Protocol

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Hematopoietic Colony Forming Cell (CFC) Assays

Adapted from M. Fero, Fred Hutchinson Cancer Research Center, 2004

Introduction

Hematopoietic cells are stem cells that can give rise to different types of blood cells through hematopoiesis. These stem cells can give rise to two different progenitors: the myeloid or the lymphoid progenitors. These progenitors then differentiate into platelets, eosinophils, erythrocytes, or into T-cells and B-cells. The study of these stem cells and their differentiation is critical for our understanding of hematopoiesis and disease. One assay that allows us to study these events is the hematopoietic colony forming cell assay. In this assay, cells are grown in the presence of cytokines, allowing them to differentiate into different lineages, which we can then classify and study. Here, we describe a protocol for a hematopoietic colony forming cell assay from spleen or bone marrow.

Materials

BSA in Iscove's Modified Dulbecco's Medium (IMDM) (100 mg/ml = 10% w/v)

- 5 g BSA (GoldBio Catalog # A-421)
- Fill to 50 ml with IMDM
- Rotate at 4°C for 1 hour to dissolve

Methylcellulose in IMDM, 2.6%

- Add glutamine prior to use

Growth Factors

- Human erythropoietin (EPO) (GoldBio Catalog # 1120-04) (2000 U/1 ml), dilute to 50 U/ml
- Murine colony stimulating factor 2 (CSF2) (GoldBio Catalog # 1320-02) (300 µg/1ml), dilute to 100 ng/ml
- Human colony stimulating factor 3 (CSF3) (GoldBio Catalog # 1120-02) (300 µg/1ml), dilute to 100 ng/ml
- Murine interleukin 3 (IL3) (GoldBio Catalog # 1310-03) or WEHI conditioned medium, dilute to 100 ng/ml

Growth Factor (GF) Mix

- Combine IMDM, serum, BSA and growth factors as shown in Table 1.
- To plate cells in triplicate, make aliquots of 4 ml per assay or 2 ml per assay for burst-forming unit-erythroid (BFU-E). These can be stored at 4°C overnight or at -80°C. Multiply this volume by the number of experimental groups and controls to be studied and by the number of cell doses that will be plated in order to calculate the total amount of GF mix needed.

Heat inactivated FBS

- Serum should be heat-inactivated to inactivate complement. This is important if the cells have been stained with antibodies and flow sorted by flow cytometry.
- Place a 50 ml serum aliquot in a 56°C water bath for 30 minutes and then cool.
- Centrifuge the tube at 700 g for 5 minutes to pellet insoluble proteins.
- Transfer the supernatant to a fresh 50 ml conical tube.

Table 1. Growth Factor Mix.

	CFC-Mix	GM-CFC	G-CFC	BFU-E	CFU-E
IMDM	38% v/v	40% v/v	40% v/v	8% v/v	49.6% v/v (or 9.6% for MC)
FBS	20% v/v	-	-	20% v/v	20% v/v
Horse Serum	-	20% v/v	20% v/v	-	-
BSA in IMDM 100 mg/ml	10% v/v	± 10% v/v	± 10% v/v	10% vol	10% vol
WEHI-3B C.M. or IL3 (100 ng/ml)	10% v/v	-	-	10% vol	-
huEPO 50 U/ml	2% v/v	-	-	2% vol	0.4% vol
muCSF2 (100 ng/ml)	-	10% v/v	-	-	-
huCSF3 (100 ng/ml)	-	-	10% v/v	-	-
Cell suspension (see Table 2)	10% v/v	10% v/v	10% v/v	10% v/v	10% v/v
3.3 % Agar	10% v/v	10% v/v	10% v/v	-	10% v/v
Methylcellulose In IMDM	-	-	-	50% v/v	50% v/v

(Kaushansky and Broudy add the following: βME (50µM final conc.), 1% Pen/Strep/Fungizone. See Furo, *et al.* 1996 Cell reference).
Abbreviations: CFC-Mix, colony-forming cells-mix; GM-CFC, granulocyte, macrophage-colony-forming cells; G-CFC, granulocyte-colony-forming cells; BFU-E, burst-forming unit-erythroid; CFU-E, colony-forming unit-erythroid.

Table 2. 10X Cell Suspensions.

Source	Bone Marrow	Spleen	Bone Marrow after 5-FU treatment
Cells/ml x 10(5)	1-10	10-100	10-100

Method

Procedure using agar

1. Prepare cell suspensions in IMDM + 2% FBS. Use heat inactivated FBS if antibody staining will be done. It is not necessary to remove RBC from spleen or marrow. Cells may be kept at 4°C for several hours.

2. Meanwhile, melt agar in a boiling water bath and then place in 55°C bath until ready for use.

3. Add 0.5 ml (1/10th of final volume) of cell suspension to 4 ml growth factor mix in a disposable tube and warm to 37°C.

4. Pipette the melted agar up/down to prewarm the tip. Add 0.5 ml of the melted agar to the cell/GF mix with continuous vortexing and quickly pipette 1.5 ml into three wells of a 6-well plate or 35 mm dishes.

5. Cool the dish at 4°C for 2-3 minutes to set the agar.

Procedure using methylcellulose

1. Prepare cell suspensions in IMDM + 2% heat inactivated FBS. It is not necessary to remove RBC from spleen or marrow. Cells may be kept at 4°C for several hours.

2. Add 0.5 ml (1/10th of final volume) of cell suspension to 2 ml media/growth factor cocktail in a disposable tube and warm to 37°C.

3. Add an equal volume (2.5 ml) of methyl cellulose/IMDM to the cell/GF mixture and plate ~1.5 ml into three wells of a 6-well plate or into 35 mm dishes.

Incubation

1. Incubate dishes in a humidified incubator at 36°C with 5% CO_2 and hypobaric oxygen (5% O_2). Refer to Table 3 for a description of colonies depending on the day and type of colony.

Note: The CO_2 maintains the pH of the media, and the lower temperature and low oxygen promote growth of hematopoietic cