gibco

PSC Dopaminergic Neuron Differentiation Kit

USER GUIDE

Catalog Numbers A3147701, A3165801, A3147401

Publication Number MAN0015919

Revision 2.0

Life Technologies Corporation | 3175 Staley Road | Grand Island, NY 14072
For descriptions of symbols on product labels or product documents, go to **thermofisher.com/symbols-definition**.

The information in this guide is subject to change without notice.

DISCLAIMER: TO THE EXTENT ALLOWED BY LAW, THERMO FISHER SCIENTIFIC INC. AND/OR ITS AFFILIATE(S) WILL NOT BE LIABLE FOR SPECIAL, INCIDENTAL, INDIRECT, PUNITIVE, MULTIPLE, OR CONSEQUENTIAL DAMAGES IN CONNECTION WITH OR ARISING FROM THIS DOCUMENT, INCLUDING YOUR USE OF IT.

Revision history: Pub. No. MAN0015919

Revision	Date	Description
2.0	09 September 2019	Add workflow to process the cells for downstream analysis.
1.0	01 June 2016	New user guide

Limited Use Label License No. 505: Stem Cell Intellectual Property Disclaimer Notice to Purchaser: Products associated with this label license are not provided with a license to any patents not owned by or licensed to Life Technologies Corporation and related to stem cells. Users of LifeTechnologies Corporation's products subject to this label license should determine for themselves whether they have all of the appropriate licenses in place. Further, no warranty is provided that the use of these products will not infringe third party intellectual property related to stem cells.

Notice: The use of the method described in the protocol 'PSC Dopaminergic Neuron Differentiation Kit (from human ES cells)' to produce dopaminergic neuronal cells for in vitro applications including drug discovery and development, toxicology testing and consumer products testing may require a license from GE Healthcare under US 7,763,463.

Table of contents

Product information

Product description

PSC Dopaminergic Neuron Differentiation Kit

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 4.

Contents and storage

Kit contents

PSC Dopaminergic Neuron Differentiation Kit (Cat. No. A3147701) contains the following components.

Component*	Part No.	Amount
Floor Plate Specification Supplement (20X)	A3146801	5 mL
Floor Plate Cell Expansion Kit contains: Floor Plate Cell Expansion Base Floor Plate Cell Expansion Supplement (50X)	A3165801 A3147201 A3147301	1 kit 500 mL 10 mL
Dopaminergic Neuron Maturation Supplement (50X)	A3147401	10 mL

* 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. For ordering information, see page 28.

Storage

Store the components of the PSC Dopaminergic Neuron Differentiation Kit as described below.

Component	Storage
Floor Plate Specification Supplement (20X)	–20°C to –5°C; Protect from Light
Floor Plate Cell Expansion Base	2°C to 8°C; Protect from Light
Floor Plate Cell Expansion Supplement (50X)	–20°C to –5°C; Protect from Light
Dopaminergic Neuron Maturation Supplement (50X)	–20°C to –5°C; Protect from Light

Precautions

Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. For instructions on obtaining SDSs, see "Customer and technical support" (page 32).

CAUTION! Human origin materials are non-reactive (donor level) for anti-HIV 1 & 2, anti-HCV and HBsAg. Handle in accordance with established bio-safety practices.

Note: See "Appendix C: Safety" (page 30) for the complete the biological and chemical safety information.

Required materials not supplied

The following materials are not included with the PSC Dopaminergic Neuron Differentiation Kit, but are necessary to perform differentiation experiments. Unless otherwise indicated, all materials are available through **thermofisher.com**. See page 28 for ordering information.

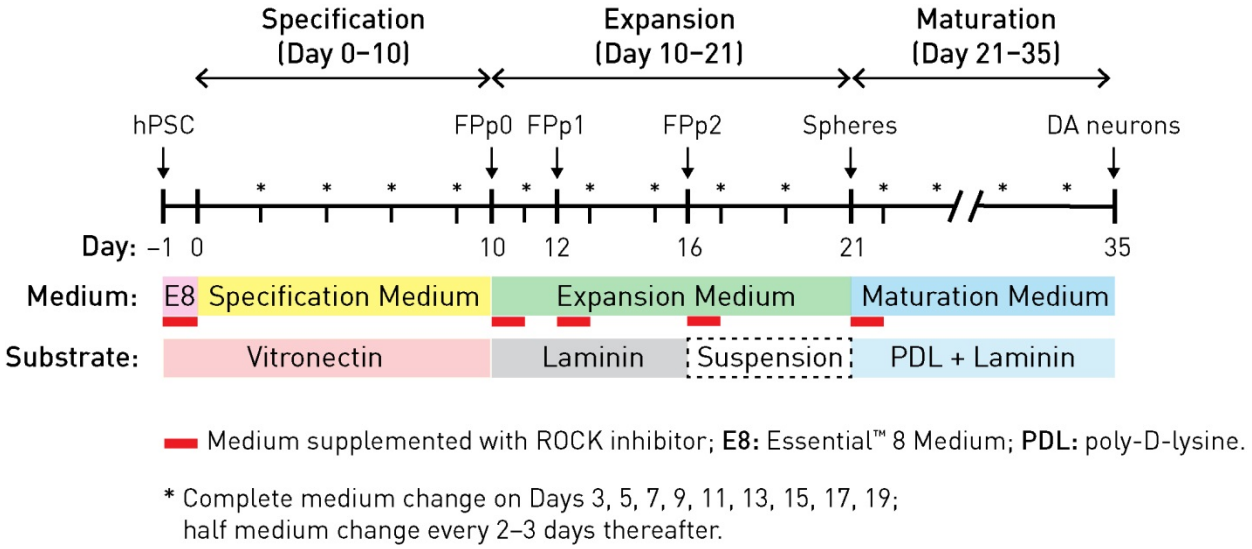
- Essential 8™ Medium (Cat. No. A1517001) or Essential 8™ Flex Medium (Cat. No. A2858501)
- Vitronectin (VTN-N), recombinant human (Cat. No. A14700)
- Neurobasal™ Medium (Cat. No. 21103049) (base medium for specification)
- DMEM/F-12 medium with GlutaMAX™ supplement (Cat. No. 10565018) (base medium for 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)
- 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)
- Nalgene™ Mr. Frosty™ Cryo 1°C Freezing Container (Thermo Scientific, 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

Workflow

The following figure summarizes the comprehensive workflow for the hPSC Dopaminergic Neuron Differentiation Kit.

During differentiation, hPSCs are first induced in Floor Plate 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.



Medium supplemented with ROCK inhibitor; **E8:** Essential™ 8 Medium; **PDL:** poly-D-lysine.

* Complete medium change on Days 3, 5, 7, 9, 11, 13, 15, 17, 19; half medium change every 2–3 days thereafter.

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.	11
2	Induce hPSCs into midbrain specified floor plate progenitor (FP) cells in complete Floor Plate Specification Medium.	12
3	Passage FP cells and start expansion as adherent culture in complete Floor Plate Cell Expansion Medium	15
4	Harvest FP cells to create a cell bank	17
5	Culture FP cells in complete Floor Plate Cell Expansion Medium to induce sphere formation	20
6	Culture cells in complete Dopaminergic Neuron Maturation Medium to achieve differentiation into mature DA neurons	22

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.

Temperature range: 36°C to 38°C

Incubator atmosphere: Humidified atmosphere of 5% CO_2. Ensure that proper gas exchange is achieved in culture vessels.

IMPORTANT! The PSC Dopaminergic Neuron Differentiation Kit is designed for hPSCs cultured in Essential 8™ or Essential 8™ Flex Media Systems. hPSCs co-cultured on MEF must be adapted to Essential 8™ culture medium for at least 4 passages before differentiation. hPSCs cultured in mTeSR™1 medium can initiate differentiation directly, but require additional supplementation at time of maturation. See the maturation section for more information (see "Note" on page 23).

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™ Medium. For details on feeder-free culture of hPSCs in Essential 8™ Medium, see **thermofisher.com/culturing-puripotent-stem-cells-essential-8-medium.**
- 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 page 3) to get double coating.
- For suspension culture of spheres, use non-tissue culture treated Nunclon™ Sphera™ culture flasks or dishes to prevent spheres from merging or adhering to the culture vessel, which can greatly compromise downstream differentiation.

  Note: T25 flask is the equivalent of 2.5 wells of a 6-well plate, and T75 flask is the equivalent of 7.5 wells of a 6-well plate.
- For the characterization of FP cells by immunocytochemistry (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 below) or troubleshoot to improve specification efficiency first (see page 26).

- 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). For ordering information, see page 28.

- 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 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 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 specification, expansion, and maturation media. On the day of use, aliquot the volume of complete needed for that day and pre-warm at 37°C before use.
- 100 mL of complete 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 Table 1 for the number of cells and volume of medium that is required when plating FP cells. Use 2.0×10^5 cells/cm^2 for expansion and sphere formation, and 1.0×10^5–2.0×10^5 cells/cm^2 for maturation as described in the protocol.

Table 1. Media volume and cell number that is required for plating FP cells

Culture vessel (approx. surface area)	Media volume (per well or dish)	Number of cells (per well or dish)	
		1.0×10^5/cm^2	2.0×10^5/cm^2
96-well plate (0.32 cm^2/well)	100 µL	3.2×10^4	6.4×10^4
48-well plate (1 cm^2/well)	250 µL	1.0×10^5	2.0×10^5
24-well plate (2 cm^2/well)	500 µL	2.0×10^5	4.0×10^5
12-well plate (4 cm^2/well)	1 mL	4.0×10^5	8.0×10^5
6-well plate (10 cm^2/well)	2 mL	1.0×10^6	2.0×10^6
35-mm dish (10 cm^2/dish)	2 mL	1.0×10^6	2.0×10^6
60-mm dish (20 cm^2/dish)	5 mL	2.0×10^6	4.0×10^6
T25 flask (25 cm^2/flask)	5 mL	2.5×10^6	5.0×10^6
100-mm dish (60 cm^2/dish)	10 mL	6.0×10^6	1.2×10^7
T75 flask (75 cm^2/flask)	15 mL	7.5×10^6	1.5×10^7

Prepare media, reagents, and culture plates

Complete Essential 8™ Medium

1. Thaw frozen Essential 8™ Supplement at room temperature for ~1 hour. **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™ Basal Medium, then aseptically transfer the entire contents of the Essential 8™ Supplement to the bottle of Essential 8™ Basal Medium. Swirl the bottle to mix.
3. Store the complete Essential 8™ 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 room temperature until it is no longer cool to the touch. **Do not warm the medium at 37°C.**

Complete Floor Plate Specification Medium

1. Thaw the Floor Plate 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 Specification Supplement to 95 mL of Neurobasal™ Medium and mix well.
3. Store the complete Floor Plate 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 Medium at 4°C and Floor Plate Cell Expansion Supplement (50X) at –20°C until use. Do not prepare 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 Medium 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 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 medium 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 Table 2, page 10, 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, pre-warm 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 Table 2, page 10, 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, pre-warm 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 Table 2 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 Table 2 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.
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, pre-warm 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.

