TECHNICAL MANUAL

Human Colony-Forming Unit (CFU) Assays
Using MethoCult™

STEMCELL™ TECHNOLOGIES

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1.0 Introduction

Mature blood cells have a limited lifespan and are continuously replaced by the proliferation and differentiation of a very small population of pluripotent hematopoietic stem cells (HSCs). The cells are found primarily in the bone marrow (BM) of healthy adults, in umbilical cord blood (CB) and in adult blood after mobilization from BM with cytokines, such as G-CSF, or other agents. HSCs have the ability to differentiate into all mature hematopoietic cells and to replenish themselves by self-renewal.[1]

During differentiation to mature blood cells the progeny of HSCs go through intermediate stages, including multi-potential progenitor cells and lineage-committed progenitor cells, prior to reaching maturity. In vitro assays are used to gain insight into the frequencies and growth properties of hematopoietic progenitor cells at various developmental stages. Although HSCs have the capacity to proliferate and differentiate in culture, most cells detected in hematopoietic culture assays consist of hematopoietic progenitor cells, which have limited self-renewal capacity and short-term hematopoietic potential. Progenitor cells detected in culture assays can either be multipotential (capable of generating progeny of multiple blood cell types) or restricted to one or two lineages (erythrocytes, granulocytes, monocytes/macrophages, or platelets).[2-4]

When cultured in a suitable semi-solid matrix (such as methylcellulose supplemented with appropriate cytokines and supplements), individual progenitor cells called colony-forming units (CFUs) proliferate and differentiate to form discrete cell clusters or colonies containing recognizable progeny. Under optimal plating and culture conditions, each colony is derived from a single progenitor cell. Thus, the number and types of colonies counted in a CFU assay provide information about the frequency and types of progenitor cells present in the original cell population and their ability to proliferate and differentiate.

Methylcellulose is the standard gelling agent used in CFU assays. It is chemically inert and its properties do not change with pH. Most importantly, cells are not exposed to high temperatures, as they are when using agar-based media.[2-4]

This Technical Manual describes procedures for the detection and counting of human CFUs in MethoCult™ methylcellulose-based media. Depending on the formulation, MethoCult™ media support optimal growth of multiple types of progenitor cells. These include:

- Erythroid progenitor cells (CFU-erythroid [CFU-E] and burst-forming unit-erythroid [BFU-E])
- Granulocyte and/or macrophage progenitor cells (CFU-granulocyte, macrophage [CFU-GM]; CFU-granulocyte [CFU-G]; and CFU-macrophage [CFU-M])
- Multi-potential progenitor cells (CFU-granulocyte, erythrocyte, macrophage, megakaryocyte [CFU-GEMM])

MethoCult™ Express (Catalog #04437) has been formulated for accelerated progenitor proliferation and colony formation and is intended for counting total CFUs in CB after much shorter culture periods than the 14 - 16 days of CFU assays in other MethoCult™ media. Colonies grown in MethoCult™ Express are larger than in standard MethoCult™ media and can be counted as early as after 7 days of culture, but without distinction of colony types. If desired, cultures in MethoCult™ Express can be maintained for the standard 14 - 16 days, after which colonies derived from BFU-E, CFU-GM, and CFU-GEMM can be counted.

Procedures for the evaluation of megakaryocyte progenitor cells (CFU-Mk) in serum-free collagen-based media are described in the MegaCult™-C Technical Manual: Assays for Quantitation of Human and Mouse Megakaryocytic Progenitor Cells (Document #28413), available at www.stemcell.com or contact us to request a copy.

2.0 Glossary of Terms

ABBREVIATION	DESCRIPTION
ACD	Acid citrate dextrose
Agar-LCM	Agar leukocyte conditioned medium
BFU-E	Burst-forming unit-erythroid
BIT	Bovine serum albumin, insulin, and transferrin
BM	Bone marrow
BSA	Bovine serum albumin
CB	Cord blood
CFU	Colony-forming unit
CFU-E	Colony-forming unit-erythroid
CFU-G	Colony-forming unit-granulocyte
CFU-GEMM	Colony-forming unit-granulocyte, erythrocyte, macrophage, megakaryocyte
CFU-GM	Colony-forming unit-granulocyte, macrophage
CFU-M	Colony-forming unit-macrophage
CFU-Mk	Colony-forming unit-megakaryocyte
D-PBS	Dulbecco's phosphate-buffered saline
EDTA	Ethylenediaminetetraacetic acid
EPO	Erythropoietin
FBS	Fetal bovine serum
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
HSC	Hematopoietic stem cell
IL-3	Interleukin 3
IL-6	Interleukin 6
IMDM	Iscove's Modified Dulbecco's Medium
MNC	Mononuclear cell
MPB	Mobilized peripheral blood
PB	Peripheral blood
RBC	Red blood cell
rh	Recombinant human
SCF	Stem cell factor
TPO	Thrombopoietin

3.0 Products for Human Colony-Forming Unit (CFU) Assays

STEMCELL Technologies rigorously screens and selects components used in the manufacture of MethoCult™ products. It is known that different batches of methylcellulose, fetal bovine serum (FBS), and bovine serum albumin (BSA) vary widely in their ability to promote CFU growth. If using medium components other than those pre-screened and available from STEMCELL Technologies, it is important to test components individually and in combination for their ability to support the optimal growth and differentiation of hematopoietic cells.

Refer to Table 1 for a list of MethoCult™ products for culture of human hematopoietic cells available from STEMCELL Technologies. For a complete list of available products, visit www.stemcell.com. Custom formulations are also available; for further information, contact us at techsupport@stemcell.com.

Table 1. MethoCult™ Products for Culture of Human Hematopoietic Cells

PRODUCT	CATALOG #	CONTAINS	APPLICATIONS
COMPLETE METHOCULT™ MEDIA			
H4034 Optimum	04034 04044	rh SCF, rh GM-CSF, rh IL-3, rh G-CSF, rh EPO	Detection of CFU-E, BFU-E, CFU-GM, CFU-GEMM in BM, CB, PB, MPB
H4035 Optimum, without EPO	04035 04045	rh SCF, rh GM-CSF, rh IL-3, rh G-CSF	Detection of CFU-GM in BM, CB, PB, MPB
H4435 Enriched	04435 04445	rh SCF, rh GM-CSF, rh IL-3, rh IL-6, rh G-CSF, rh EPO	• Detection of CFU-E, BFU-E, CFU-GM, CFU-GEMM in BM, CB, PB, MPB • Recommended for $CD34^+$ enriched cells and cells isolated by other purification methods
H4535 Enriched, without EPO	04535 04545	rh SCF, rh GM-CSF, rh IL-3, rh IL-6, rh G-CSF	• Detection of CFU-GM in BM, CB, PB, MPB • Recommended for CD34+ enriched cells and cells isolated by other purification methods
H4434 Classic	04434 04444	rh SCF, rh GM-CSF, rh IL-3, rh EPO	Detection of CFU-E, BFU-E, CFU-GM, CFU-GEMM in BM, CB, PB, MPB
H4534 Classic, without EPO	04534 04544	rh SCF, rh GM-CSF, rh IL-3	Detection of CFU-GM in BM, CB, MPB
SF H4436	04436 04446*	rh SCF, rh GM-CSF, rh IL-3, rh IL-6, rh G-CSF, rh EPO; serum-free, contains serum substitute	Detection of CFU-E, BFU-E, CFU-GM, CFU-GEMM in BM, CB, PB, MPB where a medium of defined composition is required
SF H4536	04536	rh SCF, rh GM-CSF, rh-IL-3, rh IL-6, rh G-CSF; serum-free, contains serum substitute	Detection of CFU-GM in BM, PB, CB, MPB where a medium of defined composition is required
SF H4636	04636	Cytokines, including rh EPO, supplements; serum-free, contains serum substitute	Detection of CFU-E, BFU-E, CFU-GM, CFU-GEMM in human induced pluripotent stem (iPS) cell-derived hematopoietic progenitor cells. Also suitable for detection of these colony types in human BM, PB, CB, and MPB where a medium of defined composition is required.

PRODUCT	CATALOG #	CONTAINS	APPLICATIONS
H4431	04431	Agar-LCM, rh EPO	• Detection of CFU-E, BFU-E, CFU-GM, CFU-GEMM in BM and PB • Suitable as a control medium for the detection of "EPO-independent" erythroid progenitor cells using MethoCult™ H4531
H4531	04531 04541*	Agar-LCM	• Detection of CFU-GM in BM and PB • Suitable for detection of "EPO-independent" erythroid progenitor cells
Express	04437 04447	Cytokines, including rh EPO	• Faster detection of total CFUs (count after 7 days), without distinction of colony types • Suitable for CB
INCOMPLETE METHOCULT™ MEDIA			
H4330	04330	rh EPO, **no** additional cytokines	Allows researchers to add cytokines of their choice for applications including: • Drug toxicity testing in vitro • Detection of specific hematopoietic progenitor cells • Investigating action of novel factors • Hematopoietic colony assays in other species • Detection of genetically modified hematopoietic progenitor cells • Cloning and selection of non-adherent cell lines
SF H4236	04236	**no** rh EPO, **no** additional cytokines; serum-free, contains serum substitute	
H4230	04230	**no** rh EPO, **no** additional cytokines	
H4100	04100	**no** rh EPO, **no** additional cytokines, **no** serum or serum substitutes	

*Available as special orders only.

Table 2. MethoCult™ Products Registered as In Vitro Diagnostic Devices in Select Regions*

PRODUCT	CATALOG #	CONTAINS	APPLICATIONS
GF H84434 GF H84444	84434 84444	rh SCF, rh GM-CSF, rh IL-3, rh G-CSF, rh EPO	Detection of CFU-E, BFU-E, CFU-GM, CFU-GEMM in BM, CB, PB, MPB
GF H84435 GF H84445	84435 84445	rh SCF, rh GM-CSF, rh IL-3, rh IL-6, rh G-CSF, rh EPO	• Detection of CFU-E, BFU-E, CFU-GM, CFU-GEMM in BM, CB, PB, MPB • Recommended for $CD34^+$ enriched cells and cells isolated by other purification methods
GF H84534 GF H84544	84534 84544	rh SCF, rh GM-CSF, rh IL-3, rh G-CSF	Detection of CFU-GM in BM, CB, PB, MPB
GF H84535 GF H84545	84535 84545	rh SCF, rh GM-CSF, rh IL-3, rh IL-6, rh G-CSF	• Detection of CFU-GM in BM, CB, PB, MPB • Recommended for $CD34^+$ enriched cells and cells isolated by other purification methods

*Caution: These products contain material of animal origin and should be handled as potential carriers and transmitters of disease.

Table 3. Recombinant Cytokines and Conditioned Medium for Culture of Human Hematopoietic Cells

PRODUCT	CATALOG #	UNIT SIZE	DESCRIPTION
Agar Leukocyte Conditioned Medium	02300	25 mL	• Source of growth factors such as cytokines, less defined • Useful for EPO-independence assays
Human Recombinant IL-3	78040	100 µg	Used in combination with other cytokines to promote growth of early myeloid progenitor cells of all lineages
Human Recombinant IL-6	78050	100 µg	Pleiotropic cytokine for growth and differentiation of hematopoietic progenitor cells
Human Recombinant G-CSF	78012	100 µg	For growth of granulocytic progenitor cells; increases sensitivity of CFU-GM detection, in combination with IL-3, GM-CSF, and SCF
TPO, Human, Recombinant	02922	4 x 25 µg	• Used in combination with other cytokines to promote growth of megakaryocytic progenitor cells • Megakaryocytic progenitor cells are counted in MegaCult™-C collagen-based medium
EPO, Human, Recombinant	02625	500 U	• Used in combination with other cytokines for growth of erythroid progenitor cells • Is not required for first round of proliferation but for full development in MethoCult™ of colonies derived from erythroid progenitor cells
Human Recombinant SCF	78062	100 µg	For growth of mast cells and used in combination with other cytokines to promote growth of myeloid and lymphoid progenitor cells
Human Recombinant GM-CSF	78015	100 µg	For growth of granulocytic and monocytic progenitor cells

For a complete list of available cytokines, visit www.stemcell.com.

Table 4. Support Products for Human CFU Assays Using MethoCult™

PRODUCT	CATALOG #	UNIT SIZE	DESCRIPTION
Ammonium Chloride Solution	07800 07850	100 mL 500 mL	For lysis of red blood cells
BIT 9500	09500	100 mL	Serum substitute for human CFU assays
Lymphoprep™	07801 07851	250 mL 500 mL	Density gradient medium for isolating mononuclear cells (MNCs)
SepMate™-50	85450 85460	100 tubes 500 tubes	50 mL centrifuge tube with an insert to facilitate isolation of MNCs by density gradient centrifugation
HetaSep™	07806 07906	20 mL 100 mL	For isolating human nucleated cells from peripheral blood
Dulbecco's Phosphate Buffered Saline (D-PBS) with 2% Fetal Bovine Serum	07905	500 mL	For washing and diluting nucleated cells
10% BSA Solution in Iscove's MDM	09300	100 mL	For supplementing MethoCult™ H4100 for hematopoietic progenitor CFU assays
Iscove's MDM with 2% FBS	07700	100 mL	For washing and diluting hematopoietic cells
Iscove's MDM with 25 mM HEPES	36150	500 mL	For washing and diluting hematopoietic cells in serum-free conditions
MethoCult™ Cell Wash Medium	87700	100 mL	Medium for preparation of hematopoietic cells from bone marrow, peripheral blood, or cord blood before plating in CFU assays. Registered as an In Vitro Diagnostic (IVD) device in select regions.
Trypan Blue	07050	100 mL	For counting viable cells
3% Acetic Acid with Methylene Blue	07060	100 mL	For counting nucleated cells
Atlas of Human Hematopoietic Colonies	28700	Each	Laboratory guide for identification of human hematopoietic colonies
Atlas of Hematopoietic Colonies From Cord Blood	29940	Each	Laboratory guide for identification of human hematopoietic colonies in cord blood
Human Hematopoietic Colonies in Health and Disease	28760	Each	Practical guide to using the hematopoietic CFU assay as a tool for the diagnosis and follow-up of hematopoietic stem cell disorders; shows colony morphologies from normal donors and patients with hematological disorders
STEMvision™	22000	Each	Instrument for automated imaging and colony counting
SmartDish™	27370 27371	5/Pack 50/Pack	Meniscus-free cultureware for more reliable colony counting

4.0 Equipment and Materials Required

4.1 Equipment

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

  All procedures for cell processing and setup of CFU assays should be performed using sterile technique and universal handling precautions.
- Incubator set at 37°C (or 33°C) with 5% CO_2 in air and ≥ 95% humidity

