium plus RevitaCell Supplement, and add to a 96-well plate coated with rhLaminin-521.

22. Mix the cells well and transfer half to a PCR plate. Retain the other half in the rhLaminin-521 coated plate and move the rhLaminin-521–coated plate into a humidified 37°C, 5% CO_2 incubator overnight.

23. Spin down the cells in the PCR plate and aspirate the supernatant, leaving behind about 20 µL per well to avoid aspirating the cells. The PCR plate can then be frozen until ready for PCR processing.

24. The next day, change the medium in the rhLaminin-521–coated plate with 175 µL medium and allow the cells to grow until ~80% confluency.

25. When confluent, cryopreserve the clones as follows: aspirate the media, wash with DPBS –/–, add 50 µL TrypLE Express Enzyme, and incubate at 37°C, 5% CO_2 for 3 minutes.

26. Aspirate the TrypLE Express Enzyme and resuspend in 200 µL PSC Cryopreservation Medium.

27. Wrap the plate(s) with Parafilm wrapper and store in a zippered plastic bag in –80°C.

Recovering clones and expanding cells

The clones will need to remain frozen while Sanger sequencing or NGS is in process on the PCR plates collected above. Use the protocol below to thaw and expand the desired clones from the positively identified ones.

1. Prepare rhLaminin-521–coated 12-well plates. For each clone recovered, one well of a 12-well plate is required.

2. Thaw the frozen 96-well plate inside a 37°C, 5% CO_2 incubator (typically takes about 20 minutes).

3. Identify the clones for recovery by circling the positive wells on the lid.

4. Resuspend each thawed well by adding StemFlex Medium into a 15 mL conical tube containing 2 mL medium plus RevitaCell Supplement.

5. Spin down cells, resuspend in 1 mL StemFlex Medium plus RevitaCell Supplement, and plate in one well of a 12-well plate. Repeat for as many clones as needed.

6. Expand cells until at least 1.5 x 10^6 cells have been obtained for characterization (Sanger and/or NGS confirmation, TaqMan hPSC Scorecard Panel analysis or Pluritest analysis, immunocytochemistry (TRA-1-60/Sox2), Karyostat analysis and directed differentiation and banking.

5. Differentiation of PSCs

5.1 EB formation from feeder-free PSCs

The generation of induced pluripotent stem cells (iPSCs) is often an intermediate step to reach the real experimental goals. Often, these experimental goals take advantage of the proliferative capacity and pluripotency of iPSCs to generate virtually unlimited numbers of differentiated cell types, including neurons, cardiomyocytes, beta (β) cells, or conceivably any other cell type in the body. These PSC-derived cells can then be used in a range of applications such as basic research, disease modeling, drug screening, and regenerative medicine. As such, it is critical to check new PSC clones and their ability to differentiate into the three germ lineages: ectoderm, mesoderm, and endoderm. This is often done by forming embryoid bodies (EBs) and allowing them to spontaneously differentiate in culture. Once trilineage differentiation capacity is confirmed, the differentiation of PSCs to a specific lineage is achieved by timed exposure to specific conditions via growth factors, small molecules, and substrates that mimic the sequential events that occur during embryonic development. This section provides protocols for the differentiation of PSCs to embryoid bodies, neural stem cells (NSCs), neurons, dopaminergic neurons, cardiomyocytes and definitive endoderm using Gibco reagents and kits.

EBs are floating spherical clusters of PSCs that allow you to test the *in vitro* differentiation potential of the cells. There are many different ways to generate and differentiate EBs from PSCs. This specific protocol describes the generation of EBs from feeder-free PSCs using Gibco™ KnockOut™ Serum Replacement (SR) – Multi-Species, although a similar approach can be used for feeder-dependent PSCs: EBs are set up by harvesting using collagenase at a normally scheduled passage, but the cells are initially plated onto dishes without tissue culture treatment to prevent attachment. The resulting EBs can be kept as floating spheres or eventually plated onto a matrix-coated dish for attachment and further differentiation prior to the analysis.

Materials needed

- Gibco™ DPBS, no calcium, no magnesium (Cat. No. 14190-250)
- Gibco™ Collagenase, Type IV, Powder (Cat. No. 17104-019)
- Gibco™ DMEM/F-12, GlutaMAX™ Supplement (Cat. No. 10565-018)
- Gibco™ KnockOut™ Serum Replacement – Multi-Species (Cat. No. A31815-01)
- Gibco™ FGF-basic (bFGF) (AA 1–155) Recombinant Human (Cat. No. PHG0264)
- Gibco™ MEM Non-Essential Amino Acids Solution (100X) (Cat. No. 11140-050)
- Gibco™ 2-Mercaptoethanol (55 mM) (Cat. No. 21985-023)
- **Optional:** Invitrogen™ 3 Germ Layer Immunocytochemistry Kit (Cat. No. A25538)
- Matrix:
  - Gibco™ Geltrex™ LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix (Cat. No. A1413302) for general applications
  - Vitronectin (VTN-N) Recombinant Human Protein, Truncated (Cat. No. A14700) for xeno-free applications
- Untreated culture vessels or Thermo Scientific™ Nunclon™ Sphera™ 60 mm dishes (Cat. No. 12-566-437)
- Sterile cell culture hood (i.e., biosafety cabinet)
- Inverted microscope
- Incubator set at 37°C, 5% CO_2
- Water bath set at 37°C
- Sterile serological pipettes (5 mL, 10 mL)
- Centrifuge
- 15 mL centrifuge tubes
- Cell scrapers

Preparation of media and reagents

Basic FGF solution (for 1 mL of 10 µg/mL solution)

1. To prepare 1 mL of basic FGF (bFGF) solution at a final concentration of 10 µg/mL, aseptically mix the following components:

bFGF	10 µg
DPBS, no calcium, no magnesium	990 µL
KnockOut SR	10 µL

2. Aliquot and store at –20°C for up to 3 months. Once the bFGF aliquot is thawed, store at 2–8°C and use within 7 days.

Collagenase IV solution

1. To prepare 10 mL of 5X Collagenase IV solution at a final concentration of 5 mg/mL, aseptically mix the following components:

Collagenase, Type IV, Powder	5 mg
DMEM/F-12, GlutaMAX Supplement	10 mL

2. Sterilize through 0.22 µm filter and store at 2–8°C for up to 2 weeks.

EB medium (100 mL of complete medium)

1. To prepare 100 mL of complete EB medium, aseptically mix the following components:

DMEM/F-12, GlutaMAX Supplement	79 mL
KnockOut SR	20 mL
MEM Non-Essential Amino Acids Solution	1 mL
2-Mercaptoethanol (55 mM)	100 µL

2. Sterilize through 0.22 µm filter and store at 2–8°C for up to 4 weeks.
3. When indicated (first 24 hours of the procedure), add bFGF to a final concentration of 4 ng/mL before use (e.g., 40 µL of reconstituted bFGF at 10 µg/mL per 100 mL of medium).

EB formation

The volumes given in the following procedure are for 60 mm culture dishes. For culture vessels with different sizes, adjust the volumes appropriately.

Prior to day 0

1. Culture cells under feeder-free conditions on Geltrex matrix– or vitronectin-coated culture vessels.

   Note: See section 2.2A for instructions on coating dishes. Refer to sections 2.2B and 2.2C for detailed instructions on culturing feeder-free PSCs in Gibco™ Essential 8™, Essential 8™ Flex, or StemFlex™ Medium.

Day 0: EB formation

1. When the cultures are 80–85% confluent, the cells are ready to be harvested for EB formation.

   Note: It is important that the colonies are not small and overcrowded, but rather are allowed to grow robust in individual size for about 4 days (~1,200–1,500 µm in width).

2. Aspirate the spent medium from the culture vessel and briefly wash once with 5 mL of DPBS without calcium and magnesium (for a 60 mm dish).
3. Aspirate the DPBS and add 2 mL of 5X Collagenase IV solution (5 mg/mL), prewarmed to 37°C. Ensure complete coverage of culture surface with the Collagenase IV solution.
4. Incubate the cultures grown on vitronectin-coated vessels for 10–15 minutes in a 37°C, 5% CO_2 incubator until the edges of the colonies begin to curl and detach from the plate. Do not overexpose the cultures to 5X Collagenase IV solution.

   Note: Cultures grown on Geltrex matrix may take longer to detach. Incubate cultures grown on Geltrex matrix for 15–20 minutes.

5. Aspirate off the Collagenase IV solution and add 3 mL of complete EB medium.
6. Gently dislodge the colonies from the plate using a cell scraper, and then wash by pipetting them up and down a few times in a 5 mL serological pipette.

   Note: Optimal fragment size for the colonies is critical for successful EB formation; make sure not to triturate the colonies into very small fragments to ensure good fragment size.

7. Transfer the suspended colony clusters into a 15 mL conical tube.

8. Add an additional 2 mL of complete EB medium to dislodge the remaining colonies and transfer them to the 15 mL tube.

9. Let the colony fragments sediment at the bottom of the 15 mL tube for 5–7 minutes by gravity (do not go over the 5-minute mark as clusters will begin to curl up and will not attach).

10. Gently aspirate off the supernatant, add 3 mL of complete EB medium with 4 ng/mL of bFGF, and gently resuspend the sedimented colony fragments by pipetting up and down 2 times.

11. 5 mL of complete EB medium is recommended for a 60 mm non–TC-treated dish. Transfer the 3 mL cell suspension dropwise to an untreated culture dish that has been pre-aliquotted with 2 mL of complete EB medium with 4 ng/mL of bFGF. This will give a final volume of 5 mL in the 60 mm culture dish.

12. Place the culture dish containing the cell clusters in a 37°C, 5% CO_2 incubator and incubate overnight.

Day 1

1. Transfer the contents of the untreated dish to a 15 mL conical tube. Use 2 mL of EB medium without bFGF to wash the dish to gather any remaining EBs and pool into the conical tube.

2. Collect the EBs by gravity sedimentation for 5–10 minutes.

3. Aspirate off the supernatant; this step removes bFGF and single cells.

4. Resuspend the sedimented EBs in 3 mL of EB medium (without any bFGF from here on).

5. Transfer all 3 mL of EB suspension dropwise to a new 60 mm untreated dish or the original Nunclon Sphera dish that has been pre-aliquotted with 2 mL of EB Medium without bFGF.

Option A: Analysis on day 7 using the TaqMan hPSC Scorecard Panel

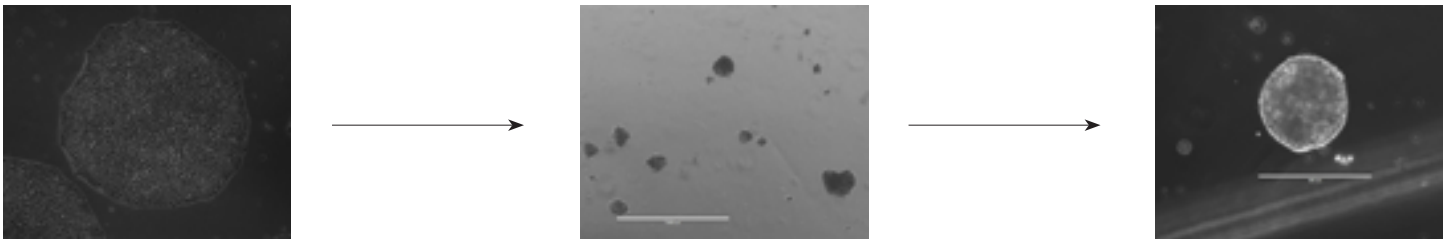
1. Continue incubation at 37°C, 5% CO_2 until day 7, repeating the day 1 harvest and feed procedure every other day.

2. On day 7, repeat step 1–3 from day 1 to harvest the EBs for analysis using the Applied Biosystems™ TaqMan® hPSC Scorecard™ Panel (Cat. No. A15870, A15871, A15872, or A15876).

   Note: For detailed instructions on sample preparation and RT-qPCR using the TaqMan hPSC Scorecard Panel, refer to the TaqMan hPSC Scorecard Panel user guide (Pub. No. MAN0008384).

Option B: Immunocytochemistry (ICC) on day 21

1. On day 4, seed the EBs on Geltrex matrix–coated TC dishes in EB medium without bFGF.
2. Change the spent medium every other day for 3 weeks.
3. On day 21, fix cells and perform ICC for trilineage markers according to standard protocols. For optimal image-based analysis of beta-III tubulin (TUJ1) for ectoderm, smooth muscle actin (SMA) for mesoderm, and alpha-fetoprotein (AFP) for endoderm, we recommend using the 3-Germ Layer Immunocytochemistry Kit.



Prior to day 0: Culture PSCs in Essential 8 Medium on vitronectin-coated dishes

Day 0: Harvest using collagenase and start EB formation in EB medium with bFGF on non-TC or Nuclon Sphera dishes

Day 1: Harvest clusters and reseed onto a new non-TC dish or the original Nuclon Sphera dish using EB medium without bFGF

Day 2–6: Continue EB formation, replace spent EB medium without bGFG every other day

Day 7: Harvest EBs and lyse with TRIzol reagent for analysis using the TaqMan hPSC Scorecard Panel

Figure 5.1. iPSC derived using Invitrogen™ iPS-Sendai reprogramming kits on feeders (BS3-C) and then cultured in Essential 8 Medium on vitronectin-coated culture vessels were harvested using 2X Collagenase IV solution (2 mg/mL) and allowed to form EBs using the procedure described above. The EBs were harvested on day 7 using Invitrogen™ TRIzol™ reagent and analyzed using the TaqMan hPSC Scorecard Panel as described in the product user guide.

	Pluri	Endo	Meso	Ecto
BS3C-p47-E8-Undiff	–0.14	–0.57	–0.72	–1.76
BS3C-E8-Col-EB-sphera	–0.77	2.16	3.06	0.93
BS3C-E8-Col-EB-petri	–0.87	2.05	3.09	0.90

Gene expression relative to the reference standard	
Above 1.5x	Upregulated
1.0x to 1.5x	
0.5x to 1.0x	
–0.5x to 0.5x	Comparable
–1.0x to –0.5x	
–1.5x to –1.0x	
Below –1.5	Downregulated

Figure 5.2. The heat map analysis shows differentially expressed markers for pluripotent cells and the three expected differentiation lineages (endoderm, mesoderm, ectoderm) between undifferentiated and day 7 EBs. Each input sample mean of *t* statistics over gene categories of pluripotent and differentiation lineages is represented with a number, with values between –0.5 and 0.5 indicating comparable expression (white), less than –0.5 indicating lower/downregulated expression (blue shades), and above 0.5 indicating higher/upregulated expression (red shades).

5.2 Induction of NSCs using PSC Neural Induction Medium

Induction of NSCs from hPSCs using PSC Neural Induction Medium

The first important step in producing various neural cells from PSCs is the induction of PSCs to neural stem cells (NSCs). Conventional methods of NSC derivation from human PSCs involving EB formation or co-cultures with stromal cell lines have several disadvantages, including a time-consuming protocol and variability in the quality of resulting NSCs. We have developed a serum-free neural induction medium that can differentiate human PSCs into NSCs in one week with high efficiency, but without the laborious processes of EB formation and mechanical NSC isolation. This can be used in conjunction with the protocol in section 5.3 to differentiate the NSCs to neurons.

Materials needed

- Gibco™ PSC Neural Induction Medium (consists of Neurobasal Medium and Neural Induction Supplement, 50X; Cat. No. A1647801)
- Gibco™ Advanced DMEM/F-12 (Cat. No. 12634) for NSC expansion
- Geltrex LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix (Cat. No. A1413301 or A1413302)
- Gibco™ Distilled Water (Cat. No. 15230)
- ROCK Inhibitor Y27632 (Sigma-Aldrich, Cat. No. Y0503)
- DPBS, no calcium, no magnesium (Cat. No. 14190)
- Gibco™ StemPro™ Accutase™ Cell Dissociation Reagent (Cat. No. A11105)
- Dimethyl sulfoxide (DMSO), Sterile (Sigma-Aldrich, Cat. No. D2650)
- Cell Scraper (Fisher Scientific, Cat. No. 08-771-1A)
- 15 mL and 50 mL sterile polypropylene conical tubes
- 6-well culture plates (Fisher Scientific, Cat. No. 08-772-1B)
- Thermo Scientific™ Nunc™ Lab-Tek™ Chamber Slide System, 4-well (Fisher Scientific, Cat. No. 1256521)
- Glass Pasteur pipettes
- 5, 10, 25, and 50 mL sterile pipettes
- 100 µm strainer (Fisher Scientific, Cat. No. 08-771-19)
- Cryovial™ vials
- Thermo Scientific™ Mr. Frosty™ Freezing Container (Fisher Scientific, Cat. No. 15-350-50)
- 37°C humidified cell culture incubator with 5% CO_2
- Liquid nitrogen storage
- Centrifuge
- 37°C water bath

Immunocytochemistry reagents:

- Invitrogen™ Human Neural Stem Cell Immunocytochemistry Kit (Cat. No. A24354)

  Note: This complete immunocytochemistry kit contains primary antibodies for four common NSC markers (nestin, Pax6, Sox1, and Sox2), a matching set of Invitrogen™ Alexa Fluor™ conjugate–labeled secondary antibodies, a nuclear DNA stain (DAPI), and all of the buffers necessary for performing the staining protocol.

- Invitrogen™ OCT4 Rabbit Monoclonal Antibody (Cat. No. MA5-14845)
- 4-well chamber slide (Fisher Scientific, Cat. No. 12-565-17)
- Cover glass, 24 x 50 mm (Fisher Scientific, Cat. No. 22-050-232)
- **Optional:** Invitrogen™ ProLong™ Gold Antifade Reagent (Cat. No. P36930)

Preparation of media and materials

PSC Neural Induction Medium (for 500 mL)

1. To prepare 500 mL of complete PSC Neural Induction Medium, aseptically mix the following components:

Neurobasal Medium	490 mL
Neural Induction Supplement	10 mL

2. Complete PSC Neural Induction Medium can be stored at 2–8°C in the dark for up to 2 weeks. Warm the Neural Induction Medium in a 37°C water bath for 5–10 minutes before using. Do not warm the Neural Induction Medium in a 37°C water bath for longer than 10 minutes, as this may cause degradation of the medium components.

