TECHNICAL MANUAL
VERSION 1.0.0

HUMAN COLONY-FORMING CELL ASSAYS USING MethoCult® Express

STEMCELL TECHNOLOGIES

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Table of Contents

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1.0 Introduction

Reliable and quantitative assays to measure the frequency and quality of hematopoietic stem cells and progenitors in bone marrow (BM), mobilized peripheral blood (MPB) and umbilical cord blood (CB) are important to assess the suitability of candidate hematopoietic grafts for transplantation. When cultured in a suitable semi-solid matrix, individual hematopoietic progenitors called colony-forming cells (CFCs) proliferate and differentiate to form discrete clusters or colonies of mature cells. CFC assays are performed by placing a cell suspension into a semi-solid medium, such as methylcellulose, supplemented with nutrients and cytokines, followed by incubation at 37°C. In standard CFC assays, the CFCs are classified and enumerated based on the morphological recognition of one or more types of hematopoietic lineage cells within the colony.

CFC assays are the most commonly used assays to measure the frequency and proliferative ability of hematopoietic cells in different cell preparations and to monitor the effects of *ex vivo* procedures, such as red blood cell removal, volume reduction and cryopreservation on the frequency and quality of the cells. Assessing the progenitor cell content of the graft prior to transplantation is particularly important in cord blood transplantation. This is because cell numbers available for transplantation are limited, there is large variability in stem cell and progenitor content between different cord blood units and *ex vivo* manipulations, such as cryopreservation and thawing of cord blood units, can have large and variable effects on cell yield and viability of the stem and progenitor cells.

Colony-forming unit-granulocyte, macrophage (CFU-GM) and total CFC numbers in hematopoietic grafts are good predictors of neutrophil and platelet engraftment and survival after transplantation.[1-6] In unrelated CB transplantation, total CFC numbers after thawing of CB units correlated more strongly with recovery and survival than other parameters, such as CFC numbers before cryopreservation, TNC counts and $CD34^+$ cell numbers.[6] In addition, the CFC content of attached segments has been shown to correlate well with the CFC content of the whole CB unit.[7] Therefore, CFC assays on attached segments can, in principle, be used to measure the CFC content of CB units prior thawing of the whole bag, assuming that proper procedures for freezing and thawing, method validation and quality control are in place.

It is not necessary to distinguish colony types in order to measure the total CFC content of a graft, and therefore neither is it necessary to wait until colonies have fully matured. MethoCult® Express (Catalog #04437/04447) is a methylcellulose-based culture medium that has been formulated for accelerated progenitor proliferation and colony formation. Colonies grown in MethoCult® Express are larger than in standard MethoCult® media and can be counted as early as after seven-days of culture. As there is no need to distinguish different colony types, colony scoring is faster and easier than in standard 14 - 16 day assays. This faster and easier CFC-assay may improve reproducibility and standardization of routine CFC assays within and between labs.

If desired, cultures in MethoCult® Express can be maintained for the standard 14 - 16 days, after which colonies derived from burst-forming unit-erythroid (BFU-E); colony-forming unit-granulocyte, macrophage (CFU-GM) and colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM) can be counted.

This Technical Manual describes procedures for the detection and enumeration of human colony-forming cells in MethoCult® Express medium. Photographs of colonies cultured in MethoCult® Express medium are shown. Results of experiments comparing seven-day assays in MethoCult® Express and fourteen-day assays in MethoCult® Express or in MethoCult® GF H4034 (Catalog #04034/04044) are also provided.

Procedures for the evaluation of colony-forming unit-erythroid (CFU-E), BFU-E, CFU-GM and CFU-GEMM using standard MethoCult® media are described in the Technical Manual for Human Colony-Forming Cell Assays Using MethoCult® (Manual Catalog #28404), available upon request and on our website at www.stemcell.com/technical/manuals.aspx.

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Procedures for the evaluation of colony-forming unit-megakaryocyte (CFU-MK) in collagen-based media are described in the Technical Manual for Assays for Quantitation of Human and Mouse Megakaryocytic Progenitors (Manual Catalog #28413), available upon request and on our website at www.stemcell.com/technical/manuals.aspx.

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2.0 Glossary of Terms Used

Abbreviation	Description
CFC	Colony-forming cell
CFU	Colony-forming unit
CFU-E	Colony-forming unit-erythroid
BFU-E	Burst-forming unit-erythroid
CFU-GM	Colony-forming unit-granulocyte, macrophage
CFU-GEMM	Colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte
BM	Bone marrow
CB	Cord blood
MPB	Mobilized peripheral blood
CFU-Mk	Colony-forming unit-megakaryocyte
RBC	Red blood cell
MNC	Mononuclear cells
FBS	Fetal bovine serum
IMDM	Iscove's Modified Dulbecco's Medium
D-PBS	Dulbecco's Phosphate Buffered Saline
TNC	Total nucleated cell

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3.0 Equipment and Materials Required

3.1 Equipment

- Biohazard safety cabinet certified for Level II handling of biological materials

  All procedures for cell processing and set-up of CFC assays should be performed using sterile technique and universal handling precautions.
- Incubator set at 37°C with 5% CO_2 in air and ≥95% humidity

  Use of water-jacketed incubators with a water pan placed in the chamber is recommended.
- Inverted microscope for colony counting

  Use of a quality inverted microscope equipped with a 10 or 12.5X eyepiece objective, 2X, 4X and 10X planar objectives and a blue filter is recommended.
- Standard light microscope for cell counting
- Laboratory centrifuge
- Vortex
- Pipette-aid
- Micropipettors
- Automated cell counter or Neubauer hemacytometer

3.2 Materials

- Sterile pipettes: 2 mL and 5 mL
- Sterile polystyrene tubes: 5 mL (12 x 75 mm), 14 mL (17 x 100 mm), 15 mL conical, 50 mL conical
- Sterile pipette tips
- Syringes with luer-lock fitting: 3 mL (Catalog #28230/28240), 6 mL, 12 mL
- 16-gauge blunt-end needles (Catalog #28110/28120)
- 35 mm pre-tested culture dishes (Catalog #27100/27150)
- 100 mm disposable petri dishes (Catalog #27125/27127)
- Optional: 245 mm square dishes (Catalog #27140/27141)
- 60 mm gridded scoring dish (Catalog #27500)
- Permanent fine-tip marker

4.0 Thawing and Dispensing MethoCult® Express

MethoCult® Express is supplied at 100 mL per bottle (Catalog #04437) or 24 x 3 mL per tube (Catalog #04447). It is formulated for the addition of cells at a 1:10 (v:v) ratio of cells:medium, which maintains the optimal viscosity of the medium. Cells can be added directly to 3 mL tubes of MethoCult® Express but bottles must be aliquoted as follows:

1. Thaw MethoCult® Express overnight under refrigeration (2 - 8°C) or at room temperature (15 - 25°C).

   Do not thaw methylcellulose-based medium at 37°C.

2. Once thawed, shake the bottle vigorously for 1 - 2 minutes, or until the medium becomes opaque with bubbles.

3. Let the bottle stand for at least 5 minutes to allow bubbles to rise to the top before aliquoting.

4. Using a luer lock syringe attached to a 16-gauge blunt-end needle (Catalog #28110/28120), aliquot 3 mL per tube for 1.1 mL duplicate cultures or 4 mL per tube for 1.1 mL triplicate cultures.

   Do not use a standard pipette to aliquot methylcellulose as the volume dispensed will not be accurate. Methylcelluse is a viscous solution and cannot be accurately dispensed using a standard pipette due to adherence of the medium to the inside of the pipette. Use blunt-end needles for dispensing to prevent needle-stick injuries.

5. To remove the air from the syringe, place the needle below the surface of the MethoCult® medium and draw up approximately 1 mL. Gently depress the plunger and expel the medium completely. Repeat until no air space is visible.

6. Draw up the desired volume of methylcellulose medium into the syringe and dispense into 14 mL (17 x 100 mm) sterile test tubes.

7. Dispense 3 mL per tube to yield duplicate cultures of 1.1 mL each. Dispense 4 mL per tube to yield triplicate cultures of 1.1 mL each.

   Do not expel the medium to the "0" mark on the syringe when aliquoting. For example, measure from 3.5 mL to 0.5 mL rather than 3.0 mL to 0 mL.

   It is preferable to dispense the entire contents of the bottle into tubes containing the appropriate volume in order to avoid repeated freezing and thawing of the bottle.

8. Tubes can be used immediately or stored for later use. Store at -20°C (-25°C to -15°C) until expiry date indicated on the label or for one month at 2 - 8°C. Ensure that the tubes are capped tightly if storing for later use.

If MethoCult® Express arrives partially thawed, the bottle can be refrozen by placing at -20°C (-25°C to -15°C) immediately, or thawed completely and aliquoted as described in steps 1 to 8.

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5.0 Human Hematopoietic CFC Assays in MethoCult® Express Media

5.1 Procedure Diagram

Step 1

Prepare Cells

- Prepare cells (red blood cell-depleted cord blood samples, whole cryopreserved cord blood samples or cord blood mononuclear cells).
- Wash cells (e.g. in Iscove's MDM plus 2% FBS), then count and adjust cell concentration.
- Two plating concentrations are recommended for each sample.

Step 2

Add Cells to MethoCult®

- Vortex tube to mix contents thoroughly.
- Let stand for at least five minutes to allow bubbles to rise to the top.

Step 3

Plate and Incubate

- Dispense MethoCult® Express mixture into 35 mm culture dishes pretested for optimal colony growth.
- Incubate for 7 days at 37°C, in 5% CO_2, with ≥95% humidity.

Step 4

Count Colonies

- Count colonies using an inverted microscope and a gridded scoring dish.
- Count all colonies containing more than 20 cells. Most colonies are immature and scoring individual colony types is not recommended after only 7 days of culture.

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5.2 Cell Processing Methods

Different methods can be used to prepare CB cell samples for CFC assays using MethoCult® Express. Mononuclear cells or cell preparations from which red blood cells (RBCs) have been depleted are recommended for the 7-day CFC assay. RBCs cause a background in the cultures, which may make it difficult to accurately count colonies after 7 days in CFC assays performed on whole, fresh, unfractionated CB samples. Cryopreserved whole CB can be cultured in MethoCult® Express without further processing as freezing and thawing results in lysis of most RBCs in the sample.

Below are guidelines for processing of fresh CB samples. It is important to note that the methods described below may increase the frequency of CFCs in the cell suspension relative to the original sample. Some loss of CFCs can also be expected as a result of cryopreservation and cell washing.

The procedures outlined below are suggested. Use the procedures operational in your institution.

5.2.1 Ammonium Chloride Lysis Procedure

1. Add buffered Ammonium Chloride Solution (NH_4Cl) (Catalog #07800/07850) to the sample to give a volume:volume ratio of 9:1 (i.e. 18 mL NH_4Cl to 2 mL sample) in a 50 mL tube.
2. Gently vortex the mixture and put on ice for 10 minutes. Continue with gentle vortexing or inversion once or twice during the incubation period.
3. Check for complete lysis. The mixture will appear translucent red in color. If not completely lysed, vortex and incubate on ice for a further 5 - 10 minutes, with intermittent gentle vortexing or inversion.
4. Add Dulbecco's Phosphate Buffered Saline (D-PBS) with 2% Fetal Bovine Serum (FBS) (Catalog #07905) to fill the tube and centrifuge at 300 x *g* for 10 minutes at room temperature with the brake on.
5. Quickly decant and discard the supernatant, taking care not to dislodge the cell pellet. Resuspend the cell pellet by adding Iscove's Modified Dulbecco's Medium (IMDM) + 2% FBS (Catalog #07700) and mix thoroughly.
6. Perform a nucleated cell count. Refer to Section 5.3.1.
7. Refer to Section 5.4 for the general method for set-up of CFC assays in MethoCult® Express media.

5.2.2 Thawing Whole Cryopreserved Cord Blood

1. Thaw the vial of frozen cells at 37°C by gently swirling.
2. When the cells are almost completely thawed, transfer dropwise to a 50 mL tube with Dulbecco's Phosphate Buffered Saline (D-PBS) + 2% Fetal Bovine Serum (FBS) (Catalog #07905). Fill the tube with D-PBS + 2% FBS and centrifuge at 300 x *g* for 10 minutes.
3. Quickly decant and discard the supernatant, taking care not to dislodge the cell pellet. Resuspend the cell pellet by adding Iscove's Modified Dulbecco's Medium (IMDM) + 2% FBS (Catalog #07700) and mix thoroughly.
4. Perform a cell count using trypan blue (Catalog #07050) dye exclusion. Refer to Section 5.3.2.

   Methods to assay viable cells (e.g. dye exclusion) should be used for cell preparations where a decrease in cell viability may be expected (e.g. cryopreserved cells).

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5. Refer to Section 5.4 for the general method for set-up of CFC assays in MethoCult® Express media.

   Cryopreserved whole CB can be cultured in MethoCult® Express without further processing as freezing and thawing results in lysis of most RBCs in the sample.

5.2.3 Isolation of Light Density (Mononuclear) Cells by Ficoll-Paque™ PLUS

Hematopoietic colony-forming cells are present in the mononuclear cell fraction of cord blood samples. Isolation of light density cells using Ficoll-Paque™ PLUS enriches CFCs and depletes mature RBC, nucleated erythroid precursors, neutrophils, and dense non-viable cells.

1. Mix sample well and remove a small volume (100 µL) to perform an initial cell count using a Neubauer hemacytometer or a calibrated automated cell counter. Perform an initial nucleated cell count to establish the number and concentration of nucleated cells in the original sample. Refer to Section 5.3.1.
2. Measure the entire volume of the sample to be processed and then transfer it into a new tube.
3. Dilute the cells at least 1:1 with Iscove's Modified Dulbecoo's Medium (IMDM) + 2% Fetal Bovine Serum (FBS) (Catalog #07700). Invert gently to mix.
4. Add 15 mL of Ficoll-Paque™ PLUS (Catalog #07907/07957/07917/07967) to a 50 mL conical tube.

   Ficoll-Paque™ PLUS should be at room temperature.

5. Slowly layer 10 mL of cell sample on top of the Ficoll-Paque™ PLUS so that a distinct layer forms. Take care not to disturb the tube.

   10 mL of diluted cell sample and 15 mL of Ficoll-Paque™ PLUS yields a 2:3 ratio of diluted cell sample:Ficoll-Paque™ PLUS. For small volumes of cell sample, scale down the volumes while keeping the ratio of Ficoll-Paque™ PLUS to sample at approximately 1:1 to 2:3 diluted cell sample:Ficoll-Paque™ PLUS.

   If the layers are disturbed such that the Ficoll-Paque™ PLUS and sample mix, distinct layers will not form after centrifugation. This will result in decreased recovery of mononuclear cells.

6. Centrifuge the tube(s) at 400 x *g* for 30 minutes at room temperature with the brake **off**.
7. Using a sterile standard pipette or sterile Pasteur pipette, remove and discard the top plasma layer, taking care not to disturb the whitish layer of mononuclear cells present at the interface of the Ficoll-Paque™ PLUS layer.
8. Remove the mononuclear cell layer and transfer it to a sterile 14 mL (17 x 100 mm) tube.

   The mononuclear layer normally contains the lymphocytes, platelets, monocytes and hematopoietic colony-forming cells.

9. Add IMDM + 2% FBS and then centrifuge at 300 x *g* for 10 minutes at room temperature with brake on.
10. Quickly but carefully decant the supernatant so as not to dislodge the cell pellet. Resuspend the cell pellet, add IMDM + 2% FBS and mix thoroughly.
11. Centrifuge at 300 x *g* at room temperature for 10 minutes with brake on.
12. Quickly but carefully decant the second supernatant.
13. Resuspend the cell pellet in 1 to 2 mL IMDM + 2% FBS (a larger volume may be desirable if the initial cell count was high). Record the final volume.

Isolation of mononuclear cells by Ficoll-Paque™ PLUS results in a final cell suspension that is enriched 2- to 4-fold in its content of primitive cells. More mature and denser myeloid cells are removed with the red blood cells. Plating concentration must be adjusted accordingly. Refer to Section 5.4 for more information about plating concentrations.

14. Record the final volume and perform a nucleated cell count in the same manner as for the initial cell count. Refer to Section 5.3.1.
15. Refer to Section 5.4 for the general method for set-up of CFC assays in MethoCult® Express media.

5.2.4 Isolation of Nucleated Cells by Sedimentation Over HetaSep™

Red cell aggregating agents such as HetaSep™ (Catalog #07806/07906) increase the red blood cell (RBC) sedimentation rate by increasing the effective size of the cells through formation of aggregates, or rouleaux. Because nucleated cells settle at a lower rate, a compact pellet consisting mainly of RBCs is formed rapidly in the presence of HetaSep™, while the nucleated cells remain suspended in the supernatant.

Gravity Sedimentation

Gravity sedimentation is a simple and reliable method of RBC depletion. A defined interface forms between the RBC fraction and the RBC-depleted (nucleated cell-rich) fraction as the RBCs sediment through the HetaSep™ solution. Approximately 99% RBC depletion is attained if the nucleated cell-rich fraction is removed carefully.

1. Add 1 part HetaSep™ solution to 5 parts blood. Mix well.
2. Allow sample to settle until the RBC interface represents approximately 40% of the total volume.
3. Harvest the supernatant containing the nucleated cells and wash once in the appropriate medium.
4. Lyse remaining RBCs with ammonium chloride solution (Catalog #07800/07850) (optional).

   A full 15 mL or 50 mL centrifuge tube will separate in approximately 45 minutes. Half-full tubes require approximately 25 minutes. Older samples will take up to 30% longer to settle.

Centrifugation

Centrifugation may be used to accelerate the sedimentation process.

1. Add 1 part HetaSep™ solution to 5 parts blood. Mix well.
2. Centrifuge for 5 minutes at 50 x *g* with the brake off.
3. Harvest the supernatant containing the nucleated cells and wash once in the appropriate medium.
4. Lyse remaining RBCs with ammonium chloride solution (Catalog #07800/07850) (optional).

   This procedure works best with fresh blood in full 50 mL or 15 mL tubes and 450 mL bags. Nucleated cell recovery will decrease substantially when using containers less than half full due to the shorter sedimentation distance. Older blood will settle more slowly. If the blood is two days old, try centrifuging for 5 minutes at 200 x g with the brake off.

After isolation of nucleated cells by sedimentation over HetaSep™, perform a nucleated cell count. Refer to Section 5.3.1.

Refer to Section 5.4 for the general method for set-up of CFC assays in MethoCult® Express media.

5.3 Manual Cell Counts

5.3.1 Nucleated Cell Count Using 3% Acetic Acid

1. Mix cell suspension thoroughly and transfer a 100 µL aliquot to a separate tube.
2. Dilute the cell sample in 3% Acetic Acid with Methylene Blue (Catalog #07060). The recommended dilution for cord blood is 1/50 to 1/100.

   Example: For a 1/50 dilution, use a micropipettor with sterile tips and add 20 µL of cells to 980 µL of 3% Acetic Acid with Methylene Blue. The 3% acetic acid solution disrupts the cytoplasmic membrane while leaving the cell nuclei intact. The methylene blue dye allows easier visualization of the nuclei. The nucleated cell count should be performed within approximately ten minutes of the cells exposure to the acetic acid solution.
3. Mix the diluted cell sample well.
4. Prepare the hemacytometer by first cleaning the chambers and coverslip with alcohol and then wiping dry using lint-free tissue.
5. Carefully position the coverslip over both chambers.
6. Draw up an aliquot of the diluted sample using a micropipettor or a capillary tube.
7. Fill both chambers of the hemacytometer using a micropipettor or a capillary tube. Do not over- or under-fill the chambers.
8. Count total nuclei in 4 large squares (1 x 1 x 0.1 mm) or ≥100 cells. See Figure 1.
9. Determine the cell count (cells per mL) as follows:

   a) AVERAGE CELL COUNT PER SQUARE x DILUTION FACTOR x 10^4 = CELL COUNT PER mL

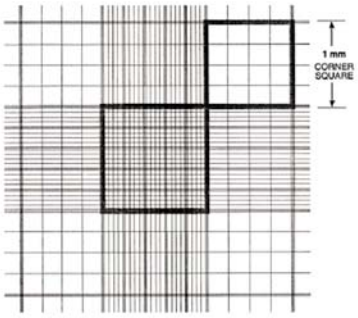

Figure 1. Neubauer hemacytometer showing dimensions of each square. Depth = 0.1 mm.

5.3.2 Viable Cell Count Using Trypan Blue Dye Exclusion

Trypan blue dye exclusion should be used for cell preparations where a decrease in cell viability may be expected (e.g. cryopreserved cells).

1. Mix cell suspension thoroughly and transfer a 100 µL aliquot to a separate tube.
2. The trypan blue dye exclusion method should be performed by diluting the cell sample in a 1:1 ratio with Trypan Blue (Catalog #07050). If additional dilution is required, the cell sample should be diluted in cell culture medium prior to dilution in Trypan Blue.

   Example: To prepare a 1/40 dilution, add 50 µL of cell suspension to 950 µL IMDM + 2% FBS (1/20 dilution) and mix well. Then add 100 µL of the diluted cell suspension to 100 µL of Trypan Blue solution (1/2 dilution for a final 1/40 dilution).
3. Mix the diluted cell sample well.
4. Allow the resulting solution to sit for 5 to 15 minutes.

   If cells are incubated for more than 15 minutes in Trypan Blue, toxic effects may occur and the viable cell count will be inaccurate (all cells will appear blue).
5. Prepare the hemacytometer by first cleaning the chambers and coverslip with alcohol and then wiping dry using lint-free tissue.
6. Carefully position the coverslip over both chambers.
7. Draw up an aliquot of the diluted sample using a micropipettor or a capillary tube.
8. Fill both chambers of the hemacytometer using a micropipettor or a capillary tube. Do not over- or under-fill the chambers.
9. Count cells in 4 large squares (1 x 1 x 0.1 mm) or ≥100 cells and keep a separate tally of the dead cells and the live cells. The dead cells are stained blue as they have taken up the trypan blue due to a decrease in cell membrane integrity. The live cells are clear and refractile as they have not taken up the trypan blue.
10. The viable cell count is calculated as follows:
    a) AVERAGE TOTAL VIABLE CELLS PER SQUARE x DILUTION FACTOR x 10^4 = VIABLE CELL COUNT PER mL
11. The percent viability is calculated as follows:
    a) # OF LIVE CELLS ÷ (# OF LIVE CELLS + # OF DEAD CELLS) x 100% = PERCENT VIABILITY

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5.4 Setting Up the CFC Assay

Cells are diluted to the appropriate cell concentration for the cell sample in Iscove's Modified Dulbecco's Medium (IMDM) + 2% Fetal Bovine Serum (FBS) (Catalog #07700). Refer to Table 1 for appropriate cell concentrations. The cell sample can be added directly to pre-aliquoted tubes of MethoCult® Express medium.

For the 7-day CFC assay with cord blood, sufficient cells should be plated to yield approximately 25 - 75 colonies per 1.1 mL culture in 35 mm dishes. The presence of too many colonies (overplating) causes inhibition of progenitor proliferation due to depletion of essential nutrients, pH changes due to accumulation of cellular metabolic products and counting errors because of difficulty in identifying individual colonies. Too few colonies (underplating) may yield statistically inaccurate data.

Two different plating concentrations are recommended for each sample. This will help ensure that at least one of the plating concentrations will yield the appropriate number of colonies in a 35 mm dish.

Six-well culture plates can be used as an alternative to 35 mm dishes. The surface area in each well of a six-well plate is different from the surface area in a 35 mm dish. The volume of MethoCult® Express plus cells plated must be adjusted accordingly when using a six-well culture plate.

Plates with smaller wells, e.g. 24-well or 96-well plates, are not recommended as the surface area is too small for sufficient colonies for statistically accurate data.

1. Thaw the required number of pre-aliquoted tubes of MethoCult® Express medium overnight under refrigeration (2 - 8°C) or at room temperature. Refer to Section 4.0.
2. Prepare culture dishes by placing two 35 mm culture dishes (Catalog #27100/27150) with lids inside a 100 mm petri dish (Catalog #27125/27127) with a lid. Add a third 35 mm culture dish without a lid as a water dish. This set of dishes is sufficient for one duplicate assay. To prepare culture dishes for triplicate assays, place three 35 mm dishes with lids in larger cultureware and add a fourth 35 mm culture dish without a lid as a water dish.

   Culture dishes for CFC assays are pre-tested for minimal cell adherence. Adherence of cells during culture can cause inhibition of colony growth and obscure visualization of colonies.
3. Label the 35 mm culture dishes at the edge with experiment and assay number using a permanent fine-tip marker.
4. Dilute the cells with IMDM + 2% FBS to 10X the final concentration(s) required for plating. Refer to Table 1 for recommended plating concentrations.

   Example: To achieve a final plating concentration of 1 x 10^4 cells per dish, a cell suspension of 1 x 10^5 cells per mL is prepared.

   When it is difficult to anticipate the plating cell concentration that will yield the appropriate number of colonies, the use of two or more cell concentrations that differ by 2 - 3-fold is advised.
   Example: 2 x 10^4 cells per dish and 1 x 10^4 cells per dish.
5. Add 0.3 mL of diluted cells to the 3 mL MethoCult® Express tube for a duplicate assay. For a triplicate assay, add 0.4 mL of diluted cells to a 4 mL MethoCult® Express tube.

