STEMdiff™ Megakaryocyte Kit

For differentiation of human ES or iPS cells to megakaryocytes and platelets

Catalog #100-0900 1 Kit

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Product Description

STEMdiff™ Megakaryocyte Kit is designed for the serum-free and feeder-free differentiation of human embryonic stem (ES) and induced pluripotent stem (iPS) cells to polyploid megakaryocytes, which express CD41a and CD41b and are capable of shedding platelets. This simple 17-day monolayer protocol includes two stages. During the first 12 days, ES/iPS cells are differentiated toward megakaryocyte-biased hematopoietic progenitor cells using STEMdiff™ Hematopoietic Basal Medium + Hematopoietic Supplement A (3 days) followed by Basal Medium + Supplement MK1 (9 days). Hematopoietic progenitor cells are then harvested from the culture supernatant, and are further differentiated to mature megakaryocytes for an additional 5 days using StemSpan™ SFEM II + Supplement MK2. By the end of the protocol (Day 17), the average frequency of viable CD41a+CD42b+ cells ranges between 56 - 77%, and the average yield of CD41a+CD42b+ cells generated per seeded human pluripotent stem cell (hPSC) ranges between 223 - 425 (n > 12 for four ES/iPS lines; results are cell line-dependent).

STEMdiff™ Megakaryocyte Kit has been optimized for differentiation of ES/iPS cells maintained in mTeSR™1 (Catalog #85850), mTeSR™ Plus (Catalog #100-0276), or TeSR™-AOF (Catalog #100-0401).

Product Information

The following components are sold as part of STEMdiff™ Megakaryocyte Kit 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™ Megakaryocyte Supplement MK1 (10X)	100-0901	10 mL	Store at -20°C.	Stable for 12 months from date of manufacture (MFG) on label.
STEMdiff™ Megakaryocyte Supplement MK2 (10X)	100-0902	10 mL	Store at -20°C.	Stable for 12 months from date of manufacture (MFG) on label.
StemSpan™ SFEM II	09605	100 mL	Store at -20°C.	Stable for 18 months from date of manufacture (MFG) on label.

Materials Required but Not Included

PRODUCT NAME	CATALOG #
12-well tissue culture-treated plates	e.g. 38052
96-well flat-bottom plate	e.g. 38022
Corning® Matrigel® hESC-Qualified Matrix	Corning 354277
DMEM/F-12 with 15 mM HEPES	36254
Gentle Cell Dissociation Reagent OR ReLeSR™ OR Dispase (1 U/mL)	100-0485 OR 100-0484 OR 07923
mTeSR™1 OR mTeSR™ Plus OR TeSR™-AOF	85850 OR 100-0276 OR 100-0401
Trypan Blue	07050

Preparation of Media

Three medium formulations are required for the megakaryocytic differentiation protocol: **Medium A (Day 0 - 3)**, **Medium MK1 (Day 3 - 12),** and **Medium MK2 (Day 12 - 17)**.

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), Medium MK1 (Basal Medium + Supplement MK1), and Medium MK2 (SFEM II + Supplement MK2). Volumes indicated are for preparing 45 mL of Medium A, 75 mL of Medium MK1, and 100 mL of Medium MK2. If preparing other volumes, adjust accordingly.

1. Thaw STEMdiff™ Hematopoietic Basal Medium and StemSpan™ SFEM II at room temperature (15 - 25°C) or overnight at 2 - 8°C. Mix thoroughly.

   NOTE: If not used immediately, store STEMdiff™ Hematopoietic Basal Medium at 2 - 8°C for up to 6 months, or aliquot and store at -20°C. For StemSpan™ SFEM II, 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 media.
2. Thaw Supplements A, MK1, and MK2 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. Prepare media as indicated in Table 1. Mix thoroughly.

