TECHNICAL MANUAL

Generation of T Cells Using STEMdiff™ or StemSpan™ T Cell Kits

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1.0 Introduction

T cells are cells of the adaptive immune system. They recognize a wide range of targets through their antigen-specific T cell receptors (TCRs), and provide protection against pathogens and cancer cells through effector functions including cytokine secretion and cytotoxic killing. Human T cells originate from $CD34^+$ hematopoietic stem and progenitor cells (HSPCs) in cord blood (CB) and bone marrow (BM). In vitro culture to promote differentiation of $CD34^+$ HSPCs to T cells is a useful tool for research into basic T cell biology, disease modeling and adoptive immunotherapy.

Human pluripotent stem cells (hPSCs), including embryonic stem (ES) and induced pluripotent stem (iPS) cells, are capable of directed differentiation to hematopoietic progenitor cells, and these can be further differentiated to various immune cells, including T cells. The in vitro differentiation of hPSCs to T cells has been challenging, due to the dependency on co-cultures with feeder cells; these introduce undefined factors, resulting in high variability. A reproducible culture system for generation of T cells from hPSCs is valuable in studies of disease modeling, gene editing, and cell therapy development.

STEMdiff™ and StemSpan™ T Cell Kits are serum- and feeder-free culture systems that enable differentiation of $CD34^+$ hematopoietic progenitor cells derived from hPSCs (STEMdiff™ T Cell Kit) or isolated from CB (StemSpan™ T Cell Generation Kit) to $CD4^+CD8^+$ double-positive (DP) T cells. An additional provided protocol enables the further maturation of DP T cells into $CD8^+$ single-positive (SP) T cells. STEMdiff™ T Cell Kit protocol involves generation of embryoid bodies (EBs) from hPSCs using STEMdiff™ Hematopoietic - EB reagents, and the isolation of $CD34^+$ hematopoietic progenitor cells from EBs after 12 days of culture. STEMdiff™ Hematopoietic - EB reagents (STEMdiff™ Hematopoietic - EB Basal Medium and supplements) are animal component-free, containing no serum or animal- or human-derived components. StemSpan™ T Cell Generation Kit may be used with $CD34^+$ cells obtained from fresh or frozen CB, or derived from hPSCs. In both kits, $CD34^+$ cells are first differentiated to $CD7^+CD5^+$ lymphoid progenitor cells that are further directed to differentiate to DP T cells.

2.0 Materials, Reagents and Equipment

2.1 STEMdiff™ T Cell Kit (Catalog #100-0194)

STEMdiff™ T Cell Kit includes components required for generation of $CD34^+$ hematopoietic progenitor cells from hPSCs (section 4.0), and differentiation of these to T cells (section 5.0).

The following components are sold as a complete kit; the StemSpan™ components are also available for individual sale. Refer to the Product Information Sheet (PIS) for component storage and stability information, available at www.stemcell.com or contact us to request a copy.

COMPONENT NAME	COMPONENT #	QUANTITY
STEMdiff™ Hematopoietic - EB Basal Medium	100-0171	120 mL
STEMdiff™ Hematopoietic - EB Supplement A	100-0172	265 µL
STEMdiff™ Hematopoietic - EB Supplement B	100-0173	7 mL
StemSpan™ Lymphoid Progenitor Expansion Supplement (10X)*	09915	5 mL
StemSpan™ Lymphoid Differentiation Coating Material (100X)*	09925	2 x 250 µL
StemSpan™ T Cell Progenitor Maturation Supplement (10X)	09930	12.5 mL
StemSpan™ SFEM II*†	09605	2 x 100 mL

*This product contains material derived from human plasma. Donors have been tested and found negative for hepatitis B surface antigen (HBsAg) and HIV-1 antibodies and/or HIV-1 antigen. However, this product should be considered potentially infectious and treated in accordance with universal handling precautions.
†500 mL format is also available (Catalog #09655).

2.2 Materials Required for STEMdiff™ T Cell Kit

PRODUCT	CATALOG #
Y-27632	72302
AggreWell™400 6-well (or 24-well) Plate	34421 (or 34411)
DMEM/F-12 with 15 mM HEPES	36254
Anti-Adherence Rinsing Solution	07010
ACCUTASE™	07920
TrypLE™ Express	Thermo Fisher 12604-013
Collagenase Type II	07418
D-PBS (Without Ca++ and Mg++)	37350
Trypan Blue	07050
EasySep™ Human CD34 Positive Selection Kit II	17856
37 µm Reversible Strainer, Large	27250
15 mL and 50 mL conical tubes	e.g. 38009 and 38010
5 mL serological pipettes	e.g. 38003
Non-tissue culture-treated 6-well plate	e.g. 38040
Non-tissue culture-treated cultureware	e.g. 38042 (24 wells)

2.3 StemSpan™ T Cell Generation Kit (Catalog #09940)

StemSpan™ T Cell Generation Kit includes components for differentiation of $CD34^+$ cells isolated from cord blood to T cells. Refer to section 5.0 for the differentiation procedure.

The following components are sold as a complete kit, and are also available for individual sale. Refer to the kit Product Information Sheet (PIS) for component storage and stability information, available at www.stemcell.com or contact us to request a copy.

COMPONENT NAME	COMPONENT #	QUANTITY
StemSpan™ Lymphoid Progenitor Expansion Supplement (10X)*	09915	5 mL
StemSpan™ Lymphoid Differentiation Coating Material (100X)*	09925	2 x 250 µL
StemSpan™ T Cell Progenitor Maturation Supplement (10X)	09930	12.5 mL
StemSpan™ SFEM II*†	09605	2 x 100 mL

*This product contains material derived from human plasma. Donors have been tested and found negative for hepatitis B surface antigen (HBsAg) and HIV-1 antibodies and/or HIV-1 antigen. However, this product should be considered potentially infectious and treated in accordance with universal handling precautions.
†500 mL format is also available (Catalog #09655).

2.4 Materials Required for StemSpan™ T Cell Generation Kit

PRODUCT	CATALOG #
D-PBS (Without Ca++ and Mg++)	37350
Trypan Blue	07050
Non-tissue culture-treated cultureware	e.g. 38042 (24 wells)

2.5 Equipment

- Biosafety cabinet certified for Level II handling of biological materials
- Incubator with humidity and gas control to maintain 37°C and 95% humidity in an atmosphere of 5% CO_2 in air
- Low-speed centrifuge with a swinging bucket rotor with an adaptor for plate holders
- Pipette-aid
- Hemocytometer
- Pipettor (e.g. Catalog #38058) with appropriate tips
- Inverted microscope
- Flow cytometer
- Optional: Fluorescence-activated cell sorting (FACS) flow cytometer
- -20°C freezer
- Refrigerator (2 - 8°C)

3.0 Preparation of Reagents and Materials

3.1 STEMdiff™ Hematopoietic - EB Media (STEMdiff™ T Cell Kit Only)

In the embryoid body (EB) hematopoietic differentiation protocol, EB Formation Medium and EB Medium A are required in Stage 1 (Day 0 - 3) and EB Medium B is required in Stage 2 (Day 3 - 12).

Use sterile technique to prepare the media as indicated in Table 1 (indicated volumes are for one well of a 6-well plate). If preparing other volumes, adjust accordingly.

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

   Note: If not used immediately, 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 STEMdiff™ Hematopoietic - EB Supplement A or 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, aliquot and store at -20°C. Do not exceed the shelf life of the supplement. After thawing aliquots, use immediately or store at 2 - 8°C for up to 2 weeks. Do not re-freeze.

3. Combine components as indicated in Table 1. Mix thoroughly. Warm to room temperature (15 - 25°C) before use.

   Note: STEMdiff™ Hematopoietic - EB Supplement A is supplied as a 200X concentrate and STEMdiff™ Hematopoietic - EB Supplement B as a 10X concentrate.

Table 1. Preparation of STEMdiff™ Hematopoietic - EB Media

MEDIUM	COMPONENTS	VOLUME	IN-USE STORAGE/STABILITY
EB Medium A (7.5 mL)	STEMdiff™ Hematopoietic - EB Basal Medium	7.5 mL	If not used immediately, store complete medium at 2 - 8°C for up to 2 weeks. Do not exceed the shelf life of the basal medium or supplement.
	STEMdiff™ Hematopoietic - EB Supplement A	37.5 µL	
EB Formation Medium (5 mL)	EB Medium A	5 mL	Use immediately.
	Y-27632 (10 µM final concentration)	10 µL of 5 mM stock solution	
EB Medium B (12.5 mL)	STEMdiff™ Hematopoietic - EB Basal Medium	11.25 mL	If not used immediately, store complete medium at 2 - 8°C for up to 2 weeks. Do not exceed the shelf life of the basal medium or supplement.
	STEMdiff™ Hematopoietic - EB Supplement B	1.25 mL	

3.2 StemSpan™ Lymphoid Progenitor Expansion Medium

Use sterile technique to prepare StemSpan™ Lymphoid Progenitor Expansion Medium (StemSpan™ SFEM II + StemSpan™ Lymphoid Progenitor Expansion Supplement [10X]). The following example is for preparing 10 mL of complete medium. If preparing other volumes, adjust accordingly.

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

   Note: If not used immediately, aliquot into tubes and store at -20°C. Do not exceed the shelf life of the medium. After thawing aliquots, use immediately. Do not re-freeze.

2. Thaw StemSpan™ Lymphoid Progenitor Expansion Supplement (10X) at room temperature (15 - 25°C). Mix thoroughly.

   Note: If not used immediately, store at 2 - 8˚C for up to 1 month. Alternatively, 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 1 mL of Expansion Supplement to 9 mL of SFEM II. Mix thoroughly.

   Note: If not used immediately, store complete medium at 2 - 8°C for up to 1 month. Do not freeze.