   This 1:10 v/v ratio of cells:medium gives the correct viscosity to ensure optimal CFC growth and morphology.
6. Vortex the tube vigorously to mix the contents thoroughly.
7. Let the tube stand for at least 5 minutes to allow the bubbles to rise to the top.

8. To dispense the MethoCult® Express mixture containing cells into culture dishes, attach a sterile 16-gauge blunt-end needle (Catalog #28110/28120) to a sterile 3 cc syringe (Catalog #28230/28240).

   For each tube plated, use a new sterile disposable 3 mL syringe fitted with a new 16-gauge blunt-end needle to prevent contamination between samples.

   Do not use a standard pipette to aliquot methylcellulose as the volume dispensed will not be accurate. Methylcelluse is a viscous solution and cannot be accurately dispensed using a standard pipette due to adherence of the medium to the inside of the pipette. Use blunt-end needles for dispensing to prevent needle-stick injuries.

9. To expel the air from the syringe, place the needle below the surface of the solution and draw up approximately 1 mL. Gently depress the plunger and expel the medium completely. Repeat until no air space is visible in the syringe.

10. Draw up the MethoCult® Express mixture containing cells into the syringe and dispense a volume of 1.1 mL into each 35 mm dish as described in the next steps.

11. While holding the syringe containing the MethoCult® Express and cells in one hand, remove the lid of a 35 mm dish with the opposite hand. Position the syringe over the center of the dish without touching the syringe to the dish. Dispense 1.1 mL and replace the lid.

12. Remove the lid of a second 35 mm dish and dispense 1.1 mL into the dish. Replace the lid.

13. Repeat dispensing procedure for the next 35 mm dish(es).

    Do not expel the medium to the "0" mark on the syringe when dispensing. For example, measure from 1.5 mL to 0.4 mL rather than 1.1 mL to 0 mL.

14. Distribute the medium evenly across the surface of each 35 mm dish by gently tilting and rotating the dish to allow the medium to attach to the wall of the dish on all sides.

    If any medium contacts the lid of the 35 mm dish while distributing the medium across the surface of the dish, replace the lid to minimize the risk of contamination.

15. Place the culture dishes into the outer dish (e.g. 100 mm petri dish, 245 mm square dish or other cultureware of an appropriate size with a loose-fitting lid). Add approximately 3 mL of sterile water to the uncovered 35 mm dish(es).

    Use of a 100 mm petri dish with lid (or other cultureware with a loose-fitting lid) and water dish(es) helps maintain humidity and minimize contamination during culture and handling.

16. Incubate at 37°C, in 5% CO_2, with ≥95% humidity for 7 days, or 14 - 16 days.

    Proper culture conditions are critical for optimal CFC growth. Use of water-jacketed incubators with water pan in chamber and routine monitoring of temperature and CO_2 levels is recommended. A suitable additive (i.e. copper sulfate crystals) can be added to the water pan to inhibit microbial growth.

    The incubator temperature should be confirmed using a thermometer placed in the incubator chamber and CO_2 levels should be routinely monitored using a Fyrite® gas analyzer.

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Table 1. Recommended Plating Concentrations

Cell Source	Cells per 35 mm Dish
Cord blood, red blood cell-depleted	2×10^4 - 5×10^4
Whole cord blood, cryopreserved	3×10^4 - 5×10^4
Cord blood mononuclear cells*	1×10^4 - 2×10^4

The progenitor content and quality of individual cord blood preparations can be highly variable. Plate cells at 2 different densities to ensure sufficient cells are plated to yield 25 - 75 colonies per 35 mm dish (1.1 mL culture).

* Mononuclear cells (MNCs) are isolated by density-based cell separation (e.g. sedimentation over Ficoll-Paque™ PLUS). Ficoll-Paque™ PLUS is a trademark of GE Healthcare Ltd.

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6.0 Enumeration of CFC Assays Using MethoCult Express®

Use a high-quality inverted microscope equipped with 2X, 4X and 10X planar objectives and stage holder for a 60 mm gridded dish (Catalog #27500). A blue filter will enhance the red color of hemoglobinized erythroblasts in BFU-E and CFU-GEMM when counting colonies at 14 - 16 days, making distinction easier.

6.1 Scoring After 7 Days

Scan the dish on low power (2X objective, 20 - 25X magnification) to evaluate relative distribution of colonies. Score colonies with a 4X objective and count all colonies containing more than 20 cells. As most colonies are immature, scoring individual colony types is not recommended after 7 days of culture.

6.2 Scoring After 14 - 16 Days

Mature BFU-E, CFU-GM and CFU-GEMM can be distinguished and counted using standard criteria. Refer to the Tutorial for Cord Blood Derived Hematopoietic Progenitors on our website at www.stemcell.com/flash/cbtutorial.htm for detailed descriptions and examples of colonies.

Colonies presented in the tutorial were cultured in MethoCult® GF H4034. Colonies in MethoCult® Express after 14 - 16 days will appear larger but the morphologies of the colony types will be similar.

Prior to counting, scan the dish on low power (2X objective, 20 - 25X magnification) to evaluate the relative distribution of colonies. Score BFU-E, CFU-GM and CFU-GEMM on low or medium power and use high power to confirm colony type as required.

Cord blood-derived colonies in MethoCult® Express can be very large after 14 days of culture and it may be difficult to accurately distinguish individual colonies in dishes plated at high cell concentrations. Plating at different cell concentrations is recommended to assess progenitor frequencies (see Table 1).

6.3 Scoring Procedure

1. Prepare a 60 mm gridded scoring dish (Catalog #27500) by drawing two perpendicular lines across the center of the dish using a permanent fine felt marker on the bottom of the dish. Count eight squares from the center on one radius and draw a short (approximately 2 mm) line the crossing the radius. Repeat for each radius.

   These lines will help to center the 35 mm culture dish to be scored and evaluated. This scoring dish can be used again to score other culture dishes.

2. Take the cultures to be scored from the 37°C incubator. Take only the number of dishes that can be scored within one hour.

3. Center a culture dish in the gridded scoring dish prepared in Step 1. Place the gridded dish on the inverted microscope stage and adjust the focus under low power (2X objective) until the colonies are in focus.

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4. Scan the entire dish on low power (2X objective, 20 - 25X magnification) for placement of colonies relative to one another. Make note of the overall appearance of the culture to help with scoring and evaluation.

   Considerations: Are the colonies close to one another or far apart? Are the colonies evenly distributed? What is the approximate number of colonies on the dish (i.e. is the dish overplated, showing >75 colonies or underplated, showing <25 colonies)? Observe the background for presence or absence of other cells or debris, general morphology and health of the colonies.

5. Count all the colonies in each dish. It is necessary to continually focus up and down to identify all colonies present in the 3-dimensional culture and to distinguish individual colonies that are close together but present on different planes.

   Move the dish up and down rather than side to side when counting. This will minimize the sensation of motion sickness common to individuals new to scoring.

   a) Score total colonies if counting after 7 days. Score at medium power (4X objective, 40 - 50X magnification).

   b) Score BFU-E, CFU-GM and CFU-GEMM if counting after 14 - 16 days. After scanning the dish on low power, count all of the colonies at low power. Low power is recommended as cord blood-derived colonies in MethoCult® Express can be very large after 14 days of culture. Switch to a higher power if necessary to help with colony identification.

6. Cultures scored after both 7 and 14 - 16 days can be placed in a 33°C incubator, with 5% CO_2 in air, and ≥95% humidity for further evaluation if necessary, for up to 7 days.

7.0 Expected Results

7.1 General Colony Descriptions

7.1.1 Seven Days

Colonies are visible after 7 days of culture in MethoCult® Express. They will be small to medium sized in comparison to colonies derived from cord blood progenitors in standard MethoCult® medium (e.g. MethoCult® GF H4034) for the 14 - 16-day assay, or colonies in MethoCult® Express after 14 - 16 days.

Different colony types cannot be distinguished after 7 days of culture in MethoCult® Express. Colonies containing more than 20 cells should be counted. If cultures are maintained for 14 - 16 days in MethoCult® Express, different colony types can be distinguished.

Colonies in MethoCult® Express after 7 days of culture can either be compact, i.e. consisting of a single cluster of cells, or composed of several clusters of cells. As some immature hematopoietic progenitors are very mobile, even in semi-solid medium, the individual clusters of one colony (i.e. derived from a single progenitor) can be as far apart from one another as the distance equivalent to several cluster diameters. This is more apparent in the 7 day assay than the 14 - 16 day assay with standard MethoCult® medium or MethoCult® Express. This is due to a shorter time for cells within the colony to proliferate and fill the spaces.

To decide whether different clusters belong to the same or different colonies, it is important to examine the context, size and morphology of the clusters. If clusters in close proximity to each other are of similar size and morphology, they are likely to belong to the same colony. If adjacent clusters are different in size and/or show different morphologies and the colony density in the dish is high (>50 colonies per 35 mm dish), the clusters likely belong to different colonies (i.e. are derived from different progenitors).

7.1.2 Fourteen Days

After culture in MethoCult® Express for 14 - 16 days, colonies derived from the following progenitors can be distinguished. Note that colony-forming unit-erythroid (CFU-E) are generally not present in cord blood samples. They are mature progenitor cells and cord blood samples tend to contain immature progenitors.

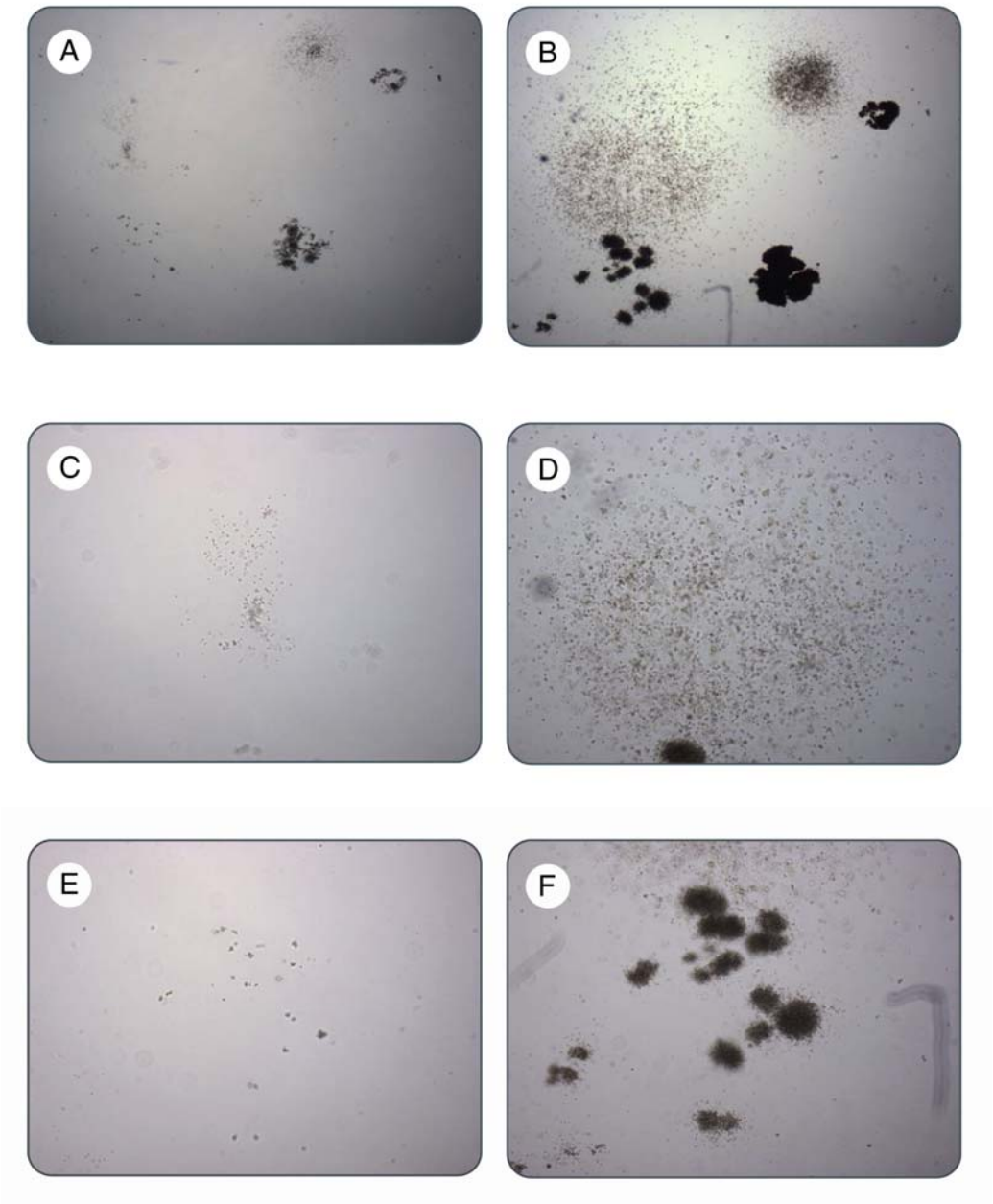
BFU-E: Burst-forming unit-erythroid. Produces a colony containing >200 erythroblasts in a single cluster or multiple clusters and can be sub-classified based on the number of cells/cell clusters per colony, if desired. BFU-E are more immature progenitors than CFU-E, which are not usually detected in cord blood. BFU-E require erythropoietin (EPO) and cytokines with burst-promoting activity such as Interleukin-3 (IL-3) and Stem Cell Factor (SCF) for optimal colony growth.

CFU-GM: Colony-forming unit-granulocyte, macrophage. Produces a colony containing at least 20 granulocyte cells (CFU-G), macrophages (CFU-M) or cells of both lineages (CFU-GM). CFU-GM colonies arising from primitive progenitors may contain thousands of cells in single or multiple clusters.

CFU-GEMM: Colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte. A multi-potential progenitor that produces a colony containing erythroblasts and cells of at least two other recognizable lineages. Due to their primitive nature, CFU-GEMM tend to produce large colonies of >500 cells.

7.2 Photographs of Colonies in MethoCult® Express

Field of view #1. Day 7 (L) and Day 14 (R).



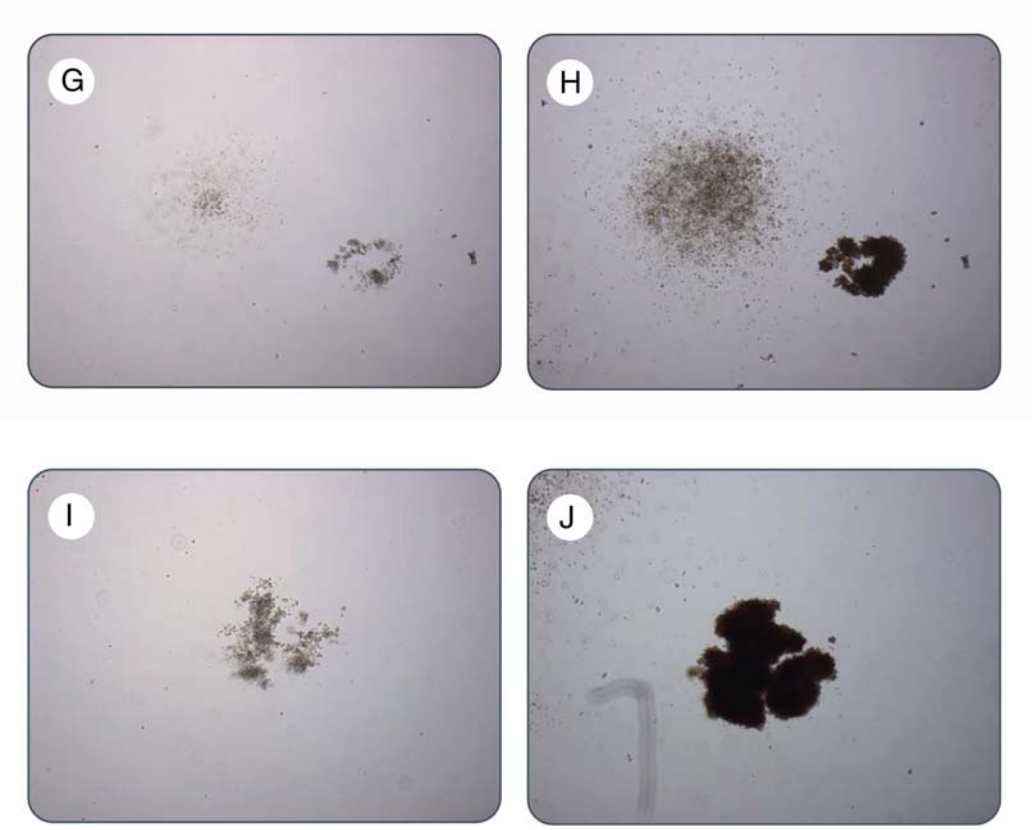


Figure 2 (Field of view #1).