   Note: Neural Induction Supplement can be thawed at 2–8°C overnight or quickly in a 37°C water bath for about 5 minutes, and then aliquotted and frozen at –5°C to –20°C to allow for preparation of smaller volumes of complete medium. Avoid repeated freezing and thawing.

Neural Expansion Medium (for 100 mL)

1. To prepare 100 mL of complete Neural Expansion Medium, aseptically mix the following components:

Neurobasal Medium	49 mL
Advanced DMEM/F-12	49 mL
Neural Induction Supplement	2 mL

2. Complete Neural Expansion Medium can be stored at 2–8°C in the dark for up to 2 weeks. Warm the Neural Expansion Medium in a 37°C water bath for 5–10 minutes before using.

ROCK inhibitor Y27632 solution (5 mM)

1. To prepare 5 mM ROCK inhibitor Y27632 solution, aseptically mix the following components:

ROCK Inhibitor Y27632	1 mg
Distilled water	0.625 mL

2. After dissolving, filter through a 0.22 µm filter, aliquot 20–50 µL into sterile tubes, and store at –5°C to –20°C.

   Note: The molecular weight of Y27632 is 320.26; however, variation in molecular weight may occur between lots, depending on the water content.

Coat culture vessels with Geltrex matrix

1. Thaw a vial of Geltrex LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix at 2–8°C overnight.

   Note: Thawed Geltrex matrix can be aliquotted and then frozen at –5°C to –20°C, or stored at 2–8°C for up to 2 weeks. Avoid repeated thawing and freezing.

2. To create a working solution, dilute the thawed Geltrex matrix solution 1:100 with cold Neurobasal Medium or DMEM/F-12 on ice.

3. Quickly cover the whole surface of each culture vessel with the Geltrex matrix solution (see Table 5.1).

4. Incubate the culture vessels in a 37°C, 5% CO_2 incubator for 1 hour.

5. The culture vessels are now ready for use. Just before use, aspirate the diluted Geltrex matrix solution from the culture vessels. Cells can be plated directly onto the Geltrex matrix–coated culture vessels without rinsing. Coated culture vessels can also be stored at 2–8°C for up to 1 week. When storing, seal culture vessels with Parafilm™ laboratory film to prevent drying. Before using, warm up the coated culture vessels stored at 2–8°C at room temperature for 30 minutes.

Table 5.1. Required volume of Geltrex matrix solution.

Culture vessel	Approximate surface area (cm^2)	Diluted Geltrex matrix volume (mL)
4-well chamber slide	1.8 cm^2/well	0.3 mL/chamber
6-well plate	9.6 cm^2/well	1 mL/well
35 mm dish	11.8 cm^2	1 mL
60 mm dish	20 cm^2	2 mL
100 mm dish	60 cm^2	5 mL

Methods

Culture and prepare hPSCs for neural induction

1. Start with high-quality hPSCs (with minimal or no differentiated colonies) cultured in feeder-free conditions such as in Essential 8 Medium on vitronectin or in StemFlex Medium on Geltrex matrix.

   Important: The quality of the PSCs (with minimal or no differentiated colonies) is critical for efficient neural induction. Remove any differentiated and partially differentiated colonies before passaging PSCs. Differentiated colonies can be marked by using a Nikon™ microscopy object marker (Nikon Instruments Inc., Cat. No. MBW10020) with a Nikon™ microscopy C-OA 15 mm objective adapter (Nikon Instruments Inc., Cat. No. MXA20750).

   Note: hPSCs cultured on mouse embryonic fibroblasts can also be used for neural induction.

2. Coat 6-well plates with the same coating material on which your PSCs are cultured.

3. When the PSCs reach ~70–80% confluency, dislodge PSCs to generate cell clumps for passaging by following the appropriate PSC subculture protocol. Follow steps 3a–e to estimate the cell concentration of the suspension of PSC clumps before plating.

   a. Generate a PSC cell suspension, then transfer a portion of the cell suspension to a 15 mL conical tube (for example, transfer 1 mL of a 6 mL PSC suspension prepared from one well of a 6-well plate) to estimate the total cell number of the PSC cell suspension.

   b. Centrifuge the 15 mL conical tube with the cells at 200 x *g* for 3 minutes, and aspirate the supernatant.

   c. Add 1 mL of prewarmed StemPro Accutase Cell Dissociation Reagent to the 15 mL conical tube containing the cells, then incubate for 5 minutes at 37°C.

d. Vigorously pipet the cells up and down with a 1 mL pipette 5 times to dissociate the cells into a single-cell suspension.

e. Determine the total number of cells using your preferred method. If the total number of cells in 1 mL of StemPro Accutase reagent is 1 x 10^6, the total number of cells in the remaining 5 mL of PSC suspension is: 1 x 10^6 x 5 = 5 x 10^6.

4. Aspirate the coating solution and add 2.5 mL PSC culture medium into each well of coated 6-well plates.

5. Gently shake the conical tube containing the PSC cell suspension and plate the PSCs into each well at 2.5 x 10^5–3 x 10^5 PSCs per well. For example, add 0.25–0.3 mL of PSC suspension to each well if the concentration of PSC suspension is 1 x 10^6 cells/mL.

6. Move the plates in several quick back-and-forth and side-to-side motions to disperse the cells across the surface, then gently place the plates in a CO_2 incubator.

Note: The split ratio varies, depending on the confluency of PSCs before splitting and variability in PSC lines. Neural induction starts on day 1 of PSC splitting (about 24 hours after passaging). The starting density of PSCs should be about 15–25% confluency. When passaging PSCs, cells should be plated as small clumps and not as a single-cell suspension. Avoid plating PSC as single cells, as that can lead to increased cell death.

Note: To prevent cell death, you may treat the cells overnight with 10 µM of ROCK inhibitor Y27632 solution by adding it to the PSC medium at the time of splitting.

Neural induction

The NSC derivation workflow is shown in Figure 5.3.

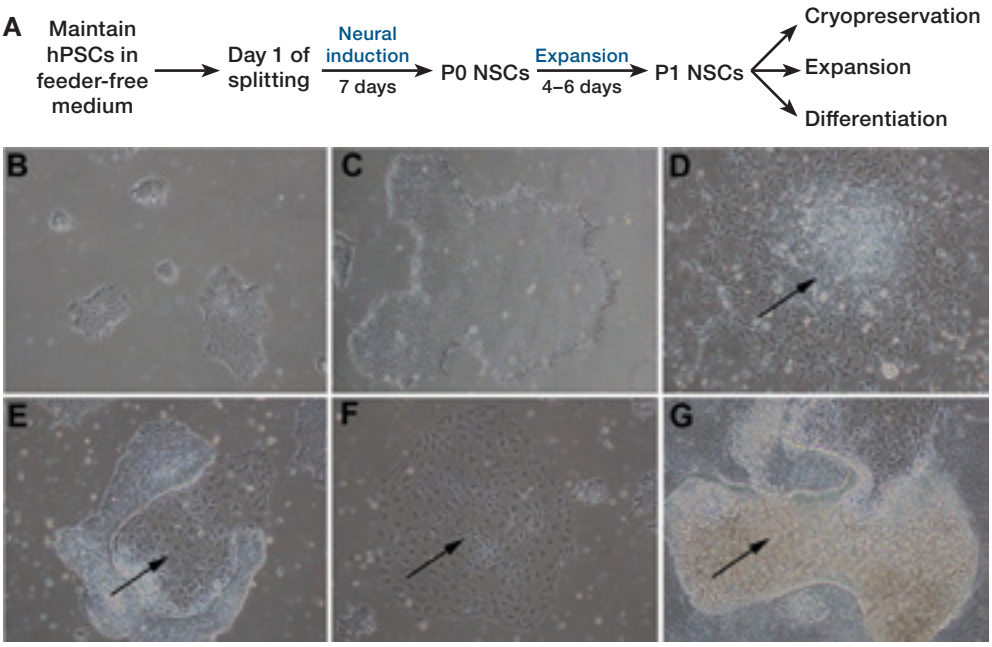


Figure 5.3. Derivation from and differentiation of PSCs. (A) Workflow of NSC derivation from PSCs. **(B)** hPSCs cultured in feeder-free condition on day 1 of splitting with 15–25% confluency. **(C)** Cells under neural differentiation on day 2 of neural induction. **(D–G)** Representative images of nonneural differentiation (indicated by arrows) on day 2 of induction, due to the use of poor-quality starter PSCs during neural induction.

1. On day 1 of PSC splitting (15–25% confluency, Figure 3B), aspirate the spent medium to remove unattached cells, and add 2.5 mL of prewarmed complete PSC Neural Induction Medium to each well of the 6-well plates. Return the plates to the incubator.

2. On day 2 of neural induction, confirm that the morphology of cell colonies is uniform (Figure 5.3C). If the quality of starting PSCs is good before neural induction, the morphology of the cells should appear as in Figure 5.3C.

   If the quality of starter PSC is poor, the cultures will be dominated by a number of nonneural colonies as shown in Figure 5.3D–G. In this case, there are two options.

   If there are few areas in dish exhibiting this morphology, cultures can be continued after removing such nonneural differentiated colonies. To remove nonneural differentiated colonies, mark all nonneural differentiation colonies with a microscopy marker. Tilt the plate and remove all unwanted colonies at the upper half of the well by using a glass Pasteur pipette to aspirate the cells from the marked colonies. Turn the plate 180 degrees, and repeat. Perform this step one well at a time to prevent cells from becoming too stressed without medium.

   If there are a large number of such nonneural colonies, we recommend discarding the cultures and starting with high-quality PSCs.

3. On day 2 (about 48 hours after switching to PSC Neural Induction Medium), change the medium by aspirating spent medium from each well. Add 2.5 mL of prewarmed complete Neural Induction Medium per well.

4. On day 4 of neural induction, cells will be reaching confluency (Figure 5.4A). Mark all colonies if any nonneural differentiation is noticed, and remove such unwanted colonies with a glass Pasteur pipette. Aspirate the spent medium from each well. Add 5 mL of prewarmed complete PSC Neural Induction Medium per well.

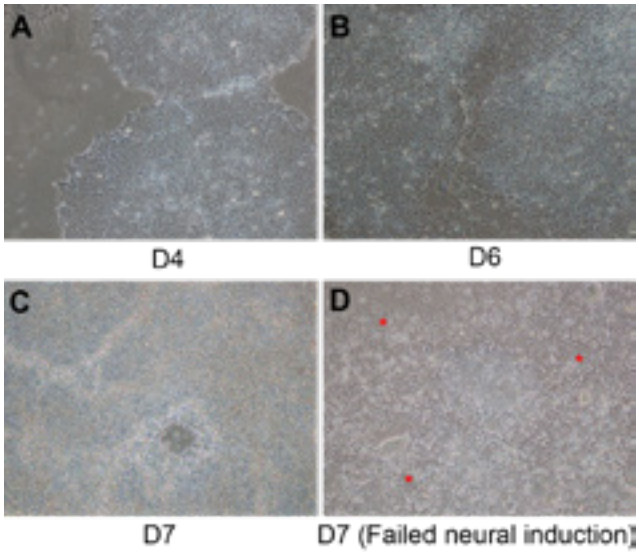


Figure 5.4. Cell morphology during neural induction. The morphology of cells on **(A)** day 4, **(B)** day 6, and **(C)** day 7 of neural induction. **(D)** The morphology of cells on day 7 of culture due to omission of key components in the neural induction supplement.

* Indicates flat nonneural cells.

5. On day 6 of neural induction, cells should be near maximal confluency (Figure 4B). Remove any nonneural differentiated cells and add 5 mL of complete PSC Neural Induction Medium into each well.

Note: Due to high cell density in the culture from day 4 onwards, doubling the volume of PSC Neural Induction Medium is very critical for cell nutrition. Also, minimal cell death should be observed from days 4 to 7 after neural induction. If the color of cells turns brownish with many floating cells during days 4 to 7 of neural induction, it indicates that the starting density of PSCs was too high. In this case, change the Neural Induction Medium every day using 5 mL per well.

Harvest and expand P0 NSC

On day 7 of neural induction (Figure 5.4C), NSCs (P0) are ready to be harvested and expanded.

1. Prepare Geltrex matrix–coated vessels before performing the next steps.
2. Use a glass Pasteur pipette to aspirate the spent PSC Neural Induction Medium from the 6-well plate(s) to be passaged.
3. Gently add 2 mL of DPBS with no calcium or magnesium to each well of a 6-well plate, and aspirate the DPBS to rinse the cells.

   Note: Add DPBS towards the wall of the well to avoid cell detachment.
4. Add 1 mL of prewarmed StemPro Accutase Cell Dissociation Reagent to each well of the 6-well plates and incubate for 5–8 minutes at 37°C until most cells detach from the surface of the culture vessels.
5. Use a cell scraper to detach the cells from the surface of the plates.
6. Use a 5 mL pipette to transfer the cell clumps to a 15 or 50 mL conical tube.
7. Add 1 mL of DPBS to each well of the 6-well plates to collect residual cells, then transfer the cell suspension to the conical tube.
8. Gently pipet the cell suspension up and down 3 times with a 5 or10 mL pipette to break up the cell clumps.
9. Pass the cell suspension through a 100 µm strainer and centrifuge the cells at 300 x *g* for 4 minutes.
10. Aspirate the supernatant, resuspend the cells with DPBS (3–5 mL of DPBS for all cells from 1 well of a 6-well plate), then centrifuge the cells at 300 x *g* for 4 minutes.
11. Aspirate the supernatant, resuspend the cells in prewarmed complete Neural Expansion Medium (for example, use 1 mL for all cells from 1 well of a 6-well plate).
12. Determine the cell concentration using your preferred method.
13. Dilute the cell suspension with prewarmed complete Neural Expansion Medium to 2 x 10^5–4 x 10^5 cells/mL.
14. Add ROCK inhibitor solution to the cell suspension to a final concentration of 5 µM.
15. Aspirate the Geltrex matrix solution from the Geltrex matrix–coated vessels and add the diluted cell suspension to each culture plate/dish to plate the cells at a density of 0.5 x 10^5 –1 x 10^5 cells/cm^2.

Culture vessel	Approximate surface area (cm^2)	Cell suspension volume (mL)
4-well chamber slide	1.8 cm^2/well	0.46 mL/chamber
6-well plate	9.6 cm^2/well	2.5 mL/well
35 mm dish	11.8 cm^2	3 mL
60 mm dish	20 cm^2	5 mL
100 mm dish	60 cm^2	15 mL

16. Move the culture vessels in several quick back-and-forth and side-to-side motions to disperse the cells across the surface, then gently place the vessels in the incubator.

    Note: Avoid splashing the medium on the outsides of the well to avoid contamination.
17. After overnight incubation, change to complete Neural Expansion Medium to eliminate the ROCK inhibitor solution. Continue to exchange the Neural Expansion Medium every other day thereafter. Do not add ROCK inhibitor solution during this step.

18. Usually, NSCs reach confluency on days 4–6 after plating (Figure 5.5). When NSCs reach confluency, they can be further expanded in complete Neural Expansion Medium. Expanded NSCs can be cryopreserved (see protocol on next page) or differentiated into specific neural cell types following the protocol of your choice.

    Note: After dissociation of P0–P4 NSCs, the overnight treatment with the ROCK inhibitor Y27632 at a final concentration of 5 µM is required at the time of plating to prevent cell death for both expansion and differentiation into glial and neuronal cells (Figure 5.5C).

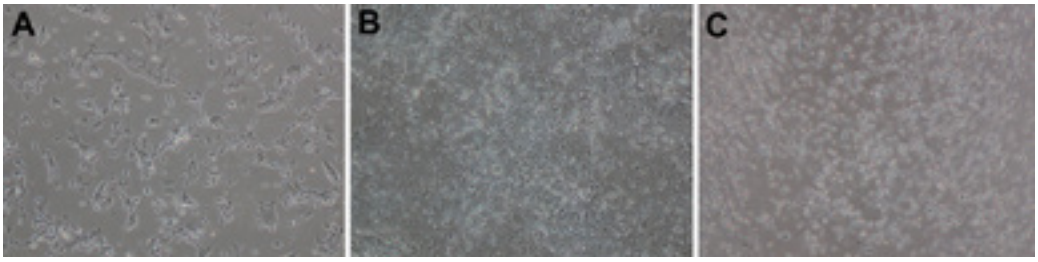


Figure 5.5. Expansion and differentiation of NSCs. (A) P0 NSCs plated at a density of 1 x 10^5 cells/cm^2 on day 1 of plating with Y27632 treatment. **(B)** NSCs reach confluency on day 5 of plating. **(C)** Without Y27632 treatment, widespread cell death takes place on day 1 of NSC plating.

Important: If you observe heterogeneous cell morphology with contaminating nonneural cells during NSC expansion (Figure 5.6A), see "Troubleshooting heterogeneous cell morphology" on page 121 and follow the procedure to diminish the number of nonneural cells.

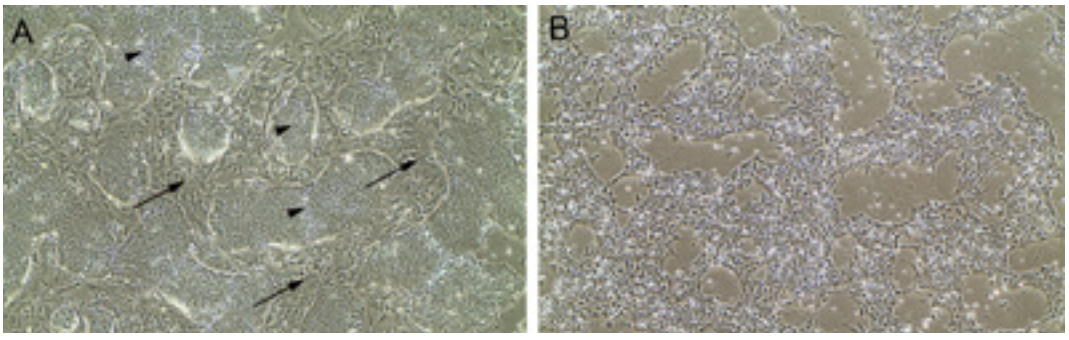


Figure 5.6. Contamination with nonneural cells. (A) P1 NSCs at day 4 of P0 NSC plating showed a heterogeneous morphology with flat nonneural cells (indicated by arrows) and compact NSCs (indicated by arrowheads) **(B)** P2 NSCs at day 2 of replating of P1 NSCs showed a relative homogeneous NSC morphology after the sequential treatment with StemPro Accutase Cell Dissociation Reagent as described on page 121.

Cryopreserve NSCs

1. Passage NSCs to at least P1 before cryopreservation.
2. When NSCs reach confluency, warm the appropriate amount of StemPro Accutase Cell Dissociation Reagent in a 37°C water bath.

Culture vessel	Approximate surface area	StemPro Accutase reagent volume
6-well plate	9.6 cm^2/well	1 mL/well
35 mm dish	11.8 cm^2	1 mL
60 mm dish	20 cm^2	2 mL
100 mm dish	60 cm^2	5 mL

3. Aspirate the spent medium and add prewarmed StemPro Accutase reagent to culture vessels according to the table above.
4. Incubate for 3–5 minutes at 37°C until all cells detach from the surface of culture vessels.
5. Transfer the cells into a 15 or 50 mL conical tube using a 5 mL pipette.
6. Add the appropriate amount of DPBS to each well of the vessel (for example, add 1 mL to 1 well of a 6-well plate) to collect the residual cells, then add the cell suspension to the tube.
7. Triturate the cell suspension 3 times with a 5 mL or 10 mL pipette to break up the cell clumps.
8. Centrifuge the cells at 300 x *g* for 4 minutes and aspirate the supernatant.
9. Resuspend the cells with the appropriate amount of DPBS and centrifuge the cells at 300 x *g* for 4 minutes.
10. Aspirate the supernatant and resuspend the cells with the appropriate amount of Neural Expansion Medium.
11. Determine the cell concentration using your preferred method.
12. Dilute the cell suspension with Neural Expansion Medium to 2 x 10^6–4 x 10^6 cells/mL.
13. Add the same volume of Neural Expansion Medium containing 20% DMSO.
14. Allocate 1 mL cell suspension into each cryotube and freeze cells at –80°C overnight in Mr. Frosty Freezing Containers with isopropanol.
15. After freezing cells overnight, transfer the cells into a liquid nitrogen tank for long-term storage.

Recover cryopreserved NSCs

1. Prepare Geltrex matrix–coated dishes as previously described.
2. Remove cryotubes of NSCs from the liquid nitrogen storage tank using metal forceps.
3. Immerse the vial in a 37°C water bath without submerging the cap. Swirl the vial gently.
4. When only an ice crystal remains, remove the vial from the water bath.
5. Spray the outside of the vial with 70% ethanol and place it in the cell culture hood.
6. Pipet the cells gently into a sterile 15 mL conical tube using a 1 mL pipette.
7. Add 1 mL of DPBS into the vial to collect the resident cells. Transfer the DPBS from the vial and add it drop-by-drop to the 15 mL conical tube. While adding, gently move the tube back and forth to mix the NSCs to reduce osmotic shock to cells.
8. Centrifuge the cells at 300 x *g* for 5 minutes and aspirate the supernatant.
9. Resuspend the cell pellet in DPBS. Centrifuge at 300 x *g* for 5 minutes and aspirate the supernatant.
10. Resuspend the cell pellet in the appropriate amount (for example, 1 mL for all NSCs from 1 vial) of prewarmed Neural Expansion Medium.
11. Determine the cell concentration using your preferred method.
12. Dilute the cell suspension with prewarmed Neural Expansion Medium into a solution containing 2 x 10^5–4 x 10^5 cells/mL.

    Note: If the NSCs were cryopreserved before passage 4, add ROCK inhibitor Y27632 solution to a final concentration of 5 µM to the cell suspension to prevent cell death.
13. Aspirate the Geltrex matrix solution from the Geltrex matrix–coated vessels and add the appropriate amount of diluted cell suspension into each culture vessel to plate cells at a density of 0.5 x 10^5–1 x 10^5 cells/cm^2.

Culture vessel	Approximate surface area (cm^2)	Cell suspension volume (mL)
4-well plate	1.8 cm^2/well	0.46 mL/chamber
6-well plate	9.6 cm^2/well	2.5 mL/well
60 mm dish	20 cm^2	5 mL
100 mm dish	60 cm^2	15 mL
T-25 flask	25 cm^2	6.5 mL
T-75 flask	75 cm^2	19.5 mL

14. Move the culture vessels in several quick back-and-forth and side-to-side motions to disperse the cells across the surface, then gently place the vessels in the incubator.

    Note: Avoid splashing the medium onto the outsides of the well to avoid contamination.
15. On day 2 of cell plating, exchange the Neural Expansion Medium. Continue to exchange the medium every other day until the NSCs reach confluency and are ready for further expansion.

    Note: If NSCs were under P4, the overnight treatment with the ROCK inhibitor Y27632 is required at the time of NSC plating to prevent cell death. At day 1 after NSC plating, replace the medium with complete Neural Expansion Medium to eliminate Y27632 from the culture. Do not add ROCK inhibitor Y27632 during this step.

Stain NSCs for pluripotent (Oct4) and NSC (nestin, Pax6, Sox1, and Sox2) markers

Note: We recommend using the Human Neural Stem Cell Immunocytochemistry Kit (Cat. No. A24354). This complete immunocytochemistry kit contains primary antibodies for four common NSC markers (nestin, Pax6, Sox1, and Sox2), a matching set of Alexa Fluor labeled secondary antibodies, a nuclear DNA stain (DAPI), and all of the buffers necessary for performing the staining protocol. In addition, pluripotency marker Oct4 (Cat. No. A13998), which is compatible with the reagents in the NSC immuncytochemistry kit, can be used as a negative control marker for NSCs.

1. Plate the dissociated NSCs at a density of 1 x 10^5–3 x 10^5 cells/cm^2 into 4-well chamber slides according to the NSC expansion protocol.

   Note: For P0–P4 NSCs, treat with 5 µM ROCK inhibitor Y27632 to prevent cell death.

2. Follow the protocol for the Human Neural Stem Cell Immunocytochemistry Kit to complete staining (Figure 5.7).

3. After staining is completed, apply a suitable quantity (about 1 drop) of ProLong Gold Antifade Reagent to the space of each well, cover with a coverslip, and air-dry the slide in the dark overnight before imaging.

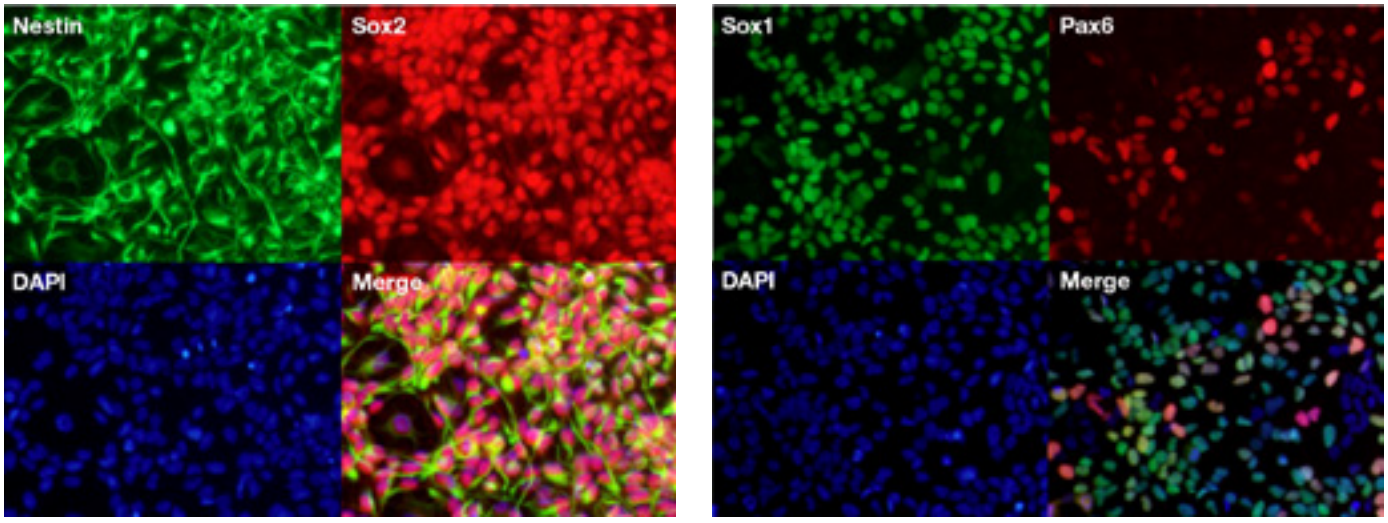


Figure 5.7. Neural stem cells derived from an iPSC line using PSC Neural Induction Medium (Cat. No. A1647801) were stained for NSC markers nestin and Sox2 (antibody combination 1) or Sox1 and Pax6 (antibody combination 2) and nuclear DNA (DAPI) using the Neural Stem Cell Immunocytochemistry Kit (Cat. No. A24354). Antibody combination 1 (left): nestin and Sox2 with additional DAPI (nuclear DNA) staining. Antibody combination 2 (right): Sox1 and Pax6 with additional DAPI (nuclear DNA) staining.

Note: Not all NSCs will stain positive for Pax6—this is normal and to be expected (i.e., only NSCs that possess forebrain fate commitment will stain positive). In our experience, ~15–50% of NSCs generated using PSC Neural Induction Medium stain positive for Pax6.

Troubleshooting

The table below lists some potential problems and solutions that may help you troubleshoot your neural induction experiments.

Observation	Recommended action
The starting density of human PSCs is too low or too high	Due to variable parameters such as the confluency of PSCs, cell clump passaging, cell attachment, and the property of different PSC lines, it may be difficult to determine the splitting ratio. By following the protocol to estimate cell number of PSC clumps before plating, PSC should be 15–25% confluent on day 1 of PSC plating (day 0 of neural induction).
Too many colonies with nonneural differentiation morphology in culture plate	Select and maintain the high quality of PSCs before starting neural induction.
Cell detachment during neural induction	For PSCs cultured in Essential 8 Medium, cells may detach during neural induction. To prevent cell detachment, coat culture plate with 10 µg/mL of vitronectin when splitting PSCs.
Extensive cell death during the late stage of neural induction	Check whether cells are overconfluent. If so, change the PSC Neural Induction Medium every day using 5 mL per well of a 6-well plate.
Extensive cell death after plating dissociated NSCs for expansion and differentiation	Check whether ROCK inhibitor Y27632 is added to the cell suspension at final concentration of 5 µM if NSCs are under P4. NSCs from some human PSC lines may be more sensitive to dissociation. For NSCs derived from those PSC lines, the overnight treatment with Y27632 after P4 decreases cell death after replating.
NSCs reach confluency in 2–3 days after passaging	NSCs derived from some human PSC lines may have an increased proliferation rate. In this case, decrease NSC plating density to 5 x 10^4 cells/cm^2.
Extensive cell death after thawing and plating cryopreserved NSCs	Check whether NSCs are treated with the ROCK inhibitor Y27632 after plating if NSCs are cryopreserved under P4.
Abnormal staining pattern of NSCs stained with antibodies against neural markers	Antibodies which are not stored or handled properly may result in loss of staining quality and should be replaced. Note that nestin should stain filaments in the cytoplasm, whereas Pax6, Sox1, and Sox2 should stain the nucleus. Oct4 should stain the nucleus of PSCs but not NSCs.

Frequently asked questions

Question	Answer
Does PSC Neural Induction Medium work for both human ESCs and iPSCs?	Yes, PSC Neural Induction Medium works for both human ESCs and iPSCs.
Does PSC Neural Induction Medium work for hPSCs cultured on feeders (mouse embryonic fibroblasts, MEFs) and feeder-free conditions?	PSC Neural Induction Medium has been tested on human PSCs cultured in both feeder-free and feeder-based conditions. To eliminate MEF contamination, we strongly recommend starting neural induction using human PSCs cultured in feeder-free conditions such as Essential 8 Medium.
Which format of cultures should I use for neural induction?	Neural induction can be started with human PSCs cultured on 6-well plates or culture dishes. We do not recommend starting neural induction using PSCs cultured in flasks, because it is difficult to remove nonneural differentiated colonies from PSCs in flasks.
Can PSC Neural Induction Medium convert mouse PSCs into NSCs?	PSC Neural Induction Medium has not been tested on mouse PSCs, and it is not recommended for the conversion of mouse PSCs into NSCs.
Can NSCs derived by PSC Neural Induction Medium be differentiated into cells in the peripheral nervous system (PNS)?	A number of NSC types derived by PSC Neural Induction Medium can be differentiated into neurons and glial cells in the CNS. Sensory, sympathetic and parasympathetic neurons, as well as Schwann cells in the PNS, are derived from precursor cells of the neural crest.

Troubleshooting heterogeneous cell morphology

If you observe heterogeneous cell morphology (see Figure 5.6A) with the contamination of nonneural cells during NSC expansion, the following steps can be used to diminish the number of nonneural cells (see Figure 5.6B).

1. At day 3–4 of P0 or P1 NSC replating with 90–100% cell confluency, aspirate the spent medium, wash once with DPBS, then add the appropriate amount of prewarmed StemPro Accutase Cell Dissociation Reagent to each culture vessel.

Culture vessel	Approximate surface area (cm^2)	StemPro Accutase reagent volume (mL)
6-well plate	9.6 cm^2/well	1 mL/well
35 mm dish	11.8 cm^2	1 mL
60 mm dish	20 cm^2	2 mL
100 mm dish	60 cm^2	5 mL

2. Mount the culture vessel under a phase contrast microscopy to observe the cells.
3. After the incubation in StemPro Accutase reagent for 3–4 minutes at room temperature, nonneural cells with flat morphology will detach from the culture vessel, while densely packed neural cells remain attached. The incubation time may be adjusted for different cell lines to avoid detaching all cells from the culture vessel.
4. Gently aspirate the StemPro Accutase reagent from the culture vessel.
5. Gently add the appropriate amount of DPBS toward the wall of the culture vessel to rinse off detached cells.
6. Aspirate the DPBS, add an appropriate amount of prewarmed StemPro Accutase reagent into each culture vessel according to the table above, then incubate at 37°C for 3–4 minutes until most of the cells detach from the surface of the culture vessels.
7. Follow steps 5 through 18 in “Harvest and Expand P0 NSC” on page 115 to further plate and expand NSCs.

5.3 Maturation and maintenance of PSC-derived neurons using the B-27 Plus Neuronal Culture System

Introduction

The Gibco™ B-27™ Plus Neuronal Culture System comprises B-27™ Plus Supplement (50X) and Neurobasal™ Plus Medium. This system represents an evolution of the neuronal cell culture products B-27™ Supplement and Neurobasal™ Medium, which are designed to provide maximum *in vitro* survival, improved maturation, and functionality of PSC-derived neurons. This section includes the recommended protocol with specific guidance for using the B-27 Plus Neuronal Culture System with the most frequently used PSC-derived neuron models, including monolayer NSC, rosette-derived NSC, and factor-driven induced neurons ("iN"). For use of these reagents with primary neuron applications, see the B-27 Plus Neuronal Culture System user guide (Pub. No. MAN0017319).

Before you begin

Prepare 200 mM ascorbic acid

Dissolve 1 g of ascorbic acid 2-phosphate sesquimagnesium salt hydrate in 17.3 mL distilled water, then filter through a 0.22 µm filter.

Note: If not using solution right away, aliquot 100–200 µL each into sterile tubes, and store at –5 to –20°C in the dark for up to 6 months.

Prepare B-27 Plus Neuronal Maturation Medium

Thaw the frozen B-27 Plus Supplement (50X) at room temperature for ~1 hour or overnight at 2–8°C.

Important: Do not thaw the frozen supplement at 37°C.

Mix the following components:

Reagent	Volume
Neurobasal Plus Medium	96 mL
B-27 Plus Supplement (50X)*	2 mL
GlutaMAX Supplement	1 mL
CultureOne Supplement (100X)*	1 mL
Ascorbic acid (200 mM)	100 µL

* Supplement can be thawed at room temperature for ~1 hour or 2–8°C overnight, and then aliquotted and frozen at –5 to –20°C to allow for the preparation of smaller volumes of complete medium. Avoid repeated thawing and freezing. Once thawed do not leave thawed supplement at 4°C for more than 2 weeks.

B-27 Plus Neuronal Maturation Medium can be stored at 2–8°C in the dark for up to 2 weeks. Warm the medium in a 37°C water bath for 5–10 minutes before using.

Coating of culture plates

Coat plates with poly-D-lysine

1. Dilute the poly-D-lysine solution in sterile DPBS to prepare a 50 µg/mL working solution.
2. Coat the surface of the culture vessel with the working solution of poly-D-lysine (e.g. 50 µL/well of a 96-well plate).
3. Incubate the vessel at room temperature for 1 hour.
4. Remove the poly-D-lysine solution and rinse culture surface 3 times with a large volume of distilled water (e.g. 100 µL/well of a 96-well plate).

   Make sure to rinse the culture vessel thoroughly as excess poly-D-lysine solution can be toxic to the cells.
5. Remove final distilled water rinse and leave the coated culture vessel uncovered in the laminar hood to dry.

   The culture surface will be fully dry after 2 hours.

   Plates can be used immediately or stored at 4°C. For storage at 4°C tightly wrap the vessel with Parafilm™ film and use within one week of coating.

Add laminin coating

1. Thaw a vial of laminin at room temperature.

   Note: Thawed laminin can be aliquotted and stored at –80°C. Avoid repeated thawing and freezing.
2. Dilute the thawed laminin solution to 3 µg/mL with sterile distilled water, to create a working solution.
3. Add laminin solution to a poly-D-lysine–coated culture vessel to cover the whole surface, and incubate in a 37°C, 5% CO_2 incubator for 1 hour.

4. Immediately prior to seeding cells aspirate laminin solution from coated culture vessel.

Culture neurons

1. Start with PSC or NSC population then differentiate cells toward a neural lineage.

   NSCs must be cultured in a neuronal differentiation medium for 3–7 days before switching to B-27 Plus Neuronal Maturation Medium for neuronal maturation and maintenance (see Figure 5.8).