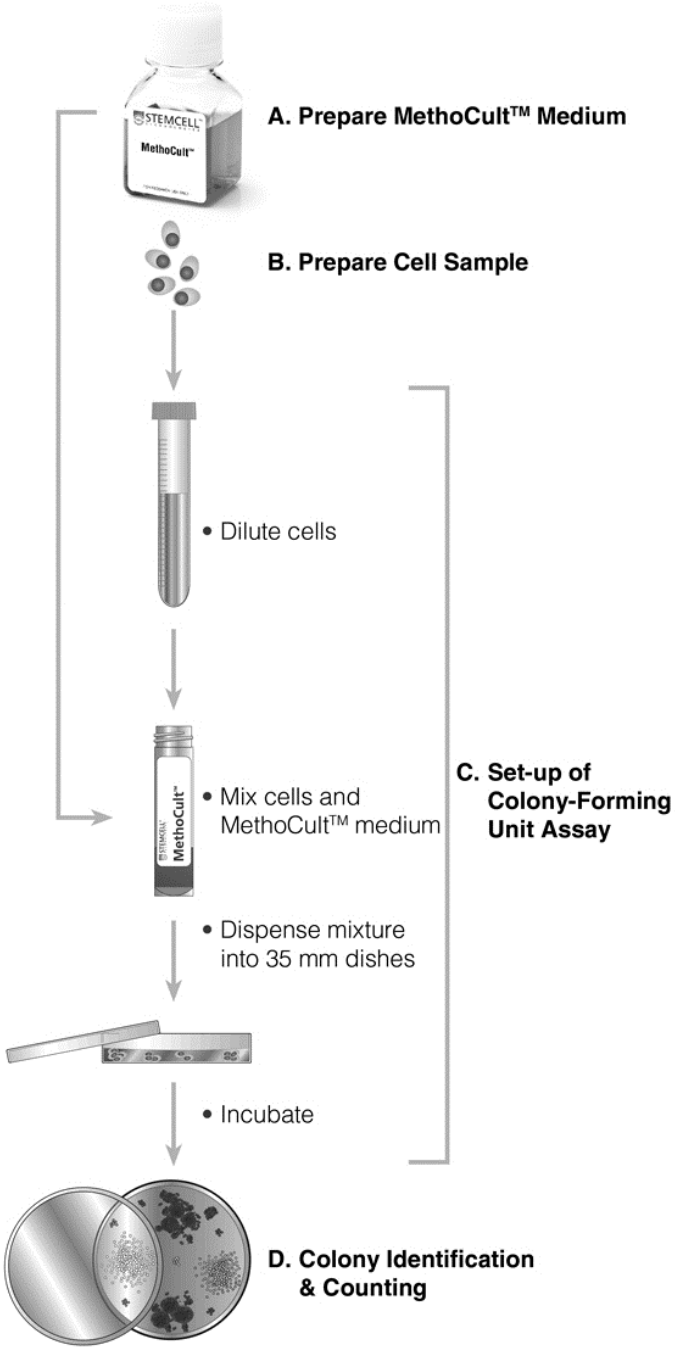
  Use of water-jacketed incubators with a water pan placed in the chamber is recommended. It is important to use medical-grade CO_2, as inhibition of CFU growth due to toxic substances present in the CO_2 gas source has been reported.
- Inverted microscope for colony counting

  Use of a quality inverted microscope equipped with a 10X 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 and micropipettors
- Automated cell counter or Neubauer hemocytometer

4.2 Materials

- Sterile serological pipettes: 2 mL (Catalog #38002), 5 mL (Catalog #38003)
- Sterile polystyrene tubes: 5 mL (12 x 75 mm; Catalog #38007), 14 mL (17 x 95 mm; Catalog #38008)
- Conical tubes: 15 mL (Catalog #38009), 50 mL (Catalog #38010)
- Sterile pipette tips
- Syringes (luer lock): 3 mL (Catalog #28230) or 6 mL
- 16 gauge Blunt-End Needles (Catalog #28110)
- 35 mm Culture Dishes (Catalog #27100) or SmartDish™ 6-well culture plates (Catalog #27370)
- 100 mm culture dishes (e.g., Treated Tissue Culture Dishes, Catalog #27125) or 245 mm square dishes (e.g., Corning® 245 mm Square Dish, Non-Treated, Catalog #38020)
- 60 mm Gridded Scoring Dishes (Catalog #27500) or STEMgrid™-6 counting grid (Catalog #27000)
- Permanent fine-tip marker

5.0 Human Hematopoietic CFU Assays in MethoCult™ Medium: Procedure Diagram



6.0 Preparation of MethoCult™ Media

For storage and stability information, refer to the Product Information Sheet (PIS) included with your MethoCult™ medium; the PIS is also available at www.stemcell.com, or contact us to request a copy.

If MethoCult™ medium arrives partially thawed, place immediately at -20°C (-25°C to -15°C), or thaw and aliquot as described in sections 6.1 and 6.2.

The preparation of complete and incomplete MethoCult™ media is summarized in Table 5 below, and further detailed in sections 6.1 and 6.2.

Table 5. Preparation of MethoCult™ Media

		COMPLETE METHOCULT™	INCOMPLETE METHOCULT™		
		04034, 04434, 04435, 04035, 04534, 04535, 84434, 84435, 84534, 84535, 04436, 04536, 04636, 04431, 04531, 04433, 04533, 04437	04330	04236, 04230	04100
MethoCult™ volume per bottle		100 mL	90 mL	80 mL	40 mL
Additional volume required for 100 mL final volume		0 mL	10 mL**	20 mL**	60 mL**
DUPLICATE CULTURES (1.1 mL each)					
Dispensing volume per tube*	MethoCult™ medium	3.0 mL	2.7 mL	2.4 mL	1.2 mL
	Additional components**	0 mL	0.3 mL**	0.6 mL**	1.8 mL**
TRIPLICATE CULTURES (1.1 mL each)					
Dispensing volume per tube*	MethoCult™ medium	4.0 mL	3.6 mL	3.2 mL	1.6 mL
	Additional components**	0 mL	0.4 mL**	0.8 mL**	2.4 mL**

*Cells are added in 0.3 mL volume to 3.0 mL MethoCult™ for duplicate cultures and 0.4 mL to 4.0 mL MethoCult™ for triplicate cultures.

**FBS, BSA, cytokines, IMDM, or other compounds.

6.1 Complete MethoCult™ Media: Thawing and Dispensing

Complete MethoCult™ media are supplied at 100 mL per bottle. They are formulated to allow the addition of cells to MethoCult™ medium at a 1:10 (v/v) ratio, which maintains the optimal viscosity of the medium.

Refer to Table 5 for a summary of preparation and dispensing volumes.

Note: Do not use pipettes to dispense methylcellulose as the volume dispensed will not be accurate. Syringes and large bore blunt-end needles should be used for accurate dispensing of viscous methylcellulose medium and to prevent needle-stick injuries.

1. Thaw bottle of complete MethoCult™ medium at room temperature (15 - 25°C) or overnight at 2 - 8°C.

   Note: Do not thaw MethoCult™ medium at 37°C.

2. Shake vigorously for 1 - 2 minutes and then let stand for at least 5 minutes to allow bubbles to rise to the top before aliquoting.
3. Use a 3 mL or 6 mL luer lock syringe attached to a 16 gauge Blunt-End Needle to dispense MethoCult™ medium into 14 mL (17 x 95 mm) sterile tubes.

   Note: Place the needle below the surface of the MethoCult™ medium and draw up approximately 1 mL to remove the air from the syringe. Gently depress the plunger and expel the medium completely. Repeat until no air space is visible.

4. Dispense 3 mL per tube for 1.1 mL duplicate cultures, or 4 mL per tube for 1.1 mL triplicate cultures.

   Note: 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.

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

5. Vortex tubes to mix well. Tubes of complete medium can be used immediately, stored at 2 - 8°C for up to 1 month, or stored at -20°C. After thawing aliquoted tubes of MethoCult™, mix well and use immediately. Do not re-freeze.

6.2 Incomplete MethoCult™ Media: Thawing and Dispensing

Incomplete MethoCult™ media allow researchers to add desired medium components in order to prepare formulations for specific cell culture requirements. Components should be added to incomplete MethoCult™ medium bottles to yield a total volume of 100 mL and then dispensed into tubes (section 6.2.1). Alternatively, appropriate volumes can be dispensed into tubes, frozen, and desired components added at the time of use (section 6.2.2).

Note: It is important to dilute MethoCult™ as described below to allow the addition of cells at a 1:10 (v/v) ratio; this will maintain optimal viscosity of the methylcellulose-based medium.

Note: Do not use pipettes to dispense methylcellulose as the volume dispensed will not be accurate. Syringes and large bore blunt-end needles should be used for accurate dispensing of viscous methylcellulose medium and to prevent needle-stick injuries.

6.2.1 Preparing 100 mL Bottle

1. Thaw bottle of incomplete MethoCult™ medium at room temperature (15 - 25°C) or overnight at 2 - 8°C.

   Note: Do not thaw MethoCult™ medium at 37°C.
2. Add desired growth factors, supplements, and IMDM with 25 mM HEPES to yield a total volume of 100 mL (see Table 5 for more information).
3. Shake vigorously for 1 - 2 minutes and then let stand for at least 5 minutes, until all bubbles rise to the top, before aliquoting.
4. Use a 3 or 6 mL luer lock syringe attached to a 16 gauge Blunt-End Needle to dispense complete MethoCult™ medium into 14 mL (17 x 95 mm) sterile tubes.

   Note: Place the needle below the surface of the MethoCult™ medium and draw up approximately 1 mL to remove the air from the syringe. Gently depress the plunger and expel the medium completely. Repeat until no air space is visible.
5. Dispense 3 mL per tube for 1.1 mL duplicate cultures, or 4 mL per tube for 1.1 mL triplicate cultures.

   Note: 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.
6. Vortex tubes to mix well. Complete MethoCult™ medium is now ready for use.

6.2.2 Preparing Individual Tubes

1. Thaw bottle of incomplete MethoCult™ medium at room temperature (15 - 25°C) or overnight at 2 - 8°C.

   Note: Do not thaw MethoCult™ medium at 37°C.
2. Shake bottle vigorously for 1 - 2 minutes and then let stand for at least 5 minutes, until all bubbles rise to the top, before aliquoting.
3. Use a 3 or 6 mL luer lock syringe attached to a 16 gauge Blunt-End Needle to dispense MethoCult™ incomplete medium into 14 mL (17 x 95 mm) sterile tubes. See Table 5 for required volumes.
4. Place the needle below the surface of the MethoCult™ medium and draw up approximately 1 mL to remove the air from the syringe. Gently depress the plunger and expel the medium completely. Repeat until no air space is visible.

   Note: Do not expel the medium to the "0" mark on the syringe when dispensing. For example, measure from 3.5 mL to 0.5 mL rather than 3.0 mL to 0 mL.
5. Add desired growth factors, supplements, and IMDM with 25 mM HEPES to tubes of MethoCult™. See Table 5 for required volumes.

   Note: If components are to be added at a later date, tubes of incomplete MethoCult™ medium may be stored at -20°C until expiry date as indicated on label. After thawing aliquoted tubes, add desired components and mix well. Refer to Table 5 for volumes of components to be added.
6. Vortex tubes to mix well. Complete MethoCult™ medium is now ready for use.
7. Dispense any remaining incomplete MethoCult™ medium (see Table 5 for required volumes), store at -20°C, then add desired components after thawing. Mix well before use.

7.0 Cell Sample Preparation

Cell samples must be processed before plating in CFU assays in order to:

- Deplete mature red blood cells (RBCs) and nucleated erythroid precursors that may be present, both of which can obscure colonies within the cultures and make colony counts inaccurate, in particular in 7-day CFU assays in MethoCult™ Express medium.
- Enrich for hematopoietic progenitor cells in cell samples where CFU frequency is expected to be low and to yield sufficient colonies for accurate CFU quantitation.
- Deplete accessory cells that produce factors that may enhance or inhibit CFU growth. For example, monocytes/macrophages can produce factors including IL-6 and TNF-alpha.

This section includes guidelines for processing fresh or frozen cell samples for hematopoietic CFU assays. Note that for **serum-free conditions**, IMDM with 25 mM HEPES should be used for cell dilution/suspension instead of IMDM with 2% FBS or MethoCult™ Cell Wash Medium.

It is important to note that the methods described below may increase the frequency of CFUs in the cell suspension relative to the original sample. Some loss of CFUs is anticipated during cell cryopreservation, cell separation, and cell washing procedural steps.

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

7.1 Anticoagulants

Anticoagulants are used to prevent clotting of the cell samples. Sodium heparin, ethylenediaminetetraacetic acid (EDTA), and acid citrate dextrose (ACD) are routinely used to collect BM, CB, and peripheral blood (PB) samples for research use and for clinical applications. If ACD or EDTA are used during sample collection, additional anticoagulant may need to be added to media for dilution and washing steps, as these non-permanent anticoagulants can be washed away, allowing clotting to initiate. The anticoagulant activity of heparin is permanent, so clotting is not a concern after dilution or washing steps.

NOTE: The media used for cell washing and counting—IMDM with 2% FBS, MethoCult™ Cell Wash Medium, or IMDM with 25 mM HEPES—do not contain anticoagulants.

Guidelines for collection of samples for research use:

- BM and CB must be transferred as quickly as possible after aspiration into a sterile tube containing 1 mL of preservative-free sodium heparin dissolved at a concentration of 800 U/mL in IMDM with 2% FBS (or IMDM with 25 mM HEPES for serum-free conditions). Mix cells and heparin solution immediately by inversion to prevent clotting.
- Collect PB in a Vacutainer® containing sodium heparin and mix immediately by inversion to prevent clotting.
- Mobilized peripheral blood (MPB): Contact institutional cell processing unit for information on collecting MPB.

7.2 Ammonium Chloride Lysis Treatment of Bone Marrow

Treatment of BM samples with Ammonium Chloride Solution yields a nucleated cell suspension depleted of mature, non-nucleated RBCs.

1. Mix sample well and remove a small volume of cells (100 µL) to perform an initial cell count using a Neubauer hemocytometer 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 (see section 8.1 for a recommended protocol).