Colonies derived from human cord blood hematopoietic progenitors in MethoCult® Express at Day 7 (left) and Day 14 (right). A and B are shown at a 25X magnification. C through J are shown at a 50X magnification. Each colony shown at a 50X magnification corresponds to a colony shown at 25X magnification. Note that the lineage of colonies cannot be distinguished at Day 7.

Field of view #2. Day 7 (L) and Day 14 (R).

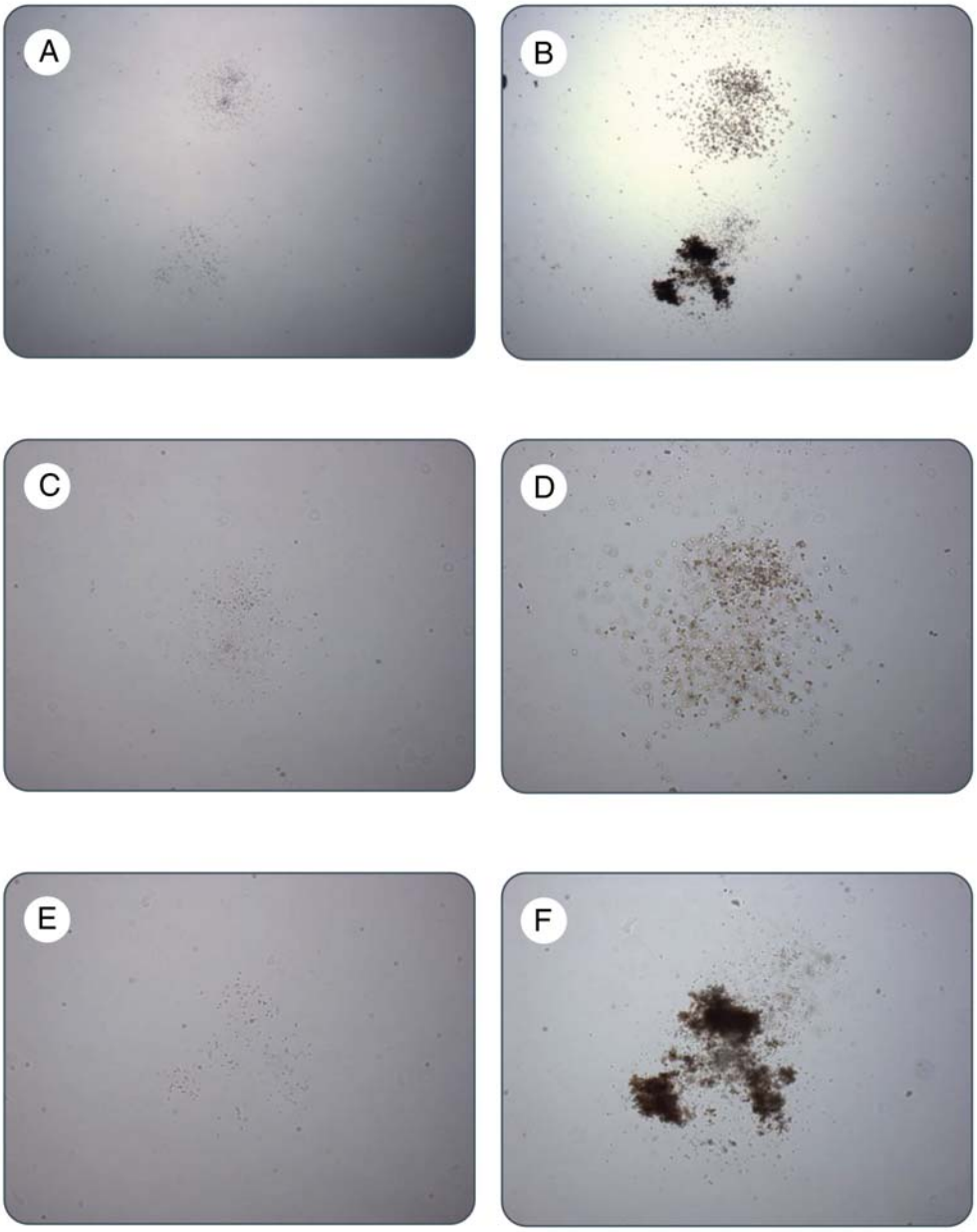


Figure 3 (Field of view #2).

Colonies derived from human cord blood hematopoietic progenitors in MethoCult® Express at Day 7 (left) and Day 14 (right). A and B are shown at a 25X magnification. C through F are shown at a 50X magnification. Each colony shown at a 50X magnification corresponds to a colony shown at 25X magnification. Note that the lineage of colonies cannot be distinguished at Day 7.

Field of view #3. Day 7 (L) and Day 14 (R).

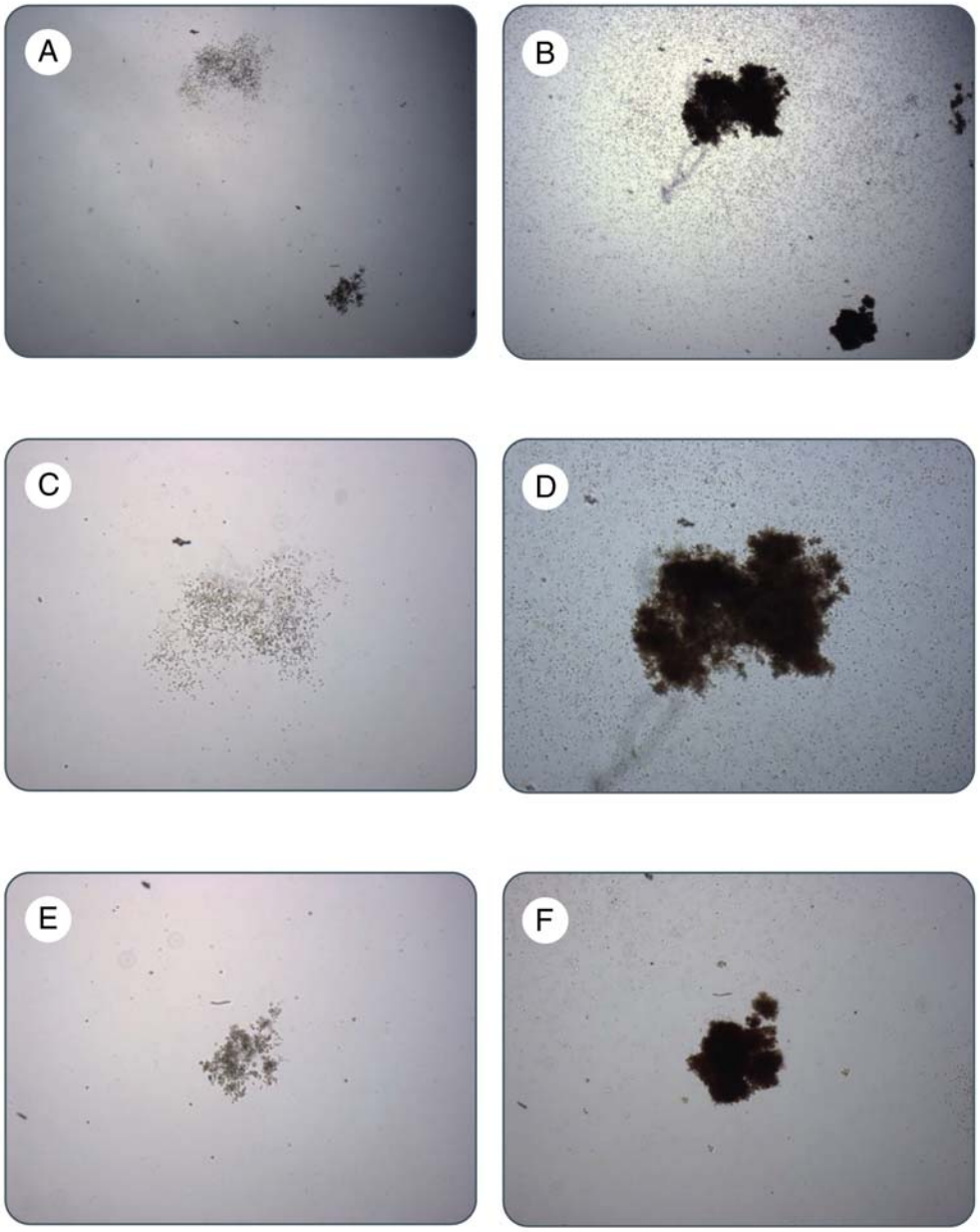


Figure 4 (Field of view #3).

Colonies derived from human cord blood hematopoietic progenitors in MethoCult® Express at Day 7 (left) and Day 14 (right). A and B are shown at a 25X magnification. C through F are shown at a 50X magnification. Each colony shown at a 50X magnification corresponds to a colony shown at 25X magnification. Note that the lineage of colonies cannot be distinguished at Day 7.