Note: For convenience, you can apply laminin (15 µg/mL) to a commercially available poly-D-lysine-coated culture plate (such as BioCoat™ Poly-D-Lysine-coated plates, see page 3) to get double coating.

Table 2. Volume of matrix solution that is required to coat various culture vessels

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
35-mm dish (10 cm^2/dish)	1 mL
6-well plate (10 cm^2/well)	1 mL
60-mm dish (20 cm^2/dish)	2.5 mL
T25 flask (25 cm^2/flask)	2.5 mL
100-mm dish (60 cm^2/dish)	5 mL
T75 flask (75 cm^2/flask)	7.5 mL

Prepare 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 9. 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 Essential 8™ Medium (see page 8) 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 Essential 8™ 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 specification medium.

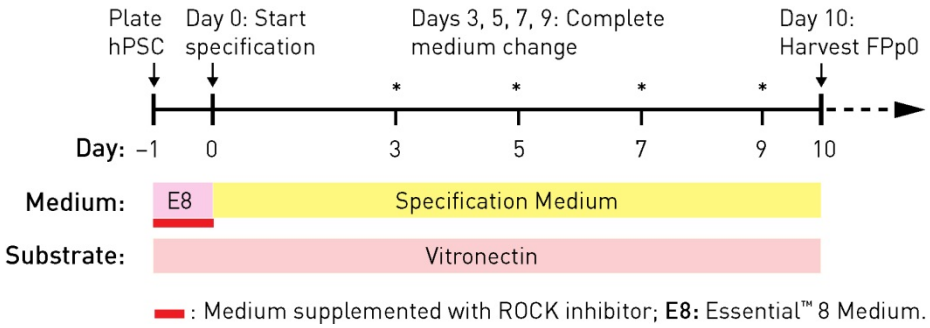
6. Incubate the cells overnight at 37°C in a humidified atmosphere of 5% CO_2.

7. Prepare complete Floor Plate Specification Medium (see page 8) 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 (FP) 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.



Day 0: Start specification

1. 24 hours after plating, the hPSC culture should be 20–40% confluent.

   IMPORTANT! Observe the quality of starting hPSC population. If the recovery (relative confluence and cell morphology) is poor, we recommend starting over with higher-quality cells. See Figure 1 for an example of a healthy hPSC culture that has recovered sufficiently to start specification.

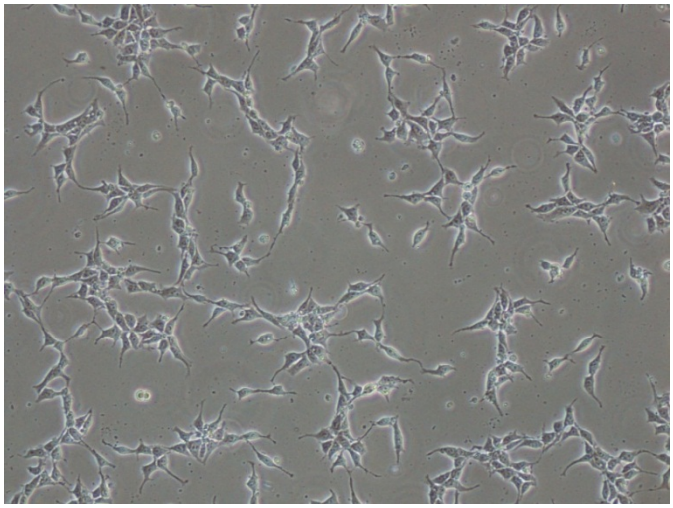
2. Aliquot the amount of complete specification medium (prepared on day –1) needed for the day and warm at 37°C (e.g., 2 mL per well of a 6-well plate; see Table 1, page 7, for the recommended volumes for other vessels).

   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 Essential 8™ medium containing the ROCK inhibitor and replace it with pre-warmed complete specification medium.

4. Incubate at 37°C in a humidified atmosphere of 5% CO_2.

Figure 1 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.