3.3 StemSpan™ T Cell Progenitor Maturation Medium

Use sterile technique to prepare StemSpan™ T Cell Progenitor Maturation Medium (StemSpan™ SFEM II + StemSpan™ T Cell Progenitor Maturation Supplement [10X]). The following example is for preparing 10 mL of complete medium. If preparing other volumes, adjust accordingly.

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

   Note: If not used immediately, aliquot into tubes and store at -20°C. Do not exceed the shelf life of the medium. After thawing aliquots, use immediately. Do not re-freeze.

2. Thaw StemSpan™ T Cell Progenitor Maturation Supplement at room temperature (15 - 25°C). Mix thoroughly.

   Note: If not used immediately, store at 2 - 8°C for up to 1 month. Alternatively, 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 1 mL of Maturation Supplement to 9 mL of SFEM II. Mix thoroughly.

   Note: If not used immediately, store complete medium at 2 - 8°C for up to 1 month. Do not freeze.

3.4 StemSpan™ Lymphoid Differentiation Coating Material

Use sterile technique to prepare StemSpan™ Lymphoid Differentiation Coating Material (Coating Material [100X] + D-PBS [Without Ca++ and Mg++]. The following example is for preparing 1 mL of Coating Material. If preparing other volumes, adjust accordingly.

1. Thaw StemSpan™ Lymphoid Differentiation Coating Material (100X) at room temperature (15 - 25°C). Mix thoroughly.

   Note: If necessary, centrifuge vial in a microfuge for 30 seconds to collect liquid from cap.

   Note: If not used immediately, store at 2 - 8°C for up to 1 month. Alternatively, aliquot and store at -20°C. Do not exceed the shelf life of the product. After thawing aliquots, use immediately or store at 2 - 8°C for up to 1 month. Do not re-freeze.

2. Add 10 µL of Coating Material to 990 µL of D-PBS (Without Ca++ and Mg++). Mix thoroughly. Use immediately.

4.0 Generating CD34+ Hematopoietic Progenitor Cells From hPSCs

The following protocol is for generating CD34+ hematopoietic progenitor cells from hPSCs using STEMdiff™ T Cell Kit. hPSCs are first induced to form mesoderm for 3 days followed by 9 days of hematopoietic specification. If using CD34+ cells isolated from cord blood (StemSpan™ T Cell Generation Kit), proceed to section 5.0.

4.1 Protocol Diagram

4.2 Generating EBs Using an AggreWell™400 Plate (Day 0)

The following instructions are for generating a single-cell suspension of hPSCs (section 4.2.1), then plating cells into one well of an AggreWell™400 6-well plate to generate EBs (section 4.2.2). If using other cultureware or number of wells, adjust volumes accordingly.

3.5 x 10^6 cells will be required for each well of an AggreWell™400 6-well plate, resulting in 500 cell aggregates per microwell.

4.2.1 Generating a Single-Cell Suspension

This protocol is for generating a single-cell suspension from hPSCs cultured in mTeSR™1, mTeSR™ Plus, or TeSR™-E8™ in a 100 mm dish. Use the medium in which the cells are routinely maintained.

Note: For complete instructions on maintaining high-quality hPSCs and 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. Thaw ACCUTASE™ overnight at 2 - 8°C, at room temperature (15 - 25°C), or in a container of cool water. Do not thaw at 37°C. Mix thoroughly.

   Note: Once thawed, use immediately or store at 2 - 8°C for 2 months. Alternatively, aliquot and store at -20°C. Do not exceed the expiry date on the label.
2. Use a microscope to visually identify regions of differentiation in the hPSC culture. Mark these using a felt tip or lens marker on the bottom of the 100 mm dish. Remove regions of differentiation by scraping with a pipette tip or by aspiration.
3. Wash the culture once with 5 - 10 mL of sterile D-PBS.
4. Aspirate D-PBS and add 5 mL of ACCUTASE™.
5. Incubate at 37°C for 10 minutes.

   Note: The incubation time may vary when using different cell lines or other non-enzymatic cell dissociation reagents; dissociation should be monitored under the microscope until the optimal time is determined.

6. Using a pipettor, pipette the cell suspension up and down 3 - 5 times to dislodge the remaining attached cells. Using a 5 mL serological pipette, collect the cells into a 15 mL or 50 mL conical tube. Ensure that any remaining cell aggregates are broken up into single cells.
7. Wash the dish with 10 mL of DMEM/F-12 with 15 mM HEPES and add to the tube containing the single-cell suspension.
8. Count viable cells using Trypan Blue and a hemocytometer.
9. Centrifuge at 300 x *g* for 5 - 10 minutes. Carefully aspirate the supernatant.
10. Resuspend cells in 2.5 mL of EB Formation Medium (section 3.1) to obtain a final concentration of 1.4 x 10^6 cells/mL (for 3.5 x 10^6 cells/well).