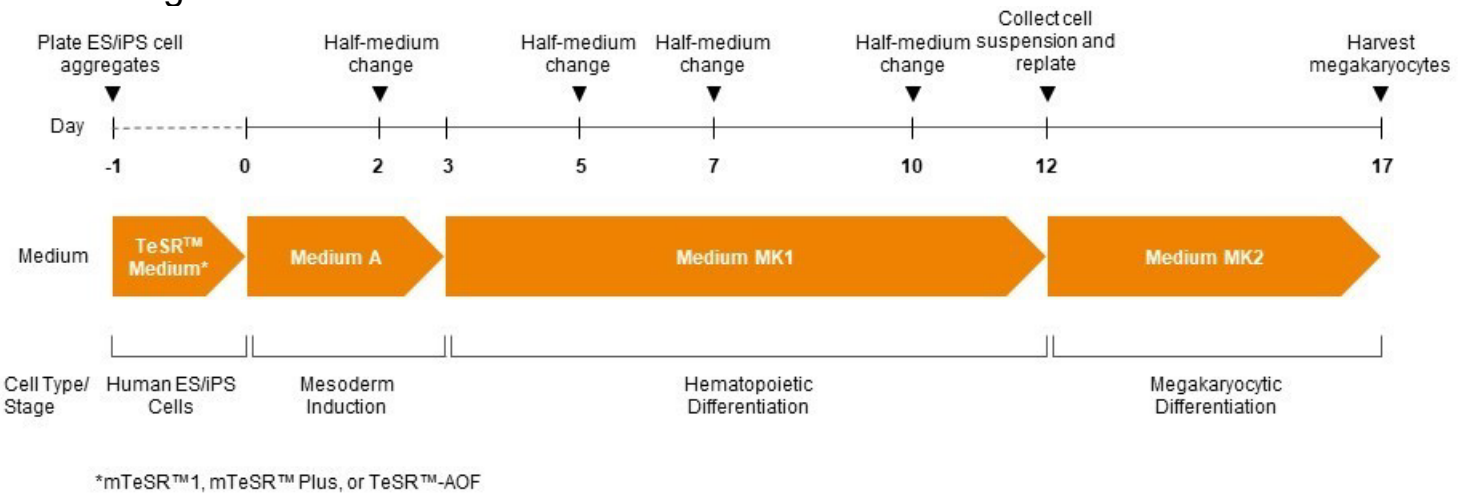
   NOTE: If not used immediately, store complete medium at 2 - 8°C for up to 4 weeks, or at -20°C for up to 6 months.

Table 1. Preparation of STEMdiff™ Megakaryocyte 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 MK1 (75 mL)	STEMdiff™ Hematopoietic Basal Medium	67.5 mL	
	STEMdiff™ Megakaryocyte Supplement MK1 (10X)	7.5 mL	
Medium MK2 (100 mL)	StemSpan™ SFEM II	90 mL	
	STEMdiff™ Megakaryocyte Supplement MK2 (10X)	10 mL	

* Do not exceed the shelf life of the components.

Protocol Diagram



*mTeSR™1, mTeSR™ Plus, or TeSR™-AOF

On Day -1, harvest and seed human ES/iPS cell colonies as small aggregates in mTeSR™1, mTeSR™ Plus, or TeSR™-AOF. On Day 0 (after confirming the number of adhered colonies is within 4 - 10/cm^2), replace TeSR™ medium with Medium A. On Day 2, perform a half-medium change with fresh Medium A. On Day 3, change to Medium MK1 and perform half-medium changes on Days 5, 7, and 10. On Day 12, passage cells at 100,000 - 350,000 cells/mL into Medium MK2 for an additional 5 days. By Day 17, high yields of large megakaryocytes and proplatelets are typically generated (results are dependent on the ES/iPS cell lines used).

Directions for Use

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

A. Passaging Aggregates and Differentiation Setup

B. Hematopoietic Differentiation

C. Megakaryocytic Differentiation

D. Assessing Differentiation

A. PASSAGING AGGREGATES AND DIFFERENTIATION SETUP

This protocol is for human ES or iPS cells cultured in mTeSR™1, mTeSR™ Plus, or TeSR™-AOF. Use the medium with which the cells are routinely maintained and use whichever passaging reagent is preferred. The following 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™-AOF, available at www.stemcell.com or contact us to request a copy.