2. Measure the entire volume of the cell sample to be processed and then transfer it into one or more 14 mL sample tube(s). Do not add more than 2 mL of sample per 14 mL tube. Use 50 mL tube(s) for volumes > 2 mL.
3. Add buffered Ammonium Chloride Solution to sample to give a minimum of 4:1 (v/v) ratio (i.e. ≥ 4 mL Ammonium Chloride Solution:1 mL of sample).
4. Gently vortex the mixture and put on ice for 10 minutes with gentle vortexing or inversion once or twice during the incubation period. All of the RBCs should be lysed within 10 minutes.
5. Check for complete lysis (mixture appears 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.
6. Make up the volume in each tube to 12 mL with IMDM with 2% FBS or MethoCult™ Cell Wash Medium (or IMDM with 25 mM HEPES for serum-free conditions) and centrifuge the contents at 300 x *g* for 10 minutes at room temperature (15 - 25°C) with the brake on.
7. Quickly, but carefully, remove and discard the supernatant so as not to dislodge the cell pellet.
8. Resuspend the cell pellet, first by gentle vortexing and then after addition of 10 mL of IMDM with 2% FBS or MethoCult™ Cell Wash Medium (or IMDM with 25 mM HEPES for serum-free conditions) by vortexing more vigorously.
9. Centrifuge the cell suspension at 300 x *g* for 10 minutes at room temperature (15 - 25°C) with the brake on. Discard the supernatant and wash the cells once more.

   Note: Cells from multiple tubes of the same sample may be pooled before this final wash.
10. Discard the supernatant from the final wash and gently resuspend the cells in 1 - 2 mL of IMDM with 2% FBS or MethoCult™ Cell Wash Medium (or IMDM with 25 mM HEPES for serum-free conditions). A larger volume may be used if the initial nucleated cell number was high.
11. Record the exact final volume and perform a final nucleated cell count in the same manner as for the initial cell count (see section 8.1 for a recommended protocol).
12. For setup of CFU assays in MethoCult™ medium, see section 9.0.

7.3 Isolation of Mononuclear Cells by Lymphoprep™

Hematopoietic colony-forming cells are present in the mononuclear cell (MNC) fraction of hematopoietic cell sources (BM, CB, PB). Isolation of light-density cells using Lymphoprep™ enriches CFUs and depletes mature RBCs, 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 hemocytometer 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 (see section 8.1 for a recommended protocol).
2. Measure the entire volume of the sample to be processed and then transfer it into a new tube.
3. Dilute the cells with at least an equal volume of IMDM with 2% FBS or MethoCult™ Cell Wash Medium (or IMDM with 25 mM HEPES for serum-free conditions). Invert gently to mix.
4. Add 15 mL of room temperature (15 - 25°C) Lymphoprep™ to a 50 mL tube.

   Note: A SepMate™-50 tube may be used for fast and easy harvesting of isolated MNCs. SepMate™ tubes contain an insert that provides a barrier between the density gradient medium and blood. Refer to the SepMate™-50 PIS (available at www.stemcell.com/sepmate) for volumes and directions for use.
5. Slowly layer 30 mL of diluted cell sample on top of the Lymphoprep™ so that a distinct layer forms. Take care not to disturb the tube.

   Note: If the layers are disturbed such that the Lymphoprep™ and sample mix, distinct layers will not form after centrifugation. This will result in decreased recovery of MNCs.

Note: For other volumes, refer to the Lymphoprep™ PIS (Document #29283), available at www.stemcell.com or contact us to request a copy.

6. Centrifuge the tube(s) at 800 x *g* for 20 minutes at room temperature (15 - 25°C) with the brake **off**.
7. Using a sterile standard pipette or Pasteur pipette, remove and discard the top plasma layer, taking care not to disturb the grey to white layer of MNCs present at the interface of the Lymphoprep™ layer.
8. Remove the MNC layer and transfer it to a 14 mL tube.

   Note: The MNC layer normally contains the lymphocytes, platelets, monocytes, and hematopoietic colony-forming cells.
9. Add IMDM + 2% FBS or MethoCult™ Cell Wash Medium (or IMDM with 25 mM HEPES for serum-free conditions) and then centrifuge at 300 x *g* for 10 minutes at room temperature (15 - 25°C) with the brake on.
10. Quickly but carefully discard the supernatant so as not to dislodge the cell pellet. Resuspend cells in IMDM + 2% FBS or MethoCult™ Cell Wash Medium (or IMDM with 25 mM HEPES for serum-free conditions) and mix thoroughly.
11. Centrifuge at 300 x *g* at room temperature (15 - 25°C) for 10 minutes with the brake on.
12. Quickly but carefully discard the second supernatant.
13. Resuspend the cell pellet in 1- 2 mL of IMDM + 2% FBS or MethoCult™ Cell Wash Medium (or IMDM with 25 mM HEPES for serum-free conditions). A larger volume may be desirable if the initial cell count was high. Record the final volume.

    Note: Isolation of MNCs by Lymphoprep™ results in a final cell suspension that is enriched 2- to 4-fold for hematopoietic progenitor cells. More mature myeloid cells are removed along with the RBCs. Plating concentration must be adjusted accordingly. Refer to section 9.0 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 (see section 8.1 for a recommended protocol).
15. For setup of CFU assays in MethoCult™ medium, see section 9.0.

7.4 Isolation of Nucleated Cells from Cord Blood by Sedimentation Over HetaSep™

Red blood cell (RBC)-aggregating agents such as HetaSep™ increase the RBC sedimentation rate by increasing the effective size of the cells through formation of aggregates, or rouleaux. Because nucleated cells settle at a slower 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 can be 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 50% of the total volume.

   Note: 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.

3. Collect the supernatant containing the nucleated cells and wash once in the appropriate medium.
4. Optional: Lyse remaining RBCs with Ammonium Chloride Solution.

Centrifugation

Centrifugation may be used to accelerate the sedimentation process.

Note: Not recommended for small sample volumes. Nucleated cell recovery will decrease substantially when using containers less than half full, due to the shorter sedimentation distance.

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.

   Note: If processing blood in a 50 mL tube, contact us at techsupport@stemcell.com for centrifuge speeds and times.

   Note: Older blood will settle more slowly. If the blood is 2 days old, try centrifuging at 200 x g for 5 minutes with the brake off.
3. Collect the supernatant containing the nucleated cells and wash once in the appropriate medium.
4. Optional: Lyse remaining RBCs with Ammonium Chloride Solution.
5. After isolation of nucleated cells by sedimentation over HetaSep™, perform a nucleated cell count (see section 8.1 for a recommended protocol).
6. For setup of CFU assays in MethoCult™ medium, see section 9.0.

7.5 Thawing Whole Cryopreserved Cord Blood

1. Thaw the vial of 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 15 mL or 50 mL conical tube.
4. Slowly (dropwise) add 10 mL of medium (D-PBS + 2% FBS, IMDM + 2% FBS, or MethoCult™ Cell Wash Medium; for serum-free conditions, use IMDM with 25 mM HEPES) to the tube containing cells while gently swirling the tube (approximately 1 - 2 minutes). Gently invert tube to mix.
5. Centrifuge the cell suspension at 300 x *g* for 10 minutes at room temperature (15 - 25°C).
6. 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.

   Optional: Repeat the wash in steps 4 - 6 to remove additional dead cells. This is not recommended for samples with low cell numbers.
7. Add 2 mL of medium to the tube (D-PBS + 2% FBS, IMDM + 2% FBS, or MethoCult™ Cell Wash Medium; for serum-free conditions, use IMDM with 25 mM HEPES).
8. Perform a cell count (e.g. using trypan blue dye exclusion; see section 8.2 for a recommended protocol).

   Note: 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).
9. For setup of CFU assays in MethoCult™ medium, see section 9.0.

7.6 Cell Preparation by Positive Selection of CD34+ Cells or Enrichment of Hematopoietic Progenitor Cells Using Negative Selection

The majority of human hematopoietic progenitor cells, including most BFU-E, CFU-GM, and CFU-GEMM, express the CD34 antigen and lack antigens present on more mature lineage-committed cells. Therefore, CFUs can be enriched from BM, CB, and MPB samples by isolation of $CD34^+$ cells or by depletion of lineage-antigen(s) positive cells using specific monoclonal antibodies and immunoseparation technologies. STEMCELL Technologies offers reagent kits and methods for the positive selection of $CD34^+$ cells and depletion of mature lineage-committed hematopoietic cells; for further information, refer to www.stemcell.com or contact us at techsupport@stemcell.com.

8.0 Manual Cell Counts

The following sections outline procedures for nucleated cell counts and viable cell counts. For further information, refer to the video “How to Perform Cell Counts with a Hemocytometer”, available at www.stemcell.com.

8.1 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 BM, CB, and MPB is 1 in 50 to 1 in 100. The recommended dilution for PB is 1 in 20 to 1 in 40. A higher dilution may be necessary if the nucleated count is elevated.

   Example: For a 1 in 50 dilution, add 20 µL of cells to 980 µL of 3% Acetic Acid with Methylene Blue.

   Note: 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 10 minutes of the cells being exposed to the acetic acid solution.
3. Mix the diluted cell sample well.
4. Prepare the hemocytometer 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 hemocytometer. Do not over- or under-fill the chambers.
8. Starting with 1 chamber of the hemocytometer, count all the nucleated cells in at least 2 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.
9. 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). Determine the cell concentration and total number of cells using the following calculations:

$$Cells\ per\ mL = Average\ count\ per\ square\ \ x\ \ Dilution\ factor\ \ x\ \ 10^4$$

$$Total\ cells = Cells\ per\ mL\ \ x\ \ Original\ start\ volume$$

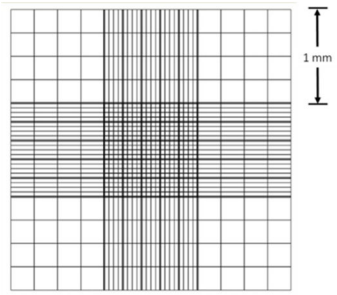


Figure 1. Neubauer Hemocytometer. Each of the 9 large squares has sides of 1 mm length and 0.1 mm depth.

8.2 Viable Cell Count Using Trypan Blue Dye Exclusion

Trypan blue dye exclusion should be used for cell preparations in which 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 with an equal volume of Trypan Blue (1 in 2 dilution) (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 in 40 dilution, add 50 µL of cell suspension to 950 µL IMDM with 2% FBS or MethoCult™ Cell Wash Medium (1 in 20 dilution) and mix well. Then add 100 µL of the diluted cell suspension to 100 µL of Trypan Blue (1 in 2 dilution for a final 1 in 40 dilution).
3. Mix the diluted sample well.
4. Allow the resulting solution to sit for 5 - 15 minutes.

   Note: 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 hemocytometer 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 hemocytometer. Do not over- or under-fill the chambers.
9. Count cells in 4 large squares 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. Calculate the **viable cell count** as follows:

$$Viable\ cell\ count\ per\ mL = Average\ total\ viable\ cells\ per\ square\ x\ Dilution\ factor\ x\ 10^4$$

11. Calculate the **percent viability** as follows:

$$\%\ Viability = \frac{Total\ number\ of\ viable\ (clear\ or\ non\text{–}\ blue)\ cells}{Total\ number\ of\ viable\ (clear\ or\ non\text{–}\ blue)\ cells + Non\text{–}\ viable\ (blue)\ cells}\ \ x\ 100\%$$

9.0 CFU Assay Setup

To set up the CFU assay, cells are diluted, mixed with MethoCult™ medium, dispensed into culture plates, and incubated. Refer to part C of the procedure diagram in section 5.0.

Cells are diluted to an appropriate cell concentration in IMDM + 2% FBS or MethoCult™ Cell Wash Medium (or IMDM with 25 mM HEPES for serum-free conditions). Recommended plating concentrations for cells from different sources are listed in Table 6 and Table 7. Once diluted, the cell sample can be added directly to pre-aliquoted tubes of complete MethoCult™ medium.

For manual colony counting, the following cultureware are recommended: Non-treated 35 mm culture dishes or 6-well culture plates, or SmartDish™ cultureware. These will have minimal cell adherence; adherence of cells during culture can cause inhibition of colony growth and can obscure visualization of colonies. Plates with smaller wells, e.g. 24-well or 96-well plates, are not recommended as the surface area is too small to obtain sufficient colonies and statistically accurate data. SmartDish™ 6-well plates have been developed as a meniscus-free alternative to 35 mm dishes. Each of the six 35 mm wells in a SmartDish™ plate has been designed to allow for a more uniform distribution of media and colonies, reducing optical distortion so that colonies located at the edge can be more easily counted. For automated colony counting with STEMvision™, SmartDish™ cultureware is required.

Sufficient cells should be plated to yield ~25 - 120 colonies per 1.1 mL culture in a 35 mm dish. The optimal number of colonies per dish depends on the cell source. For example, progenitor cells in CB samples have a high proliferative ability and will produce large colonies after 14 - 16 days of culture in complete MethoCult™ medium. Therefore, the optimal number of colonies for CFU assays on CB samples is at the low end of the range.

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. Refer to Table 6 and Table 7 for recommended plating concentrations for cell samples from different tissues.

Two or more different plating concentrations are recommended for each cell 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.

9.1 Incomplete MethoCult™ Media

This procedure is for all incomplete MethoCult™ media pre-aliquoted in tubes that still require the addition of medium components (refer to section 6.2 for preparation of pre-aliquoted tubes). For complete MethoCult™ media that have been prepared to the final desired volume, refer to section 9.2.

1. Thaw aliquoted tubes of incomplete MethoCult™ medium at room temperature (15 - 25°C) or overnight at 2 - 8°C.
2. Add desired component(s) to tubes of MethoCult™ medium. Component volumes should not exceed the volumes outlined in Table 5.

   Example: A previously dispensed 2.7 mL tube of MethoCult™ H4330 requires the addition of components to total 3 mL for duplicate cultures. For each 2.7 mL of H4330 add 0.3 mL of components.
3. Vortex tube to ensure that all components are thoroughly mixed.
4. Continue with the instructions in section 9.2, beginning at step 2.

9.2 Complete MethoCult™ Media

This procedure is for complete MethoCult™ media that have been pre-aliquoted into tubes (refer to section 6.1) and incomplete MethoCult™ media that have been supplemented with components and pre-aliquoted into tubes (refer to section 6.2). Refer to Table 1 for a listing of complete and incomplete MethoCult™ products.

1. Thaw the required number of pre-aliquoted tubes of complete MethoCult™ medium at room temperature (15 - 25°C) or overnight at 2 - 8°C.
2. Prepare culture dishes by placing 2 x 35 mm culture dishes with lids inside a 100 mm Petri dish 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 3 x 35 mm dishes with lids in larger cultureware (for example, a 245 mm square dish), and add a fourth 35 mm culture dish without a lid as a water dish.

