TECHNICAL MANUAL

STEMCELL Quality Control Kits

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1.0 Introduction

Clonogenic assays are dependent on the ability of single hematopoietic progenitor cells to divide and differentiate, forming clusters of cells (colonies) in semi-solid media containing appropriate growth factors. Since their introduction more than 40 years ago, colony assays have been used extensively for research and clinical applications including identification of stimulatory and inhibitory growth factors, supportive diagnostic assays of myeloproliferative disorders and leukemias, and evaluation of the hematopoietic potential of bone marrow, leukapheresis, and cord blood cell preparations for clinical transplantation.

The colony assay is the benchmark functional assay to assess the ability of various hematopoietic cell sources to divide and differentiate, especially following ex vivo manipulations including T cell depletion, volume reduction, $CD34^+$ cell enrichment, cryopreservation, long-term storage, thawing, and washing.

It is important to maintain a high degree of consistency in the progenitor assay setup and readout (colony counting) within a given laboratory. The STEMCELL Quality Control Kits enable laboratories to monitor their consistency on a regular basis. Colony-forming unit (CFU) assays that are set up each month using cryopreserved samples of the same human cell preparation supplied with each STEMCELL Quality Control Kit generate data that will provide a record of the laboratory's or individual technologist's reproducibility at setting up, culturing, and counting hematopoietic colonies over the course of one year. Variations in the numbers of colonies counted in successive monthly assays can reveal inconsistencies in lab performance that may need to be addressed. In addition, large variations in colony numbers may highlight equipment malfunction (e.g. freezer or incubator) that may otherwise have gone undetected.

STEMCELL Quality Control Kits are available with human cells from bone marrow (STEMCELL QC-BM) or cord blood (STEMCELL QC-CB) to allow assessment of the cell type most appropriate to a given laboratory's applications.

PRODUCT	CATALOG #	CELL TYPE PROVIDED IN KIT
STEMCELL QC-BM	00650	Human Bone Marrow
STEMCELL QC-CB	00651	Human Cord Blood

2.0 Thawing Cells, Plating, and Colony Counting

2.1 Supplies and Reagents Included in the QC Kit

- 12 frozen vials of pre-tested cells

  The source of the cells will vary depending on the catalog number ordered:
  - Catalog #00650: Frozen Human Bone Marrow, mononuclear cells
  - Catalog #00651: Frozen Human Cord Blood, mononuclear cells

- 24 tubes of MethoCult™ H4034 Optimum (3 mL per tube)

  Note: Only 12 tubes of MethoCult™ are required for use with the QC Kit. Use the remaining tubes as desired.

  Product contains the following:
  - Methylcellulose in Iscove's Modified Dulbecco's Medium (IMDM)
  - Fetal bovine serum (FBS)
  - Bovine serum albumin (BSA)
  - 2-Mercaptoethanol
  - Recombinant human (rh) stem cell factor (SCF)
  - rh Granulocyte macrophage colony-stimulating factor (GM-CSF)
  - rh Interleukin 3 (IL-3)
  - rh Granulocyte colony-stimulating factor (G-CSF)
  - rh Erythropoietin (EPO)
  - Supplements

- 15 sterile 16 gauge blunt-end needles
- 15 sterile 3 mL syringes
- 40 x 35 mm tissue culture dishes
- 15 x 100 mm dishes
- 12 x 100 mL bottles of IMDM + 2% FBS
- Trypan Blue (20 mL bottle)
- Atlas of Human Hematopoietic Colonies
- Instruction manual
- Letter, with lot-specific information that references the 10X Plating Density

2.2 Additional Reagents and Equipment Required

- Biohazard safety cabinet certified for Level II handling of biological materials
- Low-speed centrifuge equipped with biohazard containers for handling human cells
- 37°C incubator with humidity and gas control to maintain > 95% humidity and an atmosphere of 5% CO_2 in air
- Vortex mixer
- Inverted microscope with flat field objectives and eyepieces to give total object magnification of approximately 20 - 30X, 40 - 63X, and 100 - 125X.