7.3 MethoCult® Express Results

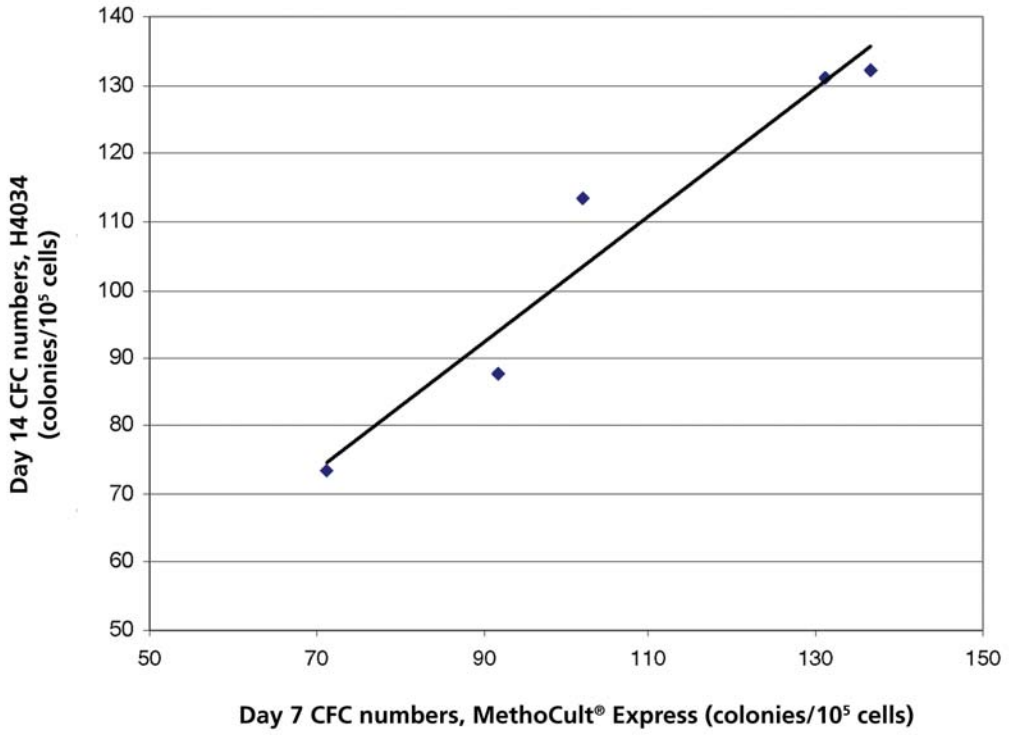


Figure 5. Correlation between colony numbers after Day 7 with MethoCult® Express and Day 14 with MethoCult® GF H4034 (Catalog #04034/04044) using cryopreserved whole cord blood.

Cryopreserved whole cord blood samples (n = 5) were thawed and set up in MethoCult® Express and MethoCult® GF H4034 without further processing. Colonies in MethoCult® Express were counted after 7 days and colonies in MethoCult® GF H4034 were counted after 14 days. Correlation coefficient: 0.973; p = 0.005 (Pearson Product Moment Correlation).

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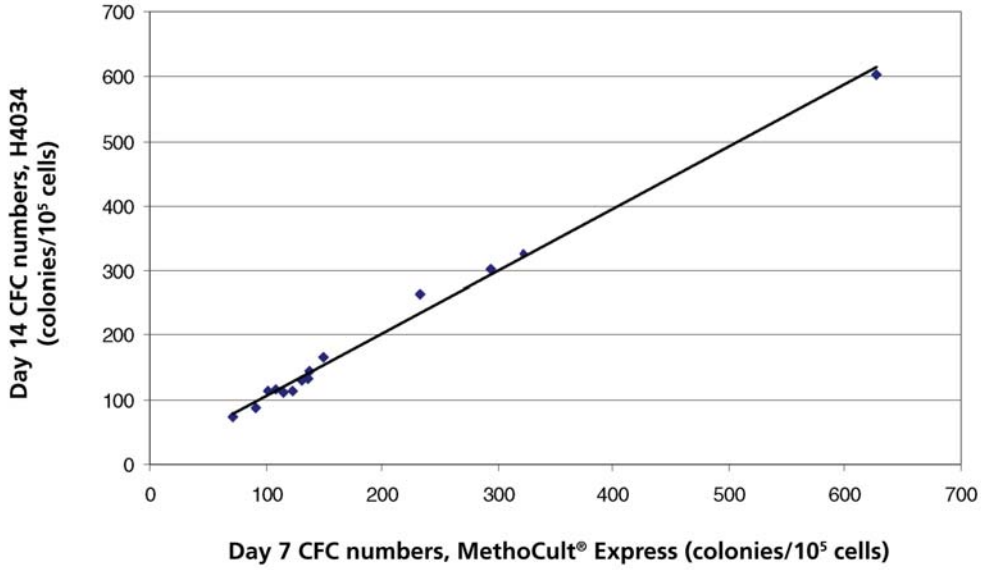

Figure 6. Correlation between colony numbers after Day 7 with MethoCult® Express and Day 14 with MethoCult® GF H4034 (Catalog #04034/04044).

Samples included cryopreserved whole cord blood (5), cryopreserved cord blood with red blood cells depleted by HetaSep™ (3), fresh cord blood with red blood cells depleted by HetaSep™ (3) and cryopreserved cord blood mononuclear cells isolated by density sedimentation over Ficoll-Paque™ PLUS (3). Samples (n = 14) were set up in MethoCult® Express and MethoCult® GF H4034 and colonies were counted after 7 and 14 days, respectively. Correlation coefficient: 0.997; $p < 0.0001$ (Pearson Product Moment Correlation).

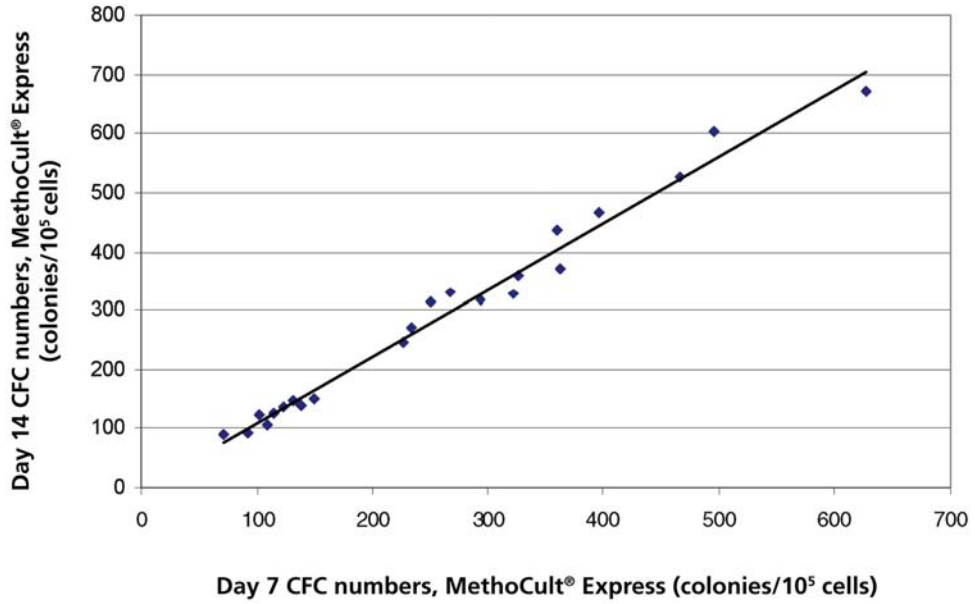

Figure 7. Correlation between colony numbers after Day 7 and Day 14 with MethoCult® Express.

Samples included cryopreserved whole cord blood samples (5), cryopreserved cord blood with red blood cells depleted by HetaSep™ (3), fresh cord blood with red blood cells depleted by HetaSep™ (3), cryopreserved cord blood mononuclear cells isolated by density sedimentation over Ficoll-Paque™ PLUS (11) and fresh cord blood mononuclear cells isolated by density sedimentation over Ficoll-Paque™ PLUS (2). Samples (n = 24) were set up in MethoCult® Express and colonies were counted after 7 and 14 days. Correlation coefficient: 0.991; $p < 0.0001$ (Pearson Product Moment Correlation).