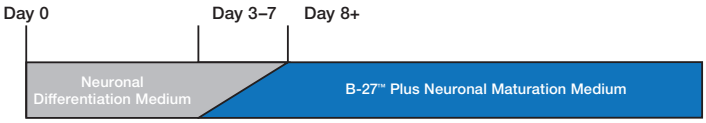


Figure 5.8. Culture NSCs for 3–7 days in Neuronal Differentiation Medium before transitioning to B-27 Plus Neuronal Maturation Medium.

2. Once cells adopt a neuronal morphology (see Figure 5.9), remove half of the spent medium and replace it with an equal volume of prewarmed fresh complete B-27 Plus Neuronal Maturation Medium.

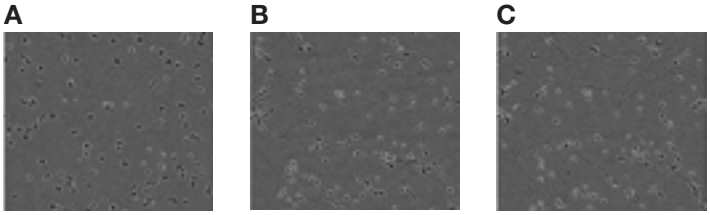


Figure 5.9. NSC differentiation and guidance on switching to B-27 Plus Neuronal Maturation Medium. (A, B) NSCs in the initial stages of neuronal differentiation. **(C)** Cells ready to switch to B-27 Plus Neuronal Maturation Medium. Cells have a neuronal-like morphology with neurites extending out and contacting neighboring cells.

3. Change spent medium every 3–4 days thereafter. (For high-density cultures, change medium every 2–3 days.)

   When changing medium, remove half of the spent medium from each well and add the same volume of prewarmed fresh B-27 Plus Neuronal Maturation Medium to each well, and return the plates to the 37°C, 5% CO_2 incubator.

4. Maintain maturing neurons with B-27 Plus Neuronal Maturation Medium for 3–10 weeks or longer, depending on NSC lines and the purpose of experiments.

   Note: Differentiating neurons detach easily. When removing spent medium, do not touch cells with pipette tips. Also, add fresh medium gently toward the wall of culture plates.

Troubleshooting

Observation	Possible cause	Recommended action
Flat cells	Nonneural cells in starting NSC population	Check the purity of NSCs derived from hPSCs. A population that is >90% Sox1-positive will reduce the occurrence of nonneural cell types.
		Treat cells with antimitotic compounds such as FUDR or AraC.
The media has turned yellow	Improved cell survival leads to greater media consumption	Change medium more frequently.
		Reduce seeding density of original number of cells.
	Serum or growth factors were added to the media	Don't add serum. B-27 Plus Neuronal Culture System has been optimized to use without added serum or growth factors.
Cultures show clumped morphology	Problem with substrate coating	Reduce laminin coating concentration (1–3 µg/mL recommended).
		Reduce seeding density of original number of cells.
		Check quality of poly-D-lysine coating.
		Adding laminin to refeed after >2 weeks may help.
	Nonneural cells displacing neurons from substrate	Check the purity of NSCs derived from hPSCs.
		Try increasing Gibco™ CultureOne™ Supplement concentration in the medium.

Guidelines for use of differentiation media and maturation media

There are a variety of methods to derive NSCs from PSC lines. Depending on the derivation method, the NSC population may have varied requirements for efficient neuronal differentiation.

When starting with NSCs we recommend an NSC neuronal differentiation medium for 3–7 days. Once cells have adopted a neuronal-like morphology (see Figure 5.9), cultures should be transitioned to B-27 Plus Neuronal Maturation Medium for continued maturation and maintenance of the neuronal cultures.

Methods to generate neural progenitors using NSC derived from either:

- Monolayer-based differentiation such as (Neural Induction Medium)
- Neural rosette formation

Here we provide recommended medium conditions for efficient neuronal differentiation of NSCs derived by the monolayer method or rosette formation. The conditions presented are guidance examples based on internal optimization using these PSC-derived neuron systems with the B-27 Plus Neuronal Culture System. Different NSC derivation methods or cell lines may require some further optimization.

Guidelines for differentiation

Prepare Monolayer-Derived NSC Differentiation Medium

Mix the following components:

Reagent	Volume
Neurobasal Medium	95.9 mL
B-27 Plus Supplement*	2 mL
GlutaMAX Supplement	1 mL
CultureOne Supplement*	1 mL
Ascorbic acid (200 mM)	100 µL

* Supplement can be thawed at 2–8°C overnight or quickly in a 37°C water bath for about 5 minutes, and then aliquotted and frozen at –5 to –20°C to allow for the preparation of smaller volumes of complete medium. Avoid repeated thawing and freezing.

Prepare complete Rosette-Derived NSC Differentiation Medium

Mix the following components:

Reagent	Volume
DMEM/F-12 Medium	86.9 mL
StemPro hESC Supplement*	2 mL
BSA (25%)	7.2 mL
GlutaMAX Supplement	2.5 mL
CultureOne Supplement (100X)*	1 mL
GDNF Recombinant Human Protein (20 µg/mL)	100 µL
BDNF Recombinant Human Protein (20 µg/mL)	100 µL
dcAMP (500mM)	100 µL
Ascorbic acid (200 mM)	100 µL

* Supplement can be thawed at 2–8°C overnight or quickly in a 37°C water bath for about 5 minutes, and then aliquotted and frozen at –5 to –20°C to allow for the preparation of smaller volumes of complete medium. Avoid repeated thawing and freezing.

Plating and differentiation of NSCs

Differentiate NSCs

1. Dissociate expanded hPSC-derived NSCs in culture with Gibco™ StemPro™ Accutase™ Cell Dissociation Reagent or thaw frozen PSC-derived NSCs.
2. Resuspend dissociated or thawed NSCs with 5–10 mL of DPBS without calcium and magnesium.
3. Centrifuge the cells at 300 x *g* for 5 minutes and aspirate the supernatant.
4. Resuspend NSCs in 1–2 mL of prewarmed Neuronal Differentiation Medium.
5. Determine the concentration of viable cells using your preferred method.
6. Dilute the NSC suspension with prewarmed Neuronal Differentiation Medium to an appropriate density. NSCs can be seeded at a density in the range of 2–10 x 10^4 cells/cm^2—e.g., for a 24-well plate, 0.5 mL per well of a cell suspension of 1.6 x 10^5 cells/mL yields 4 x 10^4 cells/cm^2.
7. Aspirate the laminin solution from poly-D-lysine– and laminin-coated plates immediately before plating cells.
8. Gently mix the tube containing NSCs and add an appropriate amount of diluted cell suspension to each well of culture plates.
9. Move the culture plates in several quick back-and-forth, side-to-side motions to disperse NSCs across the surface, and place them gently into a 37°C, 5% CO_2 incubator.
10. At 2–3 days after NSC plating, if cells are not yet showing a neuronal morphology, remove half the volume of spent medium and add the same volume of prewarmed fresh Neuronal Differentiation Medium to each well of the plates, and return them to the 37°C, 5% CO_2 incubator.

Neuronal maturation

1. Once cells adopt a neuronal-like morphology 3–7 days after NSC plating, remove half the volume of spent medium and add the same volume of prewarmed fresh B-27 Plus Neuronal Maturation Medium to each well, and return them to the 37°C, 5% CO_2 incubator.
2. Change the spent medium every 3–4 days thereafter.

   To change the medium, remove half of the spent medium from each well and add the same volume of prewarmed fresh B-27 Plus Neuronal Maturation Medium to each well, and return them to the incubator.

   Note: The CultureOne Supplement is recommended for suppressing continued and undesired NSC proliferation during neural differentiation and maturation. For additional details, see CultureOne Supplement for Neuronal Cell Culture.

   Note: The B-27 Plus Neuronal Maturation Medium can also support improved functionality of factor-driven induced neuronal "iN" cells, which typically utilize overexpression of lineage-specific factors to rapidly induce neuronal cells. B-27 Plus Neuronal Maturation Medium is recommended for use following initial induction steps (~1 week from onset of protocol), for neuronal maturation. There are many variations of protocols for generating induced neurons. Optimal timing should be empirically determined by the user.

5.4 Dopaminergic neuron differentiation using the PSC Dopaminergic Neuron Differentiation Kit

The Gibco™ PSC Dopaminergic Neuron Differentiation Kit (Cat. No. A3147701) is a media system for the efficient differentiation of human pluripotent stem cells (hPSCs) into mature dopaminergic (DA) neurons in approximately 35 days. For more information, see “Workflow overview” on page 128.

Kit contents and storage

Kit contents

PSC Dopaminergic Neuron Differentiation Kit (Cat. No. A3147701) contains the following components.

Storage

Store the components of the PSC Dopaminergic Neuron Differentiation Kit as described below.

Component*	Storage	Amount	Cat. No.
Floor Plate Cell Specification Supplement (20X)	–20 to –5°C; protect from light	5 mL	A3146801
Floor Plate Cell Expansion Kit Contains:		1 kit	A3165801
Floor Plate Cell Expansion Base	2–8°C; protect from light	500 mL	A3147201
Floor Plate Cell Expansion Supplement (50X)	–20 to –5°C; protect from light	10 mL	A3147301
Dopaminergic Neuron Maturation Supplement (50X)	–20 to –5°C; protect from light	10 mL	A3147401

* Floor Plate Specification Supplement (20X) (Cat. No. A3146801), Floor Plate Cell Expansion Kit (Cat. No. A3165801), and Dopaminergic Neuron Maturation Supplement (50X) (Cat. No. A3147401) are also available separately.

Required materials not supplied with kit

- Essential 8 Medium (Cat. No. A1517001), Essential 8 Flex Medium (Cat. No. A2858501), or StemFlex Medium (Cat. No. A3349401)
- Vitronectin (VTN-N), recombinant human (Cat. No. A14700)
- Gibco™ Neurobasal™ Medium (Cat. No. 21103049) (base medium for specification)
- DMEM/F-12, GlutaMAX Supplement (Cat. No. 10565018) (base medium for maturation)
- Laminin (Cat. No. 23017015)
- DPBS, no calcium, no magnesium (Cat. No. 14190)
- Distilled water (Cat. No. 15230)
- StemPro Accutase Cell Dissociation Reagent (Cat. No. A11105)
- ROCK inhibitor Y27632 (Sigma-Aldrich, Cat. No. Y0503)
- DMSO, Hybri-Max™ grade (Sigma-Aldrich, Cat. No. D2650)
- BioCoat™ poly-D-lysine–coated plates (Corning, Cat. No. 354413, 354414, or 354640) or poly-D-lysine (Sigma, Cat. No. p7280 or p9155)—to prepare poly-D-lysine and laminin double-coated culture plates
- Thermo Scientific™ Nunclon™ Sphera™ Cell Culture Flasks (Cat. No. 174951 or 174952) (for suspension culture)
- Nunclon Sphera Dishes (Cat. No. 174930, 174931, or 174932) (for suspension culture)
- Human Dopaminergic Neuron Immunocytochemistry Kit (Cat. No. A29515) (for image-based analysis of intermediate floor plate progenitors and mature dopaminergic neurons)
- Thermo Scientific™ Mr. Frosty™ Freezing Container (Cat. No. 5100-0001)
- Cryogenic vials (for banking floor plate progenitor cells)
- 37°C humidified cell culture incubator with 5% CO_2
- Liquid nitrogen storage
- Centrifuge
- 37°C water bath
- 15 mL and 50 mL sterile polypropylene conical tubes
- 5, 10, 25, and 50 mL sterile pipettes

Workflow overview

The following figure summarizes the comprehensive workflow for the hPSC Dopaminergic Neuron Differentiation Kit.

During differentiation, hPSCs are first induced in Floor Plate Cell Specification Medium into midbrain-specified floor plate progenitor (FP) cells. Next, FP cells are expanded as adherent cultures in Floor Plate Cell Expansion Medium and then cultured in suspension to form spheres. Finally, the spheres are differentiated into mature dopaminergic neurons in Dopaminergic Neuron Maturation Medium. The entire differentiation workflow takes approximately 35 days.

Experimental outline

The following table describes the major steps that are required to differentiate hPSCs into mature dopaminergic (DA) neurons. See the specified pages for detailed instructions to perform each step.

Step	Action	Page
1	Prepare hPSCs for differentiation	134
2	Induce hPSCs into midbrain specified floor plate progenitor (FP) cells in complete Floor Plate Cell Specification Medium	135
3	Passage FP cells and start expansion as adherent culture in complete Floor Plate Cell Expansion Medium	138
4	Harvest FP cells to create a cell bank	141
5	Culture FP cells in complete Floor Plate Cell Expansion Medium to induce sphere formation	142
6	Culture cells in complete Dopaminergic (DA) Neuron Maturation Supplement to achieve differentiation into mature DA neurons	144

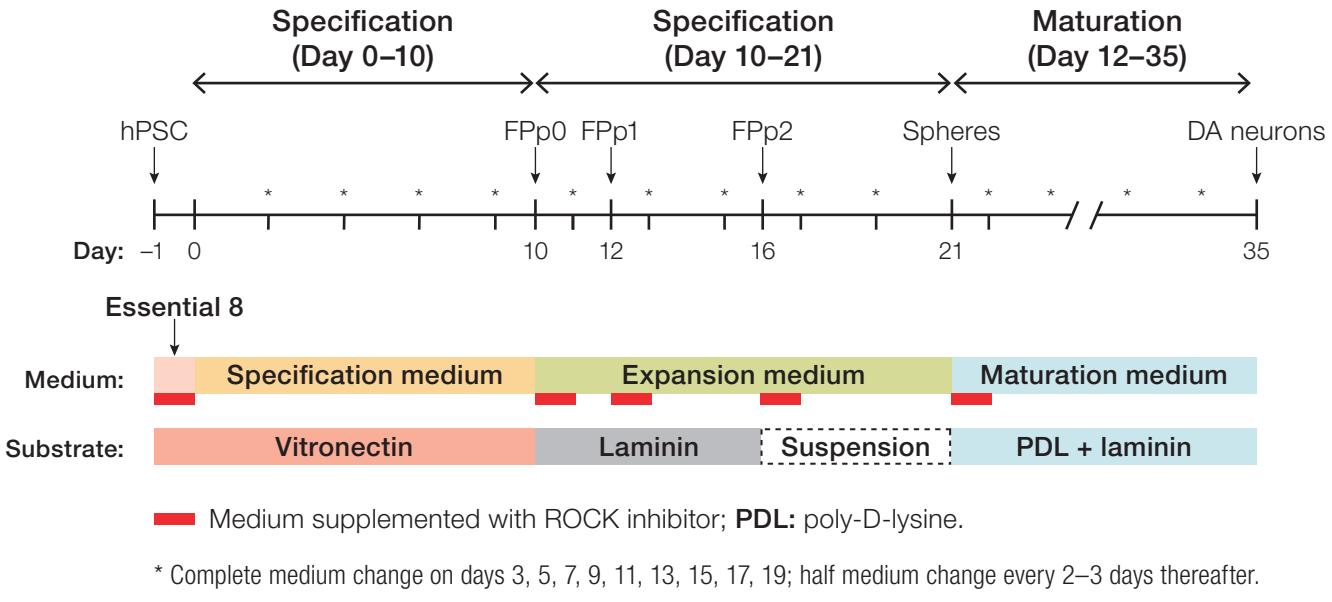


Figure 5.10. Workflow timeline for differentiation of hPSCs into mature DA neurons.

Methods: Important procedural guidelines

Culture conditions

Culture type: Adherent culture for the specification, expansion, and maturation; suspension culture for sphere formation

Recommended substrate: Vitronectin for FP specification, laminin for expansion, and poly-D-lysine and laminin for maturation

Important: The PSC Dopaminergic Neuron Differentiation Kit is compatible with hPSCs cultured in Essential 8, Essential 8 Flex, or StemFlex Media Systems. hPSCs co-cultured on MEF must be adapted to one of these culture media for at least 4 passages before differentiation. hPSCs cultured in mTeSR™1 medium (STEMCELL Technologies) can initiate differentiation directly, but require additional supplementation at the time of maturation. See the maturation section on page 144 for more information.

General cell handling and culture

Use high-quality human hPSCs (with minimal or no differentiated colonies) that are karyotypically normal and express pluripotency markers.

Before differentiation, culture hPSCs under feeder-free conditions on vitronectin-coated culture vessels in complete Essential 8, Essential 8 Flex, or StemFlex Medium. For details on feeder-free culture of hPSCs in these media systems, see section 2.2B.

Rapid media aspiration and addition can be detrimental to differentiation efficiency; add and remove media slowly, especially during specification.

For the specification of FP cells, use vitronectin-coated (10 µg/mL) culture plates (48-well plate or larger).

For the expansion of FP cells in adherent culture, use laminin-coated (10 µg/mL) culture plates (6-well plate or larger).

For the maturation of FP cells into DA neurons, use poly-D-lysine (100 µg/mL) and laminin (15 µg/mL) double-coated culture plates (96-well plate or larger).

Note: For convenience, you can apply laminin (15 µg/mL) to a commercially available poly-D-lysine–coated culture plate (see pages 132–133) to get double coating.

For suspension culture of spheres, use untreated Nunclon Sphera culture flasks or dishes to prevent spheres from merging or adhering to the culture vessel, which can greatly compromise downstream differentiation.

Note: A T-25 flask is the equivalent of 2.5 wells of a 6-well plate, and a T-75 flask is the equivalent of 7.5 wells of a 6-well plate.

For the characterization of FP cells by ICC, you can use a 48-well vitronectin-coated (1 µg/mL) plate. For downstream gene analysis by qPCR, you can use a 6-well, 12-well, or 24-well plate.

You can examine the culture for correct FP specification by ICC as early as 7 days after addition of specification medium.

Important: If marker expression is <60%, do not proceed to expansion and maturation. Instead, optimize the specification step (see “Note” on next page) or troubleshoot to improve specification efficiency first.

If the culture kinetics of your cell line differs from the culture kinetics of the reference ESC line H9, optimize the cell-seeding density to attain full confluency at day 7 for efficient specification before proceeding with the entire 35 days of differentiation.