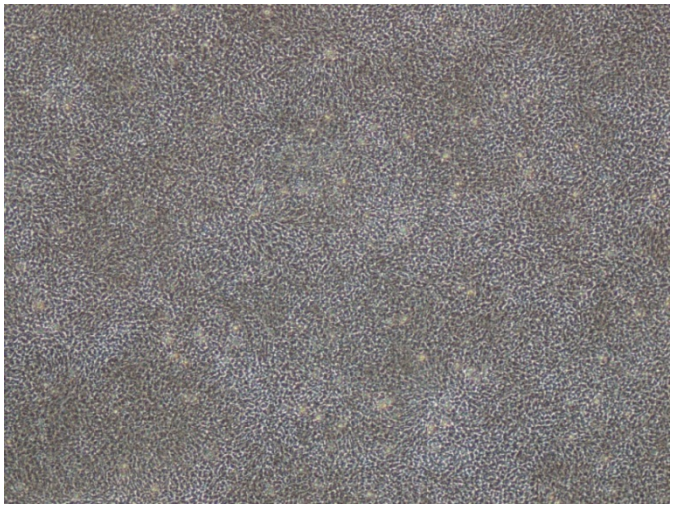


Days 3, 5, 7, 9: Complete medium change

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 Table 1, page 7, for the recommended volumes for other vessels).
2. **Days 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. **Days 7 and 9:** Cultures should be near or at 100% confluence, resulting in increased medium consumption. Replace the spent medium with 2X volume 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 floor plate progenitor phenotype as early as day 7 of specification. For more information, see "Expected results for specification", page 14.
4. On day 9, prepare complete expansion medium as described on page 8 and store at 4°C.
5. **Day 10:** Culture is now completely confluent and ready to be passaged (see Figure 2). Cells at this stage are floor plate progenitors (FP) and their numbers have increased approximately 25-fold compared to the hPSC seeded on day –1.

Figure 2. 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", page 14.

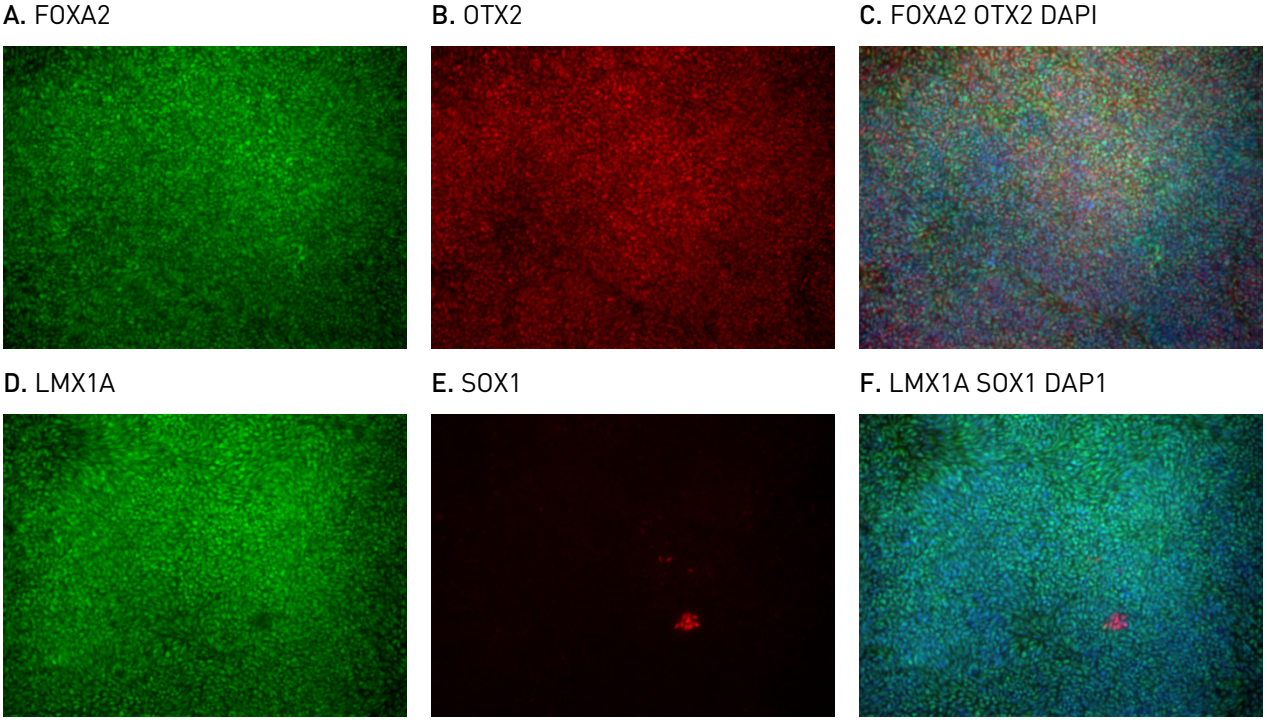
Expected results for specification

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 pre-made buffers for an optimized staining experiment.

Note: If the efficiency of FP specification (as determined by double positive expression 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", page 26).

