TECHNICAL MANUAL

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

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1.0 Introduction

Natural killer (NK) cells are innate immune cells capable of killing tumor- or virus-infected cells as well as secreting pro-inflammatory cytokines. Human NK 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 NK cells is a useful tool for research into the use of NK cells for adoptive immunotherapy as well as research into basic NK cell biology.

Human pluripotent stem cells (hPSCs), including embryonic stem (ES) and induced pluripotent stem (iPS) cells, can be induced to differentiate to hematopoietic cells, including those that constitute the immune system, such as NK cells. The in vitro differentiation of hPSCs to NK cells has been difficult, since it is often dependent on feeder cells that rely on undefined culture medium components, which cause variability. A reproducible culture system for generation of NK cells from hPSCs is valuable in studies of disease modeling, gene editing, and cell therapy development applications.

STEMdiff™ and StemSpan™ NK Cell Kits are serum-free and feeder-free culture systems that enable differentiation of $CD34^+$ hematopoietic progenitor cells derived from hPSCs (STEMdiff™ NK Cell Kit) or isolated from CB or BM (StemSpan™ NK Cell Generation Kit) to $CD56^+$ NK cells with high frequencies and yields. STEMdiff™ NK 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™ NK Cell Generation Kit may be used with fresh or frozen $CD34^+$ cells obtained from CB or BM, 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 $CD56^+$ NK cells.

2.0 Materials, Reagents and Equipment

2.1 STEMdiff™ NK Cell Kit (Catalog #100-0170)

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

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

PRODUCT	CATALOG #	SIZE
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	250 µL
StemSpan™ NK Cell Differentiation Supplement (100X)	09950	0.5 mL
StemSpan™ SFEM II*†	09605	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™ NK 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)
UM729	72332

2.3 StemSpan™ NK Cell Generation Kit (Catalog #09960)

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

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

PRODUCT	CATALOG #	SIZE
StemSpan™ Lymphoid Progenitor Expansion Supplement (10X)*	09915	5 mL
StemSpan™ Lymphoid Differentiation Coating Material (100X)*	09925	250 µL
StemSpan™ NK Cell Differentiation Supplement (100X)	09950	0.5 mL
StemSpan™ SFEM II*†	09605	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™ NK Cell Generation Kit

PRODUCT	CATALOG #
D-PBS (Without Ca++ and Mg++)	37350
Trypan Blue	07050
UM729	72332
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
- -20°C freezer
- Refrigerator (2 - 8°C)

3.0 Preparation of Reagents and Materials

3.1 STEMdiff™ Hematopoietic - EB Media (STEMdiff™ NK 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 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 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 (10 mL)	STEMdiff™ Hematopoietic - EB Basal Medium	9 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 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. 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™ NK Cell Differentiation Medium

Use sterile technique to prepare StemSpan™ NK Cell Differentiation Medium (StemSpan™ SFEM II + StemSpan™ NK Cell Differentiation Supplement [100X] + UM729). 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™ NK Cell Differentiation Supplement at room temperature. 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 supplement. After thawing aliquots, use immediately. Do not re-freeze.

3. Add 100 µL of Differentiation Supplement to 9.9 mL of SFEM II. Mix thoroughly.
4. Add UM729 to a final concentration of 1 µM. Mix thoroughly.

   Note: If not used immediately, store complete medium at 2 - 8°C for up to 3 weeks. 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.

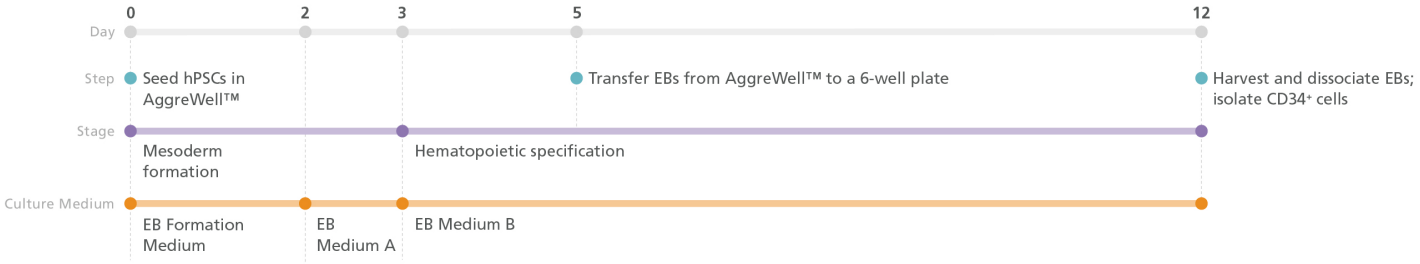
   Note: If not used immediately, store at 2 - 8°C for up to 1 month. Alternatively, aliquot and store at -20°C. Do not exceed the shelf life of the product. After thawing aliquots, use immediately or store at 2 - 8°C for up to 1 month. Do not re-freeze.

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™ NK 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 or bone marrow (StemSpan™ NK Cell Generation Kit), proceed to section 5.0 for differentiation to NK cells.

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.

3.5×10^6 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 (Catalog #85850), mTeSR™ Plus (Catalog #100-0276), or TeSR™-E8™ (Catalog #05990) in a 100 mm dish. Use the medium in which the cells are routinely maintained.

Note: For complete instructions on maintaining high-quality hPSCs, 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™. 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.

5. 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 in a 15 mL or 50 mL conical tube. Ensure that any remaining cell aggregates are broken up into single cells.

   Note: If clumping is noticeable, passing cell suspension through a 37 µm strainer may improve performance.
6. Wash the dish with 10 mL of DMEM/F-12 with 15 mM HEPES and add to the tube containing the single-cell suspension.
7. Count viable cells using Trypan Blue and a hemocytometer.
8. Centrifuge at 300 x *g* for 5 - 10 minutes. Carefully aspirate the supernatant.
9. 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™.

   Note: 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 (i.e. 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".
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 2 - 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 upward. 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.

   Note: Even distribution of EBs is important to avoid aggregation. If EBs aggregate into a few large clumps, differentiation may be affected. See Troubleshooting (section 6.0) for further details.
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 to 2 to increase yield while retaining a sufficient $CD34^+$ purity for further culture.
10. Proceed to section 5.0 for differentiation to NK cells.

4.6 Phenotype Assessment

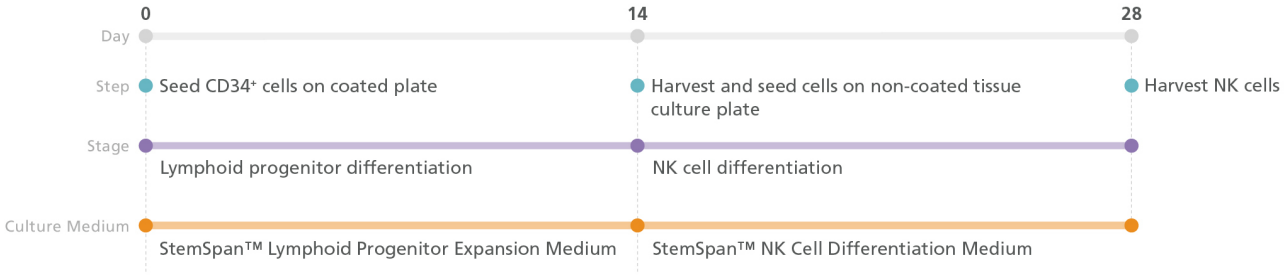
For phenotype assessment of hematopoietic progenitor cells by flow cytometry, use the following fluorochrome-conjugated antibodies:

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

5.0 Differentiation to NK 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) or bone marrow (BM), to NK cells.

5.1 Protocol Diagram



5.2 Differentiation Procedure

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 DIFFERENTIATION MEDIUM	NUMBER OF CD34+ CELLS/WELL		
			CB-DERIVED	BM-DERIVED	hPSC-DERIVED*
96-well plate (e.g. Catalog #38044)	100 µL/well	100 µL/well	1×10^3	5×10^3	5×10^3
12-well plate (e.g. Catalog #38041)	1 mL/well	1 mL/well	1×10^4	5×10^4	5×10^4
6-well plate (e.g. Catalog #38040)	2.5 mL/well	2.5 mL/well	2.5×10^4	1.25×10^5	1.25×10^5

*hPSC-derived CD34+ cells must be generated using STEMdiff™ NK Cell Kit (Catalog #100-0170) 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 or BM, thaw cells then proceed to step 5.
   - If using fresh (less than 72 hours old) human CB or BM, isolate $CD34^+$ cells using one of the EasySep™ kits listed below, then proceed to step 5.
     - CB: EasySep™ Human Cord Blood CD34 Positive Selection Kit II (Catalog #17896)
     - BM: EasySep™ Human CD34 Positive Selection Kit II (Catalog #17856)
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 the frequency of hPSC-derived $CD34^+$ cells after cell separation is < 50%, an additional magnetic separation may improve results. See Troubleshooting (section 6.0) for further details.
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)
   - BM- or 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.