   Note: If using SmartDish™ cultureware, add 4 - 8 mL of sterile water to the empty spaces between the SmartDish™ wells. Place the SmartDish™ in a 245 mm square dish, along with additional 35 mm culture dishes each containing 3 - 4 mL of sterile water. Up to 3 SmartDish™ 6-well plates can fit in a 245 mm square dish.
3. Prepare cells (refer to section 7.0).
4. Dilute the cells with IMDM with 2% FBS or MethoCult™ Cell Wash Medium (or IMDM with 25 mM HEPES for serum-free conditions) to 10X the final concentration(s) required for plating. Refer to Table 6 and Table 7 for recommended plating concentrations.

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

   Note: When it is difficult to anticipate the correct plating cell concentration, use 2 or more cell concentrations that differ by 2 - 3-fold. Example: 2 x 10^4 cells per dish and 1 x 10^4 cells per dish.

Table 6. Recommended Plating Concentrations for MethoCult™ Media

CELL SOURCE	10X CONCENTRATION TO BE PREPARED	PLATING CONCENTRATION (cells per 35 mm dish)*
BM, ammonium chloride-treated	5×10^5 (2×10^5 - 1×10^6)	5×10^4 (2×10^4 - 1×10^5)
BM MNCs	2×10^5 (1×10^5 - 5×10^5)	2×10^4 (1×10^4 - 5×10^4)
CB MNCs	1×10^5 (5×10^4 - 2×10^5)	1×10^4 (5×10^3 - 2×10^4)
PB MNCs	2×10^6 (1×10^6 - 2×10^6)	2×10^5 (1×10^5 - 2×10^5)
MPB MNCs	2×10^5 (1×10^5- 5×10^5)	2×10^4 (1×10^4- 5×10^4)
Lin-depleted ($CD34^+$ enriched BM, CB, MPB)	1×10^4 (5×10^3 - 2×10^4)	1000 (500 - 2000)
$CD34^+$ cells (BM, CB, MPB)	5×10^3 (5×10^3 - 2×10^4)	500 (500 - 2000)

*These numbers apply to MethoCult™ media containing recombinant cytokines. Recommended plating concentrations for MethoCult™ media with conditioned media (Catalog # 04431, 04531) are 1.5- to 2.5-fold higher, as plating efficiencies in these media tend to be lower.

Table 7. Recommended Plating Concentrations for MethoCult™ Express

CELL SOURCE	10X CONCENTRATION TO BE PREPARED	PLATING CONCENTRATION (cells per 35 mm dish)
CB, RBC-depleted	2×10^5 - 5×10^5	2×10^4 - 5×10^4
Whole CB, cryopreserved	3×10^5 - 5×10^5	3×10^4 - 5×10^4
CB MNCs	1×10^5 - 2×10^5	1×10^4 - 2×10^4

5. For a duplicate assay, add 0.3 mL of diluted cells to a pre-aliquoted 3 mL MethoCult™ tube. For a triplicate assay, add 0.4 mL of diluted cells to a pre-aliquoted 4 mL MethoCult™ tube.

   Note: This 1:10 (v/v) ratio of cells:medium gives the correct viscosity to ensure optimal CFU growth and morphology.
6. Vortex the tube vigorously for at least 4 seconds to mix the contents thoroughly.
7. Let stand for at least 5 minutes to allow the bubbles to rise to the top.
8. To dispense the MethoCult™ mixture containing cells into culture dishes, attach a sterile 16 gauge Blunt-End Needle to a sterile 3 mL luer lock syringe.

   Note: 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.

   Note: Do not use pipettes to dispense methylcellulose as the volume dispensed will not be accurate. Syringes and large bore blunt-end needles should be used for accurate dispensing of viscous methylcellulose medium and to prevent needle-stick injuries.

9. To expel the air from the syringe, place the needle below the surface of the MethoCult™ medium and draw up approximately 1 mL to remove the air from the syringe. Gently depress the plunger and expel the medium completely. Repeat until no air space is visible.
10. Draw up the MethoCult™ mixture containing cells into the syringe and dispense a volume of 1.1 mL into each 35 mm dish as follows:

    While holding the syringe containing the MethoCult™ 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.

    Note: 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.
11. 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.

    Note: 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.
12. Place the culture dishes into the outer dish (e.g. 100 mm Petri dish or 245 mm square dish). Add approximately 3 mL of sterile water to the uncovered 35 mm dish(es). Place lid onto outer dish.

    Note: Using 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.
13. Incubate at 37°C, in 5% CO_2 with ≥ 95% humidity for 7 days for assays in MethoCult™ Express and for 14 - 16 days for assays in other MethoCult™ media.

    Note: Proper culture conditions are critical for optimal CFU 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.
14. If cultures cannot be counted after the recommended incubation time, refill water dishes, if required, and transfer cultures to an incubator maintained at 33°C in 5% CO_2 with ≥ 95% humidity. Colony growth will be slowed; count as soon as possible, preferably within 1 week.

    Note: Most CFUs will have reached a maximal size (cells per colony) by the recommended incubation time. The lower incubation temperature will not completely inhibit proliferation or prevent cell death, but will assist in maintaining colony morphology.

10.0 Counting Human CFU Assays

10.1 Counting CFU Assays Using MethoCult™ Media

Evaluate human CFU numbers in situ following the recommended incubation period of 7 days for assays on CB cells in MethoCult™ Express medium (see section 10.2) or 14 - 16 days for cultures in other media. See Table 8 for typical progenitor cell colony frequencies obtained in MethoCult™ media after 14 - 16 days of culture.

It is important to use a high-quality inverted microscope equipped with low power (2X or 2.5X) and higher power (4 - 5X, 10X) objective lenses, 10X or 12.5X ocular eyepieces, and a blue filter to enhance the red color of hemoglobinized erythroblasts. Not all donors will have erythroid progenitor cells that hemoglobinize, so it is important to verify colony morphology.

For assistance with recognizing the various colony types, refer to section 11.0 as well as the Atlas of Human Hematopoietic Colonies (Catalog #28700) or the Atlas of Hematopoietic Colonies from Cord Blood (Catalog #29940), available at www.stemcell.com.

Table 8. Typical Progenitor Cell Colony Frequencies Obtained in MethoCult™ Media After 14 - 16 Days of Culture

CELL SOURCE	TYPICAL NUMBER OF PROGENITOR CELL COLONIES**			
	CFU-E	BFU-E	CFU-GM	CFU-GEMM
BM ammonium chloride-treated per 10^5 cells (n = 50)	31 (1 - 78)	115 (1 - 251)	100 (30 - 170)	5 (1 - 15)
BM MNC* per 10^5 cells (n = 17)	188 (1 - 506)	175 (1 - 477)	408 (1 - 990)	10 (1 - 30)
BM $CD34^+$ enriched cells per 10^3 cells (n = 15)	30 (1 - 59)	34 (1 - 74)	54 (7 - 101)	2 (1 - 5)
CB MNC* per 10^5 cells (n = 16)	9 (1 - 48)	104 (1 - 310)	115 (1 - 303)	25 (1 - 59)
PB MNC* per 10^5 cells (n = 30)	2 (1 - 10)	30 (1 - 62)	9 (1 - 18)	2 (1 - 5)
MPB MNC* per 10^5 cells (n = 19)	8 (1 - 27)	121 (1 - 257)	111 (1 - 257)	23 (1 - 67)

*MNCs are isolated by density-based cell separation (e.g. sedimentation over Lymphoprep™).

**Determined using MethoCult™ H4434 Classic. Values are expressed as means and the range is defined by mean ± 2 standard deviations.

1. Prepare a 60 mm Gridded Scoring Dish by drawing 2 perpendicular lines across the center of the dish using a permanent fine felt marker on the bottom of the dish. Count 8 squares from the center on one radius and draw a short (approximately 2 mm) line the crossing the radius. Repeat for each radius.

   Note: These lines will help to center the 35 mm culture dish to be counted and evaluated. This scoring dish can be used again to count other culture dishes. Alternatively, use STEMgrid™-6, which is designed for manual counting with SmartDish™ 6-well plates, but can also be used to assist with counting colonies in standard 35 mm dishes.

2. Remove the cultures to be counted from the 37°C incubator. Take out only the number of dishes that can be counted within 1 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, 20X - 25X magnification) until the colonies are in focus.
4. Scan the entire dish on low power for placement of colonies relative to one another. Make note of the overall appearance of the culture to help with counting 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 or underplated)? Observe the background for presence or absence of other cells or debris, and general morphology and health of the colonies.
5. Count all colonies in each dish. It is necessary to continually adjust the microscope focus to identify all colonies present in the 3-dimensional culture and to distinguish individual colonies that are close together but in different planes of focus. CFU-E and small BFU-E colonies are usually counted at medium magnification (4X objective, 40X - 50X magnification). Large BFU-E, CFU-GM, and CFU-GEMM colonies are usually counted at low magnification (2X objective, 20X - 25X magnification). Switch to high power to help with colony identification if necessary.

   Note: Move the dish from top to bottom rather than from left to right when counting. This will minimize the sensation of motion sickness common to individuals new to colony counting.
6. Counted cultures can be incubated at 33°C in 5% CO_2 with ≥ 95% humidity for further evaluation if necessary, for up to 7 days.

10.2 Counting CFU Assays Using MethoCult™ Express

10.2.1 Counting After 7 Days

Scan the dish on low power (2X objective, 20X - 25X magnification) to evaluate relative distribution of colonies. Count colonies with a 4X objective and count all colonies containing more than 20 cells. Count only total colonies if counting after 7 days. Counting individual colony types, e.g., BFU-E and CFU-GM, is not recommended as most colonies are immature and cannot be distinguished on the basis of morphology after 7 days of culture.

10.2.2 Counting After 14 - 16 Days

If desired, BFU-E, CFU-GM, and CFU-GEMM can be distinguished and counted after 14 - 16 days of culture as described in section 10.1. Counting colonies at low power is recommended, as CB-derived colonies in MethoCult™ Express can be very large after 14 - 16 days of culture. Switch to a higher power if necessary to help with colony identification. For detailed descriptions and examples of colonies, refer to the Atlas of Hematopoietic Colonies from Cord Blood (Catalog #29940). CB-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 7).

10.3 Automated Colony Counting Using STEMvision™

In addition to manual colony counting, CFU assays plated in MethoCult™ Express, MethoCult™ Optimum, or MethoCult™ Optimum without EPO can be counted with the STEMvision™ instrument. Instead of manually identifying and counting colonies using a microscope, the user simply loads a SmartDish™ 6-well plate (containing up to 6 individual CFU assays) into STEMvision™. The instrument then captures an image of each 35 mm well in approximately 1 minute, and uses highly sophisticated image analysis software to identify and classify colonies produced by myeloid (CFU-G/M/GM), erythroid (CFU-E, BFU-E), and mixed (CFU-GEMM) progenitor cells. For more information, visit www.stemvision.com.

11.0 Colony Identification

11.1 General Colony Descriptions

Human hematopoietic progenitor cells can be quantified in cell suspensions of BM, CB, MPB, and PB. The classes of human hematopoietic progenitor cells detected using various formulations of MethoCult™ media include:

CFU-E: Colony-forming unit-erythroid. Produces 1 - 2 cell clusters containing a total of 8 - 200 erythroblasts. CFU-E are relatively mature erythroid progenitor cells that require erythropoietin (EPO) for differentiation. CFU-E are usually detectable in BM, but absent in CB.

BFU-E: Burst-forming unit-erythroid. Produces a colony containing > 200 erythroblasts in single or multiple clusters and can be sub-classified based on the number of cells or cell clusters per colony, if desired. BFU-E are more immature progenitor cells than CFU-E. BFU-E require EPO and cytokines with burst-promoting activity, such as interleukin 3 (IL-3) and stem cell factor (SCF), for optimal colony growth.

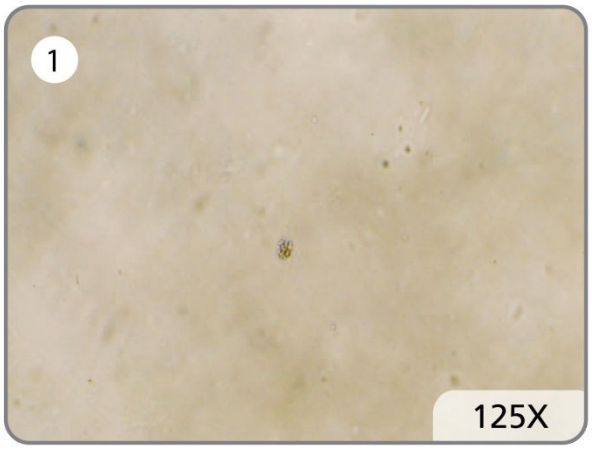
CFU-GM: Colony-forming unit-granulocyte, macrophage. Produces a colony containing at least 40 granulocytes (CFU-G), macrophages (CFU-M), or cells of both lineages (CFU-GM). Colonies arising from primitive CFU-GM may contain thousands of cells in single or multiple clusters.

CFU-GEMM: Colony-forming unit-granulocyte, erythrocyte, macrophage, megakaryocyte. A multi-potential progenitor that produces a colony containing erythroblasts and cells of at least 2 other recognizable lineages. Due to their primitive nature, CFU-GEMM tend to produce large colonies containing > 500 cells under optimal plating and culture conditions.

CFU-Mk: Colony-forming unit-megakaryocyte. Produces a colony containing 3 or more megakaryocytic cells.

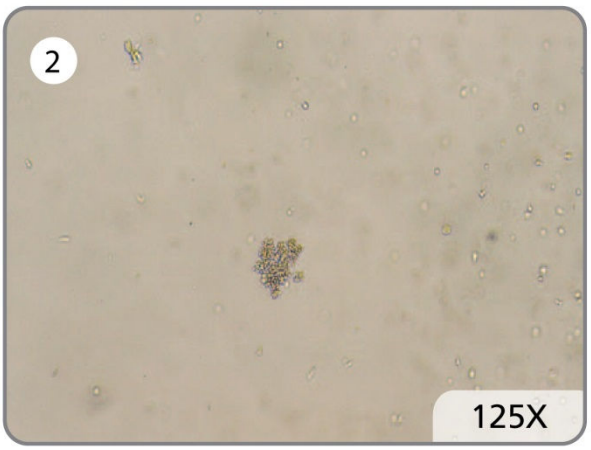
Note: Although megakaryocytic progenitor cells can be cultured in MethoCult™ containing the appropriate growth factors, it is difficult to distinguish CFU-Mk based on cellular and colony morphology. Therefore, we recommend counting CFU-Mk by culturing in collagen-based, serum-free MegaCult™-C media, followed by immunocytochemical staining in dehydrated gels. For further information, contact us at techsupport@stemcell.com or refer to the MegaCult™-C Technical Manual: Assays for Quantitation of Human and Mouse Megakaryocytic Progenitor Cells (Document # 28413), available at www.stemcell.com or contact us to request a copy.

