# 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 defined, 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 and CD45.

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 or TeSR™-E8™.

## 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 12 months from date of manufacture (MFG) on label. |
| STEMdiff™ Hematopoietic Supplement B (200X) | 05313 | 375 µL | Store at -20°C. | Stable for 12 months from date of manufacture (MFG) on label. |

## Materials Required But Not Included

| PRODUCT NAME | CATALOG # |
|---|---|
| mTeSR™1<br>OR<br>TeSR™-E8™ | 85850<br>OR<br>05940 |
| Corning® Matrigel® hESC-Qualified Matrix | Corning 354277 |
| Gentle Cell Dissociation Reagent<br>OR<br>ReLeSR™<br>OR<br>Dispase (1 U/mL) | 07174<br>OR<br>05872<br>OR<br>07923 |
| DMEM/F-12 with 15 mM HEPES | 36254 |

## Preparation of Media

Two medium formulations are required for the hematopoietic differentiation protocol: Medium A (Day 0 - 3) and Medium B (Day 3 - 12).

Use sterile techniques to prepare Medium A (Basal Medium + Supplement A) and Medium B (Basal Medium + Supplement B).

The following examples are for preparing 45 mL of Medium A and 75 mL of Medium B. If preparing other volumes, adjust accordingly.

**Medium A** (Prepare on **Day 0**)

NOTE: A total of 1.5 mL of Medium A is required during Stage 1 of Hematopoietic Differentiation, per well of a 12-well plate.

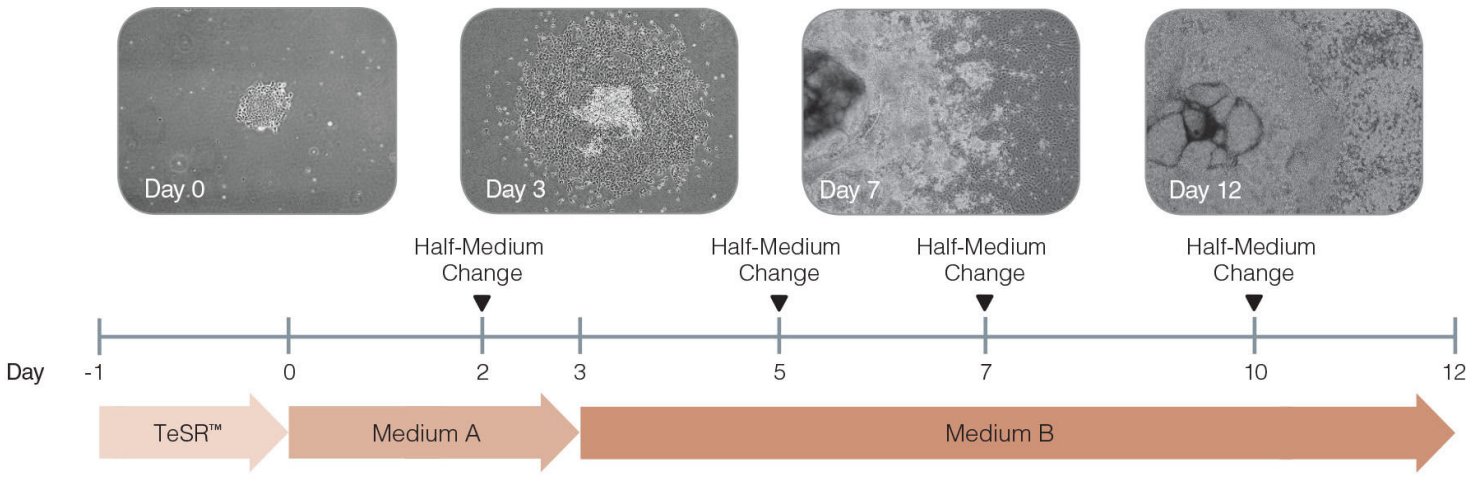
1. Thaw STEMdiff™ Hematopoietic Basal Medium at room temperature (15 - 25°C) or overnight at 2 - 8°C. Mix thoroughly.
   NOTE: If not used immediately, store as described below. Do not exceed the shelf life of the basal medium.
   - Store at 2 - 8°C for up to 6 months OR
   - Aliquot and store at -20°C. After thawing aliquots, use immediately or store at 2 - 8°C for up to 2 weeks. Do not re-freeze.
2. Thaw Supplement A at room temperature (15 - 25°C) 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, store as described below. Do not exceed the shelf life of the supplement.
   - Aliquot and store at -20°C. After thawing aliquots, use immediately. Do not re-freeze.
3. Add 225 µL of Supplement A to 45 mL of Basal Medium. Mix thoroughly.
   NOTE: If not used immediately, store as described below. Do not exceed the shelf life of the basal medium or supplement.
   - Store complete Medium A at 2 - 8°C for up to 3 weeks OR
   - Store at -20°C for up to 6 months

**Medium B** (Prepare on **Day 3**)

NOTE: A total of 2.5 mL of Medium B is required during Stage 2 of Hematopoietic Differentiation, per well of a 12-well plate.

1. Thaw Supplement B at room temperature (15 - 25°C) 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, store as described below. Do not exceed the shelf life of the supplement.
   - Aliquot and store at -20°C. After thawing aliquots, use immediately. Do not re-freeze.
2. Add 375 µL of Supplement B to 75 mL of Basal Medium. Mix thoroughly.
   NOTE: If not used immediately, store as described below. Do not exceed the shelf life of the basal medium or supplement.
   - Store complete Medium B at 2 - 8°C for up to 3 weeks OR
   - Store at -20°C for up to 6 months

## Protocol Diagram



# Directions for Use

Please read the entire protocol before proceeding. Use sterile techniques when performing the following protocols.