  Note: Total object magnification = eyepiece x objective

  i.e. 25X = 2.5 x 10
- Hemocytometer (e.g. Neubauer)
- 70% ethanol or isopropanol
- 60 mm gridded scoring dishes or STEMgrid™-6 counting grid (Catalog #27000)
- 3% Acetic Acid with Methylene Blue (Catalog #07060)
- Routine light microscope for hemocytometer cell counts
- 14 mL polystyrene tube (e.g. Catalog #38008)
- 5 mL round-bottom polystyrene tube (e.g. Catalog #38007)
- 50 mL conical tube (e.g. Catalog #38010)
- 1 mL, 2 mL, and 10 mL serological pipettes (e.g. Catalog #38001, 38002, and 38004)
- Sterile distilled water
- Hand tally counter

2.3 Product Storage

- Store bone marrow and cord blood cells at -135°C or colder, or in liquid nitrogen, for up to 2 years.
- Store MethoCult™ H4034 Optimum and IMDM + 2% FBS at -20°C (-25°C to -15°C). Do not exceed expiry date (EXP) as indicated on label.
- Store all other materials and reagents at room temperature (15 - 25°C).

2.4 Method

2.4.1 Definitions

- **Cell Stock:** The 'neat' mononuclear cell sample washed with IMDM with 2% FBS
- **Viable Cell Concentration** (cells per mL):
  Cell Stock Concentration (section 2.4.3 step A6) x **% Viability**
- **10X Plating Density** (cells per mL): The viable cell concentration of the **Cell Stock** which has been diluted in IMDM with 2% FBS to ten times (10X) the final cell plating density
- **Final Plating Density** (cells per mL): The number of viable cells per volume of semi-solid culture medium per well

2.4.2 Thawing Cells

1. Thaw cells quickly (within 2 minutes) in a 37°C water bath by gently swirling. Do not vortex cells at any time during the thawing procedure.
2. When the cells are almost completely thawed, wipe the outside of the vial with 70% ethanol or isopropanol.
3. Gently transfer cells to a 14 mL polystyrene tube.
4. Slowly (dropwise) add 10 mL of IMDM + 2% FBS while gently swirling the tube (approximately 1 - 2 minutes).
5. Gently invert tube to mix.
6. Centrifuge cells at 300 x *g* for 10 minutes at room temperature (15 - 25°C).
7. Carefully remove the supernatant, taking care not to dislodge the cell pellet. Do not pour off. Gently flick the tube to resuspend the cell pellet.
8. Add 2 mL of IMDM + 2% FBS to the tube. This is the **Cell Stock**.

2.4.3 Performing Cell Counts

The cell counting procedures outlined below are suggestions. Use the procedures that have been validated in your institution.

A) Manual Nucleated Cell Count

1. Clean coverslip and hemocytometer thoroughly with alcohol. Dry coverslip and hemocytometer with lint-free tissue before using. Place the coverslip on the hemocytometer so that it is centered over both chambers.
2. Dilute **Cell Stock** for a nucleated cell count according to your laboratory standards. Mix the diluted sample.

   Example: Place 20 µL of **Cell Stock** into 380 µL of 3% Acetic Acid with Methylene Blue to achieve a 1 in 20 dilution.
3. Draw up an aliquot of diluted sample using a micropipettor or capillary tube.
4. Fill both chambers of the hemocytometer using a micropipettor or capillary tube. Do not overfill or underfill the chambers.

5. Starting with one chamber of the hemocytometer, count all the nucleated cells in at least two of the major corner 1 mm squares using a hand tally counter or other similar device. Count the same number of squares in the opposite chamber. Keep a total count of the cells and establish the average number of cells per square. If the cell count is less than 10 cells per square, a more concentrated suspension should be prepared (i.e. 20 µL of sample into 180 µL of 3% Acetic Acid with Methylene Blue to achieve a 1 in 10 dilution).
6. Determine the **Cell Stock Concentration** as follows:

   Each of the 9 major squares of the hemocytometer, with coverslip in place, represents a total volume of 0.1 mm^3 (or 10^{-4} cm^3, which is equivalent to 10^{-4} mL). The **Cell Stock Concentration** and total number of cells can be determined using the following calculations:

   $$\textbf{\textit{Cell Stock Concentration}}\ (cells\ per\ mL) = average\ count\ per\ square\ x\ dilution\ factor\ x\ 10^4$$

   $$TOTAL\ CELLS = \textbf{\textit{Cell Stock Concentration}}\ x\ original\ start\ volume$$

B) Viable Cell Count (Trypan Blue Exclusion Test)