11.2 Photographs of Colonies in MethoCult™ Media



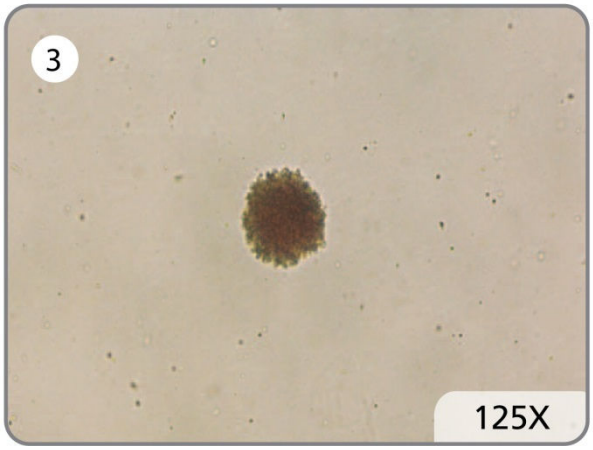
Small CFU-E-derived colony.

CFU-E-derived colony forming a single cluster containing 8 - 20 erythroblasts.



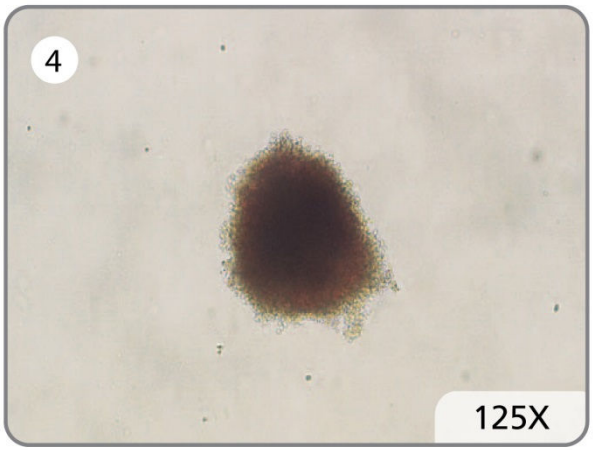
CFU-E-derived colony.

CFU-E-derived colony forming a single cluster containing ~200 cells.



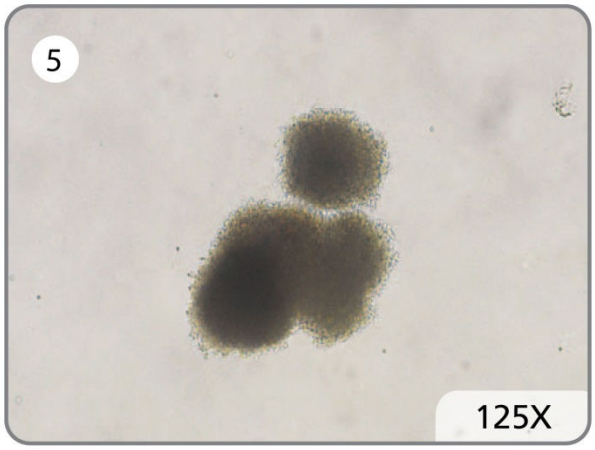
Small BFU-E-derived colony.

BFU-E-derived colony forming a single cluster and containing greater than 200 cells. Note the small size of the individual cells and the reddish color due to hemoglobinization of the erythroblasts.



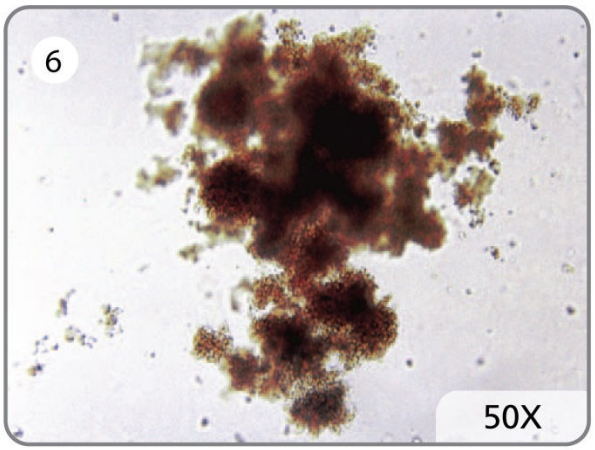
BFU-E-derived colony.

BFU-E colony, forming a single cluster and containing greater than 1000 cells.



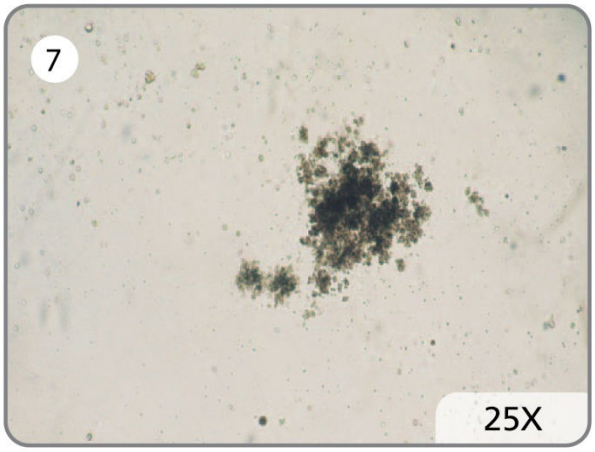
BFU-E-derived colony.

BFU-E-derived colony with 3 - 8 clusters (and containing similar cell numbers as the BFU-E-derived colony shown in Photo 4).



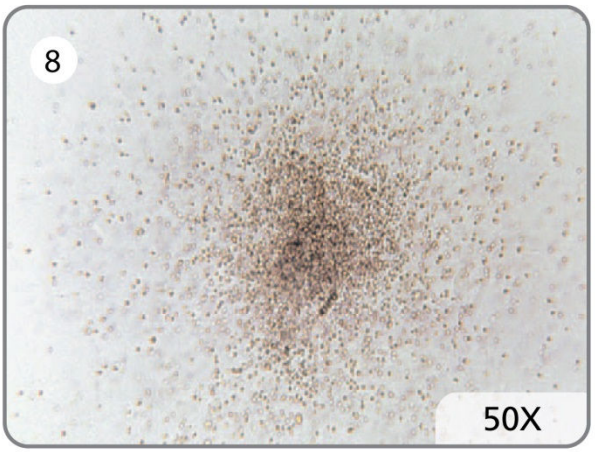
Large BFU-E-derived colony.

BFU-E-derived colony containing > 16 clusters (thousands of erythroblasts) and considered to arise from a primitive erythroid progenitor cell.



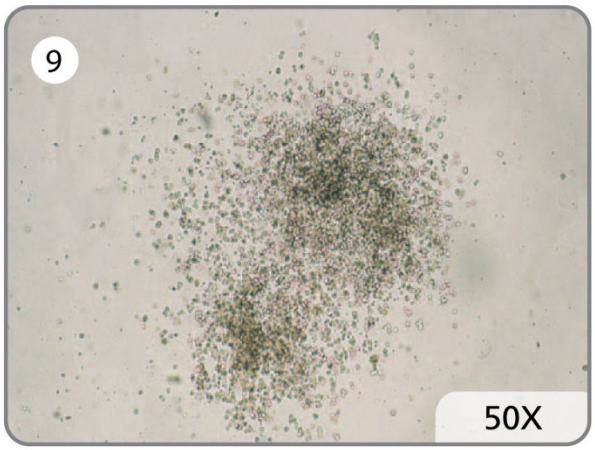
One large BFU-E-derived colony.

BFU-E-derived colony containing multiple clusters.



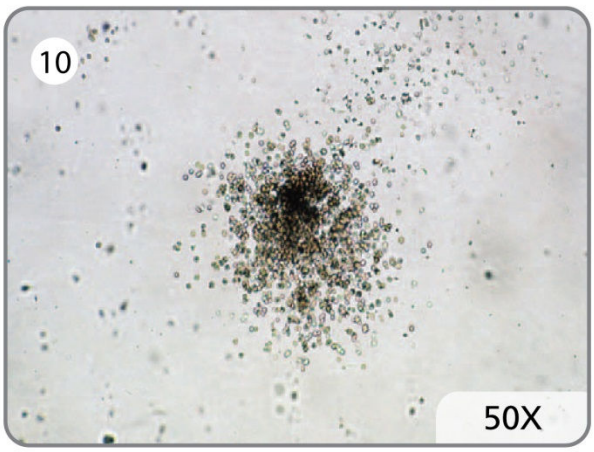
CFU-GM-derived colony.

Note the dense core of cells at the center of the colony and the individual cells distinguishable at the periphery. Monocyte/macrophage cells tend to be larger and more irregular in shape than granulocytes. The small piece of debris seen near the center of the colony does not inhibit CFU growth.

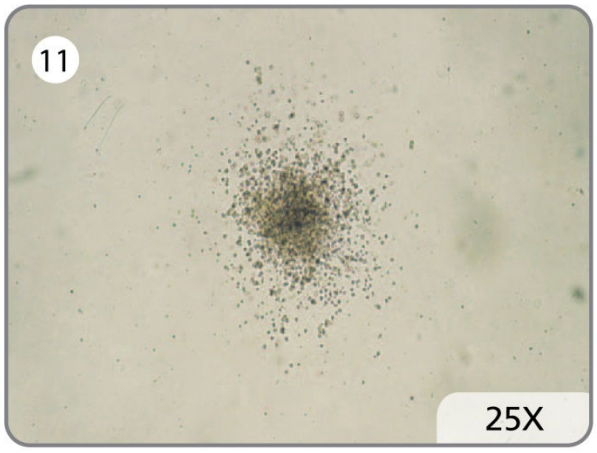


One CFU-GM-derived colony with 2 centers.

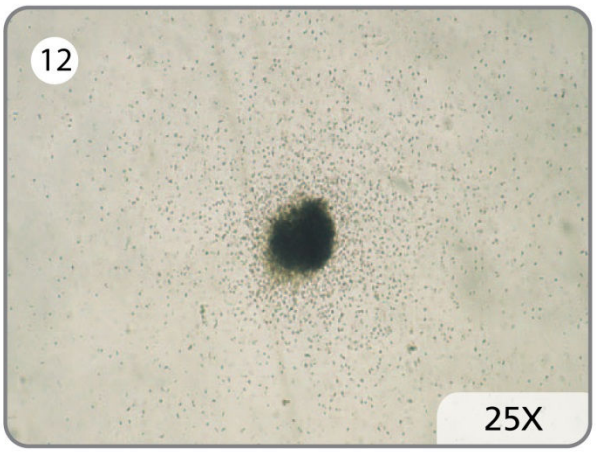
Single CFU-GM-derived colony containing two distinct clusters. Note that each cluster is present on a similar plane with comparable cellular morphology. These features can be used to assist in distinguishing between individual colonies that are close together.



CFU-GM-derived colony.

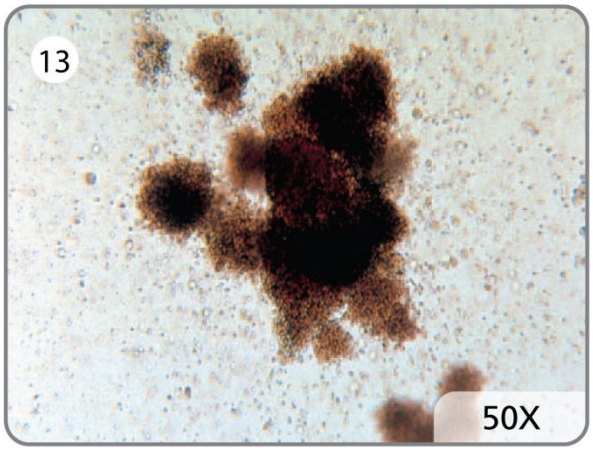


CFU-GM-derived colony.

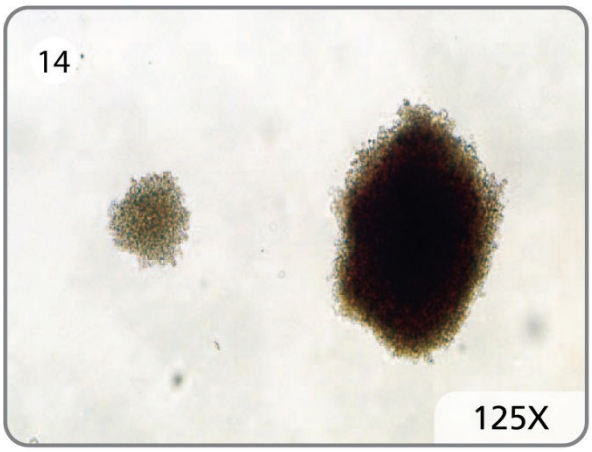


Small CFU-GEMM-derived colony.

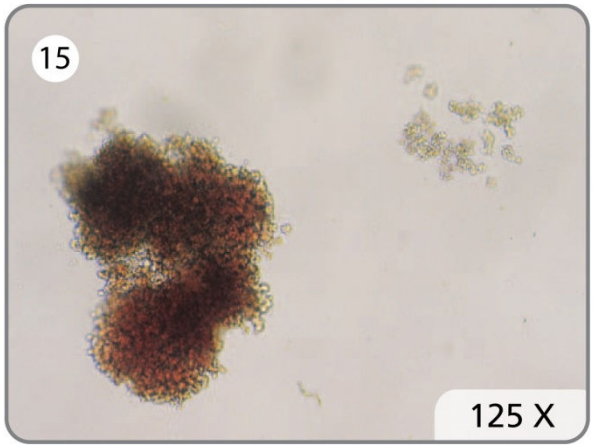
CFU-GEMM-derived colony containing a single dense cluster of erythroid cells at the center surrounded by cells of the granulocyte and monocyte/macrophage lineages. A higher magnification may be required to confirm that the colony contains reddish hemoglobinized erythroblasts. Note that the erythroid cluster is slightly out of focus.



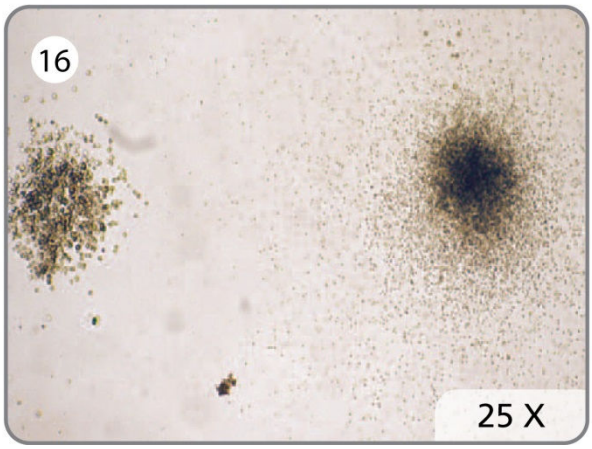
CFU-GEMM-derived colony.
A dense core of erythroid clusters and recognizable granulocyte and macrophage cells at the periphery. There may be a separate BFU-E-derived colony on the lower right-hand side of the photograph. It depends on the overall colony density in the dish to determine whether this colony should be counted separately or considered as part of the larger colony.



