TECHNICAL MANUAL

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

STEMCELL™ TECHNOLOGIES

Table of Contents

1.0 Introduction

In adult mouse bone marrow (BM), a small number of hematopoietic stem cells (HSCs) produce heterogeneous populations of actively dividing hematopoetic progenitor cells. These hematopoietic progenitor cells proliferate and differentiate, resulting in the generation of millions of mature blood cells daily.[1]

During differentiation to mature blood cells, the progeny of HSCs go through intermediate stages prior to reaching maturity, including multi-potential progenitor cells and lineage-committed progenitor cells. 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. 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 detection and counting of mouse CFUs in MethoCult™ methylcellulose-based medium. Depending on the formulation, MethoCult™ media support optimal growth of multiple types of hematopoietic 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])
- B lymphocyte progenitor cells (CFU-pre-B)

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
BIT	Bovine serum albumin, insulin, and transferrin
BM	Bone marrow
BFU-E	Burst-forming unit-erythroid
BSA	Bovine serum albumin
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
EPO	Erythropoietin
FL	Fetal liver
FBS	Fetal bovine serum
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
IL-3	Interleukin 3
IL-6	Interleukin 6
IL-7	Interleukin 7
IMDM	Iscove's Modified Dulbecco's Medium
M-CSF	Macrophage colony-stimulating factor
PB	Peripheral blood
PC	Postcoitus
rh	Recombinant human
rm	Recombinant mouse
SCF	Stem cell factor
TPO	Thrombopoietin

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

STEMCELL Technologies Inc. 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 mouse 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 Mouse Hematopoietic Cells

PRODUCT NAME	CATALOG #	CONTAINS	APPLICATIONS
COMPLETE METHOCULT™ MEDIA			
MethoCult™ GF M3434	03434/03444	rm SCF, rm IL-3, rh IL-6, rh EPO, rh insulin, human transferrin (iron-saturated)	Detection of BFU-E, CFU-GM, CFU-GEMM in BM, PB, spleen, and FL
MethoCult™ GF M3534	03534	rm SCF, rm IL-3, rh IL-6, rh insulin, human transferrin (iron-saturated)	Detection of CFU-GM in BM, PB, spleen, and FL
MethoCult™ M3630	03630	rh IL-7	Detection of CFU-pre-B in BM
MethoCult™ M3334	03334	rh insulin, human transferrin (iron-saturated), rh EPO	Detection of CFU-E
MethoCult™ SF M3436	03436	rh insulin, human transferrin (iron-saturated), cytokines (including rh EPO)	Detection of BFU-E
INCOMPLETE METHOCULT™ MEDIA			
MethoCult™ M3234	03234	rh insulin, human transferrin (iron-saturated); **no** 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
MethoCult™ M3231	03231	**no** rh insulin, **no** human transferrin, **no** rh EPO, **no** additional cytokines	
MethoCult™ SF M3236	03236	rh insulin, human transferrin (iron-saturated); serum-free, contains serum substitutes; **no** rh EPO, **no** additional cytokines	
MethoCult™ M3134	03134	Contains only methylcellulose in IMDM; **no** rh EPO, **no** additional cytokines, **no** serum or serum substitutes	

Table 2. Recombinant Cytokines for Culture of Mouse Hematopoietic Cells

PRODUCT	CATALOG #	UNIT SIZE	DESCRIPTION
Mouse Recombinant G-CSF Human Recombinant G-CSF	78014 78012	100 µg 100 µg	For growth of granulocytic progenitor cells
Mouse Recombinant GM-CSF	78017	100 µg	For growth of granulocytic and monocytic progenitor cells
Mouse Recombinant IL-3	78042	100 µg	Used in combination with other cytokines to promote growth of early myeloid progenitor cells of all lineages
Mouse Recombinant IL-6 Human Recombinant IL-6	78052 78050	100 µg 100 µg	Pleiotropic cytokine for growth and differentiation of hematopoietic progenitor cells
Mouse Recombinant M-CSF	78059	100 µg	For growth of monocytic progenitor cells
Mouse Recombinant SCF	78064	100 µg	For growth of mast cells and used in combination with other cytokines to promote growth of myeloid and lymphoid progenitor cells
Mouse Recombinant TPO TPO, Human, Recombinant	78072 02922	50 µg 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

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

Table 3. Support Products for Mouse CFU Assays Using MethoCult™

PRODUCT	CATALOG #	UNIT SIZE	DESCRIPTION
10% BSA Solution in Iscove's MDM	09300	100 mL	For supplementing incomplete methylcellulose-based media, such as MethoCult™ M3134
3% Acetic Acid with Methylene Blue	07060	100 mL	For counting nucleated cells
40 µm Cell Strainer	27305	50/case	For preparation of single-cell suspensions
Alpha MEM with Nucleosides	36450	500 mL	For washing and diluting nucleated cells
Ammonium Chloride Solution	07800 07850	100 mL 500 mL	For lysis of red blood cells
BIT 9500	09500	100 mL	Serum substitute for CFU assays and other hematopoietic cell cultures in serum-free media
Dulbecco's Phosphate Buffered Saline (D-PBS) with 2% Fetal Bovine Serum	07905	500 mL	For washing and diluting nucleated cells
Iscove's MDM with 2% FBS	07700	100 mL	For washing and diluting hematopoietic cells
Iscoves Modified Dulbecco's Medium	36150	500 mL	With 25 mM HEPES; for washing and diluting hematopoietic cells in serum-free conditions
L-Glutamine	07100	100 mL	Medium supplement; required for cell culture in vitro
Trypan Blue	07050	100 mL	For counting viable cells
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 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