1. Clean coverslip and hemocytometer thoroughly with alcohol. Dry coverslip and hemocytometer with lint-free tissues before using. Place the coverslip on the hemocytometer so that it is centered over both chambers.
2. Dispense 100 µL of **Cell Stock** into a 12 x 75 mm tube.
3. Dispense 100 µL of Trypan Blue into the same tube.
4. Agitate gently and let tube stand undisturbed for 2 minutes. Do not let the mixture stand for longer than 5 minutes as viable cells may begin to take up the stain as well.
5. Draw up an aliquot of the diluted sample using a pipettor or capillary tube.
6. Fill both chambers of the hemocytometer using a pipettor or capillary tube. Do not overfill or underfill the chambers.
7. Using a multi-channel counter or two hand tally counters, count each viable, clear (non-blue) nucleated cell and each non-viable, blue nucleated cell (cells with damaged membranes) separately. Continue to score squares in the hemocytometer until you have a counted a minimum of 100 cells.
8. Calculate the **% Viability** using the following formula:

$$\%\ \boldsymbol{Viability} = \frac{Viable\ cell\ count}{Total\ cell\ count\ (Viable + Non{-}viable)}\ x\ 100\%$$

2.4.4 Diluting Cell Stock

1. Thaw a tube of MethoCult™ H4034 Optimum at room temperature (15 - 25°C) or overnight at 2 - 8°C.
2. Refer to the lot-specific information letter (Document #29115 or 29116) included in the QC Kit. The letter references a cell concentration called **10X Plating Density** to be used to set up the CFU assay with a predetermined cell number. The following steps outline how to dilute the **Cell Stock** to prepare the **10X Plating Density**, which is then diluted 10-fold in the semi-solid culture medium to generate the **Final Plating Density**.
3. Use this formula to calculate the **Viable Cell Concentration** of the **Cell Stock**:

$$\textit{Viable Cell Concentration} = \textit{Cell Stock Concentration (section 2.4.3 step A6)} \times \textit{\% Viability (section 2.4.3 step B8)}$$

   Example:

$$\textit{Viable Cell Concentration} = 3.8 \times 10^6 \text{ cells per mL} \times 92\%$$
$$= 3.5 \times 10^6 \text{ cells per mL}$$

4. Use this formula to calculate the **Volume of Cell Stock** and **Volume of IMDM + 2% FBS** required to prepare 1 mL of the **10X Plating Density**:

$$\textbf{Volume of Cell Stock}\ (mL) = \frac{\textbf{10X Plating Density}\ (cells\ per\ mL;\ refer\ to\ Catalog\ \#29115) \times 1\ mL}{\textbf{Viable Cell Concentration}\ (cells\ per\ mL)(section\ 2.4.4\ step\ 3)}$$

$$\textit{Volume of IMDM + 2\% FBS}\ (mL) = (1\ mL) - (\textbf{Volume of Cell Stock}\ [mL])$$

5. Gently mix the **Volume of Cell Stock needed** + **Volume of IMDM with 2% FBS** (as calculated above) to prepare the **10X Plating Density** for CFU assay setup.
6. Add 0.3 mL of the **10X Plating Density** (step 5) to the 3 mL tube of MethoCult™ H4034 Optimum to obtain the **Final Plating Density**.
7. Vortex the tube vigorously for at least 4 seconds. After vortexing, let the tube stand for at least 5 minutes to allow all bubbles to rise to the surface.
8. Prepare 35 mm dishes by placing them in pairs inside a 100 mm dish. Be sure to add a third 35 mm dish (without its lid) for a water dish. The purpose of the water dish is to ensure that maximum humidity is maintained during incubation. The 35 mm dishes used for the assay cultures have been pre-tested for optimal colony growth and do not support growth of anchorage-dependent cells.
9. The package of 10 x 35 mm dishes should be resealed for future assays.
10. To plate the MethoCult™/cell mixture into the sterile dishes, attach a 16 gauge blunt-end needle to a 3 mL syringe. Draw up the MethoCult™/cell mixture to the 1.0 mL mark and slowly dispense this initial volume back into the tube in order to remove the large air bubble that is present in the syringe and needle. Draw up the mixture again to the 2.6 mL mark. Dispense 1.1 mL into a labeled 35 mm dish (plunger now at 1.5 mL mark). Dispense another 1.1 mL into the second labeled dish (plunger now at 0.4 mL mark).
11. Rotate and tilt each dish to spread the viscous mixture evenly across the surface of each dish.
12. Add 3 mL of sterile water to the water dish. Place the cultures on a level tray in a 37°C humidified incubator with 5% CO_2 in air. It is important that the correct temperature, CO_2, and humidity (> 95%) are maintained in the incubator during the entire culture period.