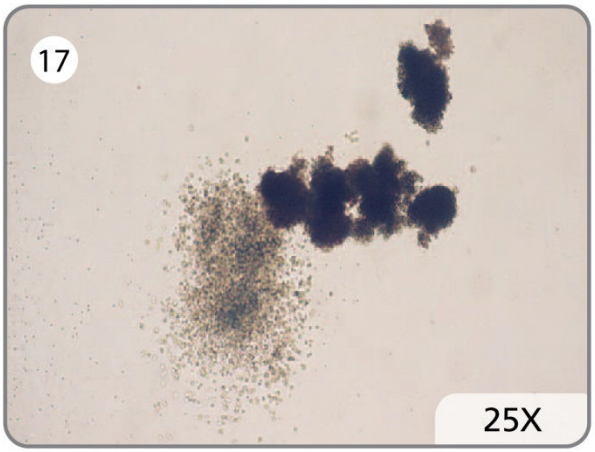
Two BFU-E-derived colonies.



Two BFU-E-derived colonies.
The colony on the left is derived from a more immature progenitor, as it is larger. The cells also show a greater degree of hemoglobinization, which is apparent by the reddish color of the colony. The colony on the right is derived from a more mature progenitor, as it is smaller. The cells show less hemoglobinization, as the color of the colony is paler.

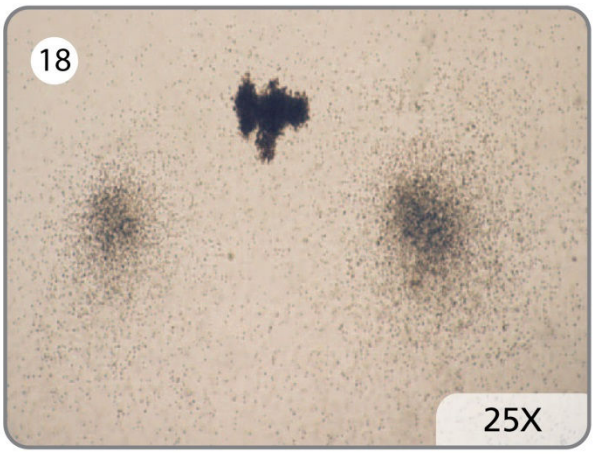


CFU-M-derived colony (left); small erythroid (center low); CFU-G-derived colony (right).
The colony at the left can be counted as a CFU-M-derived colony, as the cells are larger and more irregular in shape than the cells in the colony at the right, which can be counted as a CFU-G-derived colony. Higher magnification is required to confirm whether the small colony in the center is derived from a BFU-E or a CFU-E. This distinction would be made by estimating the number of cells within the colony.

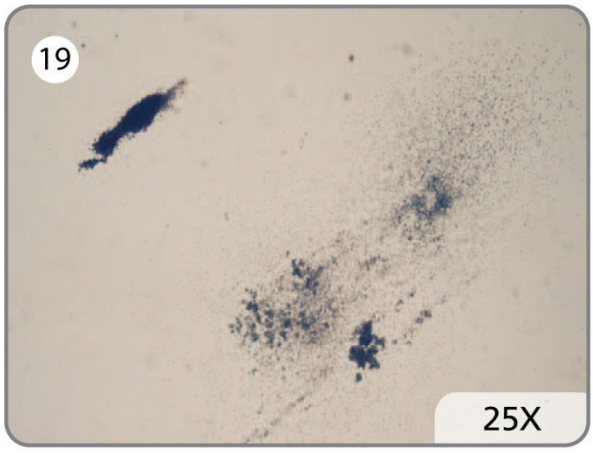


CFU-GM-derived colony (left) and a large BFU-E-derived colony (right).

This is not one colony derived from a single CFU-GEMM progenitor. Both of the colonies appear very distinct and only overlap in one area. Compare the two colonies in this photo to the CFU-GEMM-derived colony in Photo 13.

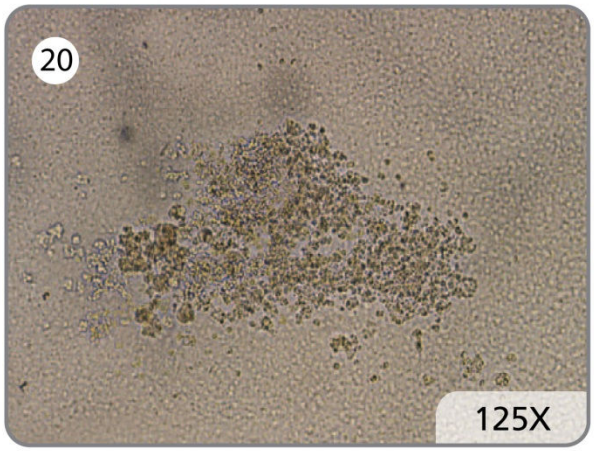


Two CFU-GM-derived colonies (left & right) and one BFU-E-derived colony (center).



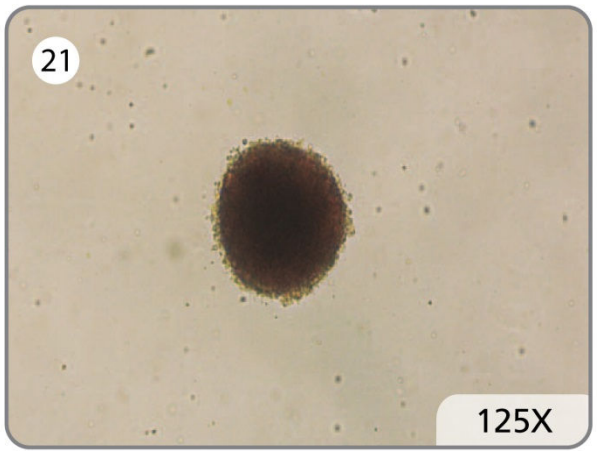
Colonies in culture with 'runny' methylcellulose.

The colonies appear distorted and "streaky." The dishes were disturbed during culture or transport.



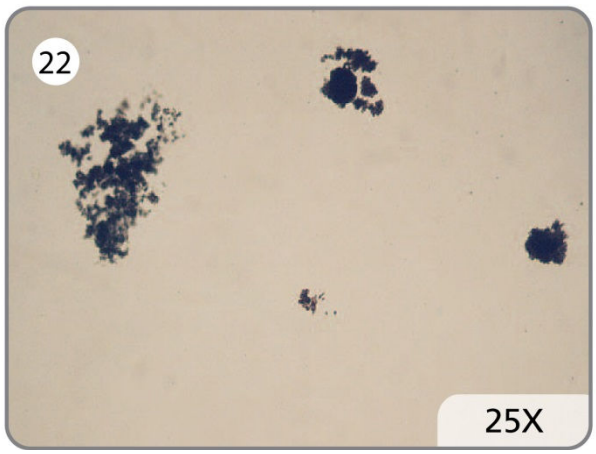
Colonies in 'dried-up' methylcellulose.

Dehydrated or dried-up culture caused by low humidity during culture. To avoid dehydration, ensure that water dishes are used and the water-jacketed incubator chamber contains a water pan filled with water.



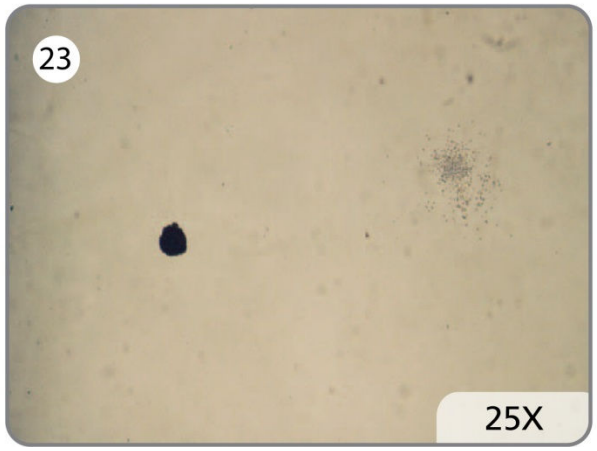
Compact BFU-E-derived colony (resembles a ball).

The morphology is often dependent on the sample.



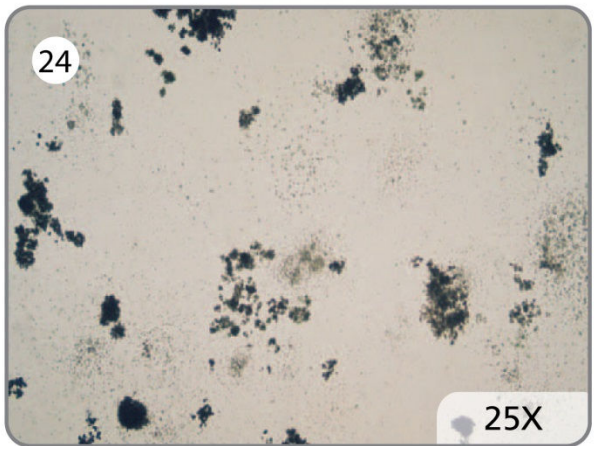
Good plating density.

Good distribution of colonies is required for ensuring optimal CFU growth and the ability to count the colony numbers accurately.



Underplating (approximately 3X too low).

'Underplating'; too few colonies for accurate CFU counting.



Overplating (approximately 5X too high).

'Overplating'; too many colonies for accurate CFU counting. CFU growth may also be inhibited.

11.3 General Descriptions of Colonies in MethoCult™ Express

11.3.1 After 7 Days of Culture

Colonies are visible after 7 days of culture in MethoCult™ Express. They will be small- to medium-sized in comparison to colonies derived from human CB hematopoietic progenitor cells cultured in other MethoCult™ media (e.g. MethoCult™ GF H4034) for the 14- to 16-day assay, or colonies in MethoCult™ Express after 14 - 16 days. Colonies containing more than 20 cells should be counted. Individual colony types cannot be distinguished after 7 days of culture in MethoCult™ Express. If cultures are maintained for 14 - 16 days in MethoCult™ Express, individual 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 progenitor cells are very mobile, even in semi-solid medium, the distance between individual clusters of one colony (i.e. derived from a single progenitor cell) can be larger than the diameter of individual clusters. This is more apparent in the 7-day assay than in the 14- to 16-day assay. This is due to a shorter time for cells within the colony to proliferate and fill the spaces. Refer to Chapter 5 in the Atlas of Hematopoietic Colonies from Cord Blood (Catalog #29940) for detailed descriptions and images of colonies after 7 days of culture in MethoCult™ Express.

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. they are derived from different progenitor cells).