8.0 Frequently Asked Questions

1. My media arrived partially thawed. What should I do?

The bottle can either be refrozen by placing at -20°C (-25°C to -15°C) immediately, or thawed completely in the refrigerator (2 - 8°C) or at room temperature. Once thawed, shake for 1 - 2 minutes to mix completely, and let it stand for 2 - 5 minutes to allow bubbles to rise to the top. The bottle can now be aliquoted into tubes. The tubes can either be used immediately or frozen for future use. See Section 4.0 for detailed instructions.

2. Why should MethoCult® methylcellulose-based media be thawed at room temperature or in the refrigerator instead of at 37°C?

The methylcellulose will not be homogeneous in frozen MethoCult® products and small "lumps" may be present if the product is thawed rapidly at 37°C. If the product is inadvertently thawed at 37°C, place the bottle on ice for 1 - 2 hours or in the refrigerator for 2 - 3 hours (the "lumps" will not dissolve at 37°C). Shake the bottle vigorously for 1 - 2 minutes before dispensing.

3. My media appears quite yellow or violet in color after thawing. Can I still use it?

Yes. This indicates that the pH of the media has been altered during transport or storage but the media performance is unaffected as long it has been stored at the recommended temperature of -20°C (-25°C to -15°C) until the expiry date indicated on label, or at 2 - 8°C for up to one month. Thaw the bottle and follow recommended protocol for CFC assay set-up. The pH will adjust once the cultures are incubated under 5% CO_2 conditions.

4. How many tubes can I expect to dispense from a 100 mL bottle of MethoCult® medium?

Due to loss of medium within the syringe and needle, approximately 30 tubes of 3 mL can be obtained.

5. The -20°C freezer is broken. Can I store MethoCult® Express at -80°C?

Yes. The recommended storage temperature is -20°C (-25°C to -15°C) but the performance of the MethoCult® Express will not be affected by storage at -80°C.

6. Is it possible to distinguish different colony types after 7 days of culture?

No. Although some erythroid colonies can be identified after 7 days of culture on the basis of morphology, attempts to count erythroid and myeloid colonies separately at day 7 tend to underestimate the number of erythroid colonies, and overestimate the number of myeloid colonies detectable after 14 days. Most colonies remain undifferentiated after 7 days of culture and immature erythroid colonies consist of large non-hemoglobinized erythroblasts that are not easily distinguishable from non-erythroid cells.

7. I only want to evaluate CFU-GM colonies. Can that be done in a 7-day assay in MethoCult® Express, or do I need a different medium?

It is not possible to distinguish colony types after 7 days of culture in Methocult® Express (see Question #6). In principle, a custom medium without EPO would selectively promote the CFU-GM present to form colonies. However, some BFU-E can develop into erythroid colonies after 7 days in the absence of EPO so that a proportion of total colonies detected after 7 days are derived from erythroid progenitors, even in the absence of EPO. This is due to the fact that the survival and first rounds of proliferation of erythroid progenitors, in particular immature BFU-E, is not dependent on EPO. The presence of EPO is only essential for the survival, proliferation and differentiation in later stages of development of hemoglobinized BFU-E colonies.[8]

8. Can I perform a 7-day assay using standard MethoCult® medium (e.g. MethoCult® GF H4034) instead of using MethoCult® Express?

No. MethoCult® Express has been formulated for accelerated progenitor proliferation and colony formation. Colonies grown in MethoCult® Express are larger than in standard MethoCult® media so that they can be counted as early as after 7 days of culture.

9. Is the total CFC number a good predictor of neutrophil engraftment ability of the graft or is it better to measure CFU-GM content only?

It has been shown that both parameters are useful in predicting the success of CB as well as mobilized peripheral blood stem cell (PBSC) grafts with respect to neutrophil and platelet engraftment and/or survival.[2,4-6] Measuring total CFC numbers rather than only CFU-GM numbers has several advantages: it does not require scoring CFU-GM colonies separately or culturing progenitors under conditions that favor colony formation by CFU-GM only. In addition, CFU-GM represent roughly one half of the progenitors present in a sample and measuring only CFU-GM may give less significant and reliable results than measuring total CFC.

10. Do CFC-assays directly measure the stem cell content of a graft?

There are no short-term culture assays that can exclusively measure stem cells. The majority of cells that are detected in CFC assays or in any other short-term assay consist of committed progenitors, most of which are not involved in hematopoietic engraftment themselves. Neutrophil and platelet engraftment and sustained lympho-hematopoietic recovery after transplantation are mediated by stem cells and primitive multipotent progenitors that are much less frequent than committed progenitors and are generally not detectable in CFC assays or other short-term assays. Measuring the frequency and growth of committed progenitors is important as their numbers in CB and MPB preparations have been shown to correlate better with the content and engraftment ability of repopulating stem cells than other parameters such as total nucleated count (TNC) and $CD34^+$ cell numbers.[1-6] For example, if a candidate CB unit has acceptable TNC numbers but lower CFC numbers than a second CB unit, it is likely that the stem cell content of the first unit is also lower than that of the second unit. In addition, if CFC assay results show that 90% of CFCs in a CB unit have been lost as a result of cell processing, e.g. freezing and thawing, it is likely that most stem cells have also been lost.

Please refer to Section 9.0 Frequently Asked Questions and Helpful Hints in the Technical Manual for Human Colony-Forming Cell Assays Using MethoCult® (Manual Catalog #28404) for additional information.

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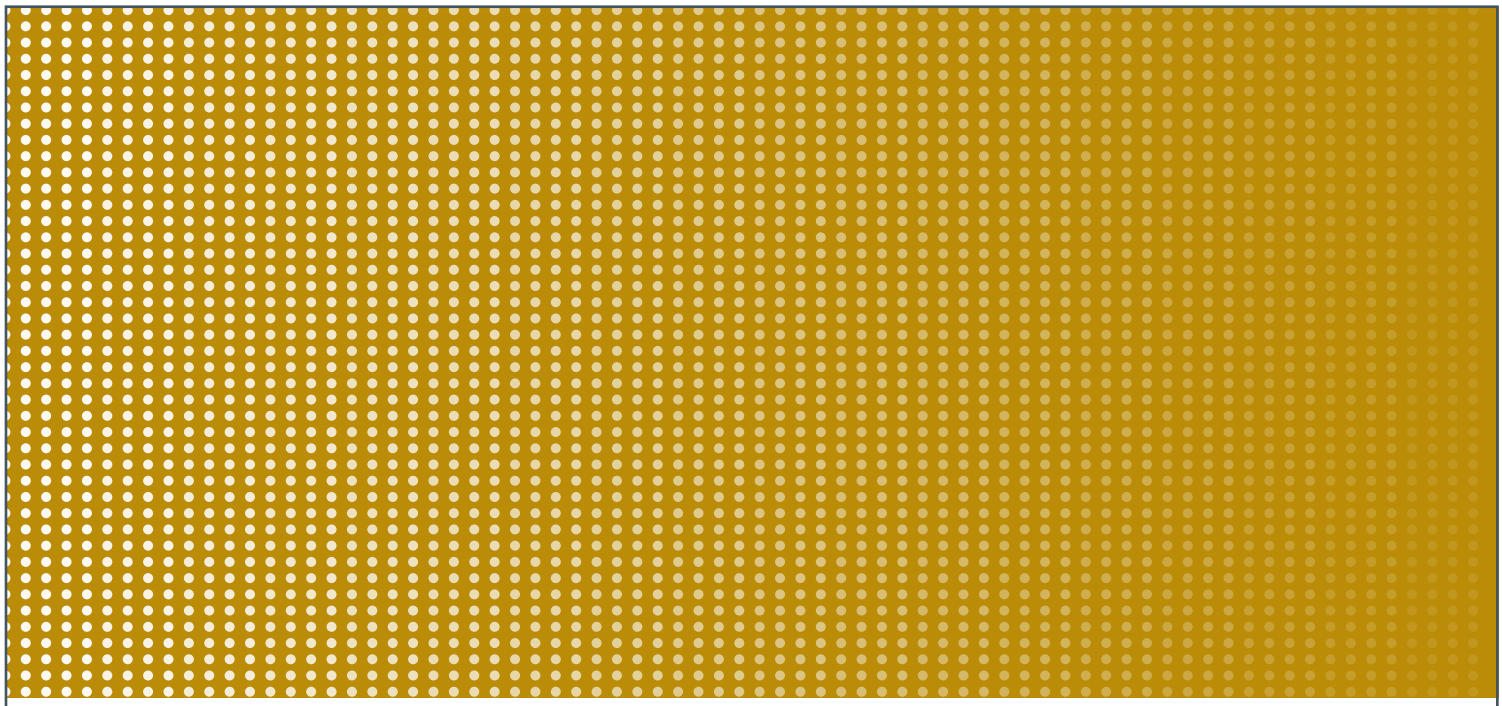

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