4.2.2 Transfer to Aggrewell™400 6-Well Plate

1. Pre-treat an AggreWell™400 6-well plate with Anti-Adherence Rinsing Solution as described in the PIS for AggreWell™; the recommended basal medium for rinsing wells is DMEM/F-12 with 15 mM HEPES.
2. Add 2.5 mL of warm (room temperature) EB Formation Medium to each well to be used.
3. Add 2.5 mL of the single-cell suspension prepared in section 4.2.1 (e.g. 3.5 x 10^6 cells) to each well containing EB Formation Medium. This will result in 500 cells/microwell in a total volume of 5 mL.

   Note: Ensure that newly plated cells are evenly dispersed across the entire surface of the well by gently pipetting up and down several times.
4. Centrifuge the AggreWell™400 plate at 100 x *g* for 3 minutes. This will capture the cells in the microwells.

   Note: Plates must be balanced. It is recommended to balance the plate against a standard 6-well plate filled with water to match the weight and position of the AggreWell™400 plate.
5. Examine the AggreWell™400 plate under a microscope to ensure that cells are evenly distributed among the microwells.
6. Incubate the plate at 37°C and 5% CO_2 for 2 days. Proceed to section 4.3.

   Note: Many cell lines form EBs within 24 hours, but some may require a longer incubation time (up to 72 hours) for optimal EB formation. Uniform EBs should be visible in the AggreWell™400 well.

4.3 Half-Medium Changes in an AggreWell™400 Plate (Days 2 & 3)

Perform a half-medium change on Day 2 (EB Medium A) and on Day 3 (EB Medium B) as described below; refer to section 3.1 for media preparation.

Day 2

1. Warm EB Medium A to room temperature.
2. Using a 5 mL serological pipette, slowly remove half of the medium (2.5 mL) from each well.

   Note: Do not disturb the EBs. Keep the pipette tip towards the upper surface of the medium in the well while removing the medium.
3. Add 2.5 mL of fresh EB Medium A.

   Note: It is important not to disturb the EBs. Do not add the medium directly onto the surface of the well. Support the pipette tip by slightly touching the side of the well at the surface level of the remaining medium inside the well. This will allow for a more controlled release of the medium. Release the medium very slowly into the well by setting the Pipette-Aid to "gravity" or "slow". Quick release of medium will dislodge the EBs from the wells.
4. Incubate at 37°C and 5% CO_2 for 24 hours.

Day 3

5. Warm EB Medium B to room temperature.
6. Perform a half-medium change with EB Medium B as described in steps 1 - 3.
7. Incubate at 37°C and 5% CO_2 for 2 days. Proceed to section 4.4.

4.4 Culturing EBs in a 6-Well Plate (Days 5 - 12)

Day 5

1. Warm DMEM/F-12 with 15 mM HEPES and EB Medium B.
2. For each AggreWell™ 400 well to be harvested, perform the following steps:
   a. Place a 37 µm reversible strainer on top of a 50 mL conical tube.
      Note: Ensure the arrow on the strainer is pointing upwards. Use a new strainer and a new tube for each AggreWell™400 well to be harvested.
   b. To dislodge EBs from the microwell, firmly pipette medium up and down around the surface of the well. Do not triturate. Transfer the EB suspension to the strainer.
      Note: Aggregates will remain on the strainer; any unincorporated single cells will flow through.
   c. Rinse the entire surface of the well with DMEM/F-12 to collect any remaining EBs. Pass rinse over the strainer.
   d. Repeat step c until all EBs have been removed from the well. One or two repeats should be sufficient to dislodge all EBs. Examine the well under a microscope to ensure that all EBs have been removed. Discard flowthrough.
   e. Flip the strainer and place on top of a fresh 50 mL conical tube. Wash with 2.5 mL of EB Medium B to collect the EBs into the tube.
   f. Using a serological pipette, mix EBs to evenly distribute them in the suspension.
   g. Using a serological pipette, add 2.5 mL of EB suspension into one well of a fresh **non-tissue culture-treated** 6-well plate.
3. Move the 6-well plate in several quick, short, back-and-forth and side-to-side motions to distribute the EBs across the surface of the wells.
4. Incubate at 37°C and 5% CO_2 for 2 days.

Day 7

5. Add EB Medium B to EBs as follows:
   a. Warm EB Medium B to room temperature.
   b. Using a 5 mL serological pipette, gently add 2.5 mL of EB Medium B to each well.
   c. Move the plate in several quick, short, back-and-forth and side-to-side motions to distribute the EBs across the surface of the wells. Place the 6-well plate in a 37°C incubator.
   d. Incubate at 37°C and 5% CO_2 for 3 days.

Day 10

6. Perform a half-medium change as follows:
   a. Warm EB Medium B to room temperature.
   b. Using a 5 mL serological pipette, slowly remove half (2.5 mL) of medium from each well.
   c. Add 2.5 mL of fresh EB Medium B.
   d. Incubate at 37°C and 5% CO_2 for 2 days. Proceed to section 4.5.

