

TECHNICAL MANUAL

# MegaCult™-C

## Assays for Quantitation of Human and Mouse Megakaryocytic Progenitor Cells



## **Special Handling Instructions**

ALKALINE PHOSPHATASE SUBSTRATE TABLETS  
A component of the MegaCult™-C Staining System



### **WARNING!**

- This material is toxic
- Do not touch with bare hands
- Wear gloves at all times
- Causes irritation to eyes, respiratory system, and skin

### **INSTRUCTIONS**

Use within one hour of preparation

### **COMPONENTS**

1. Tris tablet (gold foil packet)
2. Fast Red/Naphthol tablet (silver foil packet)

MegaCult™-C Complete Kits contain 30 sets of tablets for 1 mL where one set comprises one gold foil packet and one silver foil packet.

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## 1.0 Introduction

Colony assays for quantitating human and mouse erythroid and granulocyte/macrophage progenitor cells have been in widespread use for many years. In contrast, it has been problematic to establish conditions suitable for the routine assay of colony-forming unit-megakaryocyte (CFU-Mk).

Cells of the megakaryocyte (Mk) lineage are particularly sensitive to inhibitors such as transforming growth factor-beta (TGF- $\beta$ ), commonly present in batches of fetal bovine serum used in assays for other hematopoietic lineages.<sup>1</sup> In addition, combinations of growth factors that allow the enumeration of both erythroid and granulocyte/monocyte progenitor cells within the same assay may not promote optimal detection of CFU-Mk. These limitations have been overcome through the use of medium containing defined serum substitutes and combinations of cytokines identified as having Mk colony-stimulating activity, including thrombopoietin (TPO), interleukin 3 (IL-3), IL-6, IL-11, and stem cell factor (SCF).<sup>2-7</sup>

Megakaryopoiesis is unique in that colonies containing as few as three cells reflect the property that megakaryocytic progenitor cells may undergo cycles of endoreduplication and maturation in the absence of cell division.<sup>8</sup> In addition, megakaryocytes can be difficult to distinguish morphologically from other cell lineages, particularly macrophages. Therefore, a suitable semi-solid matrix must support the clonal growth of Mk progenitor cells and allow specific recognition of Mk colonies by fixation of the entire culture and immunocytochemical staining for Mk-specific antigens or enzyme activity. A number of different substances have been assessed for their ability to meet these criteria, including fibrin, agar or agarose, and collagen.

Fibrin clots can be fixed and stained with good preservation of cell morphology, however the cultures tend to lyse within 10 - 14 days. Agar or agarose cultures can be fixed and stained but the procedure may expose cells to potentially harmful high temperatures. The use of collagen gels, shown to be suitable for the culture of hematopoietic progenitor cells,<sup>9</sup> has distinct advantages compared to other semi-solid media. Neutralized isotonic collagen forms a suitable stable gel at a final concentration of 1 - 2% at 37°C and can be readily dehydrated for subsequent histochemical or immunocytochemical staining. Alternatively, viable cells can be obtained for further analysis by digestion of the collagen matrix with collagenase.

MegaCult™-C is a collagen-based system that has been developed for optimal detection of Mk progenitor cells in mononuclear or CD34<sup>+</sup>-enriched cell suspensions of human bone marrow, blood and cord blood. The use of collagen as a gelling agent does not require specialized laboratory equipment and also avoids exposure of the cells to high temperatures. The medium contains a defined serum substitute to minimize the inhibitory effects of factors like TGF- $\beta$ , that are found in most sera.<sup>5</sup> In addition, it contains the human recombinant cytokines IL-3, IL-6, and TPO. The same medium is also available without cytokines for investigation of additional factors influencing the growth and maturation of megakaryocytic progenitor cells. MegaCult™-C collagen-based products can also be used for quantitation of mouse CFU-Mk.

Another feature of the MegaCult™-C system is the use of chamber slides, which allows the incubation and subsequent fixation and staining of the entire culture all on the same slide. Human CFU-Mk are detected by staining of the cells with a primary antibody to the Mk-specific antigen GPIIb/IIIa (CD41) linked to a secondary biotinylated antibody-alkaline phosphatase avidin-conjugated detection system to greatly amplify the primary signal. Mouse CFU-Mk can be identified by the detection of acetylcholinesterase activity of megakaryocytes.<sup>10</sup> The slides can then be stored for examination at a later time and provide a permanent record.

All components of the MegaCult™-C system have been optimized for accurate quantitation of Mk progenitor cells.



## 2.0 MegaCult™-C Products for Human CFU-Mk Assays

### 2.1 MegaCult™-C Media Formulations and Ordering Information

MegaCult™-C serum-free media have been formulated to promote the growth of human CFU-Mk in collagen-based gels. Several formulations are available, as described in Table 1.

**Table 1. Components of MegaCult™-C Media for use with Human Cells**

*Note: See Table 2 for the MegaCult™-C kits that include MegaCult™-C media.*

MEGACULT™-C MEDIUM WITH CYTOKINES*	MEGACULT™-C MEDIUM WITHOUT CYTOKINES**	MEGACULT™-C MEDIUM WITH LIPIDS**
Catalog #04901 (24 x 2.0 mL/tube)	Catalog #04900 (24 x 1.7 mL/tube)	Catalog #04850 (50 mL/bottle)
Iscove's MDM	Iscove's MDM	Iscove's MDM
Bovine serum albumin	Bovine serum albumin	Bovine serum albumin
Recombinant human (rh) insulin	Recombinant human insulin	Recombinant human insulin
Human transferrin (iron-saturated)	Human transferrin (iron-saturated)	Human transferrin (iron-saturated)
Supplements	Supplements	Supplements
2-Mercaptoethanol	2-Mercaptoethanol	2-Mercaptoethanol
rh Thrombopoietin	n/a	Lipids
rh IL-6	n/a	n/a
rh IL-3	n/a	n/a

\*Requires the addition of Collagen Solution (Catalog #04902).

\*\*Requires the addition of Collagen Solution (Catalog #04902) and desired cytokines. See section 2.2 for recommended cytokines.



**Table 2. MegaCult™-C Medium Kits**

For a complete list of all kit components, refer to Appendix 1.

MEGACULT™-C MEDIUM	MEGACULT™-C KIT	KIT CATALOG #
<b>MegaCult™-C Medium with Cytokines</b>		
Catalog #04901 (24 x 2.0 mL/tube)	MegaCult™-C Collagen and Medium with Cytokines	04961
	MegaCult™-C Complete Kit with Cytokines	04971
<b>MegaCult™-C Medium Without Cytokines</b>		
Catalog #04900 (24 x 1.7 mL/tube)	MegaCult™-C Collagen and Medium Without Cytokines	04960
	MegaCult™-C Complete Kit Without Cytokines	04970
<b>MegaCult™-C Medium with Lipids*</b>		
Catalog #04850 (50 mL/bottle)	MegaCult™-C Collagen and Medium with Lipids	04974

\*This medium does not contain cytokines.

**Table 3. Final Composition of MegaCult™-C Medium for Human CFU-Mk Assays**

COMPONENT	VOLUME PER TUBE
MegaCult™-C Medium with Cytokines	2.0 mL *
Collagen Solution	1.2 mL
Cells	0.1 mL
<b>Total</b>	<b>3.3 mL</b>

\*For MegaCult™-C media supplied without cytokines, add 0.3 mL of Iscove's MDM with desired cytokines to 1.7 mL of medium (see section 6.1 step 2). For recommended cytokines and concentrations, see Table 4 and Table 5.



## 2.2 Recommended Cytokines

**Table 4. Recommended Cytokine Combination for Detection of Human CFU-Mk**

CYTOKINE	FINAL CONCENTRATION	CATALOG #
Human Recombinant TPO	50 ng/mL	78210
Human Recombinant IL-3	10 ng/mL	78040 or 78194
Human Recombinant IL-6	10 ng/mL	78050

**Table 5. Recommended Cytokine Combination for Detection of Human CFU-E and BFU-E**

CYTOKINE	FINAL CONCENTRATION	CATALOG #
Human Recombinant SCF	50 ng/mL	78062
Human Recombinant EPO	3 U/mL	78007

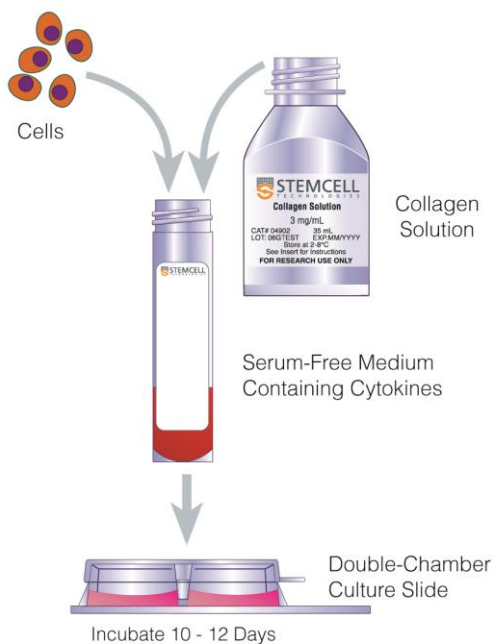
## 2.3 MegaCult™-C Staining Kit for CFU-Mk

MegaCult™-C Staining Kit for CFU-Mk (Catalog #04962) contains all the reagents needed to stain fixed and dehydrated CFU-Mk-derived colonies cultured in MegaCult™-C collagen-based medium. Megakaryocytes and platelets expressing the glycoprotein IIb/IIIa are stained with antihuman CD41 antibody and an alkaline phosphatase detection system. The kit is also suitable for detection of bipotential erythroidmegakaryocyte progenitor cells (BFU-E/Mk).