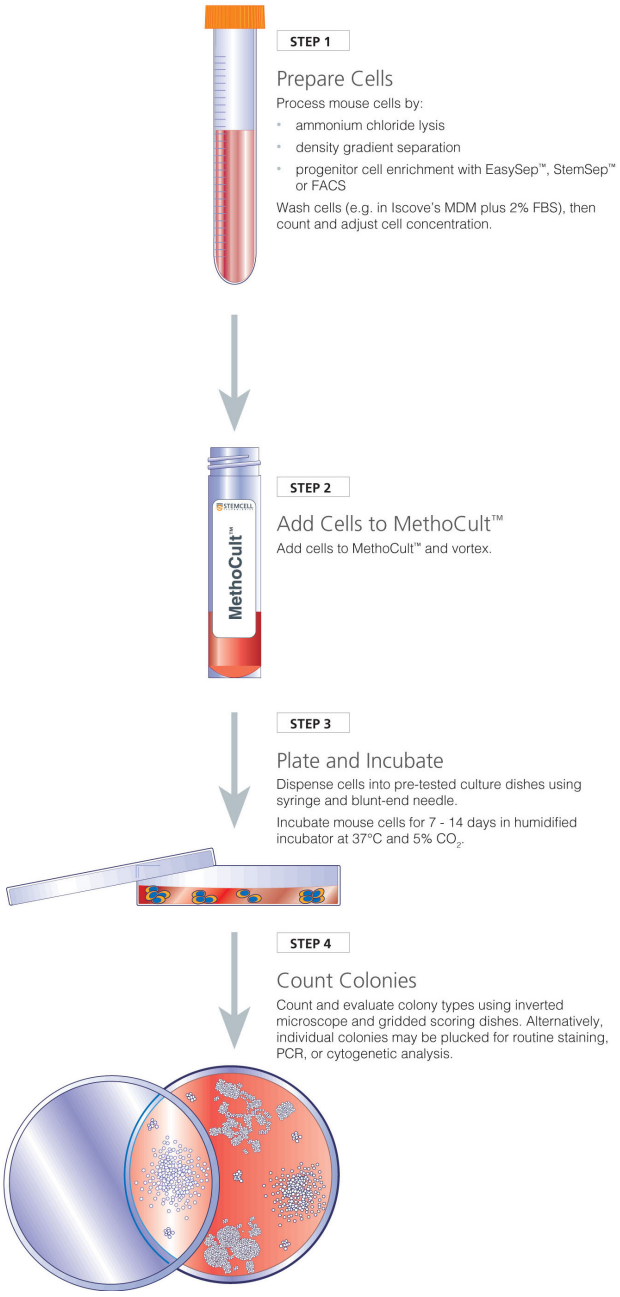
  Use of a quality inverted microscope equipped with a 10X or 12.5X eyepiece objective, 2X, 4X, and 10X planar objectives, and a blue filter is recommended.

- Standard light microscope for cell counting
- Automated cell counter or Neubauer hemocytometer
- Laboratory centrifuge
- Vortex
- Pipette-aid and micropipettors

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 Mouse Hematopoietic CFU Assays in MethoCult™ Medium: Procedure Diagram



6.0 Preparation of MethoCult™ Media

For storage and stability instructions, refer to the Product Information Sheet (PIS) included with 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 section 6.1 and 6.2.

The preparation of complete and incomplete MethoCult™ media is summarized in Table 4 below, and further detailed in sections 6.1 and 6.2.

Table 4. Preparation of MethoCult™ Media

		COMPLETE METHOCULT™	INCOMPLETE METHOCULT™		
Catalog #		03434, 03534, 03630, 03436,	03334	03234, 03236, 03231	03134
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)					
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)					
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 the MethoCult™ product at a 1:10 (v/v) ratio, which maintains the optimal viscosity of the medium.

Refer to Table 4 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 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™ bottles to yield a final 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 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 Iscove's MDM (IMDM) with 25 mM HEPES to yield a total volume of 100 mL (see Table 4 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 4 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 (Catalog #36150) to tubes of MethoCult™. See Table 4 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 4 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 4 for required volumes), store at -20°C, then add desired components after thawing. Mix well before use.

7.0 Cell Sample Preparation

Laboratory mouse strains are routinely used between 6 - 12 weeks of age. For younger or older animals, transgenic mouse strains, and compound-treated mice, it is important to use strain- and age-matched controls.

The number of nucleated cells isolated from the BM and spleen may vary depending on the age of the animal. Unless individual animals are being evaluated, the cells from two or more mice should be pooled.

Note: Mice should be maintained and sacrificed using protocols and procedures approved by the institution.

7.1 Bone Marrow Cells

1. Sacrifice mice using procedures approved by your institution.
2. Remove the femurs and tibias from each mouse. Ensure that the bones are free of skin and muscle tissues.
3. Use a scalpel to cut the ends of the bones (epiphyses) to expose the interior of the marrow shaft. Place bones into a sterile mortar.

   Note: Cells should be isolated as soon as possible after animal is sacrificed.
4. Add 5 mL of appropriate medium to mortar.

   Note: Suitable media for isolating cells include IMDM with 25 mM HEPES, IMDM with 2% FBS, Alpha MEM, PBS, or PBS with 2% FBS. Cells should be isolated in small volumes of medium.
5. Use a pestle to crack open the bones and grind, agitating mixture to free the bone marrow.
6. Pipette cell suspension into a sterile 50 mL tube through a 40 µm Cell Strainer.
7. Add another ~5 mL of medium and repeat steps 5 - 6 until the reddish color disappears from the medium and bones.
8. Centrifuge tube at 300 x *g* for 10 minutes.
9. Pour off supernatant and resuspend cells in medium at the cell concentration required for testing.
10. To make a single-cell suspension, gently draw medium and cells up and down with a 3 mL syringe and 21 gauge needle. Repeat 3 - 4 times, keeping needle below medium surface.
11. Keep the cells in medium on ice until use.