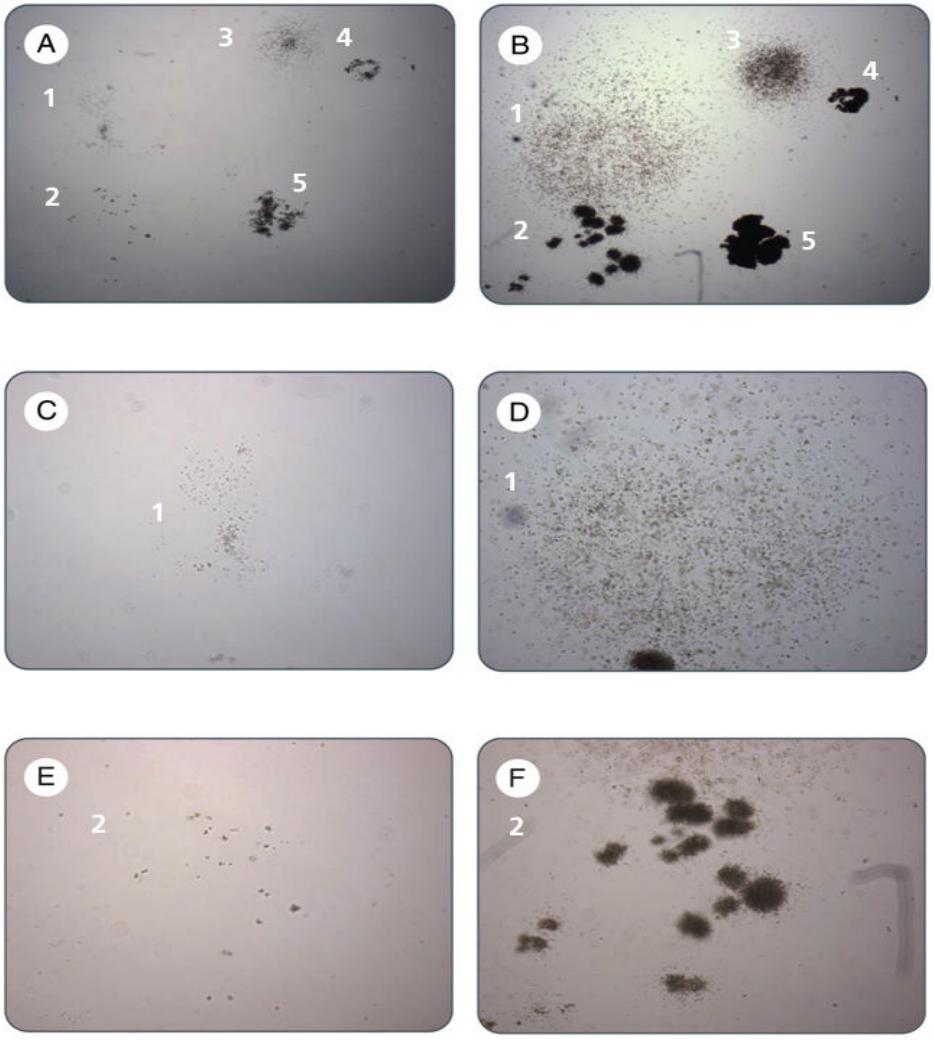
11.3.2 After 14 Days of Culture

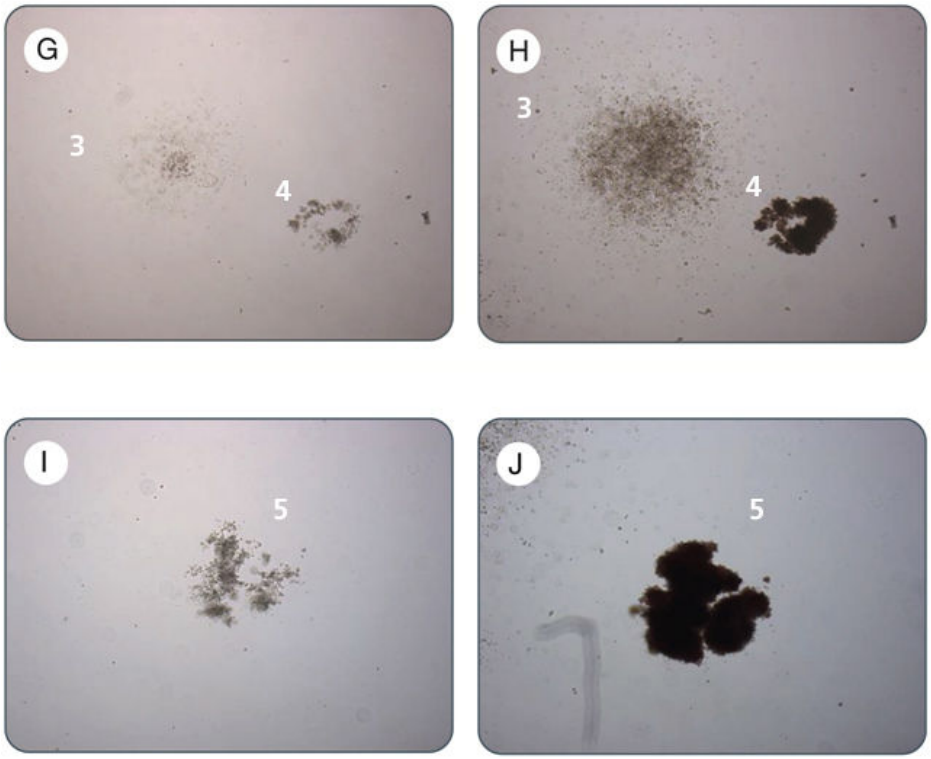
After culture in MethoCult™ Express for 14 - 16 days, colonies derived from BFU-E, CFU-GM, and CFU-GEMM can be distinguished on the basis of their size and morphology. The same counting criteria can be used as for CFU assays in other MethoCult™ media. However, after 14 - 16 days of culture in MethoCult™ Express, colonies tend to be larger than those in other MethoCult™ media and may be more difficult to distinguish from each other, in particular at high colony densities. Refer to section 11.4 for images of BFU-E-, CFU-GM-, and CFU-GEMM-derived colonies after 14 - 16 days of culture in MethoCult™ Express and the same colonies after 7 days of culture.

11.4 Photographs of Colonies in MethoCult™ Express

11.4.1 CFU-GM- and BFU-E-Derived Colonies

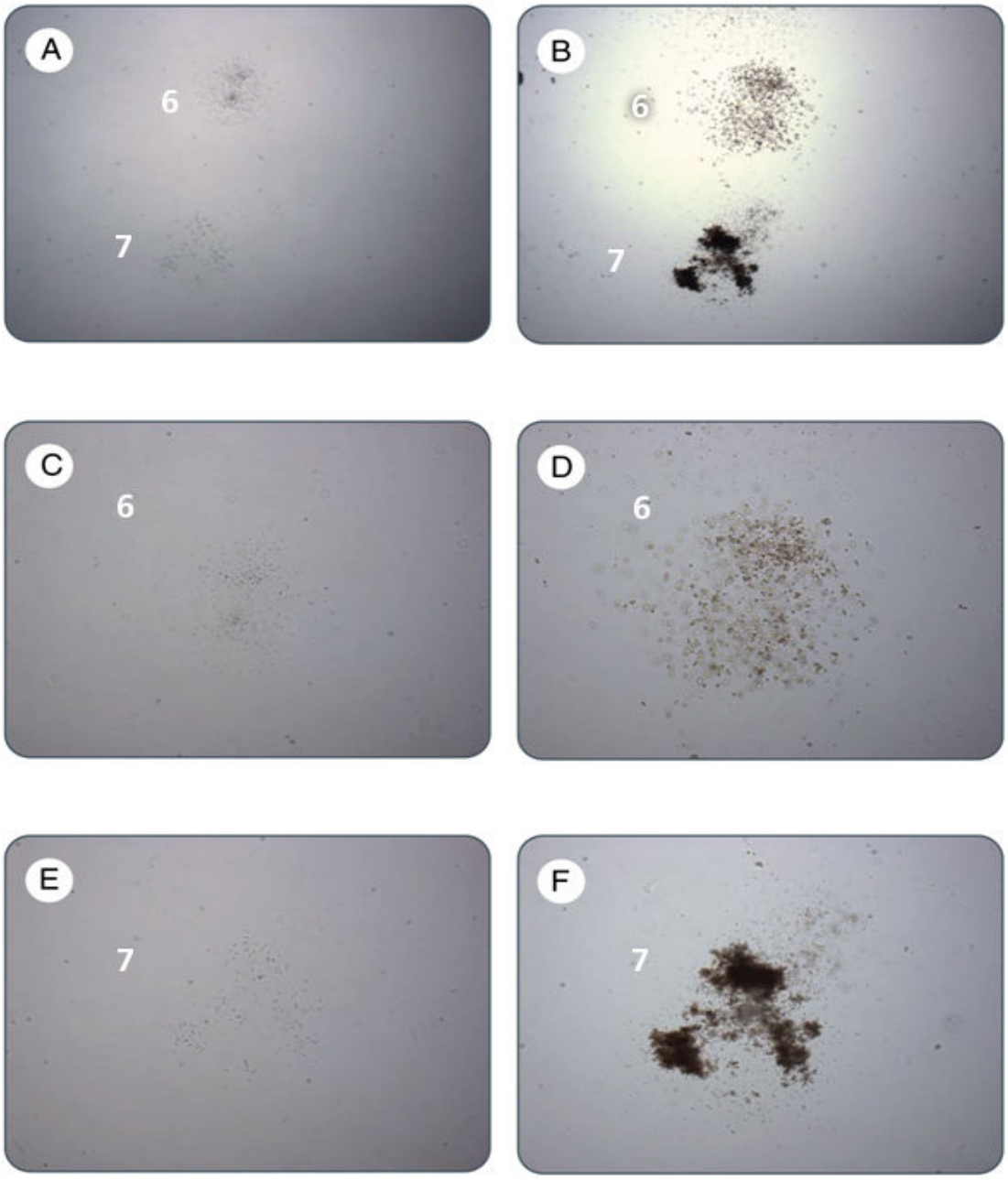
The following photographs show colonies derived from human CB hematopoietic progenitor cells in MethoCult™ Express at Day 7 (left; A, C, E, G, and I) and Day 14 (right; B, D, F, H, and J). Photos A and B show 5 colonies at a 25X magnification. Photos C through J show the same colonies as in A and B, but at a 50X magnification. There are two CFU-GM-derived colonies (colony #1 and 3) and three BFU-E-derived colonies (colony #2, 4, and 5). After 7 days of culture (left), the colonies show differences in size, number of clusters per colony, and morphology. At this point, total colonies can be counted but classification into different colony types is not possible. Classification of colony types is possible after 14 days of culture (right).





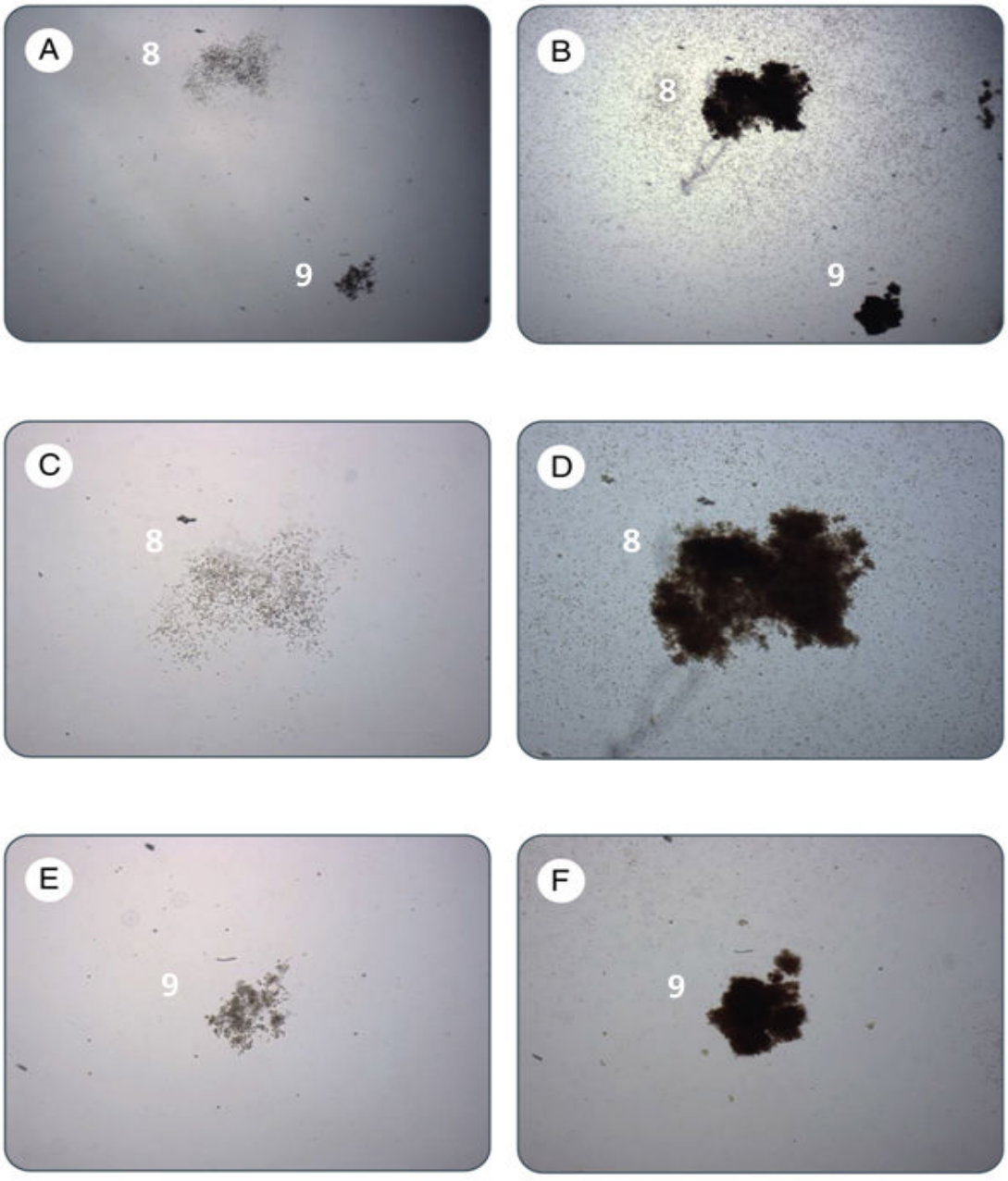
11.4.2 CFU-GM- and CFU-GEMM-Derived Colonies

The following photographs show colonies derived from human CB hematopoietic progenitor cells in MethoCult™ Express at Day 7 (left; A, C, and E) and Day 14 (right; B, D, and F). Photos A and B are shown at 25X magnification. A 50X magnification of the same colonies is shown in photos C through F. Colony 6 is a CFU-GM-derived colony. Colony 7 is a CFU-GEMM-derived colony. Note that the lineage of the colonies cannot be distinguished at Day 7.



11.4.3 CFU-GEMM- and BFU-E-Derived Colonies

The following photographs show colonies derived from human CB hematopoietic progenitor cells in MethoCult™ Express at Day 7 (left; A, C, and E) and Day 14 (right; B, D, and F). Photos A and B are shown at a 25X magnification. A 50X magnification of the same colonies is shown in photos C through F. Colony 8 is derived from a CFU-GEMM. Colony 9 is derived from a BFU-E. Note that the lineage of colonies cannot be distinguished at Day 7.



12.0 Frequently Asked Questions and Helpful Hints

12.1 MethoCult™ Media and Reagents

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

The bottle can either be refrozen at -20°C (-25°C to -15°C) or thawed completely in the refrigerator (2 - 8°C) or at room temperature (15 - 25°C). Once thawed, shake for 30 - 60 seconds to mix completely, and let it stand for at least 5 minutes until all bubbles rise to the top. The bottle can now be aliquoted into tubes. The tubes can either be used immediately or frozen for future use.

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

The methylcellulose in frozen MethoCult™ products is not homogeneous 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 medium appears yellow or violet in color after thawing. Can I still use it?

Yes. This indicates that the pH of the medium has been altered during transport or storage but the performance is unaffected as long the medium has been stored at the recommended temperature range of -25°C to -15°C and used before the expiry date indicated on label. Thaw the bottle and follow recommended protocol for CFU assay setup. 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™ at -80°C?

Yes. The recommended storage temperature range is -25°C to -15°C, but the performance of the MethoCult™ will not be affected by storage at -80°C.

6. I only want to evaluate CFU-GM colonies. Which formulation is recommended?

MethoCult™ media that do not contain EPO are used to detect CFU-GM, CFU-G, and CFU-M colonies. See Table 1 for MethoCult™ formulations that do not contain EPO.

7. Can I add antibiotics or other drugs to incomplete MethoCult™?

Antibiotics, drugs and other components can be added to the medium before the addition of cells. One important consideration is to add all components in volumes that will maintain the correct viscosity of the MethoCult™ medium. Drugs, cells and other components are added to the incomplete methylcellulose formulations as described in Table 5.

To add components to complete, ready-to-use formulations such as MethoCult™ H4434 Classic and MethoCult™ H4534 Classic without EPO, it is necessary to add the cells in a smaller volume and to maintain a 1:10 ratio of the volume of cells plus components relative to the volume of MethoCult™.

If compounds must be added in larger volumes it is recommended to use incomplete methylcellulose formulations. Refer to Table 5 and section 6.2 for details on incomplete MethoCult™ media and the volumes that are available for adding medium components, cytokines, antibiotics, drugs, and other compounds to these media.

If compounds are dissolved in solvents such as DMSO, ensure that the proper solvent-only and other appropriate controls are performed.

8. Is it necessary to add antibiotics to the medium?

Addition of antibiotics should not be required if sterile reagents, certified biosafety cabinets, and good aseptic technique are used. If necessary, penicillin (at a final concentration of 100 units/mL) and streptomycin (at a final concentration of 100 µg/mL) can be included. Anti-fungal agents like amphotericin B can potentially be used, but preliminary experiments must be performed to confirm that the anti-fungal agent does not inhibit the growth of the hematopoietic CFU of interest.