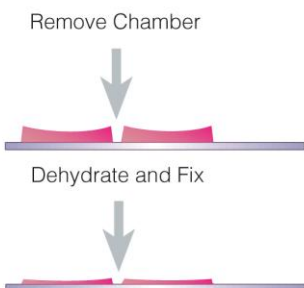
Refer to section 8.0 for components of the kit and the staining protocol.

### 3.0 MegaCult™-C Procedure Diagram for Human Cells

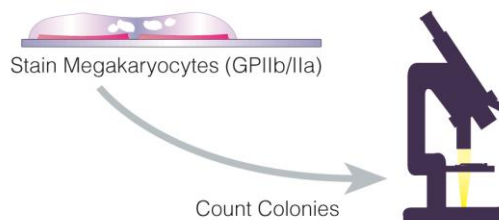
#### ① Set-up and Culture



#### ② Dehydrate and Fix



#### ③ Stain and Count



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## 4.0 Equipment and Materials Required for Human CFU-Mk Assays

### 4.1 Equipment

- Biohazard safety cabinet certified for Level II handling of biological materials
- Incubator set at 37°C with 5% CO<sub>2</sub> in air and ≥ 95% humidity

*Note: Use of water-jacketed incubators with a water pan placed in the chamber is recommended. It is important to use medical-grade CO<sub>2</sub> as inhibition of colony growth due to toxic substances present in the CO<sub>2</sub> gas source has been reported.*

- Light microscope with 5X and 10X objectives for cell counting and colony counting
- Centrifuge
- Vortex
- Pipette-aid
- Pipettors (e.g. Catalog #38058)
- Automated cell counter or Neubauer hemocytometer

### 4.2 Materials

- MegaCult™-C Medium (see section 2.0)
- Collagen Solution (Catalog #04902)
- MegaCult™-C Staining Kit for CFU-Mk (Catalog #04962)
- 35 mm Culture Dishes (Catalog #27100)
- 100 mm Culture Dish, Non-Treated (Catalog #38045)
- Iscove's MDM with 2% FBS (Catalog #07700)
- 0.05 M Tris/NaCl Buffer (see section 8.1)
  - NaCl, 5 M NaOH, and Tris/HCl (e.g. Sigma Catalog #T-3253)
- Sterile laboratory supplies (i.e. pipettes, tubes, pipette tips)
- Methanol: Methanol ACS (e.g. BDH #ACS531, 4 L/bottle), or other high-quality grade
- Acetone: Acetone OPTIMA (e.g. Fisher #A929-4, 4 L/bottle), or other high-quality grade (e.g. ACS)
- Double Chamber Slide Kit (Catalog #04963)
  - Double Chamber Slides (Catalog #04813)
  - Filter Cards and Spacers
- Containers for fixation and staining solutions (ensure that container for fixation can withstand exposure to methanol and acetone)
- 2 mL serological pipettes (e.g. Catalog #38002)
- Ice

## 5.0 Thawing and Dispensing MegaCult™-C Media

*Note: For pre-aliquoted MegaCult™-C media, proceed to section 6.0.*

### 5.1 Preparation of Bottles (MegaCult™-C Medium with Lipids)

1. Thaw bottle of MegaCult™-C Medium with Lipids at room temperature (15 - 25°C) or overnight at 2 - 8°C. Mix thoroughly.
2. Dispense 1.7 mL of MegaCult™-C Medium with Lipids into tubes.

*Note: To use immediately, proceed to section 6.0. Alternatively, store tubes of medium at -20°C.*

## 6.0 Culture of Human Cells

### 6.1 Human CFU-Mk Assay Setup

Use sterile technique when performing all culture procedures.

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

*Note: Once tubes are thawed, do not re-freeze.*

2. For MegaCult™-C Medium Without Cytokines or MegaCult™-C Medium with Lipids, add cytokines and/or additional components in Iscove's MDM in a 0.3 mL volume to achieve a total volume of 2 mL per tube. See section 2.2 for recommended cytokine concentrations.

*Note: When plating highly purified cell populations (e.g. CD34<sup>+</sup>-enriched cells), we recommend adding Human LDL (low-density lipoproteins; Catalog #02698) to the medium to achieve a final concentration of 40 µg/mL.*

3. Place Collagen Solution on ice.
4. Prepare cell suspension in Iscove's MDM at 33X the desired final cell concentration (see Table 6).

**Table 6. Recommended Plating Concentrations for Human Cells\***

CELL TYPE	CELL SUSPENSION** (cells/mL)	CELLS PER SLIDE (in 1.5 mL)
Ammonium chloride-treated bone marrow	4.4 x 10 <sup>6</sup>	2 x 10 <sup>5</sup>
Bone marrow mononuclear cells	2.2 x 10 <sup>6</sup>	1 x 10 <sup>5</sup>
Ammonium chloride-treated cord blood cells	2.2 x 10 <sup>6</sup>	1 x 10 <sup>5</sup>
Cord blood mononuclear cells	1.1 x 10 <sup>6</sup>	5 x 10 <sup>4</sup>
CD34 <sup>+</sup> -enriched cells (bone marrow, cord blood, mobilized peripheral blood)	1.1 x 10 <sup>5</sup>	5000

\*The numbers of CFU-Mk detected vary between donors. It is recommended to plate cells at two concentrations with a 2- to 3-fold difference to establish optimal plating concentrations in your laboratory.

\*\*The cell concentration given is 33X the desired final cell concentration.

5. Add 0.1 mL of the cell suspension to each tube containing 2.0 mL of medium with cytokines.
6. Mix one tube of medium containing cells (2.1 mL total volume).



7. Using a sterile 2 mL serological pipette, transfer 1.2 mL of cold Collagen Solution to the tube (3.3 mL final volume). Pipette up and down to mix.
8. Using the same 2 mL pipette, remove 1.5 mL of the final culture mixture and dispense 0.75 mL into each of the two wells of a previously labeled double chamber slide.  
*Note: Chamber slides should be labeled with a pencil or diamond point pen. Ink labeling will become illegible during the fixation process.*
9. Dispense an additional 1.5 mL in the same manner onto a second double chamber slide. Remove any air bubbles by gently touching bubble with the end of the pipette.  
*Note: The collagen will begin to gel within several minutes following addition to media containing cells. If more than one tube is being set up, collagen should be added to the first tube only and the contents of the tube dispensed into chamber slides before proceeding to the next tube.*
10. Gently tip each slide using a circular motion to allow the mixture to spread evenly over the surface of the slide.
11. Place each slide in a 100 mm Culture Dish containing an uncovered 35 mm Culture Dish with 3 mL of sterile water (to maintain optimal humidity during the incubation period). Replace the lid of the 100 mm dish.
12. Transfer the slides to a 37°C incubator with 5% CO<sub>2</sub> and ≥ 95% humidity. Gel formation will occur within approximately 1 hour. It is important not to disturb the cultures during this time.
13. Incubate cultures for 10 - 12 days. Maximum CFU-Mk colony size and numbers are typically seen at this time. Cultures should be visually assessed for overall colony growth and morphology using an inverted microscope prior to fixation and staining.

## 6.2 Helpful Hints

The following tips will assist with optimal detection of megakaryocytic progenitor cells in the human CFU-Mk assay:

- It is possible to split the medium containing growth factors into two tubes (2 x 1.0 mL) in order to carry out more tests. In this case, the volume of collagen and cells added must be reduced accordingly (i.e. 0.6 mL collagen per 1.0 mL of medium and 0.05 mL cells), and samples would be plated in one double chamber slide (2 x 0.75 mL cultures).
- Slides should be labeled with a pencil or diamond point pen. Ink labeling will become illegible during the fixation process.
- Maintain proper incubator conditions including temperature, CO<sub>2</sub> level, and humidity (37°C with 5% CO<sub>2</sub> and ≥ 95% humidity). It is also important to place cultures in a covered 100 mm Culture Dish containing an open 35 mm Culture Dish with 3 mL of sterile water.
- Ensure MegaCult™-C medium and Collagen Solution are used prior to the expiration date stated on the label and have been correctly stored.
- Collagen Solution should be stored at 2 - 8°C and placed on ice during use. Do not freeze.
- Occasionally, the medium may be yellow upon receipt due to a decrease in pH during shipping. The medium will return to the proper color within 24 hours of incubation and colony formation will not be inhibited.