13. Incubate for 14 days at 37°C, 5% CO_2 in air, and > 95% humidity.

2.4.5 Counting Colonies

1. Prepare a 60 mm gridded scoring dish by drawing two perpendicular lines across the center of the dish using a permanent fine felt marker on the bottom of the dish (refer to page 4 of the Atlas of Human Hematopoietic Colonies). This scoring dish can be used again to score other culture dishes.
2. Remove cultures from the incubator and place them (with the lid still on), one at a time, inside the 60 mm gridded tissue culture dish to count the colonies in situ using an inverted microscope. Counting is usually easier if colonies are counted in vertical rows by moving the microscope stage up and down (rather than across) the dish.
3. Counts at day 14 - 16 should include the smaller erythroid colonies, derived from the most mature types of erythroid colony-forming cells (i.e. from CFU-E); the larger erythroid colonies (from primitive BFU-E); all granulopoietic colonies (from CFU-GM); and colonies containing multiple lineages of cells (from CFU-GEMM). For detailed assistance in the recognition of various colony types, refer to the Atlas of Human Hematopoietic Colonies provided.

CFU-E	Colony-forming unit-erythroid of 1 - 2 small clusters containing 8 - 200 erythroblasts
BFU-E	Burst-forming unit-erythroid containing greater than 200 erythroblasts (may contain greater than 2 clusters)
CFU-GM	Colony-forming unit-granulocyte, macrophage containing 40 or more cells of the granulocyte and/or macrophage lineage
CFU-GEMM	Colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte containing erythroid cells and 20 or more granulocyte, macrophage, erythroid, and megakaryocyte cells

   Note: A blue filter enhances the red color of the erythroid colonies and may help in the identification of both CFU-E and BFU-E.
4. Scan the entire dish at a magnification of 20 - 30X to ensure that the plating efficiency is representative of the entire dish. Scoring or counting the number and types of colonies is best done using an inverted microscope equipped with high quality flatfield objectives and eyepieces to give total object magnification of approximately 20 - 30X, 40 - 63X, and 100 - 125X.

   Total object magnification = eyepiece magnification x objective magnification

   Example

   Total object magnification = (12.5X) x (5X)

   = 62.5X
5. Score the CFU-E using the total object magnification 40 - 63X. Once completed, score the remaining BFU-E, CFU-GEMM and CFU-GM colonies using the 20 - 30X total object magnification. Use a higher magnification to confirm colony type if uncertain.
6. Record the number of colonies in each of the culture dishes on the worksheet provided in section 4.0. Calculate the average number of each type of progenitor detected in the colony assay by dividing the sum of the number of colonies in the two dishes by two. Keep a copy of this Worksheet in your files for future use.

3.0 CD34+ Cell Counting (Optional)

Materials and recommendations for CD34+ cell counting have not been supplied with this kit. However, the cell suspension may be tested to assess the consistency of CD34+ cell counting by flow cytometry using the standard operating procedures and antibodies employed in your laboratory.

The CD34+ cell frequency determined following incubation of conjugated antibodies and flow cytometry analysis should be recorded for each test. Plots generated from the monthly analysis of CD34+ cell frequency will demonstrate the reproducibility of your protocol in counting the CD34+ cell content of a given sample.

4.0 Worksheet

STEMCELL QC LOT#		CFU-E	BFU-E	CFU-GM	CFU-GEMM	TOTAL CFU
Test 1	Plate 1					
	Plate 2					
	Average					
Test 2	Plate 1					
	Plate 2					
	Average					
Test 3	Plate 1					
	Plate 2					
	Average					
Test 4	Plate 1					
	Plate 2					
	Average					
Test 5	Plate 1					
	Plate 2					
	Average					
Test 6	Plate 1					
	Plate 2					
	Average					
Test 7	Plate 1					
	Plate 2					
	Average					
Test 8	Plate 1					
	Plate 2					
	Average					
Test 9	Plate 1					
	Plate 2					
	Average					
Test 10	Plate 1					
	Plate 2					
	Average					
Test 11	Plate 1					
	Plate 2					
	Average					
Test 12	Plate 1					
	Plate 2					
	Average					

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