9. What type of incubator should I use and what routine monitoring and cleaning should be performed?

- Culture conditions are very important to ensure optimal hematopoietic colony growth. The incubator should be maintained at 37°C, with 5% CO_2 in air and ≥ 95% humidity.
- We recommend using a water-jacketed incubator with an open pan of water placed in the incubator chamber. A suitable additive (e.g. copper sulfate crystals) can be added to the water in pan to inhibit microbial growth.
- 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.
- It is important to use medical grade CO_2, as inhibition of CFU growth due to toxic substances present in the CO_2 gas source has been reported.
- Incubator conditions should be monitored and recorded at least twice weekly.
- Periodically (e.g. every 6 months), the incubator should be cleaned by removing and autoclaving the incubator trays and wiping down the interior with 70% ethanol.
- Contaminated cultures should be removed immediately and discarded, and the incubator cleaned.

12.2 Cell Sample Preparation

10. Can I use anticoagulants besides heparin, such as EDTA or ACD, for cell sample collection?

Heparin anticoagulated samples are routinely used by researchers performing CFU assays. Other anti-coagulants can be used, but it is important to validate their use in each laboratory. If ACD or EDTA are used during sample collection, additional anticoagulant may need to be added to media for dilution and washing steps, as these non-permanent anticoagulants can be washed away allowing clotting to initiate.

11. The bone marrow arrived late in the day. Can it wait until tomorrow to be processed?

All cell samples should be set up as soon as possible after collection. Samples can be stored overnight in the refrigerator (2 - 8°C), but some loss of cell viability and CFU numbers can be expected.

12. What does a completely lysed (by ammonium chloride treatment) bone marrow sample look like?

The solution will appear translucent-red in color. To assist in evaluating whether complete RBC lysis has occurred, make a mark on the exterior of the clear tube using a permanent felt marker, preferably in black. The mark should be clearly visible through the tube and solution for samples with efficient RBC lysis.

13. After 10 minutes on ice, with intermittent inversion, the bone marrow sample still does not appear lysed. What should I do?

Gently invert the sample and replace on ice. Check at 5-minute intervals until a total of 10 additional minutes has elapsed. The sample should be lysed after this additional incubation period.

12.3 Setup and Culture

14. Why do I need to use a plating cell concentration that yields ~25 - 120 colonies per 1.1 mL culture (standard MethoCult™)?

For accurate quantitation, there should be a linear relationship between the input number of cells and the resulting number of colonies obtained. The presence of too many colonies (overplating) causes inhibition of progenitor proliferation due to depletion of essential nutrients, pH changes, and accumulation of cellular metabolic products. Overplating also causes counting errors because of difficulty in identifying individual colonies. Too few colonies (underplating) may yield statistically inaccurate data. When the number of CFUs in the starting cell suspension is expected to be low, the accuracy may be improved by setting up additional replicates or by enrichment of the cell samples by $CD34^+$ cell selection or depletion of mature, lineage antigen-positive cells.

15. How long should I wait after vortexing before plating MethoCult™ tubes with cells added?

The MethoCult™ and cell mixture can be plated as soon as the bubbles have mostly risen to the top (approximately 5 minutes after vortexing). As this is the mixture in which the cells are cultured, the cells can be left in the tube for a few hours at room temperature (15 - 25°C), if necessary, without adversely affecting subsequent colony formation. Vortex gently before plating if tubes have been left for a period of time.

16. Some of my cell samples are from patients with myeloproliferative disorders. How can I estimate the number of colonies to plate based on the normal plating concentrations provided?

In some myeloproliferative syndromes the frequency of progenitor cells relative to other cell types may be substantially increased. In such cases, it may be necessary to dilute the cells further to decrease the final cell concentration by 4- to 10-fold or more. If in doubt, always plate several concentrations, with 2- to 4-fold differences between each concentration.

17. Why are my cultures yellow?

This is due to an increase in metabolic byproducts, resulting in a decrease in pH, thereby changing the medium to a yellow color. This happens because of:

- Overplating: presence of a high number of colonies (approximately > 200)
- Contamination: presence of bacteria or fungi

Either cause can be confirmed by viewing the dish under the microscope. Very high CO_2 concentrations in the incubator can also cause medium to become acidic.

18. Why are my cultures cloudy?

This is usually caused by bacterial, fungal or yeast contamination. Scanning the cultures under high power can confirm the presence of microbial growth, sometimes visible as small grainy specks (bacteria or yeast) or branching strands (fungal). Also, look for the presence of bacterial colonies, typically appearing as round, smooth, white or yellow colonies. Methylcellulose can also appear more liquid when contaminated.

19. I have colonies that don't appear to contain hematopoietic cells. What are they?

Look at the colony under low power and also high power if necessary. If the colony does not appear to resemble a typical hematopoietic colony (presence of discernible cells), and is either:

- Solid in the center as well as the periphery, with 'leaf-like' protrusions
    - This is most likely a fungal colony.

OR

- Round, opaque, and white or yellow
    - This is most likely a bacterial colony or yeast.

20. Why are colonies present in my methylcellulose-based medium without cytokines (i.e. MethoCult™ H4230 made to the correct volume with IMDM only)?

Sufficient growth-promoting factors are present in the medium (i.e. FBS) to promote proliferation of some hematopoietic progenitor cells, often CFU-M. The hematopoietic cells initially present in the culture and those within the colonies also produce growth factors such as IL-6 that promote CFU growth within the culture (referred to as 'endogenous feeding'). The number and size of these colonies is greater when using minimally purified cell suspensions (ammonium chloride-treated samples and MNC suspensions) or when too many hematopoietic progenitor cells are present in the plated cell suspension (overplating). Control cultures (without cytokines) are not required for routine CFU counting, but should be included when evaluating the efficacy of exogenously added factors or drugs.

21. The methylcellulose in my cultures appears 'cracked' when viewed under the microscope. If viewed macroscopically from the side, the culture appears thinner than normal. What happened?

The cultures are dehydrated. Check that water has been added to water dish(es). To maintain a high humidity (≥ 95%), use of a water-jacketed incubator with a water pan placed within the incubator chamber is recommended. It may still be possible to count colonies in dehydrated cultures, assuming that dehydration occurred at a late stage of the culture. However, colony counts and identification of colony types may not be accurate.

22. The colonies in the culture are 'streaming' across the dish. What's wrong?

Cultures have been tipped or knocked while in the incubator or when transporting, or the viscosity of methylcellulose-based medium is too low. MethoCult™ medium that has not been thoroughly mixed prior to aliquoting results in some tubes containing a lower-viscosity medium (and some tubes containing a higher-viscosity medium). Lower viscosity can also be caused by adding incorrect volumes of components to incomplete medium formulations (see Table 5) or by adding cells in volumes greater than the 1:10 (v/v) ratio. MethoCult™ is formulated with optimal viscosity for colony formation at a 1:10 ratio, so > 1:10 ratio will result in colonies that are not formed in discrete clusters and will appear to 'stream' across the dish when the dish is moved. Conversely, a ratio < 1:10 will result in colonies that are extremely compact and appear as 'tight' balls of cells.

12.4 Counting Human CFU Assays

23. Why is the number of colonies lower than expected?

Possible reasons:

- Errors in cell counts or cell dilutions, resulting in too few cells being plated in the CFU assay
- Contamination of cultures by bacteria, yeast, or fungi
    - Bacterial contamination often results in the medium having a milky, orange color. Contamination is often caused by lack of good sterile technique or contaminated reagents. If contamination occurs, be sure to discard all contaminated cultures and opened bottles of media used for cell processing, and sanitize the incubator using recommended procedures.
- Improper incubator conditions
    - Dehydration of cultures can occur if high humidity (≥ 95%) is not maintained over the culture period, and even small changes in humidity can affect colony growth. Use of small chamber water-jacketed incubators, a water pan in the incubator, and dishes containing sterile water are recommended. The incubator temperature and CO_2 levels should be routinely monitored using a thermometer placed within the chamber and a Fyrite® Gas Analyzer, respectively. No CO_2 or low CO_2 levels result in the medium becoming purple in color. It is important to use medical-grade CO_2 for incubators, as inhibition of colony growth by unknown contaminants in the gas source has been reported.

- Patient samples
    - When it is difficult to anticipate the correct cell plating concentration, the use of two or more 2- to 3-fold serially diluted cell concentrations is advised.
- MethoCult™ medium has expired or has not been stored properly
- Loss of progenitor cells in the cell suspension
    - The CFU assay should be set up as soon as possible following isolation of the cells, as the viability of the progenitor cells may decline with time. If there is a long delay between processing of the cells and CFU assay setup (~6 - 8 hours), assess the viability of the cells prior to plating and adjust the amount of cells plated based on the % viable cells.

24. My cultures contain too many (> 120) colonies or too few (< 25) colonies. What should I do next time?

When it is difficult to anticipate the correct plating cell concentration, the use of two or more cell concentrations is advised.

25. I get motion sickness counting the colonies. How can I alleviate this problem?

This problem is common with individuals new to counting CFU assays. Limit the time spent at the microscope to just one hour at a time to start. Most importantly, counting is made easier if counting is done in vertical rows by moving the stage control knob up and down rather than side-to-side across the dish.

26. How can I learn to count CFU numbers accurately and reproducibly?

Practice, practice, practice. Learning tips include:

- Use training aides such as our Hematopoietic Colony Atlases (Catalog #28700, 28760, 29940) and online training tools.
- Initially, spend 1 - 2 hours per day, several days per week learning to recognize the different CFU types and count accurately. Count the same cultures on different days. Cultures placed at 33°C, with 5% CO_2 and ≥ 95% humidity will maintain good morphology for at least 1 week in addition to the initial culture period (~21 days total).
- Do comparative counting with qualified co-workers.
- Attend a STEMCELL Technologies Training Course. See section 13.1.1 for more information or visit www.stemcell.com.
- Enroll in one of the Proficiency Testing Programs offered by STEMCELL Technologies. See section 13.1.2 for more information or visit www.stemcell.com.

27. Can I use the cells in the colonies for further analysis, such as cytospins or PCR?

Colonies can be harvested ("plucked") from MethoCult™ for further analysis. For specialized applications, such as preparation of cytospins for cytotochemical staining, PCR or RNA isolation, it is often necessary to isolate individual colonies from cultures at an earlier time point to ensure a higher proportion of viable cells within the colony. Individual colonies or the cells from the entire culture can be isolated following 7 - 10 days of incubation. For detailed procedures, contact us at techsupport@stemcell.com.

28. Is it possible to distinguish different colony types in MethoCult™ Express after 7 days of culture?

No. Although some erythroid colonies can be identified after 7 days of culture in MethoCult™ Express 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. Most colonies in MethoCult™ Express 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.

29. 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?

A custom-made MethoCult™ Express medium without EPO would be more specific for CFU-GM than the regular medium with EPO. However, even in the absence of EPO, some BFU-E may form colonies after 7 days of culture; particularly for immature BFU-E, survival and first rounds of proliferation are not dependent on EPO. EPO is only essential for the survival, proliferation, and differentiation in later stages of development of hemoglobinized BFU-E colonies.[5]

30. I can't wait 14 - 16 days to count the colonies in MethoCult™ Classic, Optimum, or Enriched. Can I count the colonies after 7 days?

Most progenitor cells need approximately 14 days to fully develop into colonies containing morphologically recognizable cells. In MethoCult™ media, colonies can be detected earlier, but after only 7 days most colonies are still small and undifferentiated and it is not yet possible to accurately distinguish different colony types. In addition, the number of colonies detectable after 7 days in standard MethoCult™ media may not be representative of the number of colonies present after 14 days. For 7-day colony assays we recommend MethoCult™ Express. This medium has been formulated for accelerated progenitor cell proliferation and colony formation, resulting in larger colonies compared to standard medium. Day 7 *total* CFU numbers in MethoCult™ Express show excellent correlation with Day 14 *total* CFU numbers in MethoCult™ Express and other MethoCult™ media. For further information visit www.stemcell.com or contact us at techsupport@stemcell.com.

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

This is not recommended. 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.

32. Is the total CFU 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 MPB stem cell (PBSC) grafts with respect to neutrophil and platelet engraftment and/or survival.[6-12] Measuring total CFU numbers rather than only CFU-GM numbers has several advantages. It does not require counting CFU-GM colonies separately or culturing progenitor cells under conditions that favor only CFU-GM colony formation. In addition, CFU-GM represent roughly one half of the progenitor cells present in a sample and measuring only CFU-GM may give less significant and less reliable results than measuring total CFU.

33. Do CFU 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 CFU assays or in any other short-term assay consist of committed progenitor cells, 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 progenitor cells that have much lower frequency than committed progenitor cells and are generally not detectable in CFU assays or other short-term assays. Measuring the frequency and growth of committed progenitor cells 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 with other parameters such as total nucleated count (TNC) and $CD34^+$ cell numbers.[6-12] For example, if a candidate CB unit has acceptable TNC numbers but lower CFU 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 CFU assay results show that 90% of CFUs 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.

13.0 Appendices

13.1 Related Products and Services

STEMCELL Technologies offers a wide range of products to complement MethoCult™ media for human hematopoietic CFU assays. For more information, visit www.stemcell.com, contact your Technical Representative, or contact us at techsupport@stemcell.com.

13.1.1 Training Course

Standardization of the Hematopoietic Progenitor Assay Training Course (Catalog #00215)

The popular Hematopoietic Progenitor Assay Training Course is held over two days in order to allow for "hands-on" participation in the lab, including practice in identifying and counting colonies, and in-depth discussion of topics. The Scientists at STEMCELL Technologies will provide their knowledge and expertise to help you overcome challenges in assay setup and evaluation.

Course content is customized to meet the needs of participants, and enrolment is limited to ensure personalized instruction.

Visit www.stemcell.com or contact a Technical Representative for more information.

13.1.2 Proficiency Testing

STEMCELL Technologies is committed to standardizing hematopoietic colony assays through numerous products and services. Our Proficiency Testing Programs allow for the comparison of progenitor quantification among laboratories worldwide, with the goal of standardizing all steps of hematopoietic colony assays. Participants in the programs are assessed on their proficiency at performing CFU assays, with a focus on sample preparation, plating, and setup of the CFU assay, and colony counting and identification. Data analysis and reporting methods are designed to match the recommendations and guidelines outlined in the International Standard ISO 13528.

For further information and to register for Proficiency Testing, contact us at proficiency@stemcell.com or visit www.stemcell.com/education.

13.1.3 Contract Assay Services

Contract Assay Services is a contract research organization (CRO) established within STEMCELL Technologies that offers services in performing primary cell-based assays including the CFU assay. Their services include:

- Confidential consultation with the experts
- Custom-designed studies to meet your requirements
- Studies performed following Good Laboratory Practices, using STEMCELL's industry standard reagents manufactured under ISO13485 guidelines
- Thorough and timely reporting of data and report followup
- Customized educational and training courses to fit your needs

Visit www.contractassay.com for more information.

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DOCUMENT #28404 VERSION 4.5.0