4.5 EB Harvest and Dissociation (Day 12)

1. Add DMEM/F-12 with 15 mM HEPES to Collagenase Type II to prepare a 2500 U/mL Collagenase II Solution.
2. Gently pipette EBs and cells up and down in the wells to ensure all EBs are in suspension. Transfer the suspension from one well to a 15 mL conical tube.
3. Centrifuge the EB suspension at 300 x *g* for 5 - 10 minutes.
4. Carefully aspirate the supernatant and add 1 mL of Collagenase II Solution to the pellet from one well. Gently pipette up and down to resuspend. Incubate at 37°C for 20 minutes.
5. Add 3 mL TrypLE™ Express to the suspension. Gently pipette up and down to mix. Incubate at 37°C for 20 minutes.
6. Gently pipette the suspension up and down to break up any remaining clumps.
7. Add 6 mL of DMEM/F-12 with 15 mM HEPES. Centrifuge at 300 x *g* for 5 - 10 minutes. Remove and discard the supernatant.

   Optional: Pass the suspension through a 37 µm strainer to remove clumps and obtain a single-cell suspension.
8. Resuspend the cell pellet in EasySep™ Buffer (Catalog #20144), RoboSep™ Buffer (Catalog #20104), or PBS containing 2% fetal bovine serum and 1 mM EDTA. Medium should be free of Ca++ and Mg++.
9. Isolate $CD34^+$ cells using EasySep™ Human CD34 Positive Selection Kit II. Perform the $CD34^+$ isolation using the protocol optimized for ES or iPS cell cultures (Table 2 in the EasySep™ PIS); reduce the number of separations in the magnet from 4 to 2 to increase yield while retaining a sufficient $CD34^+$ purity for further culture.
10. Proceed to section 5.0 for differentiation to T cells.

4.6 Phenotype Assessment

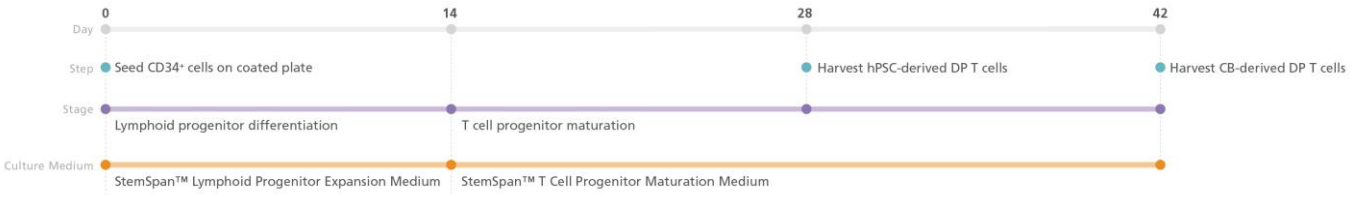
For phenotype assessment of hematopoietic progenitor cells by flow cytometry, use the following fluorochrome-conjugated antibodies:

- Anti-Human CD34 Antibody, Clone 581 (Catalog #60013)
- Anti-Human CD34 Antibody, Clone 8G12 (Catalog #60121)

5.0 Differentiation to T Cells

The following protocol is for differentiating hPSC-derived CD34+ cells (generated in section 4.0), as well as CD34+ cells isolated from cord blood (CB), to T cells.

5.1 Protocol Diagram



5.2 Differentiation Protocol

The following instructions are for one well of a 24-well plate. If using alternative cultureware, refer to Table 2 and adjust cell numbers and volumes accordingly.

For optimal performance, follow the recommended schedule of feeding and passaging. However, the schedule may be adjusted as needed, as long as a feeding interval of 3 - 4 days is maintained.

Day 0

1. Add 500 µL of StemSpan™ Lymphoid Differentiation Coating Material (section 3.4) per well of a non-tissue culture-treated 24-well plate (e.g. Catalog #38042). If using other cultureware, refer to Table 2 for volumes required.

Table 2. Recommended Volumes of Coating Material and Medium and Recommended Cell Numbers for Various Cultureware

NON-TISSUE CULTURE-TREATED CULTUREWARE	VOLUME OF COATING MATERIAL	VOLUME OF EXPANSION MEDIUM OR MATURATION MEDIUM	NUMBER OF CD34+ CELLS/WELL	
			CB-DERIVED	hPSC-DERIVED*
96-well plate (e.g. Catalog #38044)	100 µL/well	100 µL/well	1 x 10^3	5 x 10^3
12-well plate (e.g. Catalog #38041)	1 mL/well	1 mL/well	1 x 10^4	5 x 10^4
6-well plate (e.g. Catalog #38040)	2.5 mL/well	2.5 mL/well	2.5 x 10^4	1.25 x 10^5

*hPSC-derived CD34+ cells must be generated using STEMdiff™ T Cell Kit (Catalog #100-0194) as described in section 4.0.

2. Incubate at room temperature (15 - 25°C) for 2 hours.

   Note: Alternatively, incubate at 2 - 8°C overnight.

