

STEMdiff™ Hematopoietic Kit

For differentiation of human ES or iPS cells into hematopoietic progenitor cells

Catalog #05310

1 Kit



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Product Description

STEMdiff™ Hematopoietic Kit includes a serum-free basal medium and supplements for the feeder-free differentiation of human embryonic stem (ES) and induced pluripotent stem (iPS) cells into hematopoietic progenitor cells expressing CD34, CD45, and CD43.

The simple, 12-day differentiation protocol is performed in two stages. During the first 3 days, STEMdiff™ Hematopoietic Supplement A is added to the basal medium to induce cells toward mesoderm. For the subsequent 9 days, mesodermal cells are further differentiated into hematopoietic progenitor cells using basal medium supplemented with STEMdiff™ Hematopoietic Supplement B. At the end of the 12-day protocol, hematopoietic cells can be easily harvested from the culture supernatant. This population typically contains 25 - 65% (average 43%) CD34+CD45+ progenitor cells, including progenitor cells that have the capacity to form hematopoietic colonies in the colony-forming unit (CFU) assay.

STEMdiff™ Hematopoietic Kit has been optimized for differentiation of cells maintained in mTeSR™1 (Catalog #85850), mTeSR™ Plus (Catalog #100-0276), or TeSR™-E8™ (Catalog #05990).

Product Information

The following components are sold as part of STEMdiff™ Hematopoietic Kit (Catalog #05310) and are not available for individual sale.

COMPONENT NAME	COMPONENT #	SIZE	STORAGE	SHELF LIFE
STEMdiff™ Hematopoietic Basal Medium	05311	120 mL	Store at -20°C.	Stable for 12 months from date of manufacture (MFG) on label.
STEMdiff™ Hematopoietic Supplement A (200X)	05312	225 µL	Store at -20°C.	Stable for 2 years from date of manufacture (MFG) on label.
STEMdiff™ Hematopoietic Supplement B (200X)	05313	375 µL	Store at -20°C.	Stable for 2 years from date of manufacture (MFG) on label.

Materials Required but Not Included

PRODUCT NAME	CATALOG #
Corning® Matrigel® hESC-Qualified Matrix	Corning 354277
DMEM/F-12 with 15 mM HEPES	36254
Dispase (1 U/mL) OR Gentle Cell Dissociation Reagent OR ReLeSR™	07923 OR 100-0485 OR 05872
mTeSR™1 OR mTeSR™ Plus OR TeSR™-E8™	85850 OR 100-0276 OR 05990

Preparation of Media

Two different media are required for the hematopoietic differentiation protocol: **Medium A (Stage 1; Day 0 - 3)** and **Medium B (Stage 2; Day 3 - 12)**.

Prepare media as required according to section B of Directions for Use. Refer to Table 1 for medium components, volumes, and in-use storage and stability.

Use sterile technique to prepare Medium A (Basal Medium + Supplement A) and Medium B (Basal Medium + Supplement B). Volumes indicated are for preparing 45 mL of Medium A and 75 mL of Medium B. If preparing other volumes, adjust accordingly.

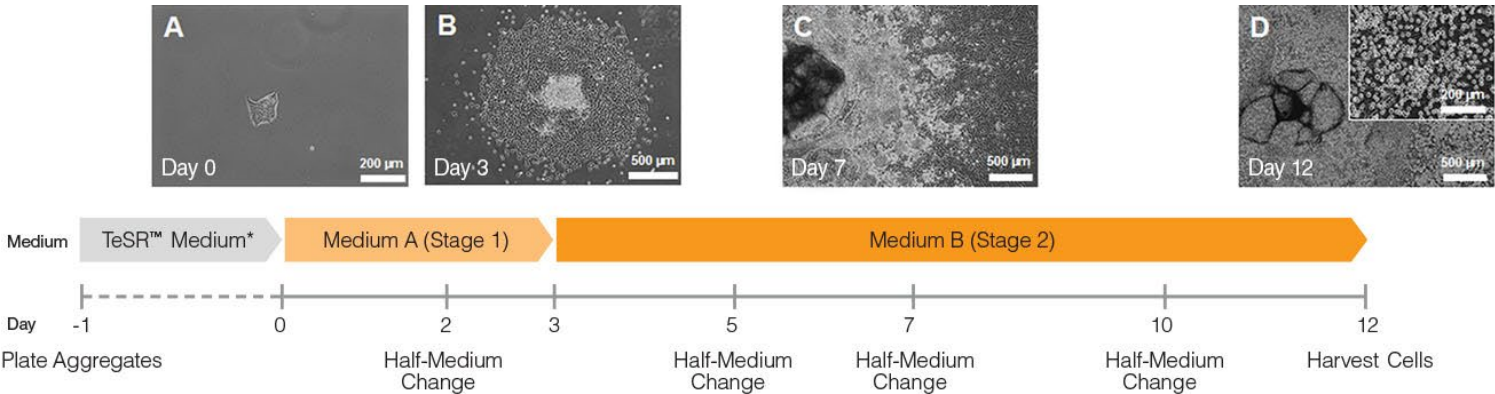
1. Thaw STEMdiff™ Hematopoietic Basal Medium at room temperature (15 - 25°C) or overnight at 2 - 8°C. Mix thoroughly.
NOTE: If not used immediately, store at 2 - 8°C for up to 4 weeks, or aliquot and store at -20°C. After thawing aliquots, use immediately or store at 2 - 8°C for up to 2 weeks. Do not re-freeze. Do not exceed the shelf life of the basal medium.
2. Thaw Supplement A or B at room temperature or at 2 - 8°C until just thawed. Mix thoroughly. If necessary, centrifuge for 30 seconds to remove liquid from cap.
NOTE: If not used immediately, aliquot and store at -20°C. Do not exceed the shelf life of the supplement. After thawing aliquots, use immediately. Do not re-freeze.
3. Add Supplement to Basal Medium as indicated in Table 1. Mix thoroughly.
NOTE: If not used immediately, store complete medium as indicated in Table 1.

Table 1. Preparation of STEMdiff™ Hematopoietic Differentiation Media

MEDIUM	COMPONENT	VOLUME	IN-USE STORAGE AND STABILITY*
Medium A (45 mL)	STEMdiff™ Hematopoietic Basal Medium	45 mL	Store at 2 - 8°C for up to 4 weeks OR Store at -20°C for up to 6 months
	STEMdiff™ Hematopoietic Supplement A (200X)	225 µL	
Medium B (75 mL)	STEMdiff™ Hematopoietic Basal Medium	75 mL	
	STEMdiff™ Hematopoietic Supplement B (200X)	375 µL	

*Do not exceed the shelf life of the basal medium or supplement.

Protocol Diagram



*mTeSR™1, mTeSR™ Plus, or TeSR™-E8™

On Day -1, harvest and seed human ES/iPS cell colonies as small aggregates in mTeSR™1, mTeSR™ Plus, or TeSR™-E8™. On Day 0 (after confirming the number of adhered colonies is within 4 - 10/cm²), replace TeSR™ medium with Medium A to induce the cells toward a mesoderm-like state. On Day 2, perform a half-medium change with fresh Medium A. On Day 3, change to Medium B and perform half-medium changes on Days 5, 7, and 10 to promote further differentiation into hematopoietic cells. Typically, by Day 12, large numbers of hematopoietic progenitor cells can be harvested from the culture supernatant.

- (A) On Day 0, sparse colonies—which may be large or contain only a few cells—should be present. A mid-sized colony is shown.
- (B) On Day 3, mesodermal cells migrate outward from the colonies. (C) On Day 7, colonies continue to grow outward and round hematopoietic cells emerge from the supporting adherent cells. (D) On Day 12, colonies are very large, with many round hematopoietic cells floating in the culture supernatant (inset).

Directions for Use

Please read all protocols before proceeding. Use sterile technique when performing the following protocols.

A. PASSAGING CELLS AS AGGREGATES AND HEMATOPOIETIC DIFFERENTIATION SETUP

This protocol is for human ES or iPS cells cultured in mTeSR™1, mTeSR™ Plus, or TeSR™-E8™. Use the medium with which the cells are routinely maintained and use whichever passaging reagent is preferred.

The setup instructions are for 12-well plates; indicated volumes are for a single well. If using other cultureware, adjust volumes accordingly.

NOTE: For complete instructions on maintaining high-quality human ES and iPS cells and for coating plates with Corning® Matrigel®, refer to the Technical Manual for mTeSR™1, mTeSR™ Plus, or TeSR™-E8™, available at www.stemcell.com, or contact us to request a copy.

1. Coat cultureware with Corning® Matrigel® prior to passaging cells.
2. Passage human ES or iPS cells as aggregates of 100 - 200 µm in diameter using one of the following reagents:
 - Gentle Cell Dissociation Reagent: Passaging protocol as described in the Technical Manual for mTeSR™1, mTeSR™ Plus, or TeSR™-E8™.
 - ReLeSR™: Passaging protocol as described in the Product Information Sheet (PIS; Document #10000008733) for ReLeSR™.
 - Dispase (1 U/mL): Passaging protocol as described in the PIS for Dispase.
3. Perform triplicate aggregate counts as described below to determine the average number of cell aggregates (≥ 50 µm in diameter) in a 5 µL sample:
 - a. Aliquot 40 µL of DMEM/F-12 into 3 wells of a 96-well flat-bottom plate. Add 5 µL of aggregate mixture to each well.
 - b. In each well, count aggregates that are ≥ 50 µm in diameter. Average the triplicate results and calculate the *Concentration of Cell Aggregates* (aggregates/µL).

NOTE: For complete instructions on cell aggregate counting, refer to the Technical Manual for mTeSR™1, mTeSR™ Plus, or TeSR™-E8™.

4. Determine the *Number of Aggregates to Plate*. It is recommended to plate **40 - 80 aggregates/well** (10 - 20 aggregates/cm²) to achieve **16 - 40 colonies/well** (4 - 10 colonies/cm²) adhered to the cultureware after 24 hours of incubation; however, multiple plating densities may need to be tested.
5. Calculate the *Plating Volume* of cell aggregate mixture for each condition in your experiment, as follows:

$$\text{Plating Volume (}\mu\text{L)} = \text{Number of Aggregates to Plate (step 4)} \div \text{Concentration of Cell Aggregates (step 3b)}$$
6. Gently mix the cell aggregate mixture. Add the calculated *Plating Volume* (step 5) to each well of a 12-well plate coated with Corning® Matrigel® (prepared in step 1) and containing 1 mL of mTeSR™1, mTeSR™ Plus, or TeSR™-E8™.
 NOTE: If using split ratios, a range of 1 in 40 to 1 in 200 may be required depending on the confluence of the passaged well.
7. Place the plate in a 37°C incubator. Move the plate in several quick, short, back-and-forth and side-to-side motions to distribute the cell aggregates. Do not disturb the plate for 24 hours.
8. Proceed to section B for hematopoietic differentiation.

B. HEMATOPOIETIC DIFFERENTIATION

The following instructions are for 12-well plates; indicated volumes are for a single well. If using other cultureware, adjust volumes accordingly.

NOTE: Throughout the protocol, warm all media to room temperature (15 - 25°C) before use. Do not leave media at room temperature for extended periods of time.

Stage 1

Day 0

1. Confirm that **16 - 40 colonies/well** are adhered to the cultureware (4 - 10 colonies/cm²). Ensure to count all colonies, including tiny colonies with only a few cells.

NOTE: To facilitate counting, aspirate medium and replace with fresh mTeSR™1, mTeSR™ Plus, or TeSR™-E8™ (this will help to remove debris).

CRITICAL: Do not proceed if cultures have < 16 colonies or > 40 colonies per well, as differentiation will be compromised.

2. Prepare Medium A (see Preparation of Media) required for Day 0 and Day 2 (total of 1.5 mL per well of a 12-well plate).
3. Aspirate medium from wells. Add 1 mL of Medium A per well. Store remaining Medium A at 2 - 8°C until required.
4. Incubate at 37°C for 2 days.

Day 2

5. Using a serological pipette or a 1 mL pipette tip, gently remove 0.5 mL of medium from each well and discard.
6. Gently add 0.5 mL of Medium A per well.
7. Incubate at 37°C for 24 hours.

Stage 2**Day 3**

8. Prepare Medium B (see Preparation of Media) required for Day 3, 5, 7, and 10 (total of 2.5 mL per well of a 12-well plate).
9. Aspirate medium from wells. Gently add 1 mL of Medium B per well. Store remaining Medium B at 2 - 8°C until required.
10. Incubate at 37°C for 2 days.

Day 5

11. Using a serological pipette or a 1 mL pipette tip, gently remove 0.5 mL of medium from each well and discard.
12. Gently add 0.5 mL of Medium B per well. Store remaining Medium B at 2 - 8°C until required.
13. Incubate at 37°C for 2 days.

Day 7

NOTE: At this point, floating cells can often be seen in culture and they will increase in number for the remainder of the protocol.

14. Keeping the plate flat, use a serological pipette or a 1 mL pipette tip to gently remove 0.5 mL of medium from each well, being careful not to disturb the floating cell population and discard.
15. Gently add 0.5 mL of Medium B per well. Store remaining Medium B at 2 - 8°C until required.
16. Incubate at 37°C for 3 days.

Day 10

NOTE: If desired, cells may be harvested now as described for Day 12. The cell yield and proportion of CD34+CD45+ cells will be much lower at Day 10 than at Day 12; however, CFUs may be present at a higher frequency in cells harvested at Day 10.

17. Keeping the plate flat, use a serological pipette or a 1 mL pipette tip to gently remove 0.5 mL of medium from each well, being careful not to disturb the floating cell population and discard.
18. Gently add 0.5 mL of Medium B per well.
19. Incubate at 37°C for 2 days.

Day 12 - Harvest hematopoietic cells

20. Harvest supernatant cells:
 - a. Using a serological pipette or a 1 mL pipette tip, vigorously pipette the cells up and down in the well to break them up as needed (triturate).
 - b. Transfer the cell suspension to a collection tube.
 - c. Add 1 mL of DMEM/F-12 to the well. Triturate vigorously in the well and add to the collection tube.
 - d. Repeat step c.
 - e. Centrifuge the collection tube at 300 x g for 5 minutes at room temperature (15 - 25°C).
 - f. Remove and discard the supernatant.
 - g. Resuspend cell pellet in desired medium for analysis or downstream assays.
21. Harvest adherent cells (OPTIONAL):

NOTE: The adherent layer is heterogeneous but will contain some additional hematopoietic progenitor cells. Typically, > 75% of all hematopoietic progenitor cells are recovered in the supernatant.

- a. Remove supernatant cells as described in step 20.
- b. Wash the well with 1 mL of D-PBS (Without Ca++ and Mg++; Catalog #37350). Discard the wash.
- c. Add 0.5 mL of ACCUTASE™ to the well.
- d. Incubate at 37°C for 20 minutes.
- e. Triturate vigorously with a 1 mL pipette tip to dislodge the adherent cells and create a single-cell suspension. Do not scrape to remove residual colonies from the cultureware surface, as these clumps will not further dissociate.
- f. Transfer the single-cell suspension to a collection tube containing 1 - 3 mL of DMEM/F-12.
- g. Wash the well with an additional 1 mL of DMEM/F-12. Add wash to the collection tube. Repeat.
- h. Centrifuge the collection tube at 300 x g for 5 minutes at room temperature.

- i. Remove and discard the supernatant.
- j. Resuspend the cell pellet in desired medium for analysis or downstream assays.

C. ASSESSING DIFFERENTIATION TO HEMATOPOIETIC PROGENITOR CELLS

Analysis of differentiated cells can be performed using methods, such as flow cytometry or CFU assays. The following antibodies are recommended for assessment of hPSC-derived hematopoietic progenitor cells by flow cytometry:

- Anti-Human CD34 Antibody, Clone 581 (Catalog #60013)
- Anti-Human CD45 Antibody, Clone HI30 (Catalog #60018)
- Anti-Human CD43 Antibody, Clone CD43-10G7 (Catalog #60085)

For CFU assays, MethoCult™ SF H4636 (Catalog #04636) is recommended for detection and quantitation of hPSC-derived hematopoietic progenitor cell subtypes, including granulocyte-macrophage progenitor cells (CFU-GM, CFU-G, and CFU-M), erythroid progenitor cells (BFU-E and CFU-E), and multipotential granulocyte, erythroid, macrophage, and megakaryocyte progenitor cells (CFU-GEMM). Serum-containing MethoCult™ H4435 Enriched (Catalog #04435) may also be used for CFU assays of hPSC-derived hematopoietic progenitor cells.

For further details, including MethoCult™ handling and plating instructions, refer to the Technical Manual: Human Colony-Forming Unit (CFU) Assays Using MethoCult™ (Document #10000005589), available at www.stemcell.com, or contact us to request a copy. For hPSC-derived CFU assays, refer to Table 2 for the recommended number of hPSC-derived hematopoietic cells to plate.

Table 2. Recommended Number of hPSC-Derived Hematopoietic Cells to Plate in MethoCult™ SF H4636 or MethoCult™ H4435 Enriched

DAY OF DIFFERENTIATION	10X CELL SUSPENSION TO BE PREPARED	CELLS PLATED PER 35 mm DISH
Day 10	30,000 viable cells (20,000 - 100,000)	3000 viable cells (2000 - 10,000)
Day 12	50,000 viable cells (30,000 - 200,000)	5000 viable cells (3000 - 20,000)

NOTE: If it is difficult to anticipate the correct number of cells to plate (e.g. when testing a new hPSC line), use two or more numbers that differ by 2- to 3-fold (e.g. 5000 cells per dish and 10,000 cells per dish).

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