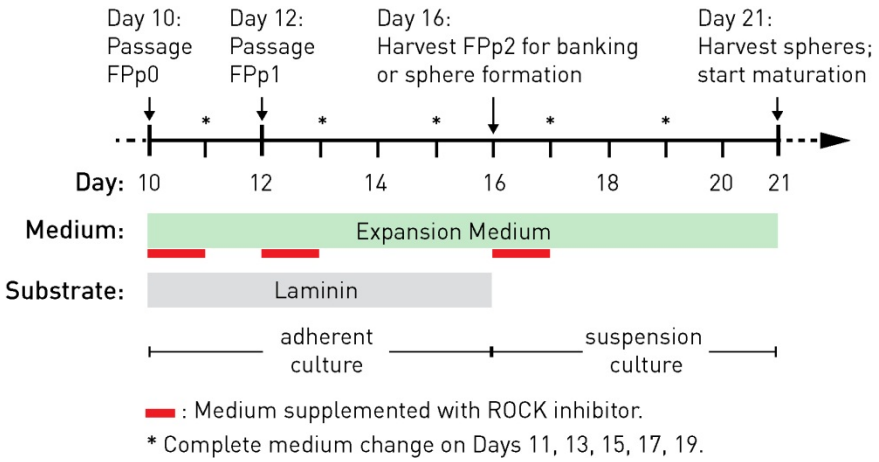
Figure 3. Marker expression of induced floor plate progenitor (FP) cells. hPSCs were treated with complete Floor Plate Specification Medium for 7 days and the cells were analyzed for the key phenotypic markers of the human dopaminergic neuron lineage using the Human Dopaminergic Neuron Immunocytochemistry 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 (page 20), which further improves the differentiation efficiency. On day 21 of differentiation, spheres are harvested for maturation.



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 9. 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 Table 1, page 7, 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 media.
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 × *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 + 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 + 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 specification medium.
12. Incubate the cells overnight at 37°C in a humidified atmosphere of 5% CO_2.

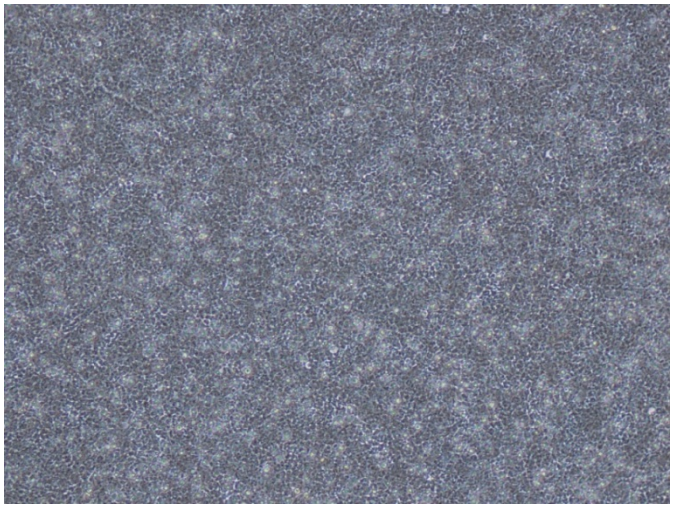
Day 11: Complete medium change

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% confluence. At this point, the culture is ready for passaging. See Figure 4 for an example of a confluent FP culture that is ready to be passaged.

    Figure 4. Floor plate progenitor culture that is ready to be passaged on day 12 of differentiation. The culture is very compact and exhibits 100% confluence.

    
2. Prepare the appropriate number of laminin-coated culture plates as described on page 9.

    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 pre-warm 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 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 × *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 1.0×10^7 viable cells/mL in complete expansion medium.
11. Plate the cell suspension at a high seeding density of 2.0×10^5 cells/cm^2 (i.e., 2.0×10^6 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.

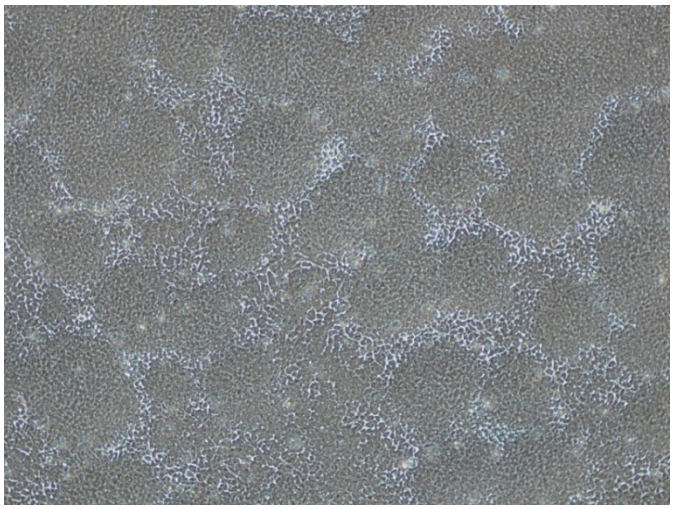
Days 13, 15: Complete medium change

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 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. Floor plate progenitor culture on day 16 of differentiation. The dense culture exhibits 100% confluence and is ready to be harvested.



2. Harvest the cells using StemPro™ Accutase™ reagent as described for FPp1 (page 16) and resuspend to 1.0×10^7 cells/mL in complete expansion medium.
3. Use the FPp2 cell suspension for cryopreservation (page 18) or proceed with sphere formation (page 20).

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 (page 26), 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 4–6-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 2×10^6– 5×10^6 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 2×10^6 cells/vial, transfer 2 mL of cells from the FPp2 harvest (at 1.0×10^7 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 2×10^6 cells/mL, add 3 mL of complete expansion medium to the 2 mL of cell suspension from the FPp2 harvest (at 1.0×10^7 cells/mL). This dilutes the cells to 4×10^6 cells/mL (2X the final frozen concentration).
4. In a drop-wise manner, add the same volume of 2X freezing medium (chilled to 4ºC) as the cell suspension while gently rocking the tube back and forth.