3. Aspirate coating material from the 24-well plate. Rinse the well with D-PBS (Without Ca++ and Mg++). Aspirate D-PBS just prior to use.
4. Prepare CD34+ cells as follows:
   - If using fresh hPSC-derived CD34+ cells (generated in section 4.0), proceed to step 5.
   - If using frozen CD34+ cells from human CB, thaw cells then proceed to step 5.
   - If using fresh (< 72 hours old) human CB, isolate CD34+ cells using EasySep™ Human Cord Blood CD34 Positive Selection Kit II (Catalog #17896), then proceed to step 5.

5. Perform a viable cell count using Trypan Blue and a hemocytometer. Determine the % $CD34^+$ cells by flow cytometry, using one of the following fluorochrome-conjugated antibodies:
   - Anti-Human CD34 Antibody, Clone 581 (Catalog #60013)
   - Anti-Human CD34 Antibody, Clone 8G12 (Catalog #60121)

   To determine the concentration of $CD34^+$ cells, multiply the % $CD34^+$ cells by the viable cell count.

   Note: Expected % $CD34^+$ cells is > 50%. If frequency is lower, do not continue.
6. Add $CD34^+$ cells (from step 4) to 500 µL of StemSpan™ Lymphoid Progenitor Expansion Medium (section 3.2) as follows:
   - CB-derived: 1 x 10^4 $CD34^+$ cells/mL (5 x 10^3 $CD34^+$ cells/well)
   - hPSC-derived: 5 x 10^4 $CD34^+$ cells/mL (2.5 x 10^4 $CD34^+$ cells/well)

   Note: This cell suspension is for one well of a 24-well plate. If using other cultureware, refer to Table 2 for volumes and cell numbers required.
7. Add 500 µL of cell suspension (prepared in step 6) to one coated well of the 24-well plate prepared in steps 1 - 3. Incubate at 37°C and 5% CO_2 for 3 or 4 days.

Day 3 or 4

8. Carefully add 500 µL of StemSpan™ Lymphoid Progenitor Expansion Medium per well of the 24-well plate. Incubate at 37°C and 5% CO_2 for 3 or 4 days.

Day 7

9. Perform a half-medium change as follows:
   a. Carefully remove 500 µL of medium from the well. Do not disturb cells.
   b. Add 500 µL of StemSpan™ Lymphoid Progenitor Expansion Medium per well.
      - For CB-derived $CD34^+$ cells: Incubate at 37°C and 5% CO_2 for 3 or 4 days, then proceed to step 10.
      - For hPSC-derived $CD34^+$ cells:
        i. Prepare a 24-well plate as described in steps 1 - 3.
        ii. Gently pipette up and down in the well to ensure all cells are in suspension.

            Note: Do not scrape the bottom of the plate or pipette too aggressively, as this will detach any adherent cells that may have developed.
        iii. Transfer suspension from one well to one well of the freshly coated plate. Repeat for all wells.
        iv. Incubate at 37°C and 5% CO_2 for 3 or 4 days, then proceed to step 10.

Day 10 or 11

10. Perform a half-medium change as follows:
    a. Carefully remove 500 µL of medium from the well. Do not disturb cells.
    b. Add 500 µL of StemSpan™ Lymphoid Progenitor Expansion Medium per well. Incubate at 37°C and 5% CO_2 for 3 or 4 days.

    Note: If cells reach confluency prior to harvest or medium changes in the remainder of the protocol, reduce cell density by pipetting up and down, removing half of the medium including cells, and replacing with fresh medium. The removed cells may be transferred to a freshly prepared 24-well plate as described in steps 1 - 3.

Day 14 - Harvest cells and reseed

11. Gently pipette cells up and down in the well to ensure all cells are in suspension. Transfer cells to an appropriate tube; these cells include lymphoid progenitor cells.

    Note: Cells can be cryopreserved at this stage using CryoStor® CS10 (Catalog #07930). Refer to the PIS for CryoStor® CS10 for cryopreserving and thawing instructions. Once cells are thawed, proceed to step 12.

12. If using hPSC-derived $CD34^+$ cells, removing dead cells using fluorescence-activated cell sorting (FACS) at this stage may improve frequency and yield of DP T cells.
13. Coat a non-tissue culture-treated 24-well plate with StemSpan™ Lymphoid Differentiation Coating Material (see steps 1 - 3).
14. Perform a viable cell count using Trypan Blue and a hemocytometer.
15. Add cells to 500 µL of StemSpan™ T Cell Progenitor Maturation Medium (section 3.3) as follows:
    - If using CB-derived $CD34^+$ cells, add 1 x 10^5 cells/mL (5 x 10^4 cells/well)
    - If using hPSC-derived $CD34^+$ cells, add 0.5 - 1 x 10^6 cells/mL (2.5 - 5 x 10^5 cells/well). Higher cell density increases the resulting frequency of DP T cells.

    Note: This cell suspension is for one well of a 24-well plate. If using other cultureware, refer to Table 2 for volumes required.

16. Add 500 µL of cell suspension (prepared in step 15) to one coated well of the 24-well plate prepared in step 13. Incubate at 37°C and 5% CO_2 for 3 or 4 days.