To do this, set up replicate ESC cultures with varying seeding densities in a 48-well plate and carry out specification for up to 7 days with the goal of attaining full confluency at day 7. On day 7, perform ICC to evaluate the efficiency of FP specification.

Note: Each well of the 48-well plate requires 0.6 mL of specification medium for 7 days of specification. Since each well of a 6-well plate requires 15 mL of medium for the entire 10 days of specification, you may require additional specification medium for your experiments if you perform optimization. You can buy additional Floor Plate Specification Supplement (Cat. No. A3146801).

We strongly recommend freezing a portion of the FP cells to prepare a cell bank before initiating sphere culture. This will enable the optimization of culture conditions and help with troubleshooting, if experimental variation is observed.

Media

Important: Floor Plate Cell Specification (20X), Floor Plate Cell Expansion (50X), and Dopaminergic Neuron Maturation (50X) Supplements are temperature-sensitive. **Do not expose the supplements to 37°C for more than 1 hour.**

When preparing complete media, thaw the Floor Plate Specification Cell Supplement (20X), Floor Plate Cell Expansion Supplement (50X), and Dopaminergic Neuron Maturation Supplement (50X) at 4°C overnight, at room temperature (15–25°C) for 30 minutes, or at 37°C until only a small ice crystal remains.

You can store the thawed supplements at 4°C for use within 2 weeks, or aliquot and refreeze them at –20°C one time. **Do not thaw and refreeze the supplements more than one time.**

After preparation, store complete specification, expansion, and maturation media at 4°C and use within 2 weeks.

Avoid repeated warming of complete cell specification, expansion, and maturation media. On the day of use, aliquot the volume of complete media needed for that day and prewarm at 37°C before use.

100 mL of complete cell specification medium is sufficient to complete the specification of cells in one 6-well plate format for further expansion or 80 wells of a 48-well plate format for ICC characterization.

Addition of the ROCK inhibitor Y27632 to the culture medium (10 µM for PCSs or 5 µM for FP cells) is essential for cell survival and recovery after passaging.

Required seeding densities

See the table below for the number of cells and volume of medium that are required when plating FP cells. Use 2.0 x 10^5 cells/cm^2 for expansion and sphere formation, and 1.0 x 10^5–2.0 x 10^5 cells/cm^2 for maturation, as described in the protocol.

Culture vessel (approx. surface area)	Media volume (per well or dish)	Number of cells	
		1.0 x 10^5/cm^2	2.0 x 10^5/cm^2
96-well plate (0.32 cm^2/well)	100 µL	3.2 x 10^4	6.4 x 10^4
48-well plate (1 cm^2/well)	250 µL	1.0 x 10^5	2.0 x 10^5
24-well plate (2 cm^2/well)	500 µL	2.0 x 10^5	4.0 x 10^5
12-well plate (4 cm^2/well)	1 mL	4.0 x 10^5	8.0 x 10^5
6-well plate (10 cm^2/well)	2 mL	1.0 x 10^6	2.0 x 10^6
35 mm dish (10 cm^2/dish)	2 mL	1.0 x 10^6	2.0 x 10^6
60 mm dish (20 cm^2/dish)	5 mL	2.0 x 10^6	4.0 x 10^6
T-25 flask (25 cm^2/flask)	5 mL	2.5 x 10^6	5.0 x 10^6
100 mm dish (60 cm^2/dish)	10 mL	6.0 x 10^6	1.2 x 10^7
T-75 flask (75 cm^2/flask)	15 mL	7.5 x 10^6	1.5 x 10^7

Preparation of media, reagents, and culture plates

Complete Floor Plate Cell Specification Medium

1. Thaw the Floor Plate Cell Specification Supplement (20X) at 4°C overnight, at room temperature (15–25°C) for 30 minutes, or at 37°C until only a small ice crystal remains.
2. Add 5 mL of 20X Floor Plate Cell Specification Supplement to 95 mL of Neurobasal Medium and mix well.
3. Store the complete Floor Plate Cell Specification Medium at 4°C and use within 2 weeks. On the day of use, aliquot the volume that is needed for that day and warm at 37°C; avoid repeated warming.

Complete Floor Plate Cell Expansion Medium

1. Store Floor Plate Cell Expansion Base at 4°C and Floor Plate Cell Expansion Supplement (50X) at –20°C until use. Do not prepare the complete expansion medium until needed.
2. Thaw the Floor Plate Cell Expansion Supplement (50X) at 4°C overnight, at room temperature (15–25°C) for 30 minutes, or at 37°C until only a small ice crystal remains.
3. Remove 10 mL of medium from the bottle of Floor Plate Cell Expansion Base and discard.
4. Add 10 mL of Floor Plate Cell Expansion Supplement (50X) to the remaining 490 mL of Floor Plate Cell Expansion Base Medium and mix well.
5. Store the complete Floor Plate Cell Expansion Medium at 4°C and use within 2 weeks. On the day of use, aliquot the volume that is needed for that day and warm at 37°C; avoid repeated warming.

Complete Dopaminergic Neuron Maturation Medium

1. Store Dopaminergic Neuron Maturation Supplement (50X) at –20°C until use. Do not prepare the complete maturation medium until needed.
2. Thaw the Dopaminergic Neuron Maturation Supplement (50X) at 4°C overnight, at room temperature (15–25°C) for 30 minutes, or at 37°C until only a small ice crystal remains.
3. Add 10 mL of Dopaminergic Neuron Maturation Supplement (50X) to 490 mL of DMEM/F-12 and mix well.
4. Store the complete Dopaminergic Neuron Maturation Medium at 4°C and use within 2 weeks. On the day of use, aliquot the volume that is needed for that day and warm at 37°C; avoid repeated warming.

ROCK inhibitor Y27632 stock solution (10 mM)

1. To prepare 10 mM ROCK inhibitor Y27632 solution, add 10 mg of Y27632 to 3.125 mL of distilled water. Mix well until dissolved.

2. After dissolving, filter through a 0.22 µm filter, aliquot 20–50 µL into sterile tubes, and store at –20°C in the dark for up to 1 year. Thawed Y27632 solution can be kept at 4°C for up to 4 weeks.

   Note: The molecular weight of Y27632 is 320.26; however, variation in molecular weight may occur between lots, depending on the water content. Therefore, the volume of distilled water that is used for the preparation of the stock solution may need to be adjusted accordingly.

Vitronectin-coated culture plates

1. Prepare a 10 µg/mL vitronectin working solution by diluting the 0.5 mg/mL vitronectin stock solution in sterile DPBS without calcium and magnesium, at room temperature (1:50 dilution). Gently mix the vitronectin dilution by pipetting it up and down.

   Note: When used to coat a 6-well plate (10 cm^2/well) at 1 mL/well, the final vitronectin concentration will be 1.0 µg/cm^2.

2. Add 1 mL of the diluted vitronectin solution to each well of a 6-well plate (see step 6 of "Poly-D-lysine and laminin double-coated culture plates" for the recommended volumes for other culture vessels).

3. Incubate the coated plates at room temperature for 1 hour. The culture vessel can now be used or stored at 4°C wrapped in laboratory film for up to one week. Do not allow the vessel to dry.

4. Before use, prewarm the culture vessel to room temperature for at least 1 hour before aspirating and discarding the vitronectin solution.

   Note: It is not necessary to rinse off the culture plate after the removal of the vitronectin solution. Cells can be plated directly onto the vitronectin-coated culture plates.

Laminin-coated culture plates

1. Thaw the required volume of 1.0 mg/mL laminin stock solution (stored at –80°C) slowly at 4°C.

   Note: Laminin may form a gel when thawed too rapidly. Thawing the stock solution in the cold (4°C) prevents this. Thawed stock solution can be stored at 4°C for up to 1 month.

2. Prepare a 10 µg/mL laminin working solution by diluting the thawed stock solution in sterile deionized water at room temperature (1:100 dilution). Gently mix the laminin dilution by pipetting it up and down.

3. Add 1 mL of the diluted laminin solution to each well of a 6-well plate (see step 6 of "Poly-D-lysine and laminin double-coated culture plates" for the recommended volumes for other culture vessels).

   Note: When used to coat a 6-well plate (10 cm^2/well) at 1 mL/well, the final laminin concentration will be 1.0 µg/cm^2.

4. Incubate the coated plates overnight at 4°C or at 37°C for 2 hours. The culture vessel can now be used or stored at 4°C wrapped in laboratory film for up to one week. Do not allow the vessel to dry.

   Important: Laminin deforms and loses attachment power upon drying. Aspirate the laminin solution just before use to prevent the laminin-coated plates from drying out.

5. Before use, prewarm the culture vessel to room temperature for at least 1 hour before aspirating and discarding the laminin solution.

   Note: It is not necessary to rinse off the culture plate after the removal of the laminin solution. Cells can be plated directly onto laminin-coated plates.

Poly-D-lysine and laminin double-coated culture plates

1. Prepare a 100 µg/mL poly-D-lysine working solution in sterile distilled water.
2. Add 1 mL of the poly-D-lysine working solution to each well of a 6-well plate (see step 6 for the recommended volumes for other vessels).

   Note: When used to coat a 6-well plate (10 cm^2/well) at 1 mL/well, the final poly-D-lysine concentration will be 10.0 µg/cm^2.
3. Incubate the culture vessel at room temperature for 1–2 hours.
4. Remove the poly-D-lysine solution and rinse 3 times with distilled water.

   Important: Make sure to rinse the culture vessel thoroughly, because excess poly-D-lysine can be toxic to the cells.
5. Prepare a 15 µg/mL working solution of laminin in sterile distilled water.

   Important: To prepare a double-coated culture plate, you need a higher working concentration of laminin (15 µg/mL instead of 10 µg/mL).
6. Add 1 mL of the 15 µg/mL laminin working solution to each well of a 6-well plate (see the table below for the recommended volumes for other vessels).

   Note: When used to coat a 6-well plate (10 cm^2/well) at 1 mL/well, the final laminin concentration will be 1.5 µg/cm^2.

Culture vessel (approx. surface area)	Volume of matrix solution (per well or dish)
96-well plate (0.32 cm^2/well)	50 µL
48-well plate (1 cm^2/well)	125 µL
24-well plate (2 cm^2/well)	250 µL
12-well plate (4 cm^2/well)	500 µL
6-well plate (10 cm^2/well)	1 mL
35 mm dish (10 cm^2/dish)	1 mL
60 mm dish (20 cm^2/dish)	2.5 mL
T-25 flask (25 cm^2/flask)	2.5 mL
100 mm dish (60 cm^2/dish)	5 mL
T-75 flask (75 cm^2/flask)	7.5 mL

7. Incubate the coated plates overnight at 4°C or at 37°C for 2 hours.

   Note: You can use the coated culture plate immediately or store it at 4°C wrapped in laboratory film for up to one week. Do not allow the plate to dry.
8. Before use, prewarm the culture plate to room temperature for at least 1 hour before aspirating and discarding the laminin solution. It is not necessary to rinse off the plate after the removal of the laminin solution.

   Important: Laminin deforms and loses attachment power upon drying. Aspirate the laminin solution just before use to prevent the laminin-coated plates from drying out.

Preparing hPSC culture for differentiation (day –1)

Set up hPSC culture

1. Coat each well of a 6-well plate with 1 mL of 10 µg/mL vitronectin working solution as described on page 132. You will use this plate for carrying over specified FP cells for further expansion.

   Similarly, coat 8 wells of a 48-well plate with 125 µL/well of 10 µg/mL vitronectin working solution. You will use this plate for ICC characterization of specified FP cells.

2. Incubate the coated plates at room temperature for 1 hour. Before use, aspirate the vitronectin solution and discard.

   Note: It is not necessary to rinse off the culture plate after the removal of vitronectin.

3. Prepare complete feeder-free PSC medium and warm to room temperature before use.

4. To seed cells for differentiation, start with a healthy hPSC culture that is ~70–85% confluent and maintained in feeder-free PSC medium on vitronectin-coated culture vessels. Alternatively, thaw a fresh vial of hPSCs to start differentiation.

   Important: The quality of the hPSCs (with minimal or no differentiated colonies) is critical for efficient differentiation. Remove any differentiated and partially differentiated colonies before passaging hPSCs. Differentiated colonies can be marked by using a Nikon microscopy object marker (Nikon Instruments Inc., Cat. No. MBW10020) with a Nikon microscopy C-OA 15 mm objective adapter (Nikon Instruments Inc., Cat. No. MXA20750).

5. Prepare a single-cell suspension of hPSCs and seed each well of the vitronectin-coated plate with 3.0×10^4–5.0×10^4 viable cells/cm^2 in complete Essential 8 Medium + 10 µM ROCK inhibitor (Y27632).

   Important: The recommended seeding density of 3.0×10^4–5.0×10^4 viable cells/cm^2 is for the reference ESC line H9 only. If the culture kinetics of your cell line differs from the culture kinetics of the H9 reference line, optimize the cell-seeding density for your cell line to attain full confluency at day 7.

   Note: Overnight treatment with the ROCK inhibitor is required for cell survival and recovery after passaging. The ROCK inhibitor is removed from the culture the following day when the spent medium is replaced with complete cell specification medium.

6. Incubate the cells overnight at 37°C in a humidified atmosphere of 5% CO_2.

7. Prepare complete Floor Plate Cell Specification Medium for use on days 0–10.

Specification (day 0–10)

Specification workflow

The first step of dopaminergic neuron differentiation is the specification of hPSCs into midbrain-specified floor plate progenitor cells in complete Floor Plate Specification Medium on vitronectin-coated plates. During specification, spent medium is replaced every other day with fresh complete specification medium, and the FP cells are harvested on day 10 to start the expansion stage.

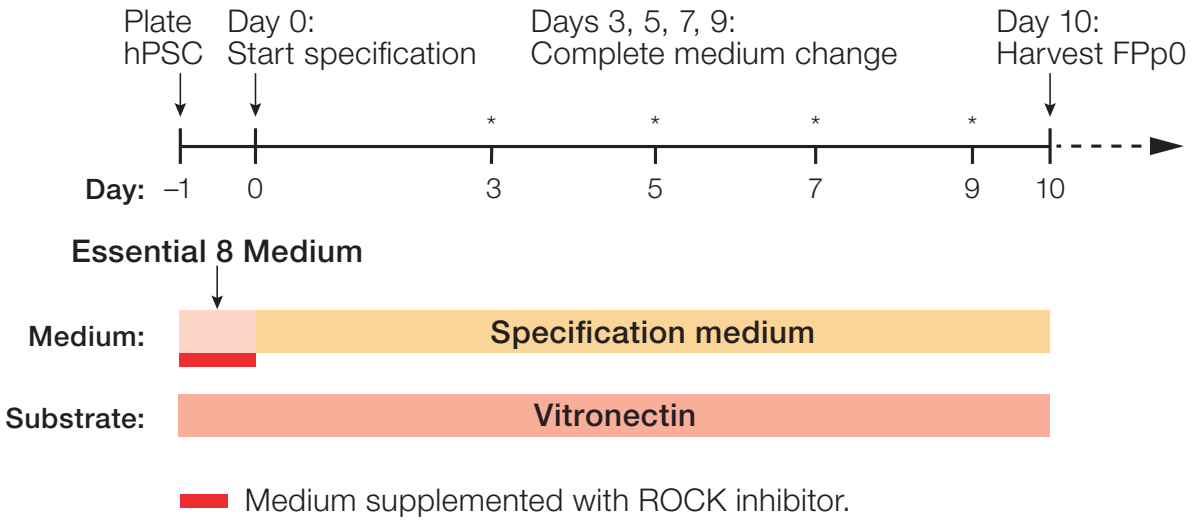


Figure 5.11. hPSCs specification workflow timeline.

Day 0: Start specification

1. At 24 hours after plating, the hPSC culture should be 20–40% confluent.

   Important: Observe the quality of the starting hPSC population. If the recovery (relative confluency and cell morphology) is poor, we recommend starting over with higher-quality cells. See Figure 5.12 for an example of a healthy hPSC culture that has been recovered sufficiently to start specification.

2. Aliquot the amount of complete specification medium (prepared on day –1) needed for the day and warm at 37°C (e.g., 2 mL per well of a 6-well plate; see "Required seeding densities" on page 131 for the recommended volumes for other vessels).

   Note: Do not expose the complete specification medium to 37°C for more than 10 minutes. Avoid warming the entire volume of prepared specification medium repeatedly.

3. Aspirate the spent feeder-free PSC medium containing the ROCK inhibitor, and replace it with prewarmed complete specification medium.

4. Incubate at 37°C in a humidified atmosphere of 5% CO_2.

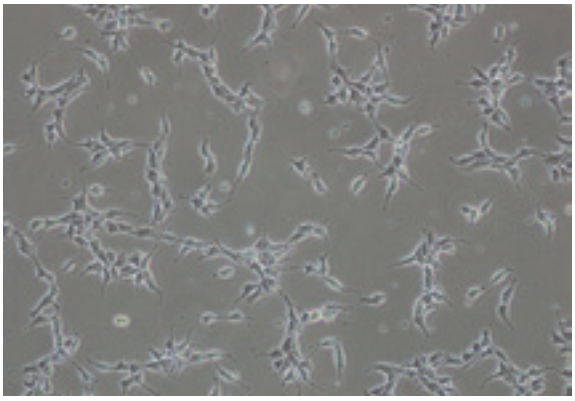


Figure 5.12. hPSCs incubated in complete Essential 8 Medium that is supplemented with 10 µM ROCK inhibitor (Y27632) for 24 hours after thaw. The culture shows robust recovery with healthy morphology and is ready for specification.

Day 3, 5, 7, and 9: Complete medium change

1. Aliquot the amount of complete specification medium that is needed for the day and warm at 37°C (e.g., 2 mL per well of a 6-well plate; see "Required seeding densities" on page 131 for the recommended volumes for other vessels).

2. **Day 3 and 5:** Aspirate the spent medium, and replace it with 1 volume of fresh complete specification medium (2–3 mL for each well of a 6-well plate).

3. **Day 7 and 9:** Cultures should be near or at 100% confluency, resulting in increased medium consumption. Replace the spent medium with 2 volumes of fresh complete specification medium (4–5 mL for each well of a 6-well plate) to compensate for high medium consumption.