   Note: If using hPSC-derived $CD34^+$ cells, monitor culture for growth of adherent cells. If adherent cells appear to reach > 50% confluence, it is advisable to perform step 9b: "For hPSC-derived $CD34^+$ cells" early to maintain optimal performance. See Troubleshooting (section 6.0) for further details.

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 human CB- or BM-derived $CD34^+$ cells: Incubate at 37°C 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 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.

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. Perform a viable cell count using Trypan Blue and a hemocytometer.
13. Add cells at 1 x 10^5 cells/mL to 500 µL of StemSpan™ NK Cell Differentiation Medium (section 3.3).

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

14. Add 500 µL of cell suspension (prepared in step 13) to one well (5 x 10^4 cells/well) of a tissue-culture treated, non-coated, 24-well plate (e.g. Catalog #38017). Incubate at 37°C and 5% CO_2 for 3 or 4 days.

Day 17 or 18

15. Carefully add 500 µL of StemSpan™ NK Cell Differentiation Medium per well. Incubate at 37°C and 5% CO_2 for 3 or 4 days.

Day 21

16. 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™ NK Cell Differentiation Medium per well. Incubate at 37°C and 5% CO_2 for 3 or 4 days.

Day 24 or 25

17. 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™ NK Cell Differentiation Medium per well. Incubate at 37°C and 5% CO_2 for 3 or 4 days.

Day 28 - Harvest cells

18. Gently pipette cells up and down to ensure all cells are in suspension. Transfer cells to an appropriate tube. These NK cells are ready for assays or analysis as required.

5.3 Phenotype Assessment

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

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

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

- Anti-Human CD16 Antibody, Clone 3G8 (Catalog #60041)
- Anti-Human CD56 Antibody, Clone HCD56 (Catalog #60021)
- Anti-human CD94 antibody, clone DX22
- Anti-human CD158 (KIR) antibody, clone 180704 and/or HP-MA4
- Anti-human CD335 (NKp46) antibody, clone 9E2
- Anti-human CD336 (NKp44) antibody, clone P44-8
- Anti-human CD337 (NKp30) antibody, clone P30-15
- Anti-human NKG2D antibody, clone 1D11

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. If large aggregates form, this may affect differentiation efficiency.
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; addition of DNase I may also be beneficial.
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.
When performing Lymphoid Progenitor Differentiation (section 5.2) using hPSC-derived $CD34^+$ hematopoietic progenitor cells, I observe growth of adherent cells prior to day 7.	Some cell lines and/or experiments may have an increased number of adherent cells. If the adherent cells grow enough to cover 50% or more of the plate surface, it is advisable to transfer the non-adherent cells to a freshly coated plate. Adherent cell overgrowth may interfere with required signaling provided by the StemSpan™ Lymphoid Differentiation Coating Material, and thus may affect differentiation efficiency.
When performing Lymphoid Progenitor Differentiation (section 5.2) using hPSC-derived $CD34^+$ hematopoietic progenitor cells, it is recommended to seed 5 x 10^4 $CD34^+$ cells/mL. Do I need to assess $CD34^+$ cell frequency prior to seeding, and what should I do with my cells in the interim?	While it is optimal to seed cultures based on number of $CD34^+$ hematopoietic progenitor cells, you can seed based on total number of cells without any significant effect on differentiation. While running flow cytometry assessment, store cells at room temperature in EasySep™ Buffer or StemSpan™ SFEM II. If the time between assessment of isolated cells and seeding into culture is prolonged, performance may be affected; in this situation, seeding based on total cells is advised. After seeding using total cells, flow cytometry is still recommended to monitor frequency of initial $CD34^+$ cells; additionally, carefully monitor cultures for adherent cell overgrowth.
The frequency of $CD34^+$ cells is low. Are there any aspects of the protocol that I could adjust to optimize results for my cell line?	Our protocol was optimized using our in-house cell lines, and some cell lines may benefit from optimizations to increase performance. Two aspects of the protocol are amenable to changes that may affect the frequency and yield of $CD34^+$ hematopoietic progenitor cells: 1. Titrating the number of cells per aggregate to create larger or smaller EBs (section 4.2); EB size has a strong impact on CD34 differentiation and can be cell line dependent. 2. The culture duration and harvest time for $CD34^+$ cells can influence the frequency of $CD34^+$ cells (section 4.5). Extending cultures may increase the frequency and yield of $CD34^+$ cells; however, this may negatively affect lymphoid potential.

TECHNICAL MANUAL

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

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