   For example, if the tube contains 5 mL of cells suspension at 4×10^6 cells/mL, add 5 mL of 2X freezing medium to dilute the cells to a final concentration of 2×10^6 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 Nalgene™ Mr. Frosty™ Freezing Container with isopropanol, and freeze them at –80°C overnight.

   Note: You can also freeze the cells in an automated or manual controlled rate freezing 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 × *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 1.0×10^6 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) (see page 14).

Expansion (Day 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 (page 17) or thaw FPp2 cells (page 19) as described, then remove a small volume of cells and perform a viable cell count.
2. Resuspend the FPp2 cells to 1.0×10^6 viable cells/mL in complete expansion medium + 5 µM ROCK inhibitor (Y27632).
3. Transfer cell suspension to a non-tissue culture treated culture vessel and adjust the volume of the cell suspension to the size of vessel. See Table 1, page 7, for the recommended seeding density and media volume for various culture vessel sizes.

   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.

Days 17, 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 × *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.

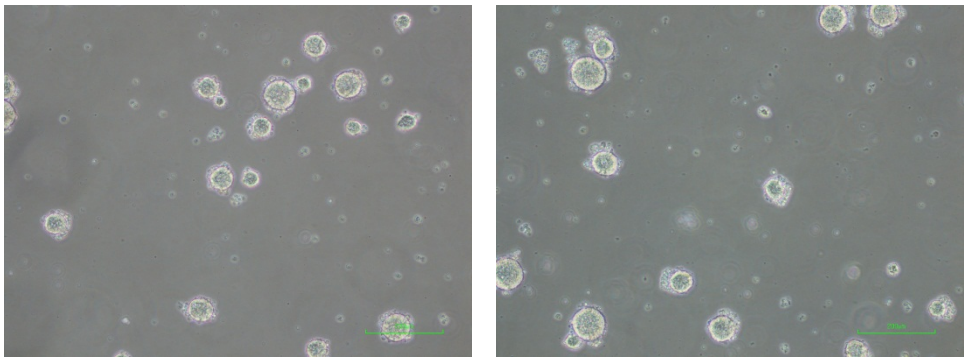
   #### Gravity method

   a. Tilt the T-flask so that most of the spheres settle down 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 6. Spheres on Day 3 (left) and Day 5 (right) of suspension culture (Day 19 and 21 of differentiation, respectively).



Day 21: Dissociate spheres

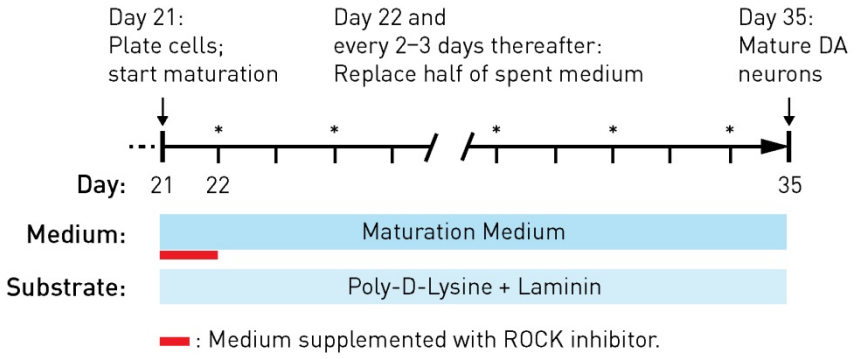
1. Transfer the sphere suspension from culture vessel to a sterile 15-mL conical tube. Allow spheres to settle to the bottom of the tube (~2–5 minutes) before proceeding to the next step.
2. Carefully aspirate the spent medium, leaving the spheres at the bottom of tube in a minimal volume (~100 µL) of 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 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 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., Countess™ II Automated Cell Counter) or a hemocytometer.
10. Centrifuge the cell suspension at 300 × *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 + 5 µM ROCK inhibitor (Y27632) and proceed to maturation step (page 22).

Maturation (Day 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.



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 10.

   Note: For convenience, you can apply laminin (15 µg/mL) to a commercially available poly-D-lysine-coated culture plate (see page 3) to get double coating.

   IMPORTANT! Make 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, page 21) at a seeding density of 1.0×10^5–2.0×10^5 cells/cm^2 in complete maturation medium + 5 µM ROCK inhibitor (Y27632). See Table 1, page 7, for the cell number and media volume required for various culture vessels.

   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.

Days 22–35: 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, 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", page 24).

Note: To maintain derived neurons after maturation, you can replace DMEM/F-12 medium (Cat. No. 10565018) with Neurobasal™ Medium (Cat. No. 21103049) as the base medium in complete maturation medium (see page 8). In some cell lines and culture conditions (for example, when using mTeSR™1 medium), maturation occurs more quickly and neurons benefit from base medium change (from DMEM/F-12 medium to Neurobasal™ Medium) and the addition of 1X B-27 supplement to the complete maturation medium.