   Note: You can examine marker expression for the midbrain FP progenitor phenotype as early as day 7 of specification. For more information, see "Expected results for specification".

4. **Day 9:** Prepare complete expansion medium as described on page 131 and store at 4°C.

5. **Day 10:** Culture is now completely confluent and ready to be passaged (see Figure 5.13). Cells at this stage are FP progenitors and their numbers have increased approximately 25-fold compared to the hPSC seeded on day –1.

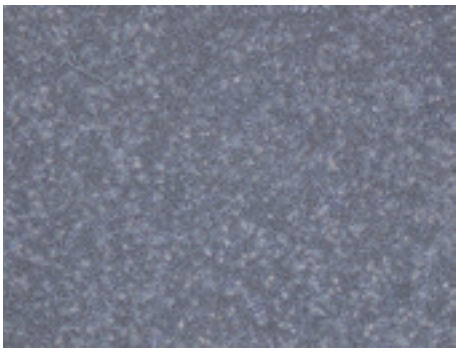


Figure 5.13. hPSC treated with Floor Plate Specification Medium for 10 days. The culture is homogeneous and dense, and consists mainly of FP cells.

Note: At this point, we recommend that you examine your cells for the expression of FOXA2 and OTX2, key markers for the midbrain floor plate progenitor phenotype, before proceeding with the expansion step. For more information, see "Expected results for specification".

Expected results for specification

You can examine the expression of FOXA2 and OTX2, key markers for the midbrain floor plate progenitor phenotype, as early as day 7 of specification.

For the image-based analysis of intermediate floor plate progenitors, we recommend using the Human Dopaminergic Neuron Immunocytochemistry Kit (Cat. No. A29515). The kit includes a complete set of primary and secondary antibodies, a nuclear DNA stain, and premade buffers for an optimized staining experiment.

Note: If the efficiency of FP specification (as determined by double-positive expression of FOXA2 and OTX2 markers) is less than 60%, do not proceed to the expansion step. Instead, repeat the specification procedure with a higher seeding density (see "Troubleshooting") at the end of this protocol.

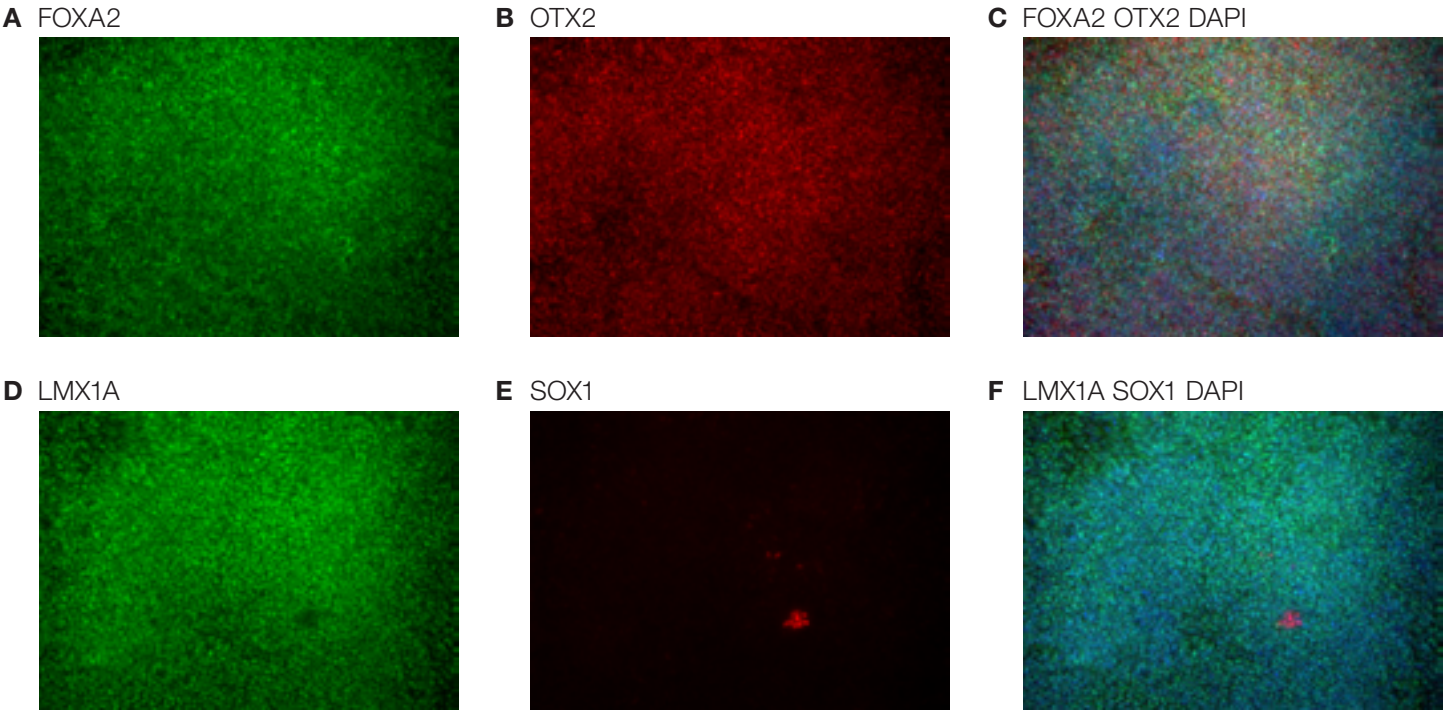


Figure 5.14. Marker expression of induced FP progenitor cells. hPSCs were treated with complete Floor Plate Specification Medium for 7 days and the cells were analyzed for the key phenotypic markers of the human dopaminergic neuron lineage using the Human Dopaminergic Neuron Immunocytochemistry Kit (Cat. No. A29515). **(A–C)** After floor plate specification of hPSCs, the cells express FP marker FOXA2 (green) and rostral marker OTX2 (red). **(D–E)** The specified FP cells are positive for the DA progenitor marker LMX1A (green), but negative for the neural stem cell marker SOX1 (red).

Expansion (day 10–16): Expand cells in adherent culture

Expansion workflow

The second step of DA neuron differentiation is the expansion of FP cells as adherent cultures in complete Floor Plate Cell Expansion Medium on laminin-coated culture vessels for two passages. This increases the cell number and improves the efficiency of differentiation towards DA neurons. A portion of the FP cells at passage 2 (FPp2; day 16 of differentiation) are frozen to create a cell bank for future use, while the remainder are used to seed a suspension culture to generate spheres, which further improves the differentiation efficiency. On day 21 of differentiation, spheres are harvested for maturation.

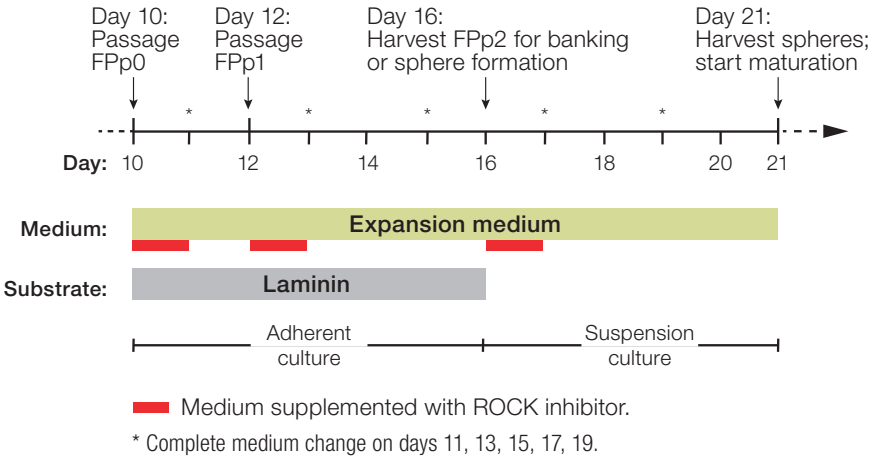


Figure 5.15. Expansion workflow timeline for FP progenitor cells.

Day 10: FP passage 0 (FPp0)

1. Coat each well of a 6-well plate with 10 µg/mL of laminin as described on page 132. Equilibrate the plate to 37°C before use.

   Note: When used to coat a 6-well plate (10 cm^2/well) at 1 mL/well, the final laminin concentration will be 1.0 µg/cm^2.

   Important: Laminin deforms and loses attachment power upon drying. Aspirate the laminin solution just before use to prevent the laminin-coated plates from drying out.

2. Aliquot the amount of complete Floor Plate Cell Expansion Medium that is needed for the day and warm at 37°C (e.g., 2 mL per well of a 6-well plate; see "Required seeding densities" on page 131 for the recommended volumes for other vessels).

3. Aspirate the spent medium from the specification culture plate, and rinse the wells with DPBS to remove any remaining medium.

4. Aspirate the DPBS and add an appropriate volume of StemPro Accutase Cell Dissociation Reagent to fully cover the surface (1 mL per well of a 6-well plate or 1 mL per 10 cm^2 of surface area).

5. Incubate the vessel at 37°C, 5% CO_2 for ~5–15 minutes, continually observing the wells for cell detachment.

6. After several minutes or when some colonies start detaching (whichever happens first), gently tap the bottom of the vessel several times. Most colonies should freely come into suspension. If all colonies do not detach, wait 1–2 minutes, and then tap the vessel again to detach the remaining colonies.

7. Transfer the cell clumps to a sterile 50 mL culture tube.

   Note: FPp0 cells do not need to be dissociated into a single-cell suspension.

8. Rinse the wells of the specification culture plate twice with DPBS, using 4X the volume of StemPro Accutase reagent used in each well (4 mL per well of a 6-well plate). After each rinse, collect the cell clumps in the same 50 mL culture tube to ensure the recovery of all colonies.

9. Centrifuge the cell suspension at 300 x *g* for 3 minutes at 4°C to pellet the cells. Carefully aspirate the supernatant, leaving the cell pellet in the culture tube.

10. Gently flick the bottom of the tube to dislodge the cell pellet and resuspend the cells in a sufficient volume of complete expansion medium plus 5 µM ROCK inhibitor (Y27632) for a 1:2 split ratio (i.e., one plate to two plates).

11. Aspirate the laminin solution from the newly coated plates and plate the FP cells at a 1:2 split ratio. Ensure that recipient wells contain sufficient final volume of complete Floor Plate Cell Expansion Medium plus 5 µM ROCK inhibitor (Y27632) (i.e., 2 mL of cell suspension per well of a 6-well plate).

    Note: Overnight treatment with the ROCK inhibitor is required. The ROCK inhibitor is removed from the culture the following day when the spent medium is replaced with complete cell specification medium.

12. Incubate the cells overnight at 37°C in a humidified atmosphere of 5% CO_2.

Day 11: Complete medium change

1. Aliquot the amount of complete expansion medium that is needed for the day and warm at 37°C.
2. Aspirate the spent medium and replace it with 1X volume of fresh complete expansion medium without the ROCK inhibitor (2–3 mL for each well of a 6-well plate).

Day 12: FP passage 1 (FPp1)

1. On day 12, the FP cultures should be very compact and at 100% confluency. At this point, the culture is ready for passaging. See Figure 5.16 for an example of a confluent FP culture that is ready to be passaged.

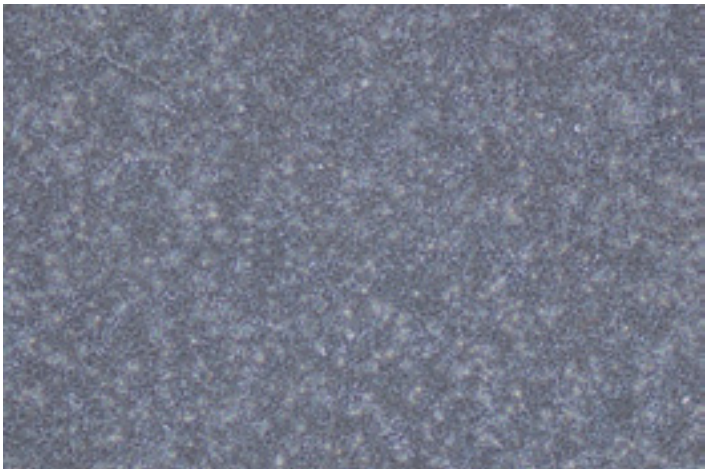


Figure 5.16. FP progenitor culture that is ready to be passaged on day 12 of differentiation. The culture is very compact and exhibits 100% confluency.

2. Prepare the appropriate number of laminin-coated culture plates as described on page 132.

   Note: You can apply a split ratio of approximately 1:4–6 (i.e., each well can be split into 4–6 wells).

3. Aliquot and prewarm to 37°C the amount of complete expansion medium that is needed for the day.
4. Aspirate the spent medium from the culture plates and rinse the wells with DPBS to remove any remaining media.
5. Add an appropriate volume of StemPro Accutase reagent to each well and incubate at 37°C, 5% CO_2, continually observing the wells for cell detachment.
6. After >80% of the cells have detached (about 5–15 minutes), pipet the cell clumps up and down several times to create a single-cell suspension, and then transfer the cell suspension to a sterile 50 mL culture tube.
7. Rinse the wells twice with DPBS, using 4x the volume of StemPro Accutase reagent that is used in each well (4 mL per well of a 6-well plate), and collect the PBS in the same 50 mL culture tube to ensure the recovery of all cells.
8. Remove a small volume of cell suspension and perform a viable cell count.
9. Centrifuge the cell suspension at 300 x *g* for 3 minutes to pellet the cells. Carefully aspirate the supernatant, taking care not to disturb the cell pellet in the culture tube.
10. Gently flick the bottom of the tube to dislodge the cell pellet and resuspend the cells to 1.0 x 10^7 viable cells per mL in complete expansion medium.
11. Plate the cell suspension at a high seeding density of 2.0 x 10^5 cells/cm^2 (i.e., 2.0 x 10^6 cells per well of the 6-well plate) in complete expansion medium + 5 µM ROCK inhibitor (Y27632).
12. Incubate the cells overnight at 37°C in a humidified atmosphere of 5% CO_2.

Day 13 and 15: Complete medium change

1. Aliquot the amount of complete expansion medium that is needed for the day and warm at 37°C.
2. Aspirate the spent medium and replace it with fresh complete expansion medium without the ROCK inhibitor (2–3 mL per well of a 6-well plate).

Day 16: Harvest FP passage 2 (FPp2) cells

1. On day 16, the FP cultures should again be very compact and 100% confluent. Figure 5.17 shows an example of a confluent FP culture at day 16 of differentiation that can be harvested for cryopreservation or for suspension culture to form spheres.

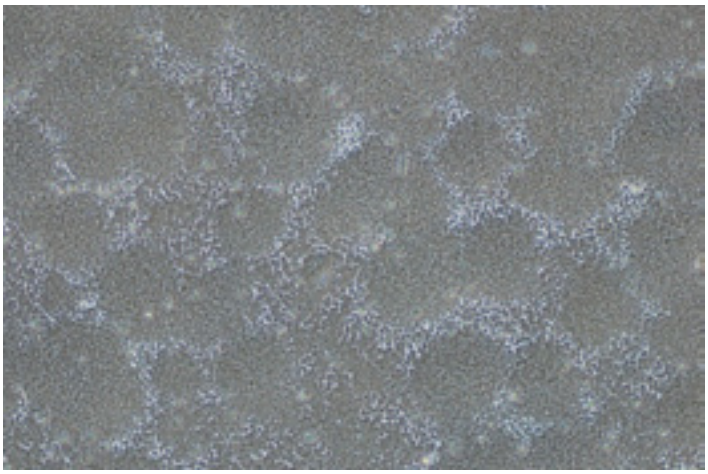


Figure 5.17. FP progenitor culture on day 16 of differentiation. The dense culture exhibits 100% confluency and is ready to be harvested.

2. Harvest the cells using StemPro Accutase reagent as described for FPp1 and resuspend to 1.0 x 10^7 cells/mL in complete expansion medium.

3. Use the FPp2 cell suspension for cryopreservation or proceed with sphere formation.

Note: We strongly recommend freezing a portion of the FPp2 cells to prepare a cell bank before initiating sphere culture. This will enable optimization of culture conditions and aid troubleshooting if experimental variation is observed.

Optional: Expansion beyond FPp2

You can expand the FP cells for another round and freeze them at FPp3 before proceeding with sphere formation. This will result in an additional 4- to 6-fold increase in the total number of FP cells without compromising the quality of TH neurons (co-expressing TH and FOXA2).

Note: Further expansion (up to passage 10) results in stable expansion of progenitor cells, but we have observed that the resulting TH neurons show decreased FOXA2 expression.

Day 16: Cryopreserve FPp2 cells

Freeze FP cells at a final density of 2 x 10^6–5 x 10^6 viable cells/mL in 90% fresh complete Floor Plate Cell Expansion Medium and 10% DMSO. When freezing FP cells, follow the procedure below:

1. Prepare freezing medium at 2X concentration (80% complete expansion medium + 20% DMSO) and chill at 4°C before use.

2. Calculate the volume of cells in the FPp2 cell suspension that corresponds to the number of cells you want to cryopreserve, and transfer to a sterile tube.

   For example, if you want to bank ten vials of cells at 2 x 10^6 cells/vial, transfer 2 mL of cells from the FPp2 harvest (at 1.0 x 10^7 cells/mL).

3. Dilute the cells to 2X the intended final frozen concentration using complete expansion medium at 4°C.

   For example, for a final frozen concentration of 2 x 10^6 cells/mL, add 3 mL of complete expansion medium to the 2 mL of cell suspension from the FPp2 harvest (at 1.0 x 10^7 cells/mL). This dilutes the cells to 4 x 10^6 cells/mL (2X the final frozen concentration).

4. Add the same volume of 2X freezing medium (chilled to 4ºC) as the cell suspension dropwise while gently rocking the tube back and forth.

   For example, if the tube contains 5 mL of cells suspension at 4 x 10^6 cells/mL, add 5 mL of 2X freezing medium to dilute the cells to a final concentration of 2 x 10^6 cells/mL for cryopreservation.

   Note: Addition of DMSO to water generates heat. Using chilled freezing medium and slow addition prevents damage to the cells from the heat.