Day 17 or 18

17. Carefully add 500 µL of StemSpan™ T Cell Progenitor Maturation Medium per well. Incubate at 37°C and 5% CO_2 for 3 or 4 days.

Day 21

18. Perform a half-medium change as follows:
    a. Carefully remove 500 µL of medium from the well. Do not disturb cells.
    b. Add 500 µL of StemSpan™ T Cell Progenitor Maturation Medium per well. Incubate at 37°C and 5% CO_2 for 3 or 4 days.

Day 24 or 25

19. Perform a half-medium change as follows:
    a. Carefully remove 500 µL of medium from the well. Do not disturb cells.
    b. Add 500 µL of T Cell Progenitor Maturation Medium per well. Incubate at 37°C and 5% CO_2 for 3 or 4 days.

Day 28 - Harvest cells

20. Gently pipette cells up and down to ensure all cells are in suspension. Transfer cells to an appropriate tube.
    - If using hPSC-derived $CD34^+$ cells, the cells are now ready for assays or analysis as required; this cell suspension will include DP cells. An optional protocol for CD8 SP T cell maturation is described in section 5.2.1.
    - If using CB-derived $CD34^+$ cells, proceed to step 21.

Reseed (CB-derived $CD34^+$ cells only)

21. Coat a non-tissue culture-treated 24-well plate with StemSpan™ Lymphoid Differentiation Coating Material (see steps 1 - 3).
22. Perform a viable cell count using Trypan Blue and a hemocytometer.

23. Add cells at 5 x 10^5 cells/mL to 500 µL of StemSpan™ T Cell Progenitor Maturation Medium.

    Note: This cell suspension is for one well of a 24-well plate. If using other cultureware, refer to Table 2 for volumes required.

24. Add 500 µL of cell suspension (prepared in step 23) to one coated well of the 24-well plate prepared in step 21 (2.5 x 10^5 cells/well). Incubate at 37°C and 5% CO_2 for 3 or 4 days.

Day 31 or 32

25. Carefully add 500 µL of StemSpan™ T Cell Progenitor Maturation Medium per well. Incubate at 37°C and 5% CO_2 for 3 or 4 days.

Day 35

26. Perform a half-medium change as follows:
    a. At this stage, there may be an accumulation of cellular debris floating at the center of the well. Carefully aspirate this debris, removing ~500 µL of medium. Be careful not to disturb cells.
    b. Add 500 µL of StemSpan™ T Cell Progenitor Maturation Medium per well. Incubate at 37°C and 5% CO_2 for 3 or 4 days.

Day 38 or 39

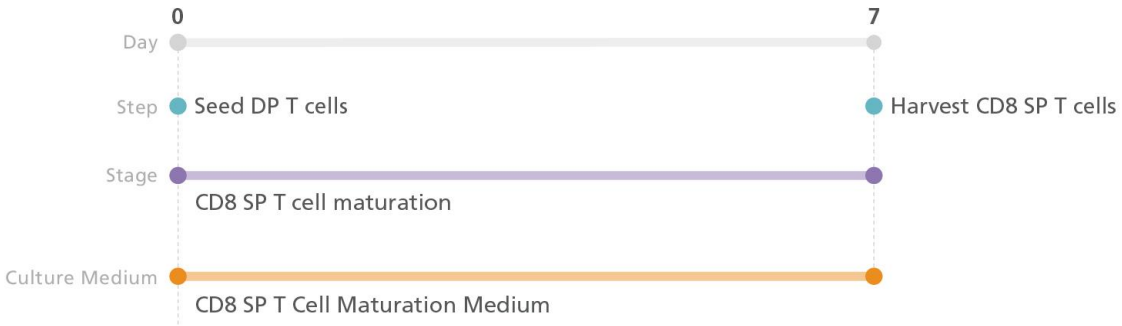
27. Perform a half-medium change as follows:
    a. At this stage, there may be an accumulation of cellular debris floating at the center of the well. Carefully aspirate this debris, removing ~500 µL of medium. Be careful not to disturb cells.
    b. Add 500 µL of StemSpan™ T Cell Progenitor Maturation Medium per well. Incubate at 37°C and 5% CO_2 for 3 or 4 days.

Day 42 - Harvest cells

28. Gently pipette cells up and down to ensure all cells are in suspension. Transfer cells to an appropriate tube. This cell suspension will include DP T cells. The cells are ready for assays or analysis as required. An optional protocol for CD8 SP T cell maturation is provided in section 5.2.1.

5.2.1 Further Maturation to CD8 SP T Cells (Optional)

Protocol Diagram



NOTE: At Day 0, either ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator (Catalog #10970) **or** ImmunoCult™ Human CD3/CD28 T Cell Activator (Catalog #10971) is added to CD8 SP T Cell Maturation Medium.

Day 0

1. Coat a non-tissue culture-treated 24-well plate with StemSpan™ Lymphoid Differentiation Coating Material (see section 5.2 steps 1 - 3).