Expected results

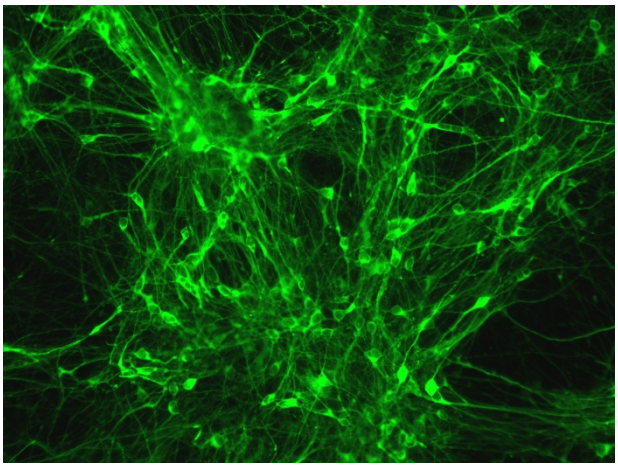
Expression of phenotypic markers for DA neurons

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.

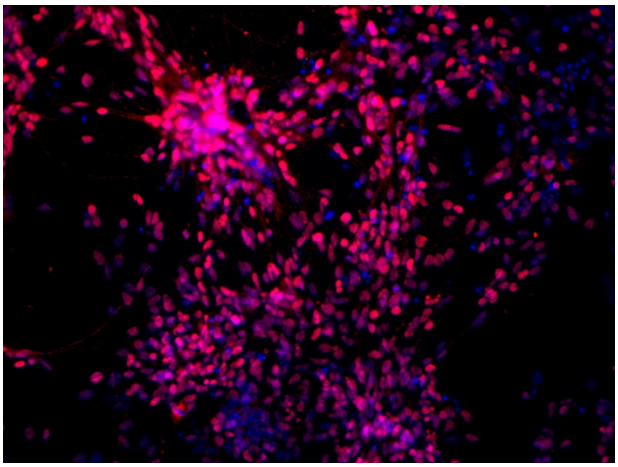
Note: For the image-based analysis of mature DA neurons, we recommend using the Human Dopaminergic Neuron Immunocytochemistry Kit (Cat. No. A29515), which includes a complete set of primary and secondary antibodies, a nuclear DNA stain, and pre-made buffers for an optimized staining experiment.

Figure 7. Representative images of mature DA neurons. The following 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 NucBlue™ (a DAPI nuclear DNA stain) (blue), **C.** Merged image with anti-TH and anti-FOXA2 (green and red).

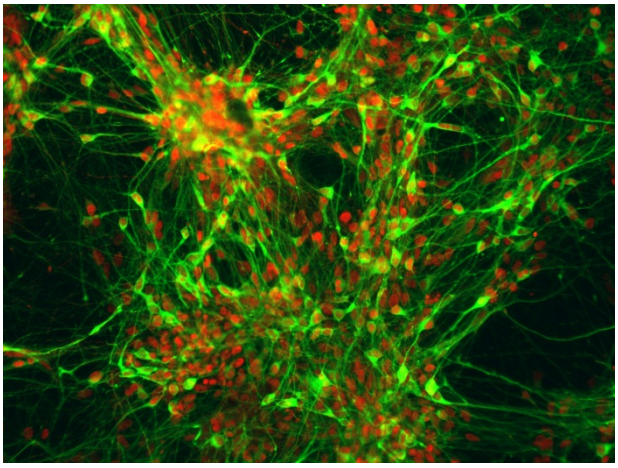
A. TH



B. FOXA2 and DAPI



C. TH and FOXA2



Replate dopaminergic neurons for downstream assays

Replate dopaminergic neurons

1. Mature floor plate progenitors, see page 19.
2. Fourteen days after maturation, the DA neuron cultures are ready to be dissociated. If desired, DA neuron yield can be checked before continuing replating by processing one well for immunostaining of TH and FOXA2.
3. Prepare poly-d-lysine/laminin (PDL/Lam) double coated plates (format of choice, 24- and 384-well format were tested extensively) as described in the PSC Dopaminergic Neuron Differentiation kit manual (MAN0015919, page 10).
4. On the day of replating, prepare DA neuron maturation medium as per the PSC Dopaminergic Neuron Differentiation kit manual (MAN0015919, page 8) and supplement the medium with 1x RevitaCell™ Supplement (Cat. No. A2644501)
5. For dissociation, gently rinse the cells with D-PBS before addition of dissociation reagents.
6. Remove D-PBS and add an appropriate volume of TrypLE™ Express Enzyme (Cat. No. 12604039) to the DA cultures. We recommend 250 µL for 24-well, 500 µL for 12-well and 1 mL for 6-well plates.
7. Incubate for 30 minutes at 37°C, with 5% CO_2.
8. After incubation, gently resuspend the cultures by triturating the cells in the TrypLE™ Express Enzyme. Minimal pipetting is needed to singularize the cultures at this point, excessive pipetting will affect viability. Add 3 volumes of DA neuron maturation medium with RevitaCell™ Supplement and carefully mix the cell suspension. At this stage clumps (if any) can be removed using a standard cell strainer (40µm).
9. Spin down the cells at 300 × *g* for 5 minutes.
10. Resuspend the cells in an appropriate volume of DA maturation medium with RevitaCell™ Supplement and count the cells.
11. Plate the cells at desired densities onto the PDL/Lam plates and place in a humidified incubator overnight (37°C, with 5% CO_2).
12. The next day, change the medium to DA maturation medium supplemented with 0.3 µM AraC (Tocris, Cat. No. 4520).