- Cells should be washed prior to culture. Serum contains substances known to inhibit the growth of CFU-Mk. The presence of large numbers of platelets may also inhibit the growth of megakaryocytes by the release of TGF- $\beta$  and platelet factor 4 (PF4) from  $\alpha$  granules.
- It may be necessary to establish the optimal plating concentrations of cells within each laboratory. There is considerable variation in the numbers of CFU-Mk detected based on the tissue source, cell purification techniques, and in vitro manipulation of cells. To establish optimal plating conditions, each cell suspension tested should be plated at two to three concentrations separated by 3- to 4-fold dilutions.
- Overplating can result in inhibition of optimal colony formation. If cultures become yellow (acidic) during culture, excessive numbers of cells may have been plated.
- Collagen gel formation is initiated by neutralization (following mixing with medium) and the increase in temperature upon incubation. Once the gelling process is initiated it is important not to manipulate or disturb the cultures. As described in Section 6.1, cultures should be set up one at a time.
- Ensure correct proportions of cells, medium, and collagen are added to each culture. For example, if the cytokines are added in a volume greater than 0.3 mL, the volume of the cell suspension must be reduced accordingly and the concentration of the cells in the suspension increased accordingly.



## 7.0 Dehydrating and Fixing Cultures of Human Cells

### 7.1 Dehydrating and Fixing Protocol

1. Examine cultures for overall colony growth using an inverted microscope prior to dehydrating and fixing.
2. Prepare a 1:3 methanol:acetone fixing solution (e.g. to prepare 200 mL, combine 50 mL methanol and 150 mL acetone). Place in a 2.5 L plastic container and tightly seal the lid to prevent evaporation of the fixative.

*Note: It is important to use a high-quality grade of methanol and acetone.*

3. Leave the cultures at 37°C until just prior to dehydration, as the collagen gel may become unstable at lower temperatures. Remove slides from the incubator singly or in small batches.

*Note: Ensure that each slide is correctly labeled with a pencil or a diamond point pen. Ink labeling will become illegible when the slide is immersed in the fixative solution.*

4. Carefully remove the plastic walls and rubber seal of the chamber slide without damaging the culture using the following procedure:
  - a) Remove chamber lid.
  - b) Partially remove the plastic chamber by pulling it up and away from the slide (use forceps to hold the slide down), starting at one corner near the labeled end of the slide.
  - c) When the rubber seal surrounding the collagen gel is visible under the plastic chamber, use forceps to grasp one corner of the rubber seal and gently stretch it over the corner of the plastic chamber so that it remains hooked there.
  - d) Use forceps to hook the rubber seal over the other corner of the plastic chamber at the same end of the slide.
  - e) The plastic chamber and rubber seal can now be easily removed in one motion by continuing to slowly pull them up and away from the slide. The collagen gel and associated liquid will remain on the slide.
5. Gently place a pre-cut spacer onto the chamber slide. Place a thick white filter card on top and allow liquid to soak the card. Filter cards and spacers are supplied with the Double Chamber Slide Kit.
6. Remove the thick white filter card as soon as it becomes fully saturated and leave the original spacer in place.
7. Place the slides with spacers into the methanol/acetone fixing solution. Ensure that the slides are completely covered with solution. Tightly seal the lid of the container.
8. The spacers should float off when slides are placed in the fixative, leaving two squares of gel on the slide.

*Note: If the spacers do not float away from the slides during fixation, gently shake the container to encourage them to detach. If a spacer remains adherent, remove that slide from the fixative solution and gently peel the gel from the spacer onto the slide using a micro spatula or tweezers, being careful not to rip the gel. Do not allow the slide to dry out during this step.*



*Note: Decreasing the time the spacer is in contact with the gel may reduce the appearance of gridlines on the slides after fixation.*

9. Leave slides in the methanol/acetone solution for 20 minutes at room temperature (15 - 25°C). Ensure that the lid of the container is tightly sealed.

*Note: The fixing solution should be changed every 12 slides.*

10. Remove the slides from the fixing solution and allow to air dry for 15 minutes.
11. Stain cultures as soon as possible after fixation (see section 8.0).

*Note: It is recommended that slides be stained within 3 days. Fixed slides can be stored at 2 - 8°C in the dark until staining can be performed. If the slides are stained more than 3 days after fixation, the intensity of the stain may be reduced.*

## 7.2 Helpful Hints

The following tips will assist with optimal detection of megakaryocytic progenitor cells in the human CFU-Mk assay:

- Cultures may appear 'runny' if left at room temperature (15 - 25°C) too long prior to fixation. Leave the slides at 37°C until just prior to fixation. Remove slides from incubator one at a time or in small batches.
- The correct ratio of methanol to acetone must be used (1:3), and it is important that these reagents are of a high grade.
- Slides should be laid flat in the fixative solution.
- If the collagen gel does not firmly adhere to slide while removing the chambers, it can be eased gently back into place.
- Fixing solution (200 mL) must be changed between each batch of slides (up to 12).



## 8.0 Staining Cultures of Human Cells

The staining protocol outlined in this manual is for the detection of megakaryocytic progenitor cells using the MegaCult™-C Staining Kit for CFU-Mk. Components of the kit are listed in Table 8; refer to the corresponding Product Information Sheet for further information.

**Table 7. MegaCult™-C Staining Kit for CFU-Mk (Catalog #04962)**

COMPONENT #	COMPONENT NAME	QUANTITY
04803	Primary Antibody	360 µL
04804	Control Antibody	100 µL
04905	Avidin-Alkaline Phosphatase Conjugate	200 µL
04906	Biotin-Conjugated Goat Anti-Mouse IgG	125 µL
04807	Human Serum	6 mL
04809	Alkaline Phosphatase Substrate Tablets	30 x 1 mL "tris buffer" tablets in gold foil packets 30 x 1 mL "naphthol" tablets in silver foil packets
04913	Evans Blue Stain	5 mL
04915	10% BSA	6 mL

### 8.1 Preparation of MegaCult™-C Staining Reagents

All solutions must be freshly prepared and used within 8 hours, except for Alkaline Phosphatase Substrate Solution, which must be used within 1 hour. The amount prepared should be based on the number of slides to be stained. Refer to Appendix 3 for volumes of staining reagents for different numbers of test slides.

#### 1. 0.05 M Tris/NaCl Buffer

*Note: 1 L of 0.05 M Tris/NaCl Buffer is sufficient for staining 48 slides. Do not use phosphate buffers, as the staining reaction may be inhibited. Discard remaining buffer at the end of the day.*

- Prepare Solution A (0.15 M isotonic saline) as follows: Dissolve 8.766 g NaCl in 1 L of deionized, distilled water. Store Solution A at room temperature (15 - 25°C) for up to 1 month.
- Prepare Solution B (0.5 M Tris/HCl, pH 7.6) as follows: Dissolve 78.8 g Tris/HCl (Sigma Catalog #T-3253) in 1 L of deionized, distilled water. Adjust pH to 7.6 using 5 M NaOH. Store Solution B at room temperature (15 - 25°C) for up to 1 month.
- On day of use, mix 9 parts Solution A and 1 part Solution B.

#### 2. 5% Human Serum (for use in steps 3 & 4)

Dilute Human Serum (Component #04807) to a concentration of 5% in **0.05 M Tris/NaCl Buffer**.

*Note: Human Serum is a source of human immunoglobulin for blocking non-specific binding of primary antibody.*

#### 3. Diluted Primary Antibody

Dilute Primary Antibody (Component #04803) 1 in 100 in **5% Human Serum**.

#### 4. **Diluted Control Antibody**

Dilute Control Antibody (Component #04804) 1 in 100 in **5% Human Serum**.

#### 5. **1% BSA** (for use in steps 6 & 7)

Dilute 10% BSA (Component #04915) 1 in 10 in **0.05 M Tris/NaCl Buffer**.

#### 6. **10 µg/mL Biotin-Conjugated Goat Anti-Mouse IgG**

*Note: Contains 15 mM sodium azide as a preservative. Please refer to the Safety Data Sheet (SDS) for hazard information.*

- a) Thaw Biotin-Conjugated Goat Anti-Mouse IgG (Component #04906) on ice and dispense 25 µL aliquots into 0.5 mL or 1.5 mL tubes. Close tightly and store at -20°C.
- b) Thaw aliquots and dilute 1 in 10 with sterile **1% BSA**. These diluted aliquots are stable for 1 month at 2 - 8°C. Do not re-freeze.
- c) Prepare a further 1 in 30 dilution of the 1 in 10 diluted IgG with sterile **1% BSA** to achieve a final 1 in 300 dilution (10 µg/mL).

#### 7. **Diluted Avidin-Alkaline Phosphatase Conjugate**

*Note: Contains 15 mM sodium azide as a preservative. Please refer to the Safety Data Sheet (SDS) for hazard information.*

- a) Dilute Avidin-Alkaline Phosphatase Conjugate (Component #04905) 1 in 10 with sterile **1% BSA**. Store 1 in 10 diluted conjugate at 2 - 8°C for up to 1 month.
- b) Prepare a further 1 in 15 dilution of the 1 in 10 diluted conjugate with sterile **1% BSA** to achieve a final dilution of 1 in 150 (approximately 18 µg/mL).

#### 8. **Alkaline Phosphatase Substrate Solution**

*Note: Alkaline Phosphatase Substrate Solution must be used within 1 hour of preparation. The substrate tablets are toxic; do not touch them with your bare hands. Please refer to the Safety Data Sheet (SDS) for hazard information.*

- a) Allow Alkaline Phosphatase Substrate Tablets (Component #04809) to come to room temperature (15 - 25°C) for 1 - 2 hours prior to preparing solution.
- b) Determine the number of Alkaline Phosphatase Substrate Tablets required to prepare the required volume of substrate (0.5 mL per slide). Each tablet set is sufficient for 1 mL of substrate solution.
- c) Add the tablet(s) marked "tris buffer" (in gold foil) to the required volume of water. Vortex until fully dissolved.
- d) Add the tablet(s) marked "naphthol" (in silver foil) and vortex until dissolved. The solution should be pale pink in color.
- e) Use the substrate solution within 1 hour.

#### 9. **Evans Blue Stain Stock Solution**

Add 3 - 4 drops of MegaCult™-C Evans Blue Stain (Component #04913) per mL of methanol.



## 8.2 Staining Protocol

Prepare staining reagents as described in section 8.1.

At each step of the staining protocol, ensure that the cultures are completely covered with solution. The use of covered containers is recommended to prevent the cultures from drying out. As much of the staining reagent as possible should be removed by gently tipping the slide over a waste container, then carefully touching the edge of the slide to an absorbent paper. Refer to section 8.3 for additional helpful hints.

1. Allow slides to come to room temperature (15 - 25°C) before staining (approximately 30 minutes). Perform all steps of the staining procedure at room temperature with the slides in a horizontal position.

*Note: Tape two plastic serological pipettes approximately 3 cm apart to bottom of staining container and allow slides to rest firmly on pipettes during staining procedure.*

2. Prepare all staining reagents *except* Alkaline Phosphatase Substrate Solution (section 8.1). Allow Alkaline Phosphatase Substrate Tablets to come to room temperature.
3. Apply approximately 1.5 mL of **0.05 M Tris/NaCl Buffer** to each slide. Incubate at room temperature for 20 minutes. The buffer can be gently applied using a wash bottle.
4. Remove solution. Apply 0.5 mL of **5% Human Serum** to each slide and incubate for 20 minutes. Ensure that the entire surface of the culture is covered with the solution.

*Note: This step is required for blocking non-specific antibody binding; do not omit.*

5. Apply 0.5 mL of either **Diluted Primary Antibody** or **Diluted Control Antibody** and incubate for 30 minutes.

*Note: Stain one slide per batch with Diluted Control Antibody.*

6. Gently rinse slides 3 times (3 minutes each) with **0.05 M Tris/NaCl Buffer**.
7. Apply 0.5 mL of **10 µg/mL Biotin-Conjugated Goat Anti-Mouse IgG** to each slide and incubate for 30 minutes.
8. Gently rinse slides 3 times (3 minutes each) with **0.05 M Tris/NaCl Buffer**.
9. Apply 0.5 mL of **Diluted Avidin-Alkaline Phosphatase Conjugate** and incubate for 30 minutes.
10. Prepare **Alkaline Phosphatase Substrate Solution** (section 8.1).
11. Gently rinse slides 3 times (3 minutes each) with **0.05 M Tris/NaCl Buffer**.
12. Apply 0.5 mL of **Alkaline Phosphatase Substrate Solution** to each slide and incubate for 15 minutes.
13. Gently rinse slides 3 times (3 minutes each) with **0.05 M Tris/NaCl Buffer**.
14. Apply 0.5 mL of **Evans Blue Stain Stock Solution** to each slide and incubate for a maximum of 10 minutes.

*Note: An incubation time > 10 minutes may result in decreased staining intensity.*

15. While holding the slide over a waste container, use a wash bottle containing distilled water to gently rinse off excess staining solution.
16. Allow the slides to air dry. They can now be stored in covered containers at room temperature (15 - 25°C) or at 2 - 8°C for prolonged storage.

*Note: If coverslips are being used, use an aqueous mounting medium. Most alkaline phosphatase substrate reaction products are soluble in organic solvents.*

### 8.3 Helpful Hints

The following tips will assist with optimal staining and detection of megakaryocytic progenitor cells in the human CFU-Mk assay:

- All solutions must be added gently to surface of slide.
- Do not allow slides to dry out at any point during the staining procedure.
- Prepare reagents as described in section 8.1. Ensure 0.05 M Tris/NaCl Buffer is at pH 7.6; prepare fresh buffer daily.
- Do not use phosphate buffers, as they can interfere with the alkaline phosphatase staining reaction.
- Do not skip the blocking step (section 8.2 step 4).
- It is critical that Alkaline Phosphatase Substrate Tablets are stored correctly (-20°C). Ensure to bring them to room temperature prior to use (1 - 2 hours).
- When preparing Alkaline Phosphatase Substrate Solution, dissolve tris buffer tablet(s) completely before adding naphthol tablet(s). Solution should be pale pink in color. To ensure solution is completely dissolved, leave for 10 minutes in the dark prior to use. The solution must be used within 1 hour of preparation.
- Do not add Evans Blue Stain to the Alkaline Phosphatase Substrate Solution; Evans Blue Stain must be done as a separate step.



## 9.0 Counting Human CFU-Mk Colonies

Scan the entire slide using a 5X objective lens, noting the distribution of the colonies on the slide. Scoring can then be performed with the same lens, and the 10X objective lens can be used to examine colonies in greater detail.

Three categories of colonies can be identified in cultures grown in MegaCult™-C medium: megakaryocytic (Mk) colonies (CFU-Mk), mixed Mk colonies (containing other lineages in addition to Mk), and non-Mk colonies. Refer to section 10.0 for representative images of human CFU-Mk colonies.

Megakaryocytes and platelets, which express Glycoprotein IIb/IIIa (CD41), will appear pink following fixation and staining. Counterstaining with Evans Blue causes the nuclei of all cells to appear pale blue, regardless of lineage. As a result, CFU-Mk appear as groups of cells which have pink membranes staining with blue nuclei. CFU-Mk colonies will range in size from three to several hundred megakaryocytes per colony. It is therefore convenient to subdivide them by colony size; for example, small (3 to 20 cells per colony), medium (21 to 49 cells per colony), or large ( $\geq 50$  cells per colony). Large Mk colonies arise from more primitive Mk progenitor cells whereas the smaller Mk colonies are produced from more mature Mk progenitor cells.

Platelet clumps are irregular in shape, stain uniformly pink, and nuclei are not visible. As the cells within the CFU-Mk mature, there may be platelet-like fragments surrounding the colony. Single megakaryocytes may also be present, but should not be scored as a colony.

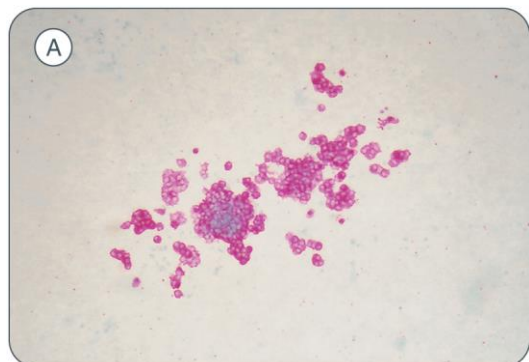
Non-Mk colonies ( $\geq 20$  cells per colony) are usually of the granulocyte/monocyte lineage and show only nuclear staining. Numbers of these colonies can be recorded if desired, but MegaCult™-C medium with TPO, IL-3, and IL-6 does not support optimal growth of CFU-GM.

Mixed Mk colonies are distinguished by the presence of both non-Mk cells and megakaryocytes within the same colony.

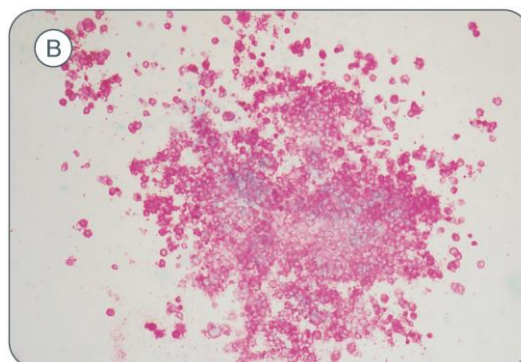
See Appendix 2 for expected numbers of CFU-Mk.