5. Aliquot 1 mL of the cell suspension into each cryogenic vial, place the vials in a Mr. Frosty Freezing Container with isopropanol, and freeze them at –80°C overnight.

   Note: You can also freeze the cells in an automated or manually controlled freezing rate apparatus following standard procedures (~1ºC decrease/minute).

6. The next day, transfer the frozen vials to liquid nitrogen (vapor phase) for long-term storage.

Day 16: Recover frozen FPp2 cells

Follow the procedure below to recover frozen FPp2 cells to initiate a suspension culture in complete expansion medium to form spheres.

1. Remove the cryogenic vial of FPp2 cells from the liquid nitrogen storage and immediately immerse it in a 37°C water bath without submerging the cap. Swirl the vial gently.

2. When only an ice crystal remains (~1–2 minutes), remove the vial from the water bath and spray the outside of it with 70% ethanol to decontaminate.

3. Pipet the cells gently into a sterile 15 mL conical tube using a 1 mL pipette.

4. Add 1 mL of DPBS into the vial to collect the remaining cells and transfer the cell suspension dropwise to the 15 mL conical tube. While adding, gently move the tube back and forth to mix the cells and prevent osmotic shock.

5. Add an additional 3 mL of DPBS to the cells to have a 5 mL suspension.

6. Remove a small volume of cell suspension and perform a viable cell count.

7. Centrifuge the cell suspension at 300 x *g* for 3 minutes to pellet the cells. Carefully aspirate the supernatant, leaving the cell pellet in the culture tube.

8. Gently flick the bottom of the tube to dislodge the cell pellet and resuspend the cells to 1.0 x 10^6 viable cells/mL in complete expansion medium + 5 µM ROCK inhibitor (Y27632).

   Note: If desired, you can confirm that your culture maintains the floor plate progenitor phenotype by analyzing the expression of key phenotypic markers (FOXA2, OTX2, and LMX1A).

Expansion (days 16–21): Sphere formation in suspension culture

Requirement for sphere formation

We recommend sphere formation as standard protocol for untested lines. However, some PSC lines can be efficiently differentiated to DA neurons without sphere formation. In such cases, FPp2 cells can be passaged adherently one additional time to FPp3, and then directly plated in maturation medium at day 21. However, we still recommend that you prepare a bank of FPp2 cells as a back-up.

Day 16: Initiate suspension culture of FP cells

1. On day 16, harvest or thaw FPp2 cells as described, then remove a small volume of cells and perform a viable cell count.

2. Resuspend the FPp2 cells to 1.0 x 10^6 viable cells per mL in complete expansion medium + 5 µM ROCK inhibitor (Y27632).

3. Transfer cell suspension to a non–TC-treated culture vessel and adjust the volume of the cell suspension to the size of vessel. See "Required seeding densities" on page 131.

   Note: For suspension culture of spheres, use low-attachment plastic culture vessels such as Nunclon Sphera cell culture dishes or flasks to prevent spheres from merging or adhering to the culture vessel, which can greatly compromise downstream differentiation.

4. Incubate the cells overnight at 37°C in a humidified atmosphere of 5% CO_2.

Day 17 and 19: Complete medium change

1. Aliquot the amount of complete expansion medium that is needed for the day and warm at 37°C.

2. Perform a complete medium change either by the centrifugation method or the gravity method.

   Note: When the spheres are small, centrifugation is the recommended method to avoid loss of the spheres (small spheres will not sediment by gravity).

   #### Centrifugation method

   a. Transfer the spheres to a 15 mL conical tube and then centrifuge at 200 x *g* for 2 minutes. Aspirate the supernatant and discard.

   b. Resuspend the spheres in fresh complete expansion medium without the ROCK inhibitor, and then transfer to original flask.

   c. Pipet the sphere suspension up and down several times to prevent them from merging with each other before plating.

   #### Gravity method

   a. Tilt the T-flask so that most of the spheres settle down at the edge of the flask.

   b. After 2–5 minutes, gently aspirate the supernatant without disturbing the spheres.

   c. Add fresh complete expansion medium without ROCK inhibitor, and then pipet the sphere suspension up and down several times to prevent them from merging with each other before plating.

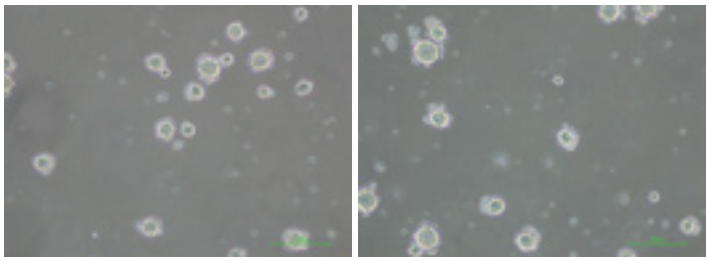


Figure 5.18. Spheres on day 3 (left) and day 5 (right) of suspension culture (days 19 and 21 of differentiation, respectively).

Day 21: Dissociation of spheres

1. Transfer the sphere suspension from culture vessel to a sterile 15 mL conical tube. Allow spheres to settle to the bottom of the tube (~2–5 minutes) before proceeding to the next step.

2. Carefully aspirate the spent medium, leaving the spheres at the bottom of the tube in a minimal volume (~100 µL) of the remaining medium.

3. Resuspend the spheres in 5 mL of DPBS without calcium and magnesium.

4. Repeat steps 2 and 3, leaving the spheres at the bottom of the tube in a minimal volume (~100 µL) of DPBS.

5. Add 1 mL of StemPro Accutase dissociation reagent to the spheres and incubate for 30 minutes at 37°C. Every 10 minutes, gently swirl the cell suspension to ensure that the spheres are exposed to the StemPro Accutase reagent evenly.

6. While the spheres are incubating with the dissociation reagent, aliquot the amount of complete Dopaminergic Neuron Maturation Medium needed for the day and warm at 37°C.

7. Gently pipet the cell suspension up and down with a P1000 pipette until all of the spheres are dispersed into a single-cell suspension.

8. Pass cell suspension through a 100 µm strainer, and then rinse the mesh with 4 mL of DPBS. If a strainer is not used, add 4 mL of DPBS to the cell suspension to stop the dissociation enzyme reaction.

   Note: Use of a mesh strainer is optional to remove undissociated clumps.

9. Remove a small volume of cell suspension to perform a viable cell count using an automated cell counter (e.g., Invitrogen™ Countess™ II Automated Cell Counter) or a hemocytometer.

10. Centrifuge the cell suspension at 300 x *g* for 3 minutes to pellet the cells. Carefully aspirate the supernatant, leaving the cell pellet in the culture tube.

11. Resuspend the cell pellet to a single-cell suspension in complete maturation medium plus 5 µM ROCK inhibitor (Y27632) and proceed to the maturation step.

Maturation (days 21–35)

Maturation workflow

The last step of dopaminergic neuron differentiation is the maturation of FP cells into mature midbrain dopaminergic neurons in complete maturation medium on poly-D-lysine and laminin double-coated culture vessels.

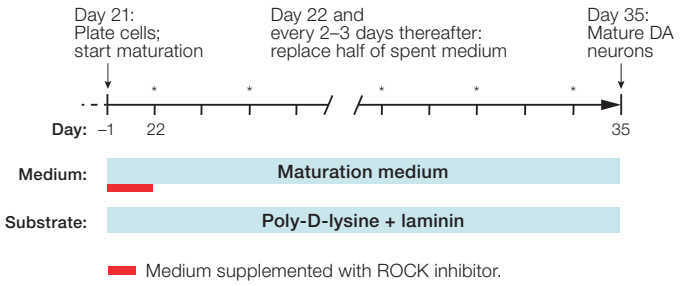


Figure 5.19. FP cell maturation workflow timeline.

Note: Do not proceed to maturation unless your FP cells have been passaged at least two times, have gone through sphere formation, and continue to express FOXA2 and OTX2 markers.

Day 21: Plate FP cells for maturation

1. Aliquot the amount of complete maturation medium that is needed for the day and warm at 37°C.

2. Prepare culture plates double-coated with poly-D-lysine and laminin, first coating the plates with 100 µg/mL poly-D-lysine and then with 15 µg/mL laminin as described on page 133.

   Note: For convenience, you can apply laminin (15 µg/mL) to a commercially available poly-D-lysine–coated culture plate to get double coating.

   Important: Be sure to rinse the culture vessel thoroughly after coating it with poly-D-lysine, because excess poly-D-lysine can be toxic to the cells.

3. Seed the double-coated culture plates with the dissociated cells (step 11) at a seeding density of 1.0 x 10^5–2.0 x 10^5 cells/cm^2 in complete maturation medium plus 5 µM ROCK inhibitor Y27632 solution. See "Required seeding densities" on page 131.

   Note: Overnight treatment with the ROCK inhibitor is required. The ROCK inhibitor will be diluted out of the culture by successive half-volume media changes over time.

4. Incubate the cells overnight at 37°C in a humidified atmosphere of 5% CO_2.

Day 22 to 35: Half-volume medium change every 2–3 days

1. The next day (day 22 of differentiation) and every 2–3 days thereafter, aliquot the amount of complete maturation medium that is needed for the day and warm at 37°C.

   Note: During maturation, a half-volume medium change (instead of complete medium change) is required every 2–3 days. This provides fresh nutrients while protecting the cells from detachment and damage from air exposure.

2. On day 22 of differentiation (first medium change), add the same volume of fresh complete maturation medium (without the ROCK inhibitor) as the existing culture volume (e.g., 2 mL for each well of a 6-well plate).

   For subsequent feeds (every 2–3 days), aspirate half of the spent medium and replace it with fresh complete maturation medium.

3. Continue incubating the cells at 37°C in a humidified atmosphere of 5% CO_2.

4. Mature neurons can be visualized as early as 10 days after the addition of maturation medium, but for optimal results, we recommend culturing cells in maturation medium for 14 days (see "Expected results").

Note: To maintain derived neurons after maturation, you can replace DMEM/F-12 (Cat. No. 10565018) with Neurobasal Medium (Cat. No. 21103049) as the base medium in complete maturation medium. In some cell lines and culture conditions (for example, when using mTeSR1 medium), maturation occurs more quickly and neurons benefit from a base medium change (from DMEM/F-12 to Neurobasal Medium) and the addition of 1X B-27 supplement to the complete maturation medium.

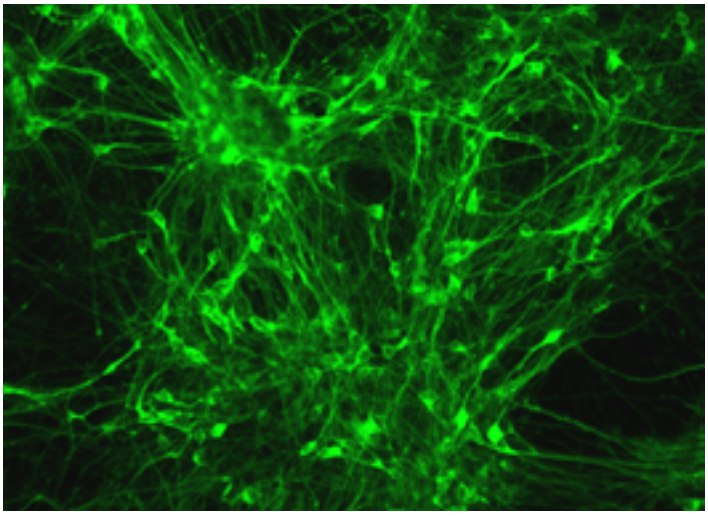
Expected results

Expression of phenotypic markers for DA neurons

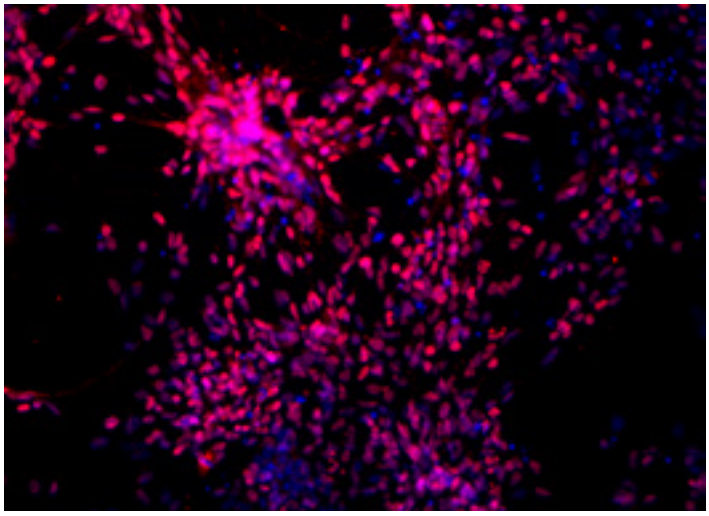
You can examine the expression of tyrosine hydroxylase (TH), the key marker for the mature dopaminergic (DA) neurons, as early as 10 days after the addition of maturation medium. However, we recommend culturing cells in maturation medium for 14 days for optimal results.

Note: For the image-based analysis of mature DA neurons, we recommend using the Human Dopaminergic Neuron Immunocytochemistry Kit (Cat. No. A29515), which includes a complete set of primary and secondary antibodies, a nuclear DNA stain, and premade buffers for an optimized staining experiment.

A TH



B FOXA2 and DAPI



C TH and FOXA2

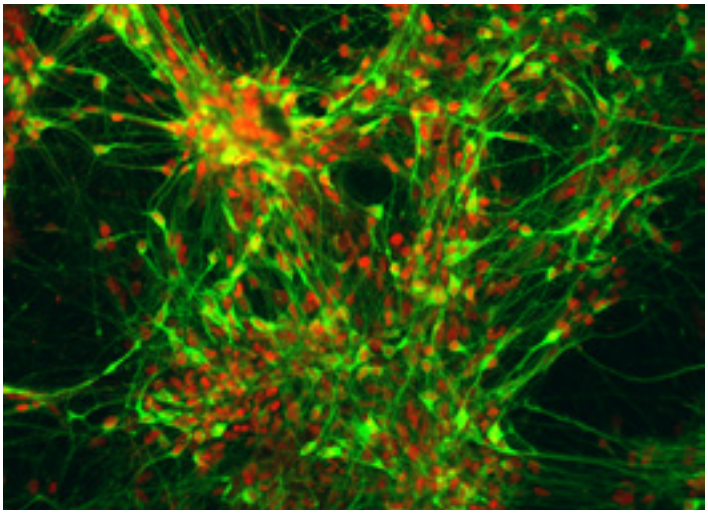


Figure 5.20. Representative images of mature DA neurons. The images were obtained from cells that were stained with the reagents provided in the Human Dopaminergic Neuron Immunocytochemistry Kit (Cat. No. A29515) 14 days after the addition of complete Dopaminergic Neuron Maturation Medium. The majority of the TH-expressing neurons also co-expressed FOXA2. **(A)** Anti-TH (green), **(B)** anti-FOXA2 (red) and Invitrogen™ NucBlue™ (a DAPI nuclear DNA stain) (blue), **(C)** merged image with anti-TH and anti-FOXA2 (green and red).

Troubleshooting

Media and supplements

Problem	Solution
Supplement (specification and maturation) has uneven (marbled) appearance.	• Uneven (marbled) appearance is due to DMSO in the supplement and will disappear upon thawing. • To prevent uneven appearance, aliquots of the supplement can be frozen first at –80°C and then stored at –20°C. • If a –80°C freezer is not available, aliquots can be frozen and stored at –20°C. In this case, the nonhomogeneous appearance of the supplements may persist when frozen, but will disappear upon thawing.

Specification

Problem	Solution
Day 0: PSC recovery or passaged cell number is lower than expected at the start of differentiation.	• Cell density is critical for efficient differentiation (optimal confluency at the start of differentiation is 20–40%). • We recommend repeating the day –1 activity and seeding a fresh hPSC culture for specification (page 134). • If you cannot seed a fresh hPSC culture, you can delay specification until the next day and feed the hPSCs with Essential 8 Medium instead. The purpose is to grow the hPSC until they reach the desired density. • However, cell growth in large colonies vs. homogeneous small cell clumps could result in decreased specification efficiency as well. Therefore, do not delay more than one day before starting specification. • In some cell lines, single-cell passaging could be more stressful than the original passaging method to which the cells have been adapted. • We recommend that you perform a small scale optimization run (48-well format) up to day 7 of specification to confirm the specification efficiency.
During specification, cells become overly confluent and peel off the dish.	• We have observed that some PSC lines require stronger attachment. • In such cases, we recommend repeating the specification procedure on Geltrex matrix–coated culture plates (1:100 dilution of Geltrex stock solution), although undefined factors in the Geltrex matrix can reduce specification efficiency.
Specification efficiency (co-stain of FOXA2 and OTX2) at day 7 is lower than 60%.	• Correct cell density is critical for efficient specification. Lower seeding densities and slower cell growth can compromise and lower the specification efficiency. In some case, culture kinetics of particular cell line is slower and the culture will not become fully confluent and dense around 6–7 days. • If you do not observe 100% confluency at day 6 of specification, repeat the specification procedure with a higher seeding density to reach 100% confluency by day 6–7 of specification.

Expansion

Problem	Solution
Day 17: Cells attach to the plastic rather than form spheres.	• It is important that the cells form spheres; otherwise the downstream differentiation process will be compromised. • Make sure to use untreated plasticware or ultralow-attachment plates. • Confirm that the correct concentration of ROCK inhibitor (5 µM) is used and that it is replaced with fresh medium after overnight treatment. • Detach the cells using a serological pipette during medium change.
During sphere culture, cells form a single large sphere instead of multiple homogeneous small spheres.	• After each medium change during sphere formation, make sure to distribute the cells homogeneously by shaking the plate or flask before returning it to the incubator. • If the cells are swirled, they will gather in the middle of the culture vessel and form a single large sphere instead of homogeneous small spheres. • If a single large sphere is formed, triturate with a P1000 pipette to break them again into small spheres.

Maturation

Problem	Solution
Day 35: Overall neuronal differentiation lower than expected (i.e., overall number of neurons is reduced).	• We found some cell lines to be more resistant to neuronal differentiation, resulting in lower neuronal population on day 35. • To improve the differentiation efficiency, increase the duration of the sphere culture from 5–10 days. When initiating suspension culture for sphere formation, reduce the seeding density by half (i.e., use 0.5 x 10^6 cells/mL) to prevent the formation of oversized spheres.
Neurons were detached from the substrate and lost after fixation.	• Make sure the coating process was followed properly. • Repeated freeze/thaw cycles decrease the attachment performance of laminin. We recommend using fresh laminin. • Neurons require gentle handling compared to established cell lines. Half medium changes and the use of the two-step fixation procedure minimize disturbance to the culture. For more information on two-step fixation, see the user guide for the Human Dopaminergic Neuron Immunocytochemistry Kit (Cat. No. A29515).
Tyrosine hydroxylase (TH) expression is dim and not prominent.	• Two-step fixation is critical to achieve and retain optimal TH staining. Multiplexing can reduce the intensity of TH staining.
Healthy neurons were initially obtained, but viability drops after maturation.	• After neurite extension is observed, change maturation base medium from DMEM/F-12 to Neurobasal Medium. Supplementation with 1X Gibco™ B-27™ Supplement can improve viability, especially for cultures initiated from PSCs maintained in mTeSR1 medium. H9-derived neurons can be maintained in Neurobasal Medium with 1X B-27 Supplement for at least 14 days after maturation (day 35 for the whole procedure).

5.5 Cardiomyocyte differentiation using the PSC Cardiomyocyte Differentiation Kit

The Gibco™ PSC Cardiomyocyte Differentiation Kit is a complete ready-to-use xeno-free system for the efficient differentiation of hPSCs into contracting cardiomyocytes within 10 days of initiating differentiation. Differentiated cardiomyocytes can be maintained in Cardiomyocyte Maintenance Medium for >30 days.

Product	Storage	Amount	Cat. No.
PSC Cardiomyocyte Differentiation Kit* contains:	2–8°C, protect from light	1 kit	A29212-01
Cardiomyocyte Differentiation Medium A		100 mL	A29209-01
Cardiomyocyte Differentiation Medium B		100 mL	A29210-01
Cardiomyocyte Maintenance Medium		500 mL	A29208-01

* The PSC Cardiomyocyte Differentiation Kit is sold as a complete kit; its components are not available separately except for Cardiomyocyte Maintenance Medium, which can be purchased separately for maintaining long-term cultures.

Culture conditions

Culture type: Adherent

Recommended substrate: For general applications, we recommend Gibco™ Geltrex™ LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix (Cat. No. A1413302). For xeno-free applications, we recommend recombinant vitronectin (Cat. No. A14700). See section 2.2A for coating protocols.

Guidelines for differentiation

Use high-quality human PSCs (with minimal or no differentiated colonies) that are karyotypically normal, express pluripotency markers, and are undergoing routine passaging. Do not use a PSC line past 100 passages.

We recommend culturing PSCs under feeder-free conditions using Essential 8, Essential 8 Flex, or StemFlex Medium on Geltrex matrix, which is an ideal substrate surface for cardiomyocyte differentiation. For details on culturing PSCs in these media, see sections 2.2B and 2.2C.

The differentiation efficiency of PSCs into cardiomyocytes varies between different PSC lines. A critical variable for the generation of robust cardiomyocyte culture is the relative confluency at the onset of differentiation. We strongly recommend performing a study to find a confluency range when using a PSC line for the first time. See “Range finding: Seeding density vs. confluency on day 4” on page 151 for guidelines.

Rapid media aspiration and addition can be detrimental to culture differentiation efficiency; we recommend slow addition and removal of media, especially during the induction phase.

Addition of 1X Gibco™ RevitaCell™ Supplement (Cat. No. A2644501) is essential for effective survival and recovery of singularized PSCs following seeding under these conditions. Alternatively, ROCK inhibitors (10 µM Y27632 or 0.5 µM thiazovivin) may also be used.

Differentiation of PSCs into cardiomyocytes

1. **PSC culture day 1:** To seed cells for differentiation, start with healthy PSC culture, approximately 70%–85% confluent. Prepare Geltrex matrix–coated 12-well plates as recommended in product manual and equilibrate to room temperature prior to use.

   Note: For reagent volumes for different culture vessels, see "Reagent volumes per well or dish" on page 151.

2. Prepare sufficient volume of Essential 8 Medium with 1X RevitaCell Supplement for use in steps 6–8, and warm to room temperature before use.

3. Aspirate the spent medium from healthy PSC culture and rinse wells once with DPBS without calcium and magnesium.

4. Aspirate the DPBS and add prewarmed Gibco™ TrypLE™ reagent to each well and incubate at 37°C until cells round up and readily detach by swirling or gentle tapping (approximately 3–5 minutes). Triturate cells 3 to 5 times while rinsing surface of well to help remove and resuspend cells. It is important not to over-digest cells. Microscopic observation to assess detachment is recommended.

5. Transfer the cell suspension to a sterile conical tube containing Essential 8 Medium according to the recommendations provided in the "Reagent volume per well or dish" table on page 151, and mix by gentle pipetting or inverting the tube.

6. Centrifuge the suspension at 200 x *g* for 4 minutes at room temperature, carefully discard the supernatant, gently flick tube 3–5 times to loosen pellet, and resuspend the pellet in an appropriate volume of Essential 8 Medium containing 1X RevitaCell Supplement.

   Note: 1–2 times the volume of TrypLE reagent used for dissociation is a good estimate for the resuspension volume of Essential 8 Medium.

7. Determine the viable cell density and percent viability using a Countess Automated Cell Counter or similar device and/or method.

   Note: Cell viability is typically >95%.

8. Aspirate Geltrex solution from plate, add Essential 8 Medium with RevitaCell Supplement to wells, and plate cells on tissue culture dish according to the following guidelines for plating cells:

   A. New or uncharacterized PSC lines—we strongly recommend performing a study to find a range to determine optimal confluency levels at the onset of differentiation (day 3 or 4). Refer to the "Range finding" table on page 151 for cell-seeding densities and expected confluency ranges using a 12-well plate.

   B. Characterized PSC lines—use previously optimized seeding densities.

9. **PSC culture day 2:** About 24 hours after plating PSCs, cells should be at 5–15% confluency. Aspirate the spent medium and add prewarmed complete Essential 8 Medium into each well. Return the plate to the incubator.

   Note: Addition of RevitaCell Supplement or other ROCK inhibitors is not required from this point onward.

10. **PSC culture day 3:** View cells under microscope and estimate confluency (see Figure 5.21). If the confluency is below target, refeed the cells with prewarmed Essential 8 Medium and return the plate to the incubator. Begin differentiation the following day, proceeding to step 11.

    If the desired confluency has been achieved, proceed to step 11.

11. **Differentiation day 1:** The PSC cultures should exhibit the approximate confluency range specified in the "Range finding" table for new lines, or optimal confluency based on prior range finding. Aspirate the spent medium and slowly add prewarmed Cardiomyocyte Differentiation Medium A into each well. Return the plate to the incubator.

    Note: Throughout the differentiation period, change the medium every 2 days, as indicated.

12. **Differentiation day 3:** The cells will start to become opaque. Some shedding of dead cells is normal. Aspirate the spent medium from each well and slowly replace with prewarmed Cardiomyocyte Differentiation Medium B. Return the plate to the incubator.

13. **Differentiation day 5:** The cells will continue to become more opaque. Shedding of dead cells is normal. Some PSC lines may shed more dead cells than others. Aspirate the spent medium from each well and slowly replace with prewarmed Cardiomyocyte Maintenance Medium. Return the plate to the incubator.

14. **Differentiation days 7, 9, and 11:** Refeed cells with Cardiomyocyte Maintenance Medium. Return the plate to the incubator. Contracting cardiomyocytes can appear as early as day 8.

15. **Differentiation day 12:** Typically, a spontaneously contracting syncytium of TNNT2/cTnT (troponin T cardiac type 2)-positive cardiomyocytes will be present and ready for use in various research applications.

    Note: Differentiated cells can be further cultured up to day 12–15 for harvesting (dissociation) and cryopreservation; beyond this time, cells are difficult to effectively harvest and recover. Alternatively, the cardiomyocytes can be maintained for a month or more for long-term studies, such as electrophysiological assays or molecular characterization. Additional Cardiomyocyte Maintenance Medium may be required beyond what is provided in this differentiation kit.
    Note: We recommend the Human Cardiomyocyte Immunocytochemistry Kit (Cat. No. A25973) for optimal image-based analysis of two key markers of the human cardiac lineage: NKX2-5 for early cardiac mesoderm and TNNT2/cTNT for cardiomyocytes.

Reagent volumes per well or dish

Culture vessel (Surface area/well)	DPBS	TrypLE Select Enzyme	Essential 8 Medium
6-well (10 cm^2/well)	2 mL	1 mL	2 mL
12-well (4 cm^2/well)	1 mL	0.5 mL	1 mL
24-well (2 cm^2/well)	0.5 mL	0.25 mL	0.5 mL
35 mm (10 cm^2/dish)	2 mL	1 mL	2 mL
60 mm (20 cm^2/dish)	4 mL	2 mL	4 mL
100 mm (60 cm^2/dish)	12 mL	6 mL	12 mL

Range finding: Seeding density vs. confluency on day 4

Culture vessel (Surface area/well)	Viable cells/well	Confluency on day 4
12-well (4 cm^2/well)	2 x 10^4	30%
	3 x 10^4	50%
	4 x 10^4	70%
	5 x 10^4	90%

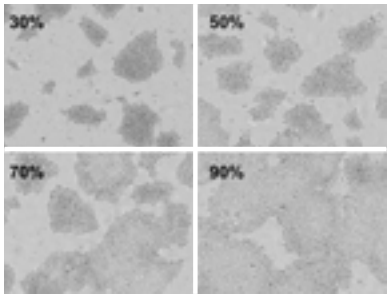


Figure 5.21. Images of H9 cultures 4 days following seeding at various densities. Confluency measurements were performed using automated image analysis and values indicated in upper left corner of each panel. For this set of H9 cultures, optimal confluency was about 60–80%.

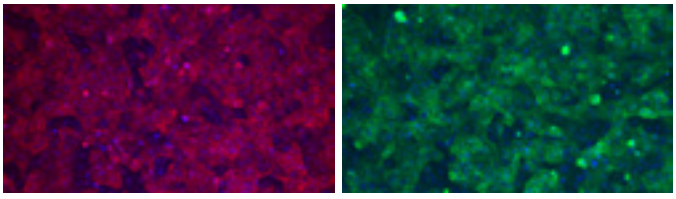


Figure 5.22. At the end of the differentiation protocol, cultures were fixed and stained for the cardiomyocyte-specific markers TNNT2 (red) and MYH6 (green).

5.6 Definitive endoderm induction using the PSC Definitive Endoderm Induction Kit

The PSC Definitive Endoderm Induction Kit is a complete ready-to-use media system for efficient induction of PSCs into the definitive endoderm (DE) lineage in 2 days.

A30626-01	Storage	Amount	Catalog No.
PSC Definitive Endoderm Induction Kit contains:		1 kit	A30626-01
Definitive Endoderm Induction Medium A	20° to –5°C, protect from light	50 mL	A30621-01
Definitive Endoderm Induction Medium B	20° to –5°C, protect from light	50 mL	A30624-01

* The PSC Definitive Endoderm Induction Kit is sold as a complete kit; its components are not available separately.

Culture conditions

Culture type: Adherent
Recommended substrate: Vitronectin (VTN-N) Recombinant Human Protein, Truncated (Cat. No. A14700)

Guidelines for differentiation

Use high-quality human PSCs that are karyotypically normal and uniformly morphologically undifferentiated. If there is spontaneous differentiation in hPSC cultures, the differentiated cells persist into directed differentiation procedures and confound downstream differentiation. Mechanically scrape morphologically differentiated cells with a pipette tip daily until only undifferentiated hPSCs remain.

Culture hPSCs under feeder-free conditions using Essential 8 Medium (Cat. No. A1517001), Essential 8 Flex Medium (Cat. No. A2858501), or StemFlex Medium (Cat. No. A3349401) on VTN-N, which is an ideal substrate surface for definitive endoderm induction. For more information on culturing hPSCs in these media, see section 2.2B. For instructions on coating dishes with VTN-N, see section 2.2A.

To promote cell survival, you can treat the cells overnight with a ROCK inhibitor such as RevitaCell™ Supplement (1X) (Cat. No. A2644501), Y27632 (10 µM), or thiazovivin (0.5 µM) at the time of splitting.

For routine passaging before induction, split the culture when the hPSC colonies occupy ~70% of the total available surface area of the well and/or when colony borders are merging with one another.

Start definitive endoderm induction when hPSC culture is 15–30% confluent. If the culture is at a higher confluency, the cells will start detaching during induction.

Preparation of DE Induction Medium

1. Thaw an entire bottle of DE Induction Medium A (for use on day 1) or DE Induction Medium B (for use on day 2) at 4°C overnight, at room temperature (15–25°C) for ~2 hours, or at 37°C for ~20 minutes, and mix thoroughly.

   Important: Ensure that the medium is prewarmed to room temperature or 37°C before use. Cold medium will significantly disrupt cell morphology of differentiating cells.

2. After thawing, use immediately (DE Induction Medium A on day 1, DE Induction Medium B on day 2) or store at 2–8°C for up to 2 weeks.

   Alternatively, aliquot and store at –20°C. After thawing the aliquots, use immediately or store at 2–8°C for up to 2 weeks. Do not refreeze.

Induction of hPSCs into DE

Day 0: Plate hPSCs

1. Aspirate the feeder-free hPSC medium (Essential 8, Essential 8 Flex, or StemFlex Medium) from the confluent hPSC culture and wash the cells with DPBS to remove any remaining media.
2. Aspirate the DPBS and add an appropriate volume of StemPro Accutase Cell Dissociation Reagent to cover the surface fully (at least 0.5 mL/well of a 12-well plate, 1 mL/well of a 6-well plate, or 3 mL per 10 cm dish).
3. Incubate the vessel at room temperature for ~5 minutes, continually observing the wells for cell detachment.
4. After several minutes or when some colonies start detaching (whichever happens first), gently tap the bottom of the vessel several times. Most hPSCs colonies should freely come into suspension. If all colonies do not detach, wait 1–2 minutes, and then tap the vessel again to liberate the remaining colonies.
5. Add feeder-free hPSC medium to the vessel to wash the colonies and dilute the StemPro Accutase reagent. After rinsing, collect the cell clumps in a 50 mL culture tube. Rinse the wells a second time with Essential 8 medium to ensure the recovery of all colonies.
6. Add sufficient feeder-free hPSC medium to the 50 mL culture tube to dilute the original volume of StemPro Accutase reagent by 1:5–1:10.
7. Centrifuge the cell suspension at 200 x *g* for 5 minutes at 4°C to pellet the hPSCs. Carefully aspirate the supernatant, leaving the cell pellet in the culture tube.
8. Gently flick the bottom of the tube to dislodge the cell pellet.

   Note: It is important to flick the tube; otherwise, the cell pellet may be difficult to subsequently resuspend into fine clumps.
9. Resuspend the cell pellet in feeder-free hPSC medium evenly into fine clumps by gently pipetting it up and down 2–3 times.

10. Seed the fine hPSCs clumps at a ~1:10 split ratio (from a 70% confluent culture) into VTN-N–coated plates. Ensure that recipient wells contain sufficient final volumes of feeder-free hPSC medium according to the table below.

    Important: For extremely confluent hPSC cultures (i.e., >90% confluent), seed the cell clumps at a 1:15–1:30 split ratio, as the optimum range for seeding density is 1 x 10^4–4 x 10^4 cells/cm^2. Otherwise, the culture will be overconfluent post-plating and the cells will detach during induction.

Culture vessel (surface area/well)	DPBS	0.5 mM EDTA in DPBS	Complete Essential 8 Medium	DE Induction Medium
6-well (10 cm^2)	2 mL	1 mL	2 mL	2 mL
12-well (4 cm^2)	1 mL	0.4 mL	1 mL	1 mL
24-well (2 cm^2)	0.5 mL	0.2 mL	0.5 mL	0.5 mL
35 mm (10 cm^2)	2 mL	1 mL	2 mL	2 mL
60 mm (20 cm^2)	4 mL	2 mL	4 mL	4 mL
100 mm (60 cm^2)	12 mL	6 mL	12 mL	12 mL
T-25 (25 cm^2)	4–5 mL	2–3 mL	4–5 mL	4–5 mL
T-75 (75 cm^2)	12–15 mL	5–8 mL	12–15 mL	12–15 mL

11. Move the plates in several back-and-forth and side-to-side motions to disperse the cells across the surface, then place them in a 37°C incubator with a humidified atmosphere of 5% CO_2.

    Note: To promote cell survival, you can treat the cells overnight with a ROCK inhibitor such as RevitaCell Supplement (1X), Y27632 (10 µM), or thiazovivin (0.5 µM) at the time of splitting.

Day 1: Start DE induction

1. Warm the DE Induction Medium A to room temperature. Mix by gently inverting the bottle several times to ensure even distribution of the components in the medium.

2. Evaluate the hPSCs; if the cells are 15–30% confluent, proceed with induction. If the culture is at a higher confluency, the cells will start detaching. If this happens, start over with a fresh hPSC culture.

3. Aspirate spent feeder-free hPSC medium from the wells completely, and add prewarmed DE Induction Medium A (see table on page 154).

   Note: Ensure that spent medium is completely removed before adding fresh medium.

4. Incubate the cells at 37°C, 5% CO_2 for 24 hours.

Day 2: Continue DE induction

1. Warm the DE Induction Medium B to room temperature. Mix by gently inverting the bottle several times to ensure even distribution of the components in the medium.

2. Aspirate the spent DE Induction Medium A from the wells completely, then add prewarmed DE Induction Medium B (see table on page 154).

   Note: Ensure that the spent medium is completely removed before adding fresh medium.

3. Incubate the cells at 37°C, 5% CO_2 for 24 hours.

Day 3: Characterize induced cells

After 24 hours of incubation in DE Induction Medium B, the cells will be ready to be assayed to evaluate their DE characteristics or be further differentiated to downstream lineages.

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Find out more at **thermofisher.com/stemcellprotocols**

Don’t want to do it yourself?
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