    Note: AraC is added to suppress the proliferation of undifferentiated floor plate progenitors.
13. Cells can be maintained by performing media changes every 2–3 days. If prolonged suppression of progenitors is required, continued addition of 0.3 µM AraC is recommended.
14. Cells can be used for neuronal assays as soon as 3 days post plating.

Appendix A: Troubleshooting

Media and supplements

Observation	Action
Supplement (Specification and Maturation) has uneven (marbly) appearance.	Uneven (marbly) 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 non-homogenous appearance of the supplements may persist when frozen, but will disappear upon thawing.

Specification

Observation	Action
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 confluence at the start of differentiation is 20–40%). We recommend repeating the Day –1 activity and seeding a fresh hPSC culture for specification (page 9). 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 instead of as 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% confluence 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

Observation	Action
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 non-tissue culture treated plasticware or ultra-low 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

Observation	Action
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 to 10 days. When initiating suspension culture for sphere formation, reduce the seeding density by half (i.e., use 0.5×10^6 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 change 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 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/F12 medium to Neurobasal™ Medium. Supplementation with 1X B27 supplement can improve viability, especially for cultures initiated from PSCs maintained in mTeSR™1 medium. H9-derived neurons can be maintained in Neurobasal™ Medium with 1X B27 supplement for at least 14 days after maturation (Day 35 for the whole procedure).

Appendix B: Ordering information

PSC Dopaminergic Neuron Differentiation Kit

The PSC Dopaminergic Neuron Differentiation Kit and some of its components are available separately from Thermo Fisher Scientific. Ordering information is provided below. For more information, see **thermofisher.com** or contact Technical Support (see page 32).

Product	Amount	Catalog No.
PSC Dopaminergic Neuron Differentiation Kit	1 kit	A3147701
Floor Plate Specification Supplement (20X)	5 mL	A3146801
Floor Plate Cell Expansion Kit	1 kit	A3165801
Dopaminergic Neuron Maturation Supplement (50X)	10 mL	A3147401

Media and reagents

The following media and reagents suitable for use with the PSC Dopaminergic Neuron Differentiation Kit are available separately from Thermo Fisher Scientific. Ordering information is provided below. For more information, see **thermofisher.com** or contact Technical Support (see page 32).

Product	Amount	Catalog No.
Essential 8™ Medium	500 mL	A1517001
Essential 8™ Flex Medium	1 kit	A2858501
Neurobasal™ Medium	500 mL	21103049
DMEM/F-12 medium, GlutaMAX™ supplement	500 mL	10565018
DPBS, no calcium, no magnesium	500 mL 1000 mL	14190144 14190136
Vitronectin (VTN-N), recombinant human	1 mL	A14700
Laminin mouse protein, natural	1 mg	23017015
StemPro™ Accutase™ Cell Dissociation Reagent	100 mL	A1110501
B-27™ Supplement (50X), serum-free	10 mL 100 mL	17504044 17504001
Distilled water	100 mL 500 mL 1000 mL	15230170 15230162 15230147
Human Dopaminergic Neuron Immunocytochemistry Kit	1 kit	A29515

Accessory products

The following accessory products suitable for use with the PSC Dopaminergic Neuron Differentiation Kit are available separately from Thermo Fisher Scientific. Ordering information is provided below. For more information, see **thermofisher.com** or contact Technical Support (see page 32).

Product	Amount	Catalog No.
Countess™ II Automated Cell Counter	1 instrument	AMQAX1000
Countess™ II FL Automated Cell Counter	1 instrument	AMQAF1000
Nalgene™ Mr. Frosty™ Cryo 1°C Freezing Container	1 each	5100-0001
Nunclon™ Sphera™ T25 Cell Culture Flask	6 units/bag, 18 units/case	174951
Nunclon™ Sphera™ T75 Cell Culture Flask	4 units/bag, 24 units/case	174952
Nunclon™ Sphera™ Multidish 24-well	1 each	174930
Nunclon™ Sphera™ Multidish 12-well	1 each	174931
Nunclon™ Sphera™ Multidish 6-well	1 each	174932

Appendix C: Safety

General safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, etc.). To obtain SDSs, see the "Documentation and support" section in this document (page 32).

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below, and consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and support" section in this document (page 32).
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.

IMPORTANT! Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological hazard safety

WARNING! Potential Biohazard. Depending on the samples used on thisinstrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Safety equipment also may include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th Edition, HHS Publication No. (CDC) 21-1112, Revised December 2009; found at:

  www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf

- World Health Organization, *Laboratory Biosafety Manual*, 3rd Edition, WHO/CDS/CSR/LYO/2004.11; found at:

  www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf

Documentation and support

Customer and technical support

Visit **www.thermofisher.com/support** for the latest in services and support, including:

- Worldwide contact telephone numbers
- Product support, including:
  - Product FAQs
  - Software, patches, and updates
  - Training for many applications and instruments
- Order and web support
- Product documentation, including:
  - User guides, manuals, and protocols
  - Certificates of Analysis
  - Safety Data Sheets (SDSs; also known as MSDSs)

Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html.** If you have any questions, please contact Life Technologies at **www.thermofisher.com/support.**