2. Perform a viable cell count on cells harvested in section 5.2 step 20 (hPSC-derived DP T cells) or step 28 (CB-derived DP T cells) using Trypan Blue and a hemocytometer.
3. Prepare CD8 SP T Cell Maturation Medium for one well of a 24-well plate, as follows:

   Combine the following:

   - 500 µL StemSpan™ T Cell Progenitor Maturation Medium
   - 10 ng/mL Human Recombinant IL-15 (Catalog #78031)

   Mix thoroughly.
4. Add 6.25 µL ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator (Catalog #10970) **or** ImmunoCult™ Human CD3/CD28 T Cell Activator (Catalog #10971) to 500 µL of CD8 SP T Cell Maturation Medium (prepared in step 3). Mix thoroughly.

   Note: This is half the concentration recommended in the activator PIS's.
5. Add cells at 1 x 10^6 cells/mL to 500 µL of CD8 SP T Cell Maturation Medium + Activator (prepared in step 4).

   Note: This cell suspension is for one well of a 24-well plate. If using other cultureware, refer to Table 2 for volumes required.
6. Add 500 µL of cell suspension (prepared in step 5) to one coated well of the 24-well plate prepared in step 1 (5 x 10^5 cells/well). Incubate at 37°C and 5% CO_2 for 3 or 4 days.

Day 3 or 4

7. Add 500 µL of CD8 SP T Cell Maturation Medium (prepared in step 3) to each well. Incubate at 37°C and 5% CO_2 for 3 or 4 days.

   Note: Do not add T Cell Activator at this step.

Day 7

8. Gently pipette cells up and down to ensure all cells are in suspension. Transfer cells to an appropriate tube; these should include CD8 SP T cells.

5.3 Phenotype Assessment

For phenotype assessment of lymphoid progenitor cells by flow cytometry, use the following fluorochrome-conjugated antibodies:

- Anti-Human CD5 Antibody, Clone UCHT2 (Catalog #60082)
- Anti-human CD7 antibody, clone CD7-6B7

For phenotype assessment of more mature T cells by flow cytometry, use the following fluorochrome-conjugated antibodies:

- Anti-Human CD3 Antibody, Clone UCHT1 (Catalog #60011)
- Anti-human CD4 antibody, clone RPA-T4
- Anti-Human CD8a Antibody, Clone RPA-T8 (Catalog #60022)
- Anti-human CD8β antibody, clone SIDI8BEE
- Anti-human TCRαβ antibody, clone IP26

6.0 Troubleshooting

PROBLEM/QUESTION	SOLUTION/ANSWER
The EBs plated in a non-tissue culture-treated 6-well plate appear to have stuck down and adherent cells are growing around them.	EBs may end up loosely adhering to the non-tissue culture-treated plate. When this happens, some adherent cells may grow out from the attached EB. This does not appear to affect performance and we do not recommend detaching them.
Will aggregation of EBs be an issue in this protocol? If yes, how does it influence differentiation? Should aggregates be broken down when noticed?	We have not noticed significant aggregation of EBs. After transferring EBs to a non-tissue culture-treated plate, distribute EBs when placing the plate into the incubator by moving the plate back-and-forth and side-to-side several times. We typically do not break up the small aggregates that may form.
After dissociating EBs (section 4.5), there are still small clumps remaining in the suspension.	Released DNA from apoptotic cells during dissociation may lead to unwanted cell aggregation. These clumps can negatively affect cell yield if not removed before cell separation. Remove clumps using a 37 µm strainer or directly with a pipettor.
Are there any indicators of normal performance at various stages during culture? If yes, what are they?	There is a wide range of performance between various PSC lines. However, at Day 12 cultures are expected to have ≥ 15% $CD34^+$ cells and a yield of ≥ 1 x 10^5 $CD34^+$ cells/well (of a 6-well AggreWell™ 400 plate) harvested before cell separation. When differentiating hPSC-derived $CD34^+$ cells (as described in section 5.2) on Day 14, cultures typically have ≥ 20% $CD5^+CD7^+$ cells and a yield of ≥ 5 $CD5^+CD7^+$ cells per input $CD34^+$ cell.
Why are hPSC-derived T cells harvested after 28 days and CB-derived T cells harvested after 42 days?	hPSC-derived T cells have faster developmental kinetics, and reach the double-positive stage before comparable CB cultures. CB cultures require the additional 2 weeks to reliably differentiate to DP T cells.
What are the non-adherent cells floating in the center of the well?	During culture in T Cell Progenitor Maturation Medium (section 5.2), a region of floating cells and/or cellular debris may develop in the center of the well. During feeds, carefully aspirate this debris, being careful not to disturb cells. Many of these cells are dead/necrotic cells. Most viable cells remain close to the plate surface to contact the coating material. The TCR rearrangement process may result in non-productive rearrangements in developing cells, impacting their survival. This also occurs in the thymus where many thymocytes fail to become T cells.

TECHNICAL MANUAL

Generation of T Cells Using STEMdiff™ or StemSpan™ T Cell Kits

STEMCELL™ TECHNOLOGIES

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