7.2 Spleen Cells

1. Sacrifice mouse using procedures approved by your institution.
2. Remove spleen and place the spleen in a sterile Petri dish on ice or in cold sterile culture medium.

   Note: Suitable media for isolating cells include IMDM with 25 mM HEPES, IMDM with 2% FBS, Alpha MEM, PBS, or PBS with 2% FBS. Cells should be isolated in small volumes of medium.
3. To prepare a single-cell suspension, place spleen(s) on a 40 µm Cell Strainer within a 35 mm dish. One strainer can be used for up to 3 spleens at once.
4. Mince the spleen(s) using fine sterile scissors.
5. Place the strainer on top of a 50 mL tube containing 2 - 3 mL of medium with 2% FBS. Gently press tissue through strainer using the end of a sterile plunger from a 3 mL syringe into the medium.
6. Rinse cells in strainer using an additional small volume of medium.
7. Cell aggregates can be disrupted by drawing the cell suspension up and down 3 - 4 times using a 3 mL syringe and 21 gauge needle if required.
8. Place cells in a 14 mL sterile culture tube and let stand for 3 - 5 minutes to allow tissue fragments to settle. Transfer cell suspension to a new sterile culture tube.
9. If desired, wash cell suspension twice and resuspend in a small volume of medium with 2% FBS or 0.1% BSA.

7.3 Peripheral Blood

1. Attach a 21 gauge needle to a 1 mL sterile syringe. Draw up small volume of anticoagulant solution (e.g. 100 IU/mL heparin) to wet the interior of the syringe. Expel the solution, leaving approximately 20 - 50 µL of anticoagulant in the needle and syringe.
2. Sacrifice mouse according to procedures approved by your institution.
3. Collect blood according to the regulations at the institution using the needle prepared in step 1.
4. Place peripheral blood (PB) in a 14 mL culture tube containing sufficient heparin to give a final concentration of 15 - 20 IU/mL.
5. Lysis of red blood cells in PB samples is recommended. Mix 1 part blood with 9 parts Ammonium Chloride Solution. Cap tightly and mix by inverting tube 3 - 4 times.
6. Incubate on ice for 5 - 15 minutes, mixing several times.
7. Centrifuge at 300 x *g* for 6 minutes. Carefully pour off or aspirate the Ammonium Chloride Solution.
8. Resuspend cells in a small volume of medium using a sterile pipette.

   Note: Suitable media for isolating cells include IMDM with 25 mM HEPES, IMDM with 2% FBS, Alpha MEM, PBS, or PBS with 2% FBS.
9. Wash cells twice and resuspend in a small volume of medium.

7.4 Fetal Liver, Day 10 - 16 PC

1. Sacrifice mouse according to procedures approved by your institution. Remove placenta-fetuses from the animal and place in a sterile Petri dish on ice. If there will be a delay in isolating cells, place placenta-fetuses in culture medium in a sterile 50 mL tube.

   Note: Suitable media for isolating cells include IMDM with 25 mM HEPES, IMDM with 2% FBS, Alpha MEM, PBS, or PBS with 2% FBS. Cells should be isolated in small volumes of medium.

2. Place placenta-fetuses in a Petri dish with cold medium. Using sterile fine scissors and forceps, isolate individual fetuses and place in new Petri dish with medium.
3. To isolate the fetal liver, place a fetus in a new Petri dish (no medium). Isolate fetal livers and transfer to a new dish containing ~1 mL of medium.
4. Using scissors, finely mince fetal livers. Add 1 - 2 mL of medium. Cell aggregates can be disrupted by drawing the cell suspension up and down 3 - 4 times using a 3 mL syringe and 21 gauge needle.
5. Place cells in a 14 mL sterile culture tube and let stand for 3 - 5 minutes to allow tissue fragments to settle. Transfer cell suspension to new sterile culture tube. Fill tube with medium and centrifuge at 300 x *g* for 10 minutes. Resuspend cells in a small volume of medium and mix using a sterile pipette.

   Note: In normal Day 14.5 fetal livers, > 80% of the cells are nucleated erythroid precursors that are not removed by ammonium chloride lysis.

6. Wash cells twice and resuspend in a small volume of medium with 2% FBS or 0.1% BSA.

7.5 Manual Cell Counts

For a manual nucleated cell count procedure using a hemocytometer, refer to the Product Information Sheet (PIS) for 3% Acetic Acid with Methylene Blue (Document #29604), available at www.stemcell.com, or contact us to request a copy. The expected numbers of nucleated cells for various tissue types are given in Table 5 below.

Table 5. Expected Numbers of Nucleated Cells

TISSUE TYPE	TOTAL NUCLEATED CELLS
Femur	$1 - 2 \times 10^7$
Tibia	$0.6 - 1 \times 10^7$
Spleen	$1 - 2 \times 10^8$
Peripheral blood (1 mL)	$3 - 5 \times 10^6$
Day 14.5 PC fetal liver	1×10^7

8.0 CFU Assay Setup

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

Cells are diluted to the appropriate cell concentration for the cell sample in IMDM + 2% FBS or IMDM supplemented with 0.1% BSA for serum-free conditions. Recommended plating concentrations for cells from different sources are listed in Table 6. Once diluted, the cell sample can be added directly to the 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 - 100 colonies per 1.1 mL culture in a 35 mm dish. 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 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.

8.1 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 8.2.

1. Thaw aliquoted tubes of incomplete MethoCult™ medium at room temperature (15 - 25°C) or overnight at 2 - 8°C. Refer to section 6.2.
2. Add desired component(s) to tubes of MethoCult™ medium. Component volumes should not exceed the volumes outlined in Table 4.

