TECHNICAL MANUAL

Culture and Quantification of CFU-Hill Colonies Using CFU-Hill Liquid Medium Kit

STEMCELL™ TECHNOLOGIES

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1.0 Introduction

Blood vessel development is a regulated process involving the proliferation and migration of endothelial cells from adjacent pre-existing blood vessels (angiogenesis) or following differentiation of angioblasts or endothelial progenitor cells (EPCs) from mesodermal precursors (vasculogenesis).[1-3] EPCs were originally thought to be present only during embryonic development. However evidence accumulated in the past several years suggests that they can persist in the bone marrow and/or in circulation into adult life.[3] This has generated interest in the use of EPCs for neovascularization of ischemic or injured tissue and for the clinical assessment of risk factors for various diseases.[4-6]

Subsequently, many distinct populations of cells that appear to correlate with or influence postnatal vasculogenesis have been identified, and these distinct cells have all been referred to as EPCs.[1,4,7-10] The mechanisms by which these putative EPCs contribute to vasculogenesis remain unclear, but may include direct contribution via proliferation and integration as endothelial cells, or by indirect means through secretion of angiogenic growth factors (paracrine effect). These latter cells may be more properly referred to as "angiogenic cells", i.e. cells that support or augment angiogenesis and/or vasculogenesis, without actually differentiating into cells that form part of the vascular network. Therefore while numerous sources of cells provide measureable angiogenic function after transplantation, they may not directly produce endothelial cells and/or they may not be integrated into the endothelium.[11,12]

Studies to purify and characterize EPCs and other angiogenic cells have been difficult due to the lack of cell surface antigens or markers that distinguish these cells from mature vascular wall-derived endothelial cells and from subsets of hematopoietic cells.[1,2] Many of the markers associated with EPCs, including LDL uptake, lectin binding, and CD34/CD133/VEGFR2/CD31/CD105/CD144 expression are also found on hematopoietic cells, making the distinction between EPCs and other specific cell populations difficult.[1,3] Alternatively, some investigators have defined putative EPCs based on their different culture properties *in vitro*. Yoder *et al*. have delineated a hierarchy of putative EPCs by determining their proliferative potential, analogous to the hierarchy established for the hematopoietic system.[9]

A cell culture assay has been developed by Hill et al.[4] to assess the correlation between the frequency of a specific population of circulating putative angiogenic cells and multiple clinical factors. We have standardized this 5-day assay and refer to it as the 5-Day CFU-Hill Colony Assay. Unique colonies that are formed in the 5-Day CFU-Hill Colony Assay are referred to as colony-forming unit-Hill colonies or CFU-Hill colonies. The CFU-Hill Liquid Medium Kit has been developed specifically to support the culture and quantification of CFU-Hill colonies. In healthy individuals, the number of CFU-Hill colonies has been found to negatively correlate with the Framingham cardiovascular risk score, and to positively correlate with vascular function as measured by flow-mediated brachial artery reactivity.[4] A number of recent reports have used the 5-Day CFU-Hill Colony Assay and/or CFU-Hill Liquid Medium Kit (formerly known as EndoCult™) in investigations into CFU-Hill colony frequency and coronary artery disease,[7, 14,15] rheumatoid arthritis,[16] peripheral arterial disease,[17] diabetes,[18] and chronic obstructive pulmonary disease.[19]

The CFU-Hill Liquid Medium does not support the culture of mature endothelial cell lines, including human umbilical vein - endothelial cells (HUV-EC-C; ATCC CRL-1730).

The CFU-Hill Liquid Medium Kit includes CFU-Hill Basal Medium and CFU-Hill Medium Supplements that have been pre-screened and tested in the 5-Day CFU-Hill Colony Assay. In this assay, mononuclear cells are isolated by density gradient centrifugation and plated on fibronectin-coated 6-well plates in CFU-Hill Liquid Medium for two days to remove mature endothelial cells and some monocytes. After two days, the non-adherent cells are harvested, enumerated and replated on fibronectin-coated 24-well plates. Colonies are evaluated and enumerated 3 days later. A CFU-Hill colony is defined as a central core of round cells with radiating elongated spindle-like cells at the periphery.