1. Prior to passaging cells, coat a tissue culture-treated 12-well plate with Corning® Matrigel®.
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™-AOF.
   - ReLeSR™: Passaging protocol as described in the Product Information Sheet (PIS) for ReLeSR™.
   - Dispase (1 U/mL): Passaging protocol as described in the PIS for Dispase (1 U/mL).
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 the 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™-AOF, available at www.stemcell.com or contact us to request a copy.
4. Determine the *Number of Aggregates to Plate*. It is recommended to plate **40 - 80 aggregates/well** (10 - 20 aggregates/cm^2) to achieve **16 - 40 colonies/well** (4 - 10 colonies/cm^2) adhered to the cultureware after 24 hours of incubation; however, multiple plating densities may need to be tested.
5. Calculate the *Plating Volume* of the cell aggregate mixture for each condition in your experiment, as follows:

   Plating Volume (µL) = Number of Aggregates to Plate (step 4) ÷ *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 either mTeSR™1, mTeSR™ Plus, or TeSR™-AOF.

   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 and 5% CO_2 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.

Day 0

1. Confirm that **16 - 40 colonies/well** are adhered to the cultureware (4 - 10 colonies/cm^2). Ensure to count all colonies, including tiny colonies with only a few cells.

   NOTE: To remove debris and facilitate counting, aspirate medium and replace with fresh mTeSR™1, mTeSR™ Plus, or TeSR™-AOF.

   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 and 5% CO_2 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; discard.
6. Gently add 0.5 mL of Medium A per well.
7. Incubate at 37°C for 24 hours.

Day 3

8. Prepare Medium MK1 (see Preparation of Media) required for Days 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 MK1 per well. Store remaining Medium MK1 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; discard.
12. Gently add 0.5 mL of Medium MK1 per well. Store remaining Medium MK1 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; discard.
15. Gently add 0.5 mL of Medium MK1 per well. Store remaining Medium MK1 at 2 - 8°C until required.
16. Incubate at 37°C for 3 days.

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; discard.
18. Gently add 0.5 mL of Medium MK1 per well. Store remaining Medium MK1 at 2 - 8°C until required.
19. Incubate at 37°C for 2 days.

Day 12

20. Harvest hematopoietic cells (supernatant cells) as follows:
    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 StemSpan™ SFEM II to the well. Triturate vigorously in the well and add to the collection tube.
    d. Repeat step c.
    e. Centrifuge the 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 StemSpan™ SFEM II and perform a viable cell count using Trypan Blue and a hemocytometer.
21. Proceed to section C for megakaryocytic differentiation.

C. MEGAKARYOCYTIC DIFFERENTIATION

Day 12

22. Prepare Medium MK2 (see Preparation of Media) required for Day 12 (total of 1 mL per well of a 12-well plate).
23. Add 1 mL of Medium MK2 per well of a tissue culture-treated 12-well plate.
24. Add harvested cells from step 20 to each well at 100,000 - 350,000 cells/mL.
25. Incubate at 37°C for 5 days.

Day 17

26. Harvest megakaryocytes as follows:
    a. Using a serological pipette or a 1 mL pipette tip, gently pipette the cells up and down to wash the well.
    b. Transfer the cell suspension to a collection tube.
    c. Centrifuge the tube at 300 x *g* for 5 minutes at room temperature.
    d. Remove and discard the supernatant.
    e. Resuspend cell pellet in desired medium and perform a viable cell count for analysis or downstream assays. If desired, purity of the $CD41a^+CD42b^+$ megakaryocytic cell population can be determined by flow cytometry.

D. ASSESSING DIFFERENTIATION

The following antibodies are recommended for assessment of human pluripotent stem cell-derived megakaryocytes by flow cytometry:

- Anti-Human CD41a Antibody, Clone HIP8 (Catalog #60114)
- Anti-human CD42b antibody, clone HIP1