Protocol for expansion of frozen-down CD34+ cells (from cord blood) on VeraVec ECs.

This protocol is based on the expansion of 50,000 CD34+ cells (previously frozen down), using VeraVec ECs as the expansion platform. The assay lasts for 12 days, with fresh cytokines being administered every two days until its conclusion (Day 0 = start of expansion of 50,000 CD34+ cells that have gone through one “recovery” day following thaw). Unlike most other expansion protocols, VeraVec mediated stem cell expansion does not result in a lineage bias or lack of long term engrafting material. VeraVec expanded stem cells demonstrate a balanced reconstitution of lineages with enhanced long term engraftment compared to alternative mechanisms of stem cell expansion (Butler et al., 2012).

Note that fetal sources of CD34+ cells (umbilical cord blood) have a higher proliferative capacity than adult CD34+ cells (bone marrow and blood). Modifications to this protocol may be performed to accommodate alternative types of stem cells, partly done by supplementing alternative cytokines in the expansion media.

Illustration Overview

The following protocol guidelines include a number of graphics that are color-coded. These color illustrations are not representative of the actual colors of the materials. Rather they are designed as a visual guide when implementing the detailed protocol guidelines.

Legend

VeraVec Cultivation Media - Red
VeraVec Expansion Media - Green
VeraVec Conditioning Media - Blue
Harvest solution Accutase - Purple
CD34+ cells in wells

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CONTACT INFORMATION

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Illustrative Protocol Steps

Day minus 2: SEEDING ALL VERAVEC ECs
1—VeraVec ECs (i.e. hVera101) should have been subcultured until reaching two confluent T75 flasks (or equivalent) for sufficient starting material. Instructions for VeraVec culturing can be found in the corresponding product instruction sheet. Plate approximately 150,000 VeraVec ECs per well in three 6-well plates* in VeraVec Cultivation Media, and place cells in a 37°C incubator set for 5% CO2. Label these wells “#1” thru “#18”. Additionally, seed three T75 flasks with approximately 500,000 VeraVec ECs per flask in VeraVec Cultivation Media, and place cells in a 37°C incubator set for 5% CO2. Lastly, plate approximately 250,000 VeraVec ECs with VeraVec Cultivation Media in one well of a fourth 6-well plate, and label this well “R”. Place this in a 37°C incubator set for 5% CO2. (This extra well will be used for “Recovery” of frozen CD34+ cells, described below in Step 3.)

NOTES: The number of 6-well VeraVec plates and T75 flasks needed for the initial stages of an expansion experiment will depend on the initial number of CD34+ cells that are available for expansion. Generally, three 6-well plates and three T75 flasks will be more than sufficient to expand a starting population of 50,000 CD34+ cells. For this initial plating of VeraVec ECs, VeraVec cultivation media should be used. One confluent T75 flask of VeraVec ECs can be used to seed three 6-well plates, which can then be used in two days. Similarly, another confluent T75 flask of VeraVec ECs can be used to seed three T75 flasks, which will be used later on in the protocol.

*Volumes for wells/flasks
6-well Plates (each well): 1.5ml
T75 Plates (each flask): 12ml
The above volumes apply to subsequent sections where cells are to be transferred.

Day minus 1: CONDITIONING THE INITIAL WELL AND CD34+ CELLS
2—Aspirate media from well #1 of the first 6-well plate and add 1.5 ml VeraVec Conditioning Media. Keep VeraVec Cultivation Media in remaining wells of the three 6-well plates and three T75 flasks.

3—Thaw an aliquot of previously frozen CD34+ cells from cord blood that contains 100,000 – 300,000 cells. Spin down and resuspend cell pellet in 1.5ml of HSC Expansion Media. Aspirate media from the “R” well of the fourth 6-well plate, and add the 1.5ml CD34+ cell sample to the “R” well. Return this plate to the incubator
**Day 0: SEEDING THE FIRST WELL OF EXPANSION**

4— Collect supernatant from the “R” well of the fourth 6-well plate, and count the cells. From this cell count, aliquot and spin down 50,000 CD34+ cells, and resuspend these cells in 1.5ml HSC Expansion Media. Aspirate media from well #1 of the first 6-well plate and add the 1.5ml cell sample (i.e. 50,000 CD34+ cells in HSC Expansion Media). Return the first 6-well plate to the incubator, and discard the 6-well plate-1 with the “R” well (this “R” is no longer needed).