   Example: A previously dispensed 2.4 mL tube of MethoCult™ M3234 requires the addition of components to a total of 3 mL for duplicate cultures. For each 2.4 mL of MethoCult™ M3234, add 0.6 mL of components.
3. Vortex tube to ensure that all components are thoroughly mixed.
4. Continue with the instructions in section 8.2, starting at step 2.

8.2 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 + 2% FBS (or IMDM supplemented with 0.1% BSA for serum-free conditions) to 10X the final concentration(s) required for plating. Refer to Table 6 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. When it is difficult to anticipate the correct plating cell concentration, use 2 or more cell concentrations that differ by 2- to 3-fold. Example: 2 x 10^4 cells per dish and 1 x 10^4 cells per dish.*

   Note: The plating concentration may be difficult to anticipate with strains other than C57BL/6 and BALB/c, as well as genetically modified strains.

Table 6. Recommended Plating Concentrations and Culture Incubation Times

METHOCULT™ FORMULATION	PROGENITOR CELLS DETECTED	TISSUE	INCUBATION TIME	10X CONCENTRATION TO BE PREPARED*	PLATING CONCENTRATION (cells per 35 mm dish)*
MethoCult™ GF M3434	BFU-E, CFU-GM, CFU-GEMM	Bone marrow	10 - 12 days	2 - 3 x 10^5	2 - 3 x 10^4
		Spleen		1 - 3 x 10^6	1 - 3 x 10^5
		Peripheral blood		1 - 3 x 10^6	1 - 3 x 10^5
		Fetal liver		2 x 10^5	2 x 10^4
MethoCult™ GF M3534	CFU-GM	Bone marrow	10 - 12 days	2 - 3 x 10^5	2 - 3 x 10^4
		Spleen		1 - 3 x 10^6	1 - 3 x 10^5
		Peripheral blood		1 - 3 x 10^6	1 - 3 x 10^5
		Fetal liver		2 x 10^5	2 x 10^4
MethoCult™ SF M3436	BFU-E	Bone marrow	10 - 14 days	3 - 8 x 10^5	3 - 8 x 10^4
		Spleen		1.5 - 5 x 10^5	1.5 - 5 x 10^4
		Fetal liver		1 - 3 x 10^5	1 - 3 x 10^4
MethoCult™ M3334	CFU-E	Bone marrow	48 hours	1 - 2 x 10^6	1 - 2 x 10^5
		Spleen		2 - 4 x 10^6	2 - 4 x 10^5
MethoCult™ M3630	Pre-B	Bone marrow	7 days	0.5 - 2 x 10^6	0.5 - 2 x 10^5
MethoCult™ M3234, M3236, M3231, M3134	Dependent on added cytokines and cell source**				

*Plating concentrations are shown for C57BL/6 and BALB/c mice. Plating concentration will vary with other strains or genetically modified mice.

**Plating concentrations will vary depending on the cytokines added to the media and the cell source of the progenitor cells. For hints and tips, contact techsupport@stemcell.com.

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 4 mL MethoCult™ tube.

   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 to mix the contents thoroughly.
7. Let the tube stand for at least 5 minutes to allow the bubbles to rise to the top.
8. To dispense the MethoCult™ 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 solution and draw up approximately 1 mL. Gently depress the plunger and expel 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, 245 mm square dish or other cultureware of an appropriate size with a loose-fitting lid). Add approximately 3 mL of sterile water to the uncovered 35 mm dish(es).

    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. Incubation times are dependent on the MethoCult™ medium formulation. Refer to Table 6.

    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.

Specific notes on MethoCult™ M3630 cultures:

- The number of CFU-pre-B (in MethoCult™ M3630) may vary between mouse strains and with age. Two different plating concentrations are recommended for each sample if variability is expected with respect to the number of progenitor cells in the 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.
- Addition of 5 - 20 ng/mL Mouse Recombinant SCF to MethoCult™ M3630 may increase the number of CFU-pre-B detected. However, addition of Mouse Recombinant SCF also promotes myeloid growth within the cultures, which can inhibit the growth of CFU-pre-B. The number of myeloid CFU in MethoCult™ M3630 also increases when higher input cell numbers are used due to endogenous cytokine production. It is recommended to perform preliminary experiments using 2 - 3 different plating concentrations in the presence and absence of Mouse Recombinant SCF to establish optimal conditions for the application.

9.0 Counting Mouse CFU Assays

Evaluate mouse CFU numbers in situ following the recommended incubation period (see Table 6). The expected number of progenitor cell colonies for different cell sources and MethoCult™ formulations are given in Table 7.

It is important to use a high-quality inverted microscope equipped with low power (2X) and higher power (4X - 5X, 10X) objective lenses, and 10X or 12.5X ocular eyepieces.

For specialized applications, such as preparation of cytospins for cytochemical staining 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. For example, individual BFU-E, CFU-GM, and CFU-GEMM or the cells from the entire culture can be isolated following 7 - 10 days of incubation in MethoCult™ GF M3434, instead of the recommended 10 - 12 days of incubation.

For assistance with identifying various colony types, refer to section 10.0.

Table 7. Expected Numbers of Progenitor Cell Colonies

CELL SOURCE	METHOCULT™ FORMULATION	PLATING CONCENTRATION (cells per 35 mm dish)	PROGENITOR CELL COLONY TYPE	COLONIES PER CULTURE*
Bone marrow	MethoCult™ M3334	2×10^5	CFU-E	340 ± 80
Bone marrow	MethoCult™ GF M3434	2×10^4	BFU-E	8 ± 3
			CFU-GM	64 ± 16
			CFU-GEMM	3 ± 1
Fetal liver	MethoCult™ GF M3434	2×10^4	BFU-E	9 ± 3
			CFU-GM	55 ± 10
			CFU-GEMM	3 ± 2
Bone marrow	MethoCult™ SF M3436	1×10^5	BFU-E	72 ± 23
Spleen	MethoCult™ SF M3436	1×10^5	BFU-E	16 ± 5
Fetal liver	MethoCult™ SF M3436	1×10^5	BFU-E	220 ± 71
Peripheral blood	MethoCult™ SF M3436	1×10^5	BFU-E	26 ± 4
Bone marrow	MethoCult™ M3630	1×10^5	CFU pre-B	92 ± 12
Fetal liver	MethoCult™ M3630	1×10^5	CFU pre-B	5 ± 2

*Values are representative for C57BL/6 mice at 8 - 12 weeks of age and day 14.5 PC fetal liver.