*Note: Gridlines may be visible on the slides (in a honeycomb pattern) and some colonies (particularly non-Mk colonies) will appear to be “trapped” in the grid, which can make scoring more challenging. However, the positively stained CFU-Mk colonies should be easy to enumerate in between the gridlines. Plating at lower cell densities should make scoring easier and minimize the number of colonies associated with the gridlines. In addition, to minimize the appearance of the gridlines, be sure not to apply pressure to the gels during the dehydration step and to ensure the spacers are removed promptly during the fixation step.*

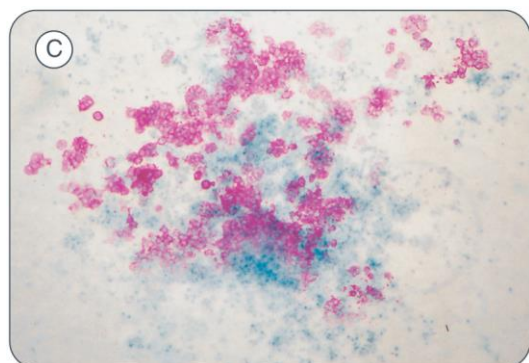
## 10.0 Representative Images of Human CFU-Mk Colonies



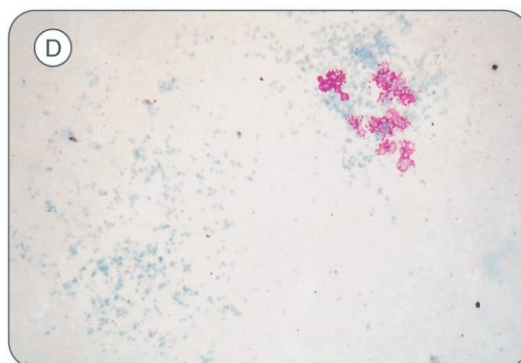
CFU-Mk; 21 - 49 cells (80X)



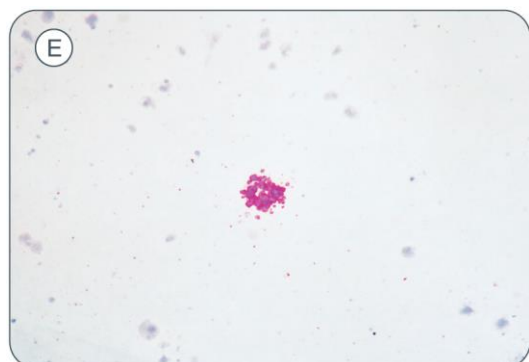
CFU-Mk; > 50 cells (80X)



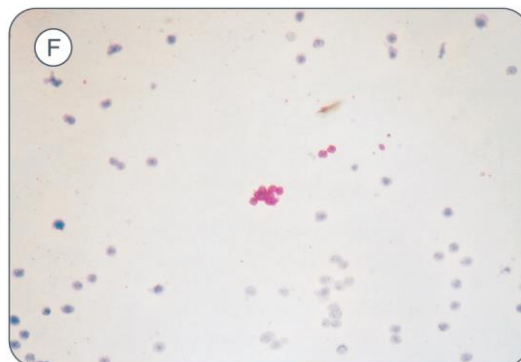
Mixed CFU-Mk/non-Mk (80X)



Non-MK and Mixed CFU-Mk/non-Mk (80X)



CFU-Mk; 3 - 20 cells (200X)



CFU-Mk; 3 - 20 cells (125X)

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## 11.0 MegaCult™-C Products for Mouse CFU-Mk Assays

### 11.1 MegaCult™-C Media Formulations and Ordering Information

MegaCult™-C serum-free media have been formulated to promote the growth of mouse CFU-Mk in collagen-based gels. The media formulations shown in Table 8 are available to support mouse megakaryocytic progenitor growth, with the addition of desired cytokines. See section 11.2 for recommended cytokines.

**Table 8. Components of MegaCult™-C Media Formulations for use with Mouse Cells**

*Note: See Table 9 for the MegaCult™-C kits that include MegaCult™-C media for use with mouse cells.*

MEGACULT™-C MEDIUM WITHOUT CYTOKINES*	MEGACULT™-C MEDIUM WITH LIPIDS*
Catalog #04900 (24 x 1.7 mL/tube)	Catalog #04850 (50 mL/bottle)
Iscove's MDM	Iscove's MDM
Bovine serum albumin	Bovine serum albumin
Recombinant human (rh) insulin	Recombinant human (rh) insulin
Human transferrin (iron-saturated)	Human transferrin (iron-saturated)
Supplements	Supplements
2-Mercaptoethanol	2-Mercaptoethanol
n/a	Lipids

\*Requires the addition of Collagen Solution (Catalog #04902) and desired cytokines.

**Table 9. MegaCult™-C Medium Kits**

For a complete list of all kit components, refer to Appendix 1.

MEGACULT™-C MEDIUM	MEGACULT™-C KIT	KIT CATALOG #
<b>MegaCult™-C Medium Without Cytokines</b>		
Catalog #04900 (24 x 1.7 mL/tube)	MegaCult™-C Collagen and Medium Without Cytokines Kit	04960
<b>MegaCult™-C Medium with Lipids*</b>		
Catalog #04850 (50 mL/bottle)	MegaCult™-C Collagen and Medium with Lipids Kit	04974

\*This medium does not contain cytokines.

*Note: MegaCult™-C Complete Kits, including staining reagents, are not suitable for mouse CFU-Mk assays, as the method for staining differs from human CFU-Mk.*



**Table 10. Final Composition of MegaCult™-C Medium for Mouse CFU-Mk Assays**

COMPONENT	VOLUME PER TUBE
MegaCult™-C Medium Without Cytokines OR MegaCult™-C Medium with Lipids	1.7 mL
Cytokines in Iscove's MDM**	0.3 mL
Collagen Solution	1.2 mL
Cells	0.1 mL
<b>Total</b>	<b>3.3 mL</b>

\*\*See Table 11 for recommended cytokine combinations and concentrations.

## 11.2 Recommended Cytokines

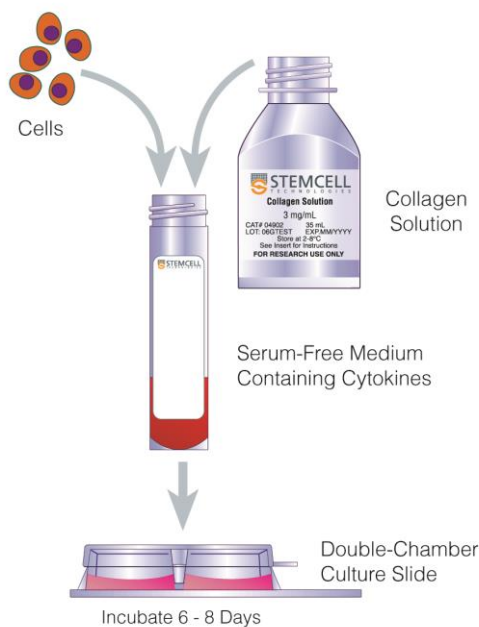
**Table 11. Recommended Cytokine Combinations for Detection of Mouse CFU-Mk**

CYTOKINE	FINAL CONCENTRATION	CATALOG #
<b>Cytokine Combination #1</b>		
Human Recombinant TPO	50 ng/mL	78210
Human Recombinant IL-6	20 ng/mL	78050
Human Recombinant IL-11	50 ng/mL	78025
Mouse Recombinant IL-3	10 ng/mL	78042
<b>Cytokine Combination #2</b>		
Human Recombinant TPO	50 ng/mL	78210
Human Recombinant IL-6	20 ng/mL	78050
Mouse Recombinant IL-3	10 ng/mL	78042
<b>Cytokine Combination #3</b>		
Human Recombinant TPO	50 ng/mL	78210
Mouse Recombinant IL-3	10 ng/mL	78042

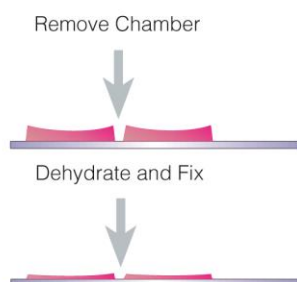
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## 12.0 MegaCult™-C Procedure Diagram for Mouse Cells

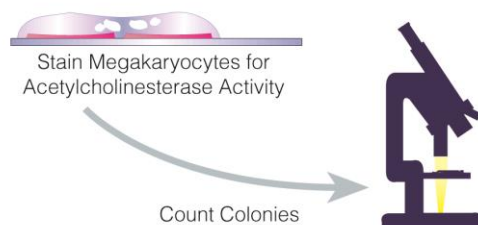
### ① Set-up and Culture



### ② Dehydrate and Fix



### ③ Stain and Count



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## 13.0 Equipment and Materials Required for Mouse CFU-Mk Assays

### 13.1 Equipment

- Biohazard safety cabinet certified for Level II handling of biological materials
- Incubator set at 37°C with 5% CO<sub>2</sub> 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<sub>2</sub> as inhibition of colony growth due to toxic substances present in the CO<sub>2</sub> gas source has been reported.*

- Light microscope with 5X and 10X objectives for cell counting and colony counting
- Centrifuge
- Vortex
- Pipette-aid
- Pipettors (e.g. Catalog #38058)
- Automated cell counter or Neubauer hemocytometer

## 13.2 Materials

- MegaCult™-C Medium (see section 11.1)
- Collagen Solution (Catalog #04902)
- Staining system for mouse CFU-Mk

STEMCELL Technologies does not supply reagents required for staining of mouse megakaryocytes in the CFU-Mk assay. A suitable protocol for staining of acetylcholinesterase in mouse megakaryocytes is described in section 17.0.
- Recombinant cytokines (see Table 11)
- 35 mm Culture Dishes (Catalog #27100)
- 100 mm Culture Dishes, Non-Treated (Catalog #38045)
- Iscove's MDM with 2% FBS (Catalog #07700)
- Iscove's Modified Dulbecco's Medium (Catalog #36150)
- 3% Acetic Acid with Methylene Blue (Catalog #07060)
- Sterile laboratory supplies (i.e. pipettes, tubes, pipette tips)
- Acetone: Acetone OPTIMA (e.g. Fisher #A929-4, 4 L/bottle), or other high-quality grade (e.g. ACS)
- Double Chamber Slide Kit (Catalog #04963)
  - Double Chamber Slides (Catalog #04813)
  - Filter Cards and Spacers
- Containers for fixation and staining solutions (ensure that container for fixation can withstand exposure to acetone)
- 2 mL serological pipettes (e.g. Catalog #38002)
- 70% isopropyl alcohol
- 3 cc Syringe (e.g. Catalog #28240) and 21 gauge needle
- Ice

## 14.0 Thawing and Dispensing MegaCult™-C Media

*Note: For pre-aliquoted MegaCult™-C media, proceed to section 15.0.*

### 14.1 Preparation of Bottles

1. Thaw bottle of MegaCult™-C Medium with Lipids at room temperature (15 - 25°C) or overnight at 2 - 8°C. Mix thoroughly.
2. Dispense 1.7 mL of MegaCult™-C Medium with Lipids into tubes.

*Note: To use immediately, proceed to section 15.0. Alternatively, store tubes of medium at -20°C.*

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## 15.0 Culture of Mouse Cells

### 15.1 Isolation of Mouse Bone Marrow Cells

*Note: Animals should be sacrificed using procedures approved by your institution.*

1. Wet the pelt thoroughly with 70% isopropyl alcohol, then clip and peel back to expose hind limbs. Using sterile sharp scissors (to avoid splitting of the bone), cut the knee joint in the center and remove ligaments and excess tissue.
2. Trim the ends of the long bones to expose the interior of the marrow shaft.
3. Collect the marrow cells in 1 to 2 mL of Iscove's MDM with 2% FBS. Flush the marrow from the femoral shaft using a 21 gauge needle attached to a 3 cc Syringe. A smaller needle (22 or 23 gauge) may be required to flush marrow from the tibia. Use the same medium to flush bones from one to three animals.
4. Prepare a single-cell suspension by gently aspirating several times using the same needle and syringe.
5. Keep the cells on ice. It is not necessary to process the cells for most applications.
6. Perform a manual nucleated cell count using 3% Acetic Acid with Methylene Blue. See Table 12 for the expected number of nucleated cells.

**Table 12. Expected Recovery of Nucleated Cells**

BONES FLUSHED	NUMBER OF NUCLEATED CELLS
1 femur	1 - 2 x 10 <sup>7</sup>
1 tibia	0.6 - 1.2 x 10 <sup>7</sup>
2 femur and 2 tibia	3 - 6 x 10 <sup>7</sup>

7. Prior to culture, wash the cells in Iscove's MDM (without FBS).

### 15.2 Mouse CFU-Mk Assay Setup

Use sterile technique when performing all culture procedures.

1. Thaw tubes of MegaCult™-C Medium Without Cytokines or MegaCult™-C Medium with Lipids (1.7 mL/tube) at room temperature (15 - 25°C) or overnight at 2 - 8°C.

*Note: Once tubes are thawed, do not re-freeze.*

2. Add the desired cytokines and Iscove's MDM in a 0.3 mL volume to the MegaCult™-C medium to achieve a final volume of 2.0 mL. See Table 11 for recommended cytokine combinations.
3. Place Collagen Solution on ice.
4. Prepare cell suspension in Iscove's MDM at 33X the desired final cell concentration (see Table 13).

**Table 13. Recommended Plating Concentrations for Mouse Cells**

CELL TYPE	CELL SUSPENSION (cells/mL)**	CELLS PER SLIDE (in 1.5 mL)
Unseparated bone marrow	$2.2 \times 10^6$	$1.5 \times 10^5$
Lineage-depleted bone marrow*	$1.1 \times 10^5$	5000

\*Lineage-depleted bone marrow suspension: Lineage-positive cells are depleted using StemSep™ Mouse Progenitor Cell Enrichment Kit (Catalog #13056) or EasySep™ Mouse Progenitor Cell Enrichment Kit (Catalog #19756).

\*\*The cell concentration given is 33X the desired final cell concentration.

5. Add 0.1 mL of the cell suspension to each tube containing 2.0 mL of medium with cytokines.
6. Mix one tube of medium containing cells (2.1 mL total volume).
7. Using a sterile 2 mL serological pipette, transfer 1.2 mL of cold Collagen Solution to the tube (3.3 mL final volume). Pipette up and down to mix.
8. Using the same 2 mL pipette, remove 1.5 mL of the final culture mixture and dispense 0.75 mL into each of the two wells of a previously labeled double chamber slide.  
*Note: Chamber slides should be labeled with a pencil or diamond point pen. Ink labeling will become illegible during the fixation process.*
9. Dispense an additional 1.5 mL in the same manner onto a second double chamber slide. Remove any air bubbles by gently touching bubble with the end of the pipette.  
*Note: The collagen will begin to gel within several minutes following addition to medium containing cells. If more than one tube is being set up, collagen should be added to the first tube only and the contents of the tube dispensed into chamber slides before proceeding to the next tube.*
10. Gently tip each slide using a circular motion to allow the mixture to spread evenly over the surface of the slide.
11. Place each slide in a 100 mm Culture Dish containing an uncovered 35 mm Culture Dish with 3 mL of sterile water to maintain optimal humidity during the incubation period. Replace the lid of the 100 mm dish.
12. Transfer the slides to a 37°C incubator with 5% CO<sub>2</sub> and ≥ 95% humidity. Gel formation will occur within approximately 1 hour. It is important not to disturb the cultures during this time.
13. Incubate cultures for 6 - 8 days. Maximum CFU-Mk colony size and numbers are typically seen at this time. Cultures should be visually assessed for overall colony growth and morphology using an inverted microscope prior to fixation and staining.

## 15.3 Helpful Hints

The following tips will assist with optimal detection of megakaryocytic progenitor cells in the mouse CFU-Mk assay:

- It is possible to split the medium containing cytokines into two tubes (2 x 1.0 mL) in order to carry out more tests. In this case, the volume of collagen and cells added must be reduced accordingly (i.e. 0.6 mL collagen per 1.0 mL of medium and 0.05 mL cells), and samples would be plated in one double chamber slide (2 x 0.75 mL).
- Label slides with a pencil or diamond point pen. Ink labeling will become illegible during the fixation process.
- Maintain proper incubator conditions including temperature, CO<sub>2</sub> level, and humidity (37°C with 5% CO<sub>2</sub> and ≥ 95% humidity). It is also important to place cultures in a covered 100 mm Culture Dish containing an open 35 mm Culture Dish with 3 mL sterile water.
- Ensure MegaCult™-C Medium and Collagen Solution are used prior to the expiration date stated on the label and have been correctly stored.
- Collagen Solution should be stored at 2 - 8°C and placed on ice during use. Collagen Solution should not be frozen.
- Occasionally, the medium may be yellow upon receipt due to a decrease in pH during shipping. The medium will return to proper color within 24 hours of incubation and colony formation will not be inhibited.
- Cells should be washed prior to culture. Serum contains substances known to inhibit the growth of CFU-Mk.
- The presence of large numbers of platelets may inhibit the growth of megakaryocytes by the release of TGF-β and platelet factor 4 (PF4) from a granules. Typically, mouse bone marrow contains low numbers of platelets and can be plated unprocessed.
- It may be necessary to establish the optimal plating concentrations of cells within each laboratory. There is considerable variation in the numbers of CFU-Mk detected based on the tissue source, cell purification techniques, and in vitro manipulation of cells. To establish optimal plating conditions, each cell suspension tested should be plated at 2 - 3 concentrations separated by 3- to 4-fold dilutions.
- Overplating can result in inhibition of optimal colony formation. If cultures become yellow (acidic) during culture, excessive numbers of cells may have been plated.
- Collagen gel formation is initiated by neutralization (following mixing with medium) and the increase in temperature upon incubation. Once the gelling process is initiated, it is important not to manipulate or disturb the cultures. As described in section 15.2, cultures should be set up one at a time.
- Ensure correct proportions of cells, medium, and collagen are added to each culture. For example, if the cytokines are added in a volume greater than 0.3 mL, the volume of the cell suspension must be reduced accordingly and the concentration of the cells in the suspension increased accordingly.

## 16.0 Dehydrating and Fixing Cultures of Mouse Cells

### 16.1 Dehydrating and Fixing Protocol

1. Examine cultures for overall colony growth using an inverted microscope prior to dehydrating and fixing.
2. Place 200 mL of acetone in a 2.5 L container on ice and seal the lid tightly to prevent evaporation. Place on ice for a minimum of 15 minutes (30 minutes or more is preferable).

*Note: It is important to use high-quality acetone.*

*Note: It is critical to use ice-cold acetone for the fixation procedure. If acetone is not properly cooled prior to fixation (for > 15 minutes on ice), opaque areas will appear around the periphery of the gels, which can make cultures difficult to view under the microscope.*

3. Leave the cultures at 37°C until just prior to dehydration, as the collagen gel may become unstable at lower temperatures. Remove slides from the incubator singly or in small batches.

*Note: Ensure that each slide is labeled with a pencil or a diamond point pen. Ink labeling will become illegible when the slide is immersed in the fixing solution.*

4. Carefully remove the plastic walls and rubber seal of the chamber slide without damaging the culture, using the following procedure:
  - a) Remove chamber lid.
  - b) Partially remove the plastic chamber by pulling it up and away from the slide (use forceps to hold the slide down) starting at one corner near the labeled end of the slide.
  - c) When the rubber seal surrounding the collagen gel is visible under the plastic chamber, use forceps to grasp one corner of the rubber seal and gently stretch it over the corner of the plastic chamber so that it remains hooked there.
  - d) Use forceps to hook the rubber seal over the other corner of the plastic chamber at the same end of the slide.
  - e) The plastic chamber and rubber seal can now be easily removed in one motion by continuing to slowly pull them up and away from the slide. The collagen gel and associated liquid will remain on the slide.
5. Gently place a pre-cut spacer onto the chamber slide. Place a thick white filter card on top and allow liquid to soak the card. Filter cards and spacers are supplied with the Double Chamber Slide Kit.

*Note: Do not apply pressure to the filter card. The liquid should start to wick onto the filter card immediately. If it does not, gently apply a small amount of pressure to the corner of the filter card on the opposite end of the slide from the gel. Applying too much pressure will lead to a grid pattern on the slides.*
6. Remove the thick white filter card as soon as it becomes fully saturated and leave the original spacer in place.
7. Place the slides with spacers into the fixing solution. Ensure that the slides are completely covered with solution. Tightly seal the lid of the container.
8. The spacers should float off when slides are placed in the fixative, leaving two squares of gel on the slide.

*Note: If the spacers do not float away from the slides during fixation, gently shake the container to encourage them to detach. If a spacer remains adherent, remove that slide from the fixative solution and*



*gently peel the gel from the spacer onto the slide using a micro spatula or tweezers, being careful not to rip the gel. Do not allow the slide to dry out during this step.*

*Note: Decreasing the time the spacer is in contact with the gel may reduce the appearance of gridlines on the slides after fixation.*

9. Leave slides in ice-cold acetone for 5 minutes. Keep the acetone on ice for the entire fixation procedure. Ensure that the lid of the container is tightly sealed.

*Note: Change the fixing solution every 12 slides.*

10. Remove the slides from the fixing solution and allow to air dry for 15 minutes.

11. Proceed to section 17.0 for a recommended staining protocol.

*Note: Fixed cultures can be stored at 2 - 8°C or at -20°C in the dark for up to 1 month, until staining can be performed.*

## 16.2 Helpful Hints

The following tips will assist with optimal detection of megakaryocytic progenitor cells in the CFU-Mk assay:

- Cultures may appear 'runny' if left at room temperature (15 - 25°C) too long prior to fixation. Leave the slides at 37°C until just prior to fixation. Remove slides from incubator one at a time or in small batches.
- If acetone is not properly cooled prior to fixation, opaque areas will appear around the periphery of the gels, which can make cultures difficult to view under the microscope.
- Slides should be laid flat in the fixing solution.
- If the collagen gel does not firmly adhere to slide while removing the chambers, it can be eased gently back into place.
- Change the fixing solution (200 mL) between each batch of slides (up to 12).

## 17.0 Staining Cultures of Mouse Cells

### 17.1 Preparation of Staining Reagents

1. **0.1 M Sodium Phosphate Buffer, pH 6.0**
  - Dissolve 13.40 g sodium phosphate ( $\text{Na}_2\text{HPO}_4$ , FW 268.07; e.g. Fisher Catalog #S373) in approximately 490 mL water. Adjust to pH 6.0 with 0.1 M HCl or 0.1 M NaOH and adjust final volume to 500 mL.
2. **Acetylthiocholiniodide Solution**
  - Dissolve 10 mg of acetylthiocholiniodide (e.g. Sigma Catalog #A5751) in 15 mL of **0.1 M Sodium Phosphate Buffer** (prepared in step 1).
3. **0.1 M Sodium Citrate Solution, pH 6.0**
  - Dissolve 2.94 g sodium citrate ( $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7$ , FW 294.1; e.g. Sigma Catalog #S4641) in approximately 95 mL water. Adjust to pH 6.0 with 0.1 M citric acid and adjust final volume to 100 mL.
4. **30 mM Copper Sulfate Solution**
  - Dissolve 749 mg copper sulfate ( $\text{CuSO}_4$ , FW 249.68; e.g. Fisher Catalog #C493) in approximately 95 mL water and adjust final volume to 100 mL.
5. **5 mM Potassium Ferricyanide Solution**
  - Dissolve 165 mg potassium ferricyanide ( $\text{K}_3\text{Fe}(\text{CN})_6$ , FW 329.24; e.g. Sigma ACS Reagent Catalog #244023) in 95 mL water and adjust final volume to 100 mL.

*Note: Solution decays slowly on standing; protect from light.*
6. **95% Ethanol**
7. **Harris' Hematoxylin Solution** (e.g. Sigma Catalog #HHS16)

### 17.2 Staining Protocol

Prepare staining reagents as described in section 17.1.

At each step of the staining protocol, remove as much of the staining reagent as possible by gently tipping the slide over a waste container and then carefully touching the edge of the slide to an absorbent paper. Refer to section 17.3 for additional helpful hints.

1. Allow slides to come to room temperature (15 - 25°C) before staining (approximately 30 minutes). Perform all steps of the staining procedure with the slides in a horizontal position. All solutions must be added gently to the surface of the slide.

*Note: Tape two plastic serological pipettes approximately 3 cm apart to bottom of staining container and allow slides to rest firmly on pipettes during staining procedure.*
2. To prepare **Staining Solution**, add the following to 15 mL of **Acetylthiocholiniodide Solution**, in order, with constant stirring:
  - a) 1 mL of **0.1 M Sodium Citrate Solution**
  - b) 2 mL of **30 mM Copper Sulfate Solution**
  - c) 2 mL of **5 mM Potassium Ferricyanide Solution**

The total volume will be 20 mL (sufficient for approximately 10 - 15 slides).



3. Flood sample slides with **Staining Solution** and incubate for approximately 5 hours in a humid chamber. Megakaryocytes will appear brown.

*Note: Ensure that the cultures are completely covered with Staining Solution. The use of a covered container is recommended to prevent the cultures from drying out.*

*Note: It has been reported that 3 - 4 hours is enough time to detect 100% of larger mature megakaryocytes, but only 30 - 60% of immature megakaryocytes. Therefore slides can be stained for up to 6 hours to detect all megakaryocytic progenitor cells.<sup>11</sup> Granules appear light red-brown in cells with low acetylcholinesterase content and dark brown-black in the intensely stained megakaryocytes. The longer the Staining Solution is left on, the darker the megakaryocytes stain.*

4. Pour off **Staining Solution**. Apply **95% Ethanol** (fixing solution) and incubate at room temperature (15 - 25°C) for 10 minutes.
5. Rinse with lukewarm water and completely air dry before proceeding to the next step.
6. Apply **Harris' Hematoxylin Solution** (counterstain) and incubate for 30 seconds at room temperature.

*Note: Do not leave slides in Harris' Hematoxylin Solution for longer than 30 seconds.*
7. Rinse with lukewarm water and allow the slides to air dry. They can now be stored in covered containers at room temperature.

### 17.3 Helpful Hints

The following tips will assist with optimal detection of megakaryocytic progenitor cells in the mouse CFU-Mk assay:

- All solutions must be added gently to surface of the slide.
- Do not allow slides to dry out at any point during staining procedure.
- Prepare reagents as described in section 17.1 and ensure they are at the correct pH. Prepare fresh reagents daily.
- Allow slides to completely air dry before counterstaining with Harris' Hematoxylin Solution.

## 18.0 Counting Mouse CFU-Mk Colonies

Scan the entire slide using a 5X objective lens, noting the relative proximity of colonies to each other. Scoring can then be performed with the same lens, and the 10X objective lens can be used to examine colonies in greater detail. See section 19.0 for representative images of mouse CFU-Mk colonies.

Mouse megakaryocytes and early megakaryocyte progenitor cells express acetylcholinesterase and have brown granular deposits of copper ferrocyanide in the cytoplasm resulting from the enzymatic reaction. Granules may appear light red-brown in cells with low acetylcholinesterase content and may range from orange-brown to dark brown/black in cells with high acetylcholinesterase content. Also, the longer the stain is left on, the more intense the staining. Counterstaining with Harris' hematoxylin solution results in violet-stained nuclei in all cells. However, this is less noticeable in megakaryocytes, as the copper ferrocyanide precipitate from the acetylcholinesterase stain can mask the counterstain.