## A. PASSAGING CELLS AS AGGREGATES FOR HEMATOPOIETIC DIFFERENTIATION

This protocol is for human ES or iPS cells cultured in either mTeSR™1 or TeSR™-E8™ medium. Use the medium with which the cells are routinely maintained and whichever passaging reagent is preferred. The differentiation protocol instructions are for use with 12-well plates. Indicated volumes are for a single well. If using alternative 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: Maintenance of Human Pluripotent Stem Cells in mTeSR™1 (Document #28315) or TeSR™-E8™ (Document #29267), 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: Maintenance of Human Pluripotent Stem Cells in mTeSR™1 (Document #28315) or TeSR™-E8™ (Document #29267).
   - ReLeSR™: Passaging protocol as described in the ReLeSR™ Product Information Sheet (Document #28207).
   - Dispase (1 U/mL): Passaging protocol as described in the Dispase Product Information Sheet (Document #29639).
3. Plate aggregates; to achieve the required colony density for differentiation, first perform cell aggregate counts as described below.

   **CRITICAL**: Plate sufficient aggregates so that after 24 hours of incubation there are approximately 16 - 40 colonies/well (4 - 10 colonies/cm$^2$) adhered to the cultureware. A plating density of 40 - 80 aggregates/well (10 - 20 aggregates/cm$^2$) is recommended; however, multiple plating densities may need to be tested to achieve the correct colony density.

   a. Draw a "+" centered on the bottom 3 wells of a 96-well flat-bottom plate to serve as a counting grid.

   b. Aliquot 40 µL of DMEM/F-12 (Catalog #36254) into each well.

   c. Add 5 µL of freshly resuspended cell aggregate mixture to each well.

   d. Count the aggregates in each well that are ≥ 50 µm in diameter. Average the results from the 3 wells to obtain the average number of cell aggregates ($N_A$) in the 5 µL sample.

   NOTE: Aggregate diameters ranging from 100 - 200 µm are ideal for use with STEMdiff™ Hematopoietic Kit; however, since aggregates ≥ 50 µm in diameter are also likely to grow into colonies, count any aggregate ≥ 50 µm.

   e. Calculate the concentration of cell aggregates (C) and the total number of cell aggregates in the mixture ($N_T$) using the total volume of the aggregate suspension ($V_T$):

   $$C = (N_A / 5\ \mu L)$$

   $$N_T = C \times V_T$$

   f. Determine the target number of aggregates to plate ($N_P$). Ensure that the total target number of cell aggregates to plate for all conditions in your experiment (i.e. $N_P$ x number of conditions) does not exceed $N_T$.

   g. Calculate the volume of cell aggregate mixture to plate ($V_p$) for each condition in your experiment:

   $$V_P = N_P / C$$

   h. Gently mix the cell aggregate mixture prior to plating to ensure a uniform suspension.

   i. Add calculated volume of cell aggregate mixture ($V_p$) to each well of a 12-well plate pre-coated with Corning® Matrigel® and containing 1 mL of either mTeSR™1 or TeSR™-E8™.

   NOTE: If using split ratios is desired, a range of 1 in 40 to 1 in 200 may be required depending on the confluence of the passaged well.
4. 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. Proceed to section B.

## B. HEMATOPOIETIC DIFFERENTIATION

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

**CRITICAL**: Confirm that 16 - 40 small colonies are attached to the cultureware (4 - 10 colonies/cm$^2$). Do not proceed if cultures have insufficient or overplated colonies, as differentiation will be compromised.

1. 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).
2. Aspirate medium from wells. Add 1 mL of Medium A per well. Store remaining Medium A at 2 - 8°C until required.

3. Incubate at 37°C for 2 days.

### Day 2

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

## Stage 2

### Day 3

7. 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).
8. Aspirate medium from wells. Gently add 1 mL of Medium B per well. Store remaining Medium B at 2 - 8°C until required.
9. Incubate at 37°C for 2 days.

### Day 5

10. Using a serological pipette or a 1 mL pipette tip, gently remove 0.5 mL of medium from each well. Discard.
11. Gently add 0.5 mL of Medium B per well. Store remaining Medium B at 2 - 8°C until required.
12. 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.

13. Using a serological pipette or a 1 mL pipette tip, gently remove 0.5 mL of medium from each well, being careful not to disturb the floating cell population. Discard.
14. Gently add 0.5 mL of Medium B per well. Store remaining Medium B at 2 - 8°C until required.
15. 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.

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

### Day 12 - Harvest hematopoietic cells

19. 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.
20. Harvest adherent cells (OPTIONAL):

NOTE: The adherent layer is very 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 19.
    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™ (Catalog #07920) 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 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 (15 - 25°C).
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 instructions and MethoCult™ plating protocol, refer to the Technical Manual: Human Colony-Forming Unit (CFU) Assays Using MethoCult™ (Document #28404), available at www.stemcell.com or contact us to request a copy. For hPSC-derived CFU assays, refer to Table 1 for the recommended number of hPSC-derived hematopoietic cells to plate.

**Table 1. 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<br>(20,000 - 100,000) | 3000 viable cells<br>(2000 - 10,000) |
| Day 12 | 50,000 viable cells<br>(30,000 - 200,000) | 5000 viable cells<br>(3000 - 20,000) |

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