1. Prepare a 60 mm Gridded Scoring Dish by drawing two perpendicular lines across the center of the dish using a permanent fine felt marker on the bottom of the dish. Count 8 squares from the center on one radius and draw a short (approximately 2 mm) line 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 the colonies in each dish. It is necessary to continually focus up and down to identify all colonies present in the 3-dimensional culture and to distinguish individual colonies that are close together but in different planes of focus. The size of the colonies will affect the magnification used to count. The colonies should be easily visible so they are not missed but should not entirely fill the field of view.

   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.

9.1 Automated Colony Counting Using STEMvision™

In addition to manual colony counting, CFU assays plated in MethoCult™ GF M3434, MethoCult™ GF M3534, or MethoCult™ SF M3436 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 count total, myeloid, or erythroid progenitor cell colonies. For more information, visit www.stemvision.com.

10.0 Colony Identification

10.1 General Colony Descriptions

Mouse hematopoietic progenitor cells can be quantitated in cell suspensions of adult bone marrow, spleen, and peripheral blood, and during development in tissues such as fetal liver. The classes of mouse hematopoietic progenitor cells detected using MethoCult™ media are described below, and photographs are in section 10.2.

CFU-E: Colony-forming unit-erythroid. These are mature erythroid progenitor cells that form 1 - 2 clusters of maturing erythroblasts in the presence of erythropoietin (EPO). Colonies derived from CFU-E are counted after 48 hours of culture. These colonies are very small and should be counted under 40X - 50X magnification. One cluster contains at least 8 erythroblast cells. Erythroblast cells within the cluster are irregular in shape and appear fused together. There is no picture shown, however a colony derived from a CFU-E looks like a single cluster of a colony derived from a BFU-E (contains approximately 250 clusters) as seen in Photo 1.

BFU-E: Burst-forming unit-erythroid. BFU-E require EPO and cytokines with burst-promoting activity such as interleukin 3 (IL-3) and stem cell factor (SCF) for optimal growth. BFU-E are counted after 10 - 14 days of culture, depending on which MethoCult™ formulation is used. Colonies derived from BFU-E are made up of erythroid clusters. Each individual cluster contains a group of cells that are tiny, irregular in shape, difficult to distinguish and appear fused together. BFU-E do not usually have a dense core. However, it is best to confirm BFU-E by looking at the individual clusters within each colony at high magnification. Refer to Photos 1 and 2.

In MethoCult™ SF M3436, colonies derived from BFU-E become very large. The cells often do not fully mature and therefore do not have the typical erythroid morphology, but they do hemoglobinize, giving the colony a red or brown appearance. Refer to Photos 3 and 4.

CFU-GM: Colony-forming unit-granulocyte, macrophage, including colony-forming unit-granulocyte (CFU-G) and colony-forming unit-macrophage (CFU-M). Colonies contain 30 to thousands of granulocytes (CFU-G), macrophages (CFU-M) or both cell types (CFU-GM). Colonies derived from CFU-GM often contain multiple cell clusters with dense cores. The monocytic lineage cells are large cells with an oval to round shape and appear to have a grainy or grey center (see Photos 6, 8, 9, and 10). The granulocytic lineage cells are round, bright, and are much smaller and more uniform in size than macrophage cells (see Photos 2, 5, and 7). It is easy to see individual cells of a CFU-GM colony, especially in the periphery of the colony, as seen in Photos 5 and 7.