CFU-Mk colonies will range in size from 3 to approximately 50 megakaryocytes per colony. CFU-Mk must contain at least 3 megakaryocytes. Single megakaryocytes are not scored as CFU-Mk.

Non-Mk colonies are groups of cells with violet nuclei but no brown precipitate. These are primarily granulocyte/macrophage colonies (> 30 cells), and can be scored if desired. It should be noted that cytokine combinations selected for optimal detection of CFU-Mk may be unsuitable for optimal detection of CFU-GM.

Mixed Mk colonies are distinguished by the presence of non-megakaryocytic cells as well as brown-stained megakaryocytes in the same cluster of cells. It is sometimes difficult to identify mixed Mk colonies, especially when the culture contains large numbers of CFU-Mk colonies and non-Mk colonies close together. Plating concentrations should be adjusted accordingly.

See Appendix 2 for expected numbers of CFU-Mk.

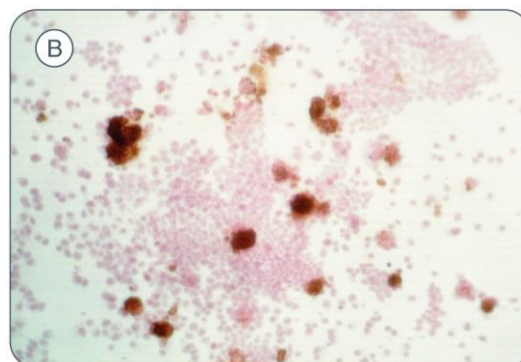
*Note: Gridlines may be visible on the slides (in a honeycomb pattern) and some colonies (particularly non-Mk colonies) will appear to be "trapped" in the grid, which can make scoring more challenging. However, the positively stained CFU-Mk colonies should be easy to count between the gridlines. Plating at lower cell densities should make scoring easier and minimize the number of colonies associated with the gridlines. In addition, to minimize the appearance of the gridlines, be sure not to apply pressure to the gels during the dehydration step and to ensure the spacers are removed promptly during the fixation step.*

## 19.0 Representative Images of Mouse CFU-Mk Colonies

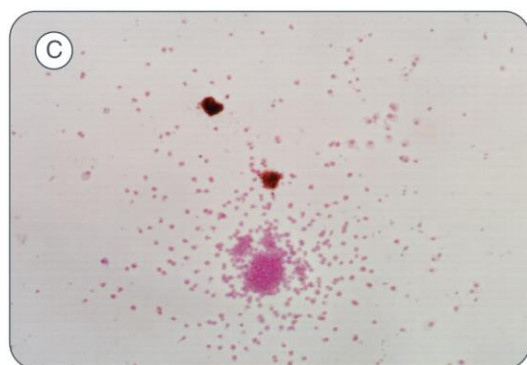
C57BL/6J mouse bone marrow was plated at  $1 \times 10^5$  cells/slide and incubated for 6 days prior to dehydration and staining.



**CFU-Mk (60X)**



**Mixed CFU-Mk/Non-CFU-Mk (100X)**



**Non-Mk colony with 2 adjacent megakaryocytes (60X)**

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## 20.0 Appendices

### 20.1 Appendix 1: MegaCult™-C Culture Media Kits

**Table 14. Components of Each MegaCult™-C Kit**

KIT CATALOG #	MEGACULT™-C KIT NAME	KIT COMPONENTS					
		MegaCult™-C Medium with Cytokines	MegaCult™-C Medium Without Cytokines	MegaCult™-C Medium with Lipids	Collagen Solution	Staining Kit for CFU-Mk	Double Chamber Slide Kit
		04901 (24 x 2.0 mL)	04900 (24 x 2.0 mL)	04850 (50 mL bottle)	04902	04962	04963
04971	MegaCult™-C Complete Kit with Cytokines	•			•	•	•
04970	MegaCult™-C Complete Kit Without Cytokines		•		•	•	•
04961	MegaCult™-C Collagen and Medium with Cytokines	•			•		
04960	MegaCult™-C Collagen and Medium Without Cytokines		•		•		
04974	MegaCult™-C Collagen and Medium with Lipids			•	•		

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## 20.2 Appendix 2: Expected Values for CFU-Mk Assays

### 20.2.1 Expected Values - Human CFU-Mk

The MegaCult™-C assay has been used to determine the range of megakaryocyte progenitor cells present in a series of cord blood samples and bone marrow samples obtained from normal adults. Note that there can be variability between individual donors.

**Table 15. Expected Number of CFU-Mk in Human Bone Marrow and Cord Blood Samples**

CELL SOURCE	n	CFU-Mk Mean (95% confidence limits)	MIXED Mk/NON-CFU-Mk Mean (95% confidence limits)
Bone marrow (per 10 <sup>5</sup> mononuclear cells)	16	68 (10 - 150)	3 (1 - 10)
Bone marrow (per 10 <sup>4</sup> CD34 <sup>+</sup> enriched cells)	6	130 (1 - 280)	5 (1 - 15)
Cord blood (per 10 <sup>5</sup> mononuclear cells)	13	51 (1 - 140)	4 (1 - 14)

### 20.2.2 Expected Values - Mouse CFU-Mk

The MegaCult™-C assay has been used to determine the range of megakaryocyte progenitor cells present in a series of mouse bone marrow samples obtained from 6- to 12-week-old C57Bl/6J mice. Variability between mouse strains and age is likely to be observed.

**Table 16. Expected Frequency of Megakaryocytic Progenitor Cells in 1 x 10<sup>5</sup> Mouse Bone Marrow Cells**

CYTOKINE COMBINATION	n	CFU-Mk Mean (95% confidence limits)
50 ng/mL rh TPO 10 ng/mL rm IL-3 20 ng/mL rh IL-6 50 ng/mL rh IL-11	12	48 (24 - 72)
50 ng/mL rh TPO 10 ng/mL rm IL-3 20 ng/mL rh IL-6	8	38 (18 - 58)
50 ng/mL rh TPO 10 ng/mL rm IL-3	8	26 (2 - 50)

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## 20.3 Appendix 3: Staining Reagent Volumes for Human CFU-Mk

Refer to section 8.0 for complete instructions for staining cultures of human cells using the MegaCult™-C Staining Kit for CFU-Mk. Table 17 provides the volumes of staining reagent components required for different numbers of test slides.

**Table 17. Staining Reagent Volumes for Human CFU-Mk**

STAINING REAGENT	COMPONENTS	NUMBER OF TEST SLIDES (TOTAL SLIDES)*				
		2 (3)	4 (5)	6 (7)	8 (9)	12 (13)
		VOLUME OF COMPONENT (mL) (unless otherwise indicated)				
Total Buffer Required	Tris/NaCl Buffer	150	250	350	450	650
5% Human Serum <i>-for blocking and dilution</i>	Human Serum	0.25	0.35	0.5	0.6	1.0
	Tris/NaCl Buffer	4.75	6.65	9.5	11.4	19.0
Primary Antibody, 1 in 100 dilution <i>-for test slides</i>	Primary Antibody	0.015	0.025	0.035	0.045	0.065
	5% Human Serum	1.49	2.48	3.47	4.46	6.44
Control Antibody, 1 in 100 dilution <i>-for negative control slides</i>	Control Antibody	0.01	0.01	0.01	0.01	0.01
	5% Human Serum	0.99	0.99	0.99	0.99	0.99
1% BSA <i>-for dilution</i>	10% BSA	0.5	0.7	0.9	1.1	1.5
	Tris/NaCl Buffer	4.5	6.3	8.1	9.9	13.5
Biotin-Conjugated Goat Anti-Mouse IgG, 1 in 300 dilution <i>-for all slides</i>	1 in 10 dilution of Biotin-Conjugated Goat Anti-Mouse IgG	0.067	0.100	0.133	0.167	0.233
	1% BSA	1.93	2.90	3.87	4.84	6.77
Avidin-Alkaline Phosphatase Conjugate, 1 in 150 dilution <i>-for all slides</i>	1 in 10 dilution of Avidin-Alkaline Phosphatase Conjugate	0.133	0.200	0.267	0.333	0.467
	1% BSA	1.87	2.80	3.74	4.67	6.53
Alkaline Phosphatase Substrate <i>-for all slides</i>	Water	2	3	4	5	7
	Alkaline Phosphatase Substrate Tablets	2 sets	3 sets	4 sets	5 sets	7 sets
Evans Blue Stain Stock Solution <i>-for all slides</i>	Evans Blue Stain	8 drops	12 drops	16 drops	20 drops	28 drops
	Methanol	2	3	4	5	7

\*Total slides = test slides + negative control. Each calculation allows sufficient reagents for one negative control slide per staining experiment. Calculated volumes allow for approximately 0.5 mL of each solution per slide with 0.5 mL extra volume.



## 21.0 References

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TECHNICAL MANUAL

# MegaCult™-C

## Assays for Quantitation of Human and Mouse Megakaryocytic Progenitor Cells



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