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2.0 Equipment and Materials Required

2.1 CFU-Hill Liquid Medium Kits

The CFU-Hill Liquid Medium Kits* (Catalog #05900 and #05950) include the following components:

COMPONENT	SIZE (CATALOG #05900)	SIZE (CATALOG #05950)
CFU-Hill Basal Medium	80 mL	5 x 80 mL
CFU-Hill Supplements	20 mL	5 x 20 mL

*CFU-Hill Liquid Medium was formerly known as EndoCult™. The name was changed to accurately reflect the application of this product.

2.2 Equipment

- Biohazard safety cabinet certified for Level II handling of biological materials
- Low speed centrifuge. To convert x *g* to rpm, use the following formula:

$$RPM = \sqrt{\frac{RCF}{(1.118 \times 10^{-5}) \times (Radius)}}$$

Where: RCF = relative centrifugal force (*g*)
RPM = centrifuge speed in revolutions per minute
Radius = radius of rotor in cm

- 37°C incubator with humidity and gas control to maintain > 95% humidity and an atmosphere of 5% CO_2 in air
- Pipette-aid
- Micropipettors P10, P200, and P1000 with sterile disposable plastic tips
- Hemacytometer
- Routine light microscope for hemacytometer cell counts
- Inverted microscope with flatfield objectives and eye pieces to give object magnification of approximately 25X - 50X

2.3 Materials

- 50 mL centrifuge tube (Corning Catalog #352070)
- 15 mL centrifuge tube (Corning Catalog #430053)
- 5 mL test tube (Corning Catalog #352058)
- 3% Acetic Acid with Methylene Blue (Catalog #07060)
- Lymphoprep™ (Catalog #07861)
- D-PBS Without Ca++ and Mg++ (PBS; Catalog #37350)
- D-PBS with 2% FBS (Catalog #07905)
- 6-well fibronectin-coated plate (Corning Catalog #354402)*
- 24-well fibronectin-coated plate (Corning Catalog #354411)*
- Methanol (Anachemia Catalog #56902-546)
- Giemsa Staining Solution (EMD Chemicals Catalog #R03055)
- Atlas of CFU-Hill Colonies (Catalog #28711)

*Fibronectin-coated plates are stored at 2 - 8°C and should be brought to room temperature (15 - 25°C) prior to use.

3.0 Preparation of Complete CFU-Hill Liquid Medium

1. Thaw CFU-Hill Supplements at 2 - 8°C overnight or at room temperature (15 - 25°C).

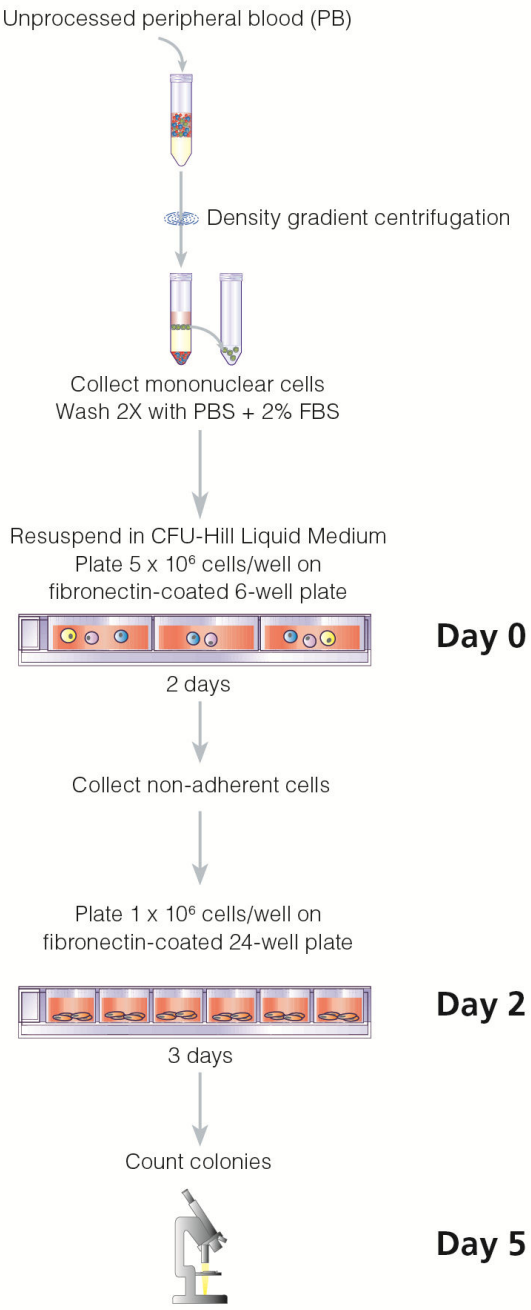
   Note: Use immediately or aliquot and store at -20°C. Once aliquots are thawed, use immediately or store at 2 - 8°C for up to 1 month. Do not re-freeze.