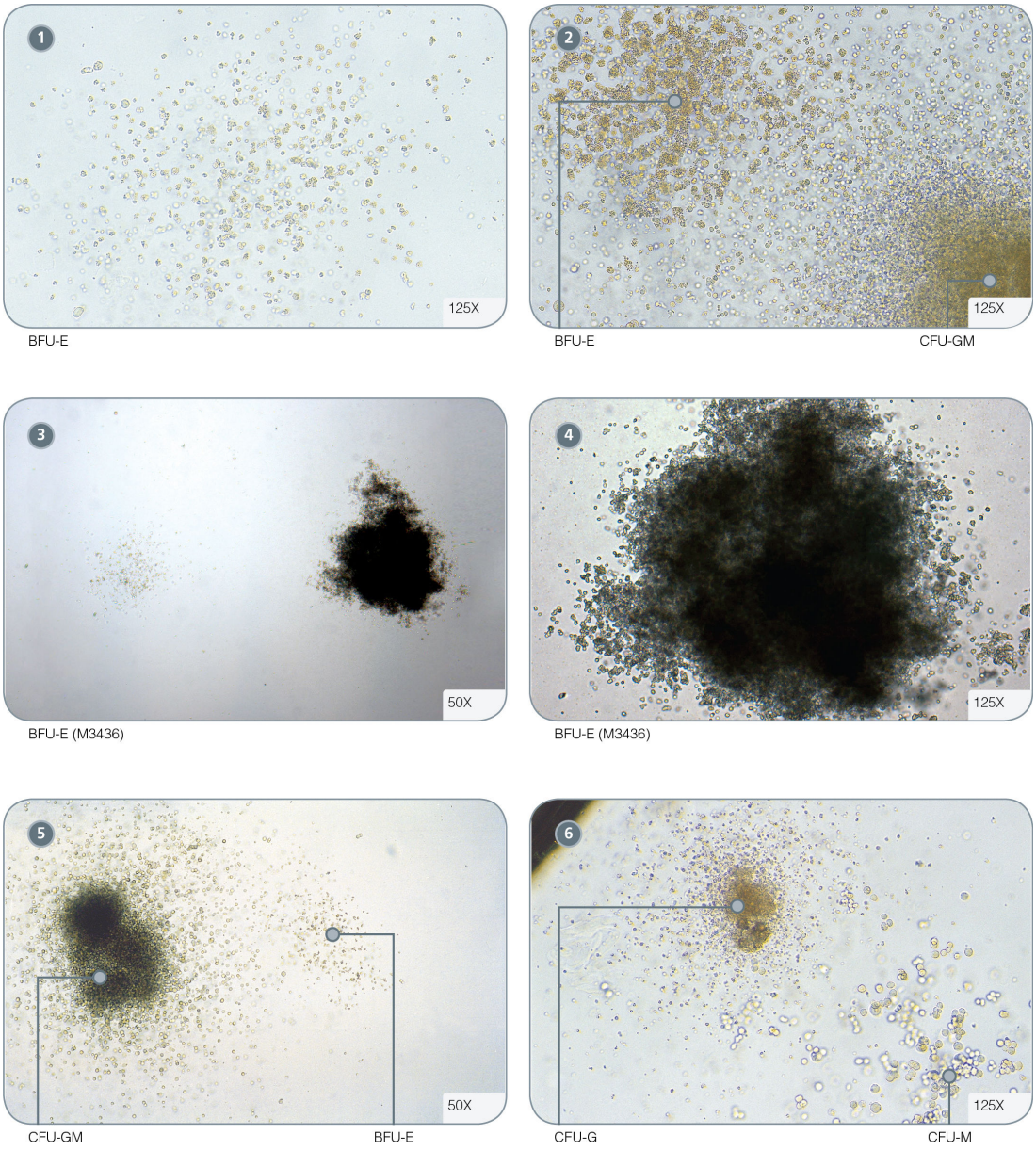
CFU-GEMM: Colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte. CFU-GEMM are multi-potential progenitor cells that require EPO and 2 or more cytokines to support the growth and differentiation of lineage-committed daughter cells within the forming colony. Because of their primitive nature, CFU-GEMM tend to produce large colonies of > 500 cells containing erythroblasts and recognizable cells of at least two other lineages. Colonies derived from CFU-GEMM have a highly dense core with an indistinct border between the core and peripheral cells. Erythroblast clusters should be visible at the periphery of the colony. Monocytic and granulocytic cells (see CFU-GM) should be easily identifiable and clusters of large megakaryocytic cells are usually seen. Refer to Photos 11 and 12.

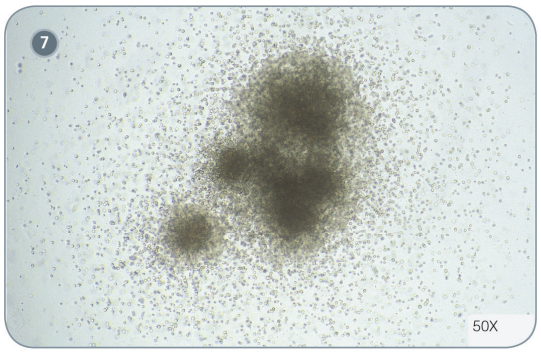
CFU-pre-B: A subset of B-lymphoid progenitor cells can be detected in the presence of interleukin 7 (IL-7). Colonies derived from CFU-pre-B are made up of at least 30 cells. Although colonies vary in size and morphology, individual cells appear tiny and irregular to oval in shape. Some CFU-pre-B colonies are very dense with very few cells in the periphery, while some have a smaller core with more cells in the periphery. Refer to Photos 13 and 14.

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

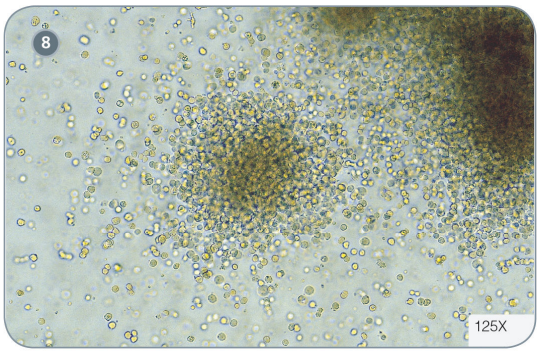
Although megakaryocytic progenitor cells can be cultured in methylcellulose-based medium containing the appropriate growth factors, it is difficult to distinguish colonies derived from CFU-Mk based on cellular and colony morphology. Therefore, we recommend that mouse CFU-Mk are counted by culturing in collagen-based, serum-free, MegaCult™-C medium, then dehydrated and fixed for subsequent staining (no picture shown). For further information, refer to Appendix 12.2.1 or 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.

10.2 Photographs of Mouse Hematopoietic CFUs

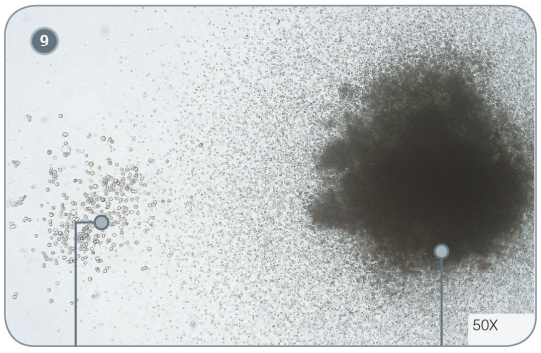




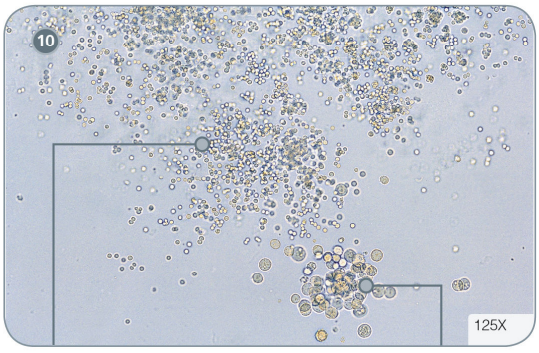
CFU-GM



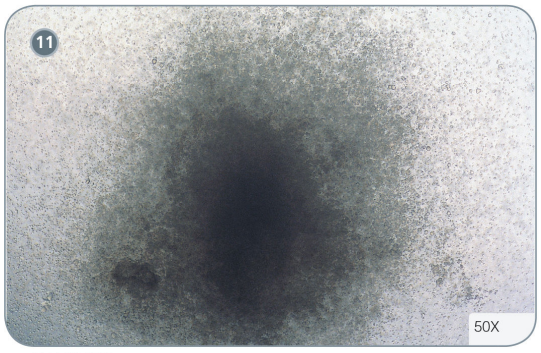
CFU-M



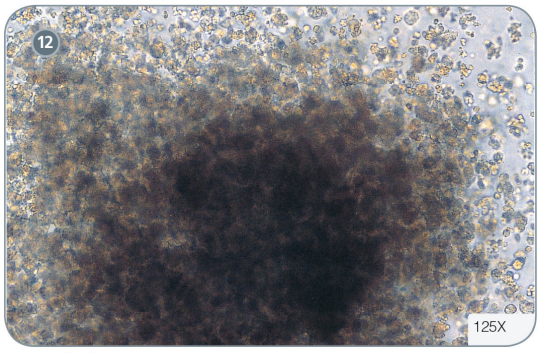
CFU-M CFU-GEMM



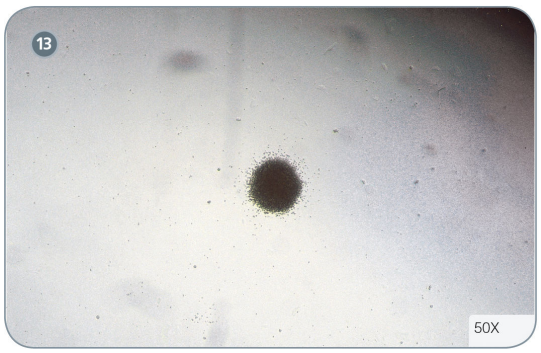
CFU-G CFU-M

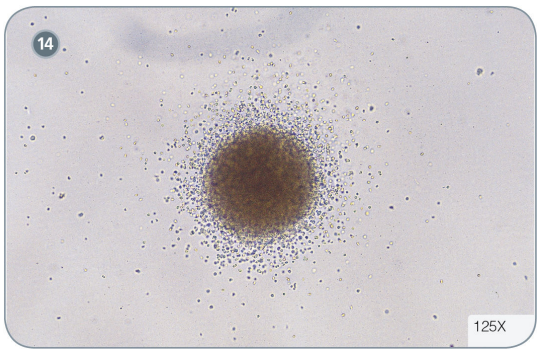


CFU-GEMM



CFU-GEMM





11.0 Frequently Asked Questions and Helpful Hints

11.1 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. Once thawed, shake for 1 - 2 minutes to mix completely and let stand for at least 5 minutes to allow bubbles to rise to the top. The bottle can now be aliquoted into tubes, which 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™ is not homogeneous and small “lumps” may be present if the medium 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 medium performance is unaffected as long it has been stored at the recommended temperature of -20°C (-25°C to -15°C) until the expiry date indicated on the label, or at 2 - 8°C for up to 1 month. Thaw the bottle and follow the 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. Although the recommended storage is at -20°C (-25°C to -15°C), the performance of 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 erythropoietin (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 MethoCult™?

Antibiotics, drugs and other compounds 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. Compounds can be added to complete, ready-to-use formulations such as MethoCult™ GF M3434, but it will be necessary to add the cells in a smaller volume, and to maintain a 1:10 ratio of the volume of cells plus compounds 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 4 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.

11.2 Cell Sample Preparation

10. Is it necessary to eliminate red blood cells (RBCs) from cell suspensions?

It is usually not necessary to lyse or remove RBCs in normal bone marrow suspensions. The low numbers of RBCs that will be present in the CFU cultures do not make the colonies difficult to see or inhibit colony growth.

Lysis of RBCs in PB samples by ammonium chloride treatment is necessary as the large number of RBCs present in PB samples can obscure the colonies and make them difficult to see.

Day 10 - 16 PC fetal liver contains a high percentage of nucleated erythroid cells (greater than 80%). BFU-E, CFU-GM, and CFU-GEMM analysis can be done on unseparated fetal liver suspensions. If desired, erythroid cells can be removed by density gradient centrifugation or by depletion of $TER119^+$ cells.

11. Should I do viable counts or total nucleated cell counts on mouse cell samples?

Nucleated cell counts using 3% Acetic Acid with Methylene Blue (Catalog #07060) are routinely performed, as cell viability should be high in most freshly isolated cell suspensions. Viable cell counts with Trypan Blue (Catalog #07050) should be used for cell preparations where a decrease in cell viability may be expected (e.g. ex vivo manipulation).

11.3 Setup and Culture

12. Why do I need to use a plating cell concentration that yields ~25 - 100 colonies per 1.1 mL culture?

For accurate quantitation of progenitor cells, 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 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. 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.

13. 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, if necessary, without adversely affecting subsequent colony formation. Vortex gently before plating if tubes have been left for a period of time.