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**Day 1: REFEEDING THE VERAVEC ECs**

5— All the wells and flasks of VeraVec ECs should be confluent at this point. VeraVec ECs are growth inhibited at contact. There is no need to split the cells any further. Aspirate media from wells #2-#18 (i.e. aspirate all wells EXCEPT well #1). Additionally, aspirate media from the three T75 flasks. Add VeraVec Cultivation Media to wells #2-#18 and to the three T75 flasks. Return the 6-well plates and T75 flasks to the incubator.

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**Day 2: REPLATE THE EXPANDING CELLS**

6— Examine well #1 under a microscope – expansion should still be relative low at this time point. Collect and transfer supernatant (i.e. media with expanding CD34+ cells – do not remove the VeraVec ECs layer) to a 15ml conical tube. Spin cells at 400g for 5 min at 4°C. Aspirate supernatant and resuspend the CD34+ pellet in 1.5ml HSC Expansion Media. Transfer 1.5ml of CD34+ cells/HSC Expansion Media to well #1. Return the 6-well plate to the incubator.
Day 3: CONDITIONING ADDITIONAL WELLS FOR EXPANSION

7— Aspirate media from wells #2-#3 in the first 6-well plate and add 1.5 VeraVec Conditioning Media to wells #2 and #3. Return the 6-well plate to the incubator.

Day 4: REPLATE THE EXPANDING CELLS

8— Examine well #1 under a microscope – expansion may now be rampant at this time point. Collect and transfer supernatant from well #1 (i.e. approximately 1.5 ml media with expanding CD34+ cells – do not remove the VeraVec ECs layer) to a 15ml conical tube. Spin cells at 400g for 5 min at 4°C. (While these cells are spinning, aspirate media from wells #2-#3.) When cells are done spinning, aspirate supernatant and resuspend the CD34+ pellet in 4.5ml HSC Expansion Media. Add 1.5ml of CD34+ cells/ HSC Expansion Media to wells #1-#3. Return the 6-well plate to the incubator.

Day 5: CONDITIONING AND REFEEDING THE VERAVEC ECs

9— Aspirate media from wells #4-#18 in the 6-well plates (i.e. aspirate media from all the wells EXCEPT wells #1-#3). Add 1.5 ml VeraVec Cultivation Media to wells #4-#18. Additionally, aspirate media from the three T75 flasks. Add VeraVec cultivation media to the three T75 flasks. Return the 6-well plates and T75 flasks to the incubator.
**Day 6: REPLATE THE EXPANDING CELLS**
10— Examine wells #1-#3 under a microscope – expansion should again be rampant in these three wells. Collect and transfer supernatant from wells #1-#3 (i.e. approximately 4.5ml media with expanding CD34+ cells – do not remove the VeraVec ECs layer) to a 50ml conical tube (the cells/media from all three wells can be combined into one 50ml conical tube). Spin cells at 400g for 5 min at 4°C. (While these cells are spinning, aspirate media from wells #4-#18.) When cells are done spinning, aspirate supernatant and resuspend the CD34+ pellet in 27ml HSC Expansion Media. Add 1.5ml of CD34+ cells/HSC Expansion Media to wells #1-#18. Return the 6-well plates to the incubator.

**Day 7: REFEEDING THE VERAVEC ECs**
11— Aspirate media from the three T75 flasks. Add VeraVec Cultivation Media to the three T75 flasks. Return the T75 flasks to the incubator.

**Day 8: REPLATE THE EXPANDING CELLS**
12— Examine wells #1 – #18 under a microscope – expansion should again be rampant in all wells. Collect and transfer supernatant from wells #1-#18 (i.e. approximately 27ml media with expanding CD34+ cells – do not remove the VeraVec ECs layer) to a 50ml conical tube (the cells/media from all wells can be combined into one 50ml conical tube). Split this 27ml sample into two new 50ml conical tubes: a “9ml sample” and a “18ml sample”. Spin both tubes of cells at 400g for 5 min at 4°C. (While these cells are spinning, aspirate media from the three T75 flasks.) When cells are done spinning, aspirate supernatant and resuspend each CD34+ pellet as follows: resuspend the “9ml sample” with 27ml HSC Expansion Media, and add 1.5ml of this to wells #1-#18; resuspend the “18ml sample” with 36ml HSC Expansion Media, and add 12ml of this to each of the three T75 flasks. Return the 6-well plates and T75 flasks to the incubator.
Day 10: REPLATE THE EXPANDING CELLS
13— Examine wells #1 – #18 and the three T75 flasks under a microscope – expansion should again be rampant in all wells of the 6-well plates, but not as rampant in the T75 flasks. Collect and transfer supernatant from wells #1-#18 (ie. approximately 27ml media with expanding CD34+ cells – do not remove the VeraVec ECs layer) to a 50ml conical tube (the cells/media from all wells can be combined into one 50ml conical tube). Split this 27ml sample into two new 50ml conical tubes: a “9ml sample” and a “18ml sample”. Spin both tubes of cells at 400g for 5 min at 4°C. When cells are done spinning, aspirate supernatant and resuspend each CD34+ pellet as follows: resuspend the “9ml sample” with 27ml HSC Expansion Media, and add 1.5ml of this to wells #1-#18; resuspend the “18ml sample” with 36ml HSC Expansion Media, and put this tube aside. Collect and transfer supernatant from the three T75 flasks (ie. approximately 36ml media with expanding CD34+ cells – do not remove the VeraVec ECs layer) to a 50ml conical tube. Spin cells at 400g for 5 min at 4°C. When cells are done spinning, aspirate supernatant and resuspend the CD34+ pellet using the “18ml sample” (resuspended in 36 ml HSC Expansion Media that was previously put aside), and add 12ml of this to each of the three T75 flasks. Return the 6-well plates and T75 flasks to the incubator.

Day 12: END OF EXPANSION
14— For each plate and flask, collect and combine all the cells in the wells and flasks (both in the media and attached to the plate – use Accutase to retrieve all cells) and count. Record total cell counts. Cells should then be kept on ice, and setup for analysis (i.e. staining for Flow, etc.) should begin. For transplantation studies, the removal of the VeraVec ECs is optional.
“CONTROL” Note:
When performing this assay initially, it is advisable to run a “control” sample in parallel with the VeraVec-expanded sample. This sample is processed in the exact same manner as described above, with the only difference being that these CD34+ cells are not co-cultured on VeraVec cells. Rather, they are simply cultured alone. The plates, flasks, media, and cytokine treatments should all be identical between the control-expanded and VeraVec-expanded CD34+ cells.

**Materials Required:**

1— 1 ampule of VeraVec HUVEC ECs
[Angiocrine Bioscience #: hVera101]

2— VeraVec Cultivation Media
Medium 199 (Hyclone: #SH30253.01)
Endothelial cell supplement (Biomedical Technologies: #BT-203)
(final concentration: 50µg/ml)
Antibiotic-Antimycotic 100X-solution (Invitrogen: # 15240-062)
Fetal Bovine Serum (Omega Scientific: FB-11)
(final concentration: 20%)
HEPES buffer (Invitrogen: 15630-080) (final concentration: 10mM)
Heparin (Sigma: # H3149-100KU) (final concentration: 50µg/ml)
Glutamax 100X-solution (Life Technologies: # 35050061)

3— VeraVec Conditioning Media:
StemSpan (StemCell: #09650)
Antibiotic-Antimycotic 100X-solution (Invitrogen: # 15240-062)

4— HSC Expansion Media (for expansion of CD34+ cells, either alone or on VeraVec ECs):
StemSpan (StemCell: #09650)
Antibiotic-Antimycotic 100X-solution (Invitrogen: # 15240-062)
Full cytokine cocktail (see below)

*5— Cytokines:
Full cytokine cocktail (final concentration given):
300ng/ml SCF
300ng/ml FLT3
100ng/ml TPO
100ng/ml IL-6
10ng/ml IL-3
Minimal cytokine cocktail (final concentration given):
50ng/ml SCF (kit-ligand)
50ng/ml FLT3
50ng/ml TPO

6— Harvest Solution, Accutase (Life Technologies A11105-01)

*To generate the maximum amount of total CD45+ cells, the full cytokine cocktail should be used. However, use of either the full cytokine cocktail or the minimal cytokine cocktail will result in similar levels of hematopoietic stem and progenitor cells (HSPCs).

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T75 #2 Plate 500,000 VeraVecs Replace Media Replace Media Replace Media Replate Expanding Cells Add Additional Expanding Cells Harvest
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VeraVec Cultivation Media
VeraVec Conditioning Media
HSC Expansion Media
Accutase

Legend