2. Add 20 mL of CFU-Hill Supplements to 80 mL of CFU-Hill Basal Medium.

   Note: If not used immediately, store complete CFU-Hill Liquid Medium at 2 - 8°C for up to 1 month.

   Note: This product does not contain antibiotics. If desired, add Penicillin and Streptomycin to achieve a final concentration of 100 U/mL of Penicillin and 100 µg/mL of Streptomycin.

4.0 5-Day CFU-Hill Assay Protocol Diagram



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5.0 5-Day CFU-Hill Colony Assay

5.1 Day 0

Note: For optimal results, use only fresh peripheral blood (PB) in the in vitro culture assay.

1. Collect PB aseptically using an anti-coagulant to avoid clotting or clumping of the sample. Sodium heparin, ACD (acid citrate dextrose), K_3 EDTA or equivalent may be used.

   Note: This protocol requires 10^7 mononuclear cells per sample for each experiment. For each PB sample, two Vacutainer® Collection Tubes (6 - 7 mL volume) should be sufficient for each experiment.

2. Add anti-coagulant treated PB to a 50 mL polypropylene tube. Count nucleated cells using 3% Acetic Acid with Methylene Blue. Perform a 1 in 20 dilution on a sample of the cells (e.g. 10 µL of cells and 190 µL of Acetic Acid) and count cells using a hemacytometer.

   Note: This provides a total blood cell count prior to density gradient centrifugation.

3. Prepare a mononuclear cell suspension by density gradient centrifugation using Lymphoprep™, as outlined below. For a more detailed protocol refer to the Product Information Sheet (PIS) for Lymphoprep™ (Document #29283) on our website at www.stemcell.com or contact us to request a copy.

   a. Add an equal volume of 1X Phosphate Buffered Saline to the 50 mL tube containing the PB sample (e.g. For PB samples, two Vacutainer® Collection tubes will provide 11 - 14 mL of blood, therefore add 11 - 14 mL of PBS).

      Note: Refer to the PIS for Lymphoprep™ for recommended volumes of blood and Lymphoprep™ and appropriate tube sizes.

   b. Add 15 mL of Lymphoprep™ to a new 50 mL centrifuge tube.

   c. Carefully layer the diluted PB on top of the 15 mL of Lymphoprep™.

      Note: Do not mix the Lymphoprep™ and diluted PB sample.

   d. Centrifuge tube(s) at 300 x *g* (see note in section 2.2) for 25 minutes at room temperature (15 - 25°C) with the brake off. With the brake off, the mononuclear cells will remain as a distinct layer for ease of collection.

   e. After centrifugation, carefully collect the mononuclear cells (layer between the Lymphoprep™ and plasma) into a new 50 mL centrifuge tube. Bring to a final volume of 40 mL with PBS with 2% FBS. Centrifuge at 300 x *g* for approximately 7 minutes at room temperature (15 - 25°C) with the brake on to pellet the cell suspension.

4. Remove supernatant, tap the tube to resuspend cells and add 6 mL of PBS with 2% FBS to the tube. Transfer the cell suspension to a new 15 mL centrifuge tube. Rinse the 50 mL tube with an additional 6 mL of PBS with 2% FBS and transfer the cell suspension to the same 15 mL tube. Spin at 300 x *g* for 7 minutes at room temperature (15 - 25°C) with the brake on to pellet the cell suspension.

5. Remove supernatant and resuspend pellet in 1 - 3 mL (depending on size of the cell pellet) of CFU-Hill Liquid Medium (section 3.0).

6. Count nucleated cells using 3% Acetic Acid With Methylene Blue by performing a 1 in 20 dilution on a sample of the cells (e.g. 10 µL cells and 190 µL 3% Acetic Acid With Methylene Blue) and count cells using a hemacytometer.

   Note: The percent recovery following density gradient centrifugation can be calculated by: (total cell count after density gradient centrifugation / total cell count before density gradient centrifugation) x 100%.

7. Add 2 mL of CFU-Hill Liquid Medium per well to a 6-well fibronectin-coated plate. Perform the experiment in duplicate using 2 wells of a 6-well plate per sample.

8. Plate 5 x10^6 mononuclear cells per well in the 6-well fibronectin-coated plate in duplicate and incubate at 37°C in 5% CO_2 with ≥ 95% humidity for 2 days. This step removes adherent cells.

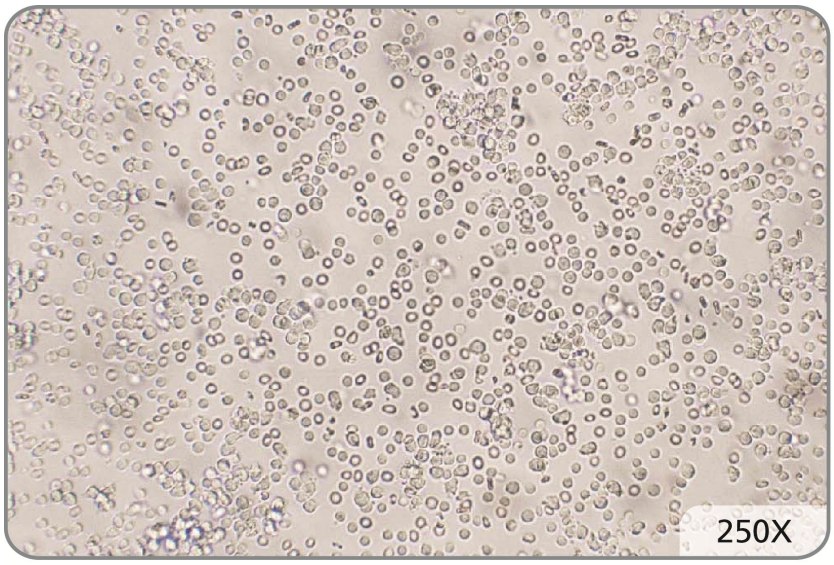

Figure 1. Peripheral Blood Mononuclear Cells in CFU-Hill Liquid Medium at Day 1

5.2 Day 2

After two days, numerous cells will adhere to the bottom of the well. The non-adherent cells contain the CFU-Hill colony-forming cells, which will be harvested at day 2 and further cultured for an additional 3 days to allow formation of CFU-Hill colonies.

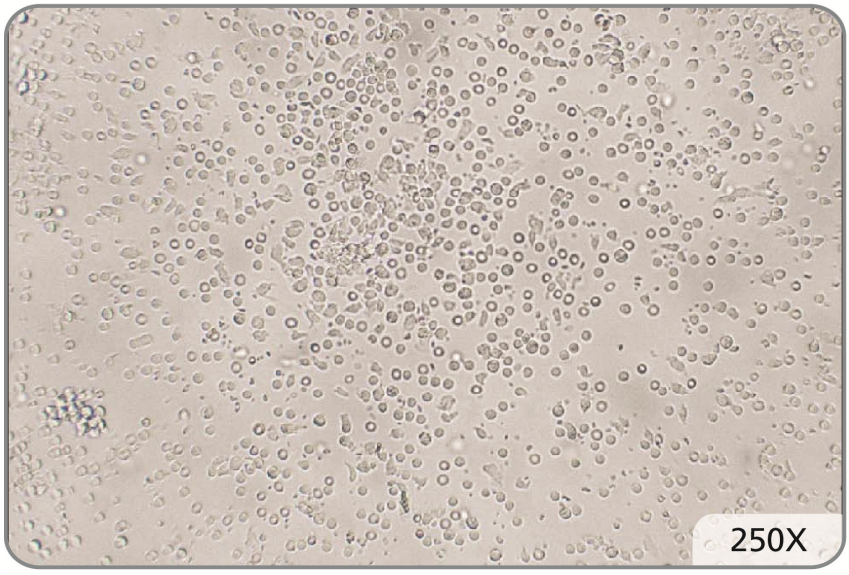

Figure 2. Peripheral Blood Mononuclear Cells in CFU-Hill Liquid Medium at Day 2

1. Collect the non-adherent cells by pipetting the medium in each well up and down 3 - 4 times using a 2 mL pipette. This will help to remove any non-adherent cells that transiently attach to the adherent population. Transfer the non-adherent cells from each well into individual 5 mL tubes and count nucleated cells using 3% Acetic Acid with Methylene Blue at a 1 in 10 dilution (e.g. 10 µL sample in 90 µL acetic acid).

   Note: Do not wash or centrifuge the cells at this stage.

2. From each well, plate 1 x 10^6 cells/well in duplicate in a 24-well fibronectin-coated plate. Add fresh CFU-Hill Liquid Medium to a final volume of 1.0 mL per well.
3. Incubate at 37°C in 5% CO2 with ≥ 95% humidity for 3 days.

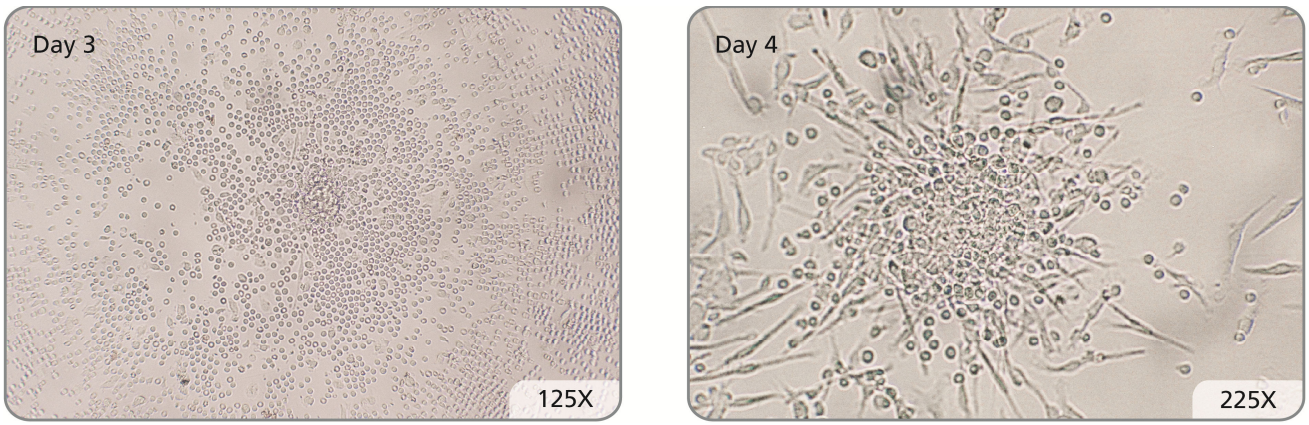

Figure 3. CFU-Hill Colonies in CFU-Hill Liquid Medium at Days 3 and 4

5.3 Day 5

On day 5 of the assay, count the number of colonies per well for each sample. CFU-Hill colonies are defined as a central core of round cells with radiating elongated spindle-like cells at the periphery (Figure 4).

Notes:

- Colonies without the CFU-Hill morphology may also be present but are not scored as CFU-Hill colonies.
- Cultures grown for more than 5 days may become difficult or impossible to score.
- Fixing and staining cultures at day 5 can make colony scoring easier and generates a permanent record that can be scored at a later date. Refer to section 6.0 for a protocol on fixing and staining of the colonies.

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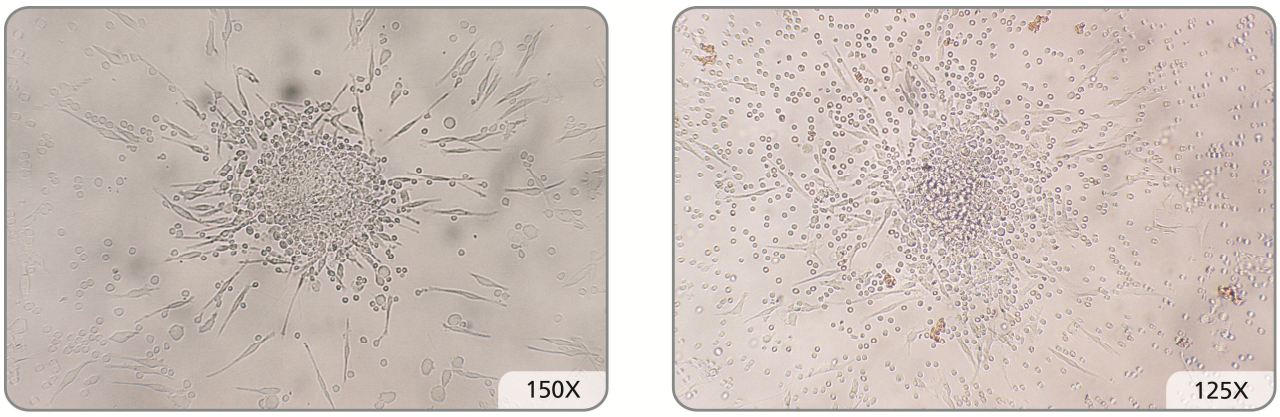

Figure 4. CFU-Hill Colonies in CFU-Hill Liquid Medium from Peripheral Blood at Day 5

The Atlas of CFU-Hill Colonies contains additional photographs of representative CFU-Hill colonies derived from human peripheral blood to further assist in their identification and evaluation.

6.0 Fixing and Staining CFU-Hill Colonies

1. At day 5, pipette the medium in one well of a 24-well plate up and down gently to suspend non-adherent cells. Remove and discard medium containing non-adherent cells.
2. Wash the wells by gently adding approximately 1 mL of PBS and pipetting up and down gently. Remove the PBS and discard.
3. Fix the cultures by gently adding 300 µL methanol by 'dribbling' the methanol down the side of wells.

   Note: Methanol is hazardous. Take appropriate precautions.
4. Incubate at room temperature (15 - 25°C) for 2 - 5 minutes.
5. Remove methanol and discard in appropriate organic waste container.
6. Stain the cultures after fixation by adding approximately 300 µL of Giemsa Staining Solution diluted 1 in 20 in distilled water.
7. Incubate at room temperature (15 - 25°C) for approximately 3 - 5 minutes.
8. Aspirate Giemsa Staining Solution.
9. Rinse wells gently with water. The amount of residual stain is controlled by the amount of washing. The plate can be tilted under the tap so that water gently runs into each well, gets swirled and then discarded. Repeat as necessary until colonies are clearly visible with limited background staining.
10. Plates can be stored indefinitely at room temperature (15 - 25°C) for future colony counting.
11. Count colonies using a standard light microscope.

    Note: A grid photocopied onto a transparency can improve counting accuracy.

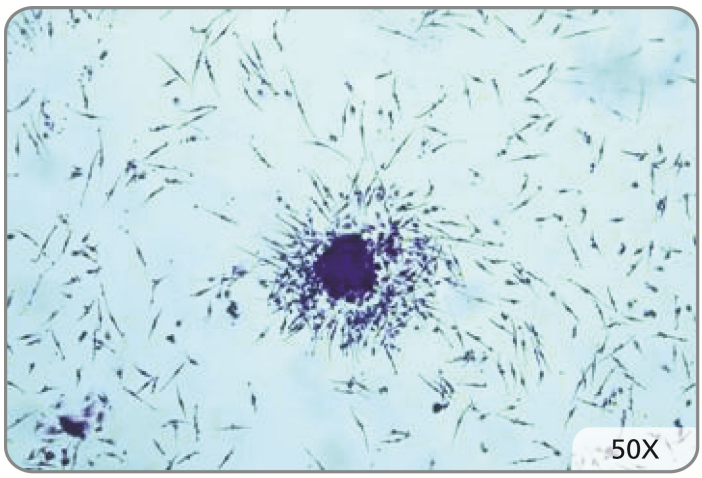

Figure 5. CFU-Hill Colony Stained with Giemsa

7.0 Peripheral Blood-Derived CFU-Hill Colony Frequencies in the General Population

There is no significant age or gender-related variation in average CFU-Hill frequency in human peripheral blood, however variability in the number of CFU-Hill per 10^6 cells was observed between individual donors (see Table 1).

Table 1. Peripheral Blood CFU-Hill Colony Frequencies

GENDER	NUMBER OF DONORS	AGE RANGE	CFU-HILL NUMBER/10^6 CELLS (RANGE)	CFU-HILL NUMBER/mL OF BLOOD
Male (mean ± SD)	15	23 - 54	21 ± 18 (1 - 67)	35 ± 35
Female (mean ± SD)	13	24 - 54	20 ± 17 (2 - 58)	26 ± 23

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