14. 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 microscopically. Very high CO_2 concentrations in the incubator can also cause medium to become acidic.

15. Why are my cultures cloudy?

Bacterial, fungal, or yeast contamination is usually the cause of cloudy cultures. 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). Look for the presence of bacterial colonies, typically appearing as round, smooth, white or yellow colonies. Methylcellulose can also appear more liquid when contaminated.

16. 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.

17. 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 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.

18. Why does the MethoCult™ appear to be runny and the colonies are floating or smearing?

There are several potential reasons:

- Thawed MethoCult™ medium was not thoroughly mixed before dispensing into tubes.
- Too-large volumes of components or cells were added to the MethoCult™.
- Tubes containing cells and the MethoCult™ medium were not thoroughly mixed before plating.

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

Cultures have been tipped or knocked in the incubator or when transporting cultures, or the viscosity of methylcellulose-based medium is too low. MethoCult™ medium not 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 4) 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.

11.4 Counting Mouse CFU Assays

20. 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 MethoCult™
- 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 medium 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.
- 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.

21. My cultures contain > 100 colonies or < 25 colonies. What should I do next time?

When it is difficult to anticipate the correct plating cell concentration, the use of 2 or more cell concentrations is advised.

22. 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. For many people counting is made easier by counting in vertical rows by moving the stage control knob up and down rather than side-to-side across the dish.

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

Practice, practice, practice. Learning tips include:

- Initially, spend 1 - 2 hours per day, several days per week learning to recognize the different CFU types and to count accurately. Count the same cultures on different days. Cultures placed at 33°C, 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 colleagues.
- Attend a STEMCELL Technologies Training Course (see Appendix 12.2.2). Refer to www.stemcell.com or contact us for course dates and availability.

24. 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.

12.0 Appendices

12.1 Enrichment of Hematopoietic Progenitor Cells or Isolation of c-KIT$^+$ or SCA1$^+$ Cells

Table 8. Kits for Preparing Lineage-Depleted (Lin$^-$) Cells by Depletion of Mature Lineage-Committed Hematopoietic Cells

PRODUCT	CATALOG #	KIT COMPONENTS
EasySep™ Mouse Hematopoietic Progenitor Cell Enrichment Kit	19756	EasySep™ Mouse Hematopoietic Progenitor Enrichment Cocktail EasySep™ Biotin Selection Cocktail EasySep™ Mouse Progenitor Magnetic Microparticles Normal Rat Serum
RoboSep™ Mouse Hematopoietic Progenitor Cell Enrichment Kit	19756RF	19756 with RoboSep™ Buffer and RoboSep™ Filter Tips

Table 9. Kits for Isolating c-KIT$^+$ or SCA1$^+$ Cells by Positive Selection

PRODUCT	CATALOG #	KIT COMPONENTS
EasySep™ Mouse SCA1 Positive Selection Kit	18756	EasySep™ Mouse SCA1 PE Labeling Reagent EasySep™ PE Selection Cocktail EasySep™ Magnetic Particles
RoboSep™ Mouse SCA1 Positive Selection Kit	18756RF	18756 with RoboSep™ Buffer and RoboSep™ Filter Tips
EasySep™ Mouse SCA1 Biotin Positive Selection Kit	18856	EasySep™ Mouse SCA1 Biotin Labeling Reagent EasySep™ Biotin Selection Cocktail EasySep™ Magnetic Particles
RoboSep™ Mouse SCA1 Biotin Positive Selection Kit	18856RF	18856 with RoboSep™ Buffer and RoboSep™ Filter Tips
EasySep™ Mouse CD117 (cKIT) Positive Selection Kit	18757	EasySep™ Mouse CD117 (cKIT) PE Labeling Reagent EasySep™ PE Selection Cocktail EasySep™ Magnetic Particles
RoboSep™ Mouse CD117 (cKIT) Positive Selection Kit	18757RF	18757 with RoboSep™ Buffer and RoboSep™ Filter Tips

12.2 Related Products and Services

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

12.2.1 MegaCult™-C

Megakaryocyte colonies cannot be readily distinguished morphologically from colonies of other cell types such as macrophages. Specific staining procedures are needed to identify megakaryocyte colonies. Methylcellulose-based cultures cannot be fixed, and further cellular or molecular analysis of colonies would require that colonies be individually plucked and processed. Use of collagen gels is an attractive alternative to methylcellulose-based semi-solid cultures. Collagen gels have been shown to support the growth of a variety of cell types, including hematopoietic cells. Following incubation, the entire culture can be dehydrated and fixed for subsequent staining. The resulting slides can be stored long-term, providing an archive of your assay results.

To meet the varying needs of researchers, STEMCELL Technologies has developed MegaCult™-C, a collagen-based medium for the quantitation of megakaryocyte progenitor cell colonies (CFU-Mk). Refer to Table 10 for ordering information. For further details, 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.

Table 10. MegaCult™-C Collagen-Based Media For Mouse CFU-Mk Assays

PRODUCT NAME	CATALOG #	SIZE	DESCRIPTION
MegaCult™-C Collagen and Medium with Lipids Without Cytokines	04974	50 mL	For assays of mouse megakaryocytic progenitor cells. Kit includes serum-free medium and collagen; does not include double chamber slides or enzymatic staining reagents. Addition of recombinant cytokines is required.
MegaCult™-C Collagen and Medium Without Cytokines	04960	1.7 mL/tube (24 tubes/rack)	

12.2.2 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.

12.2.3 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 ISO13485:2003 guidelines
- Thorough and timely reporting of data and report followup
- Customized educational and training courses optimized to fit your needs

Visit www.contractassay.com for more information.

13.0 References

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TOLL FREE PHONE 1 800 667 0322 · PHONE +1 604 877 0713
INFO@STEMCELL.COM · TECHSUPPORT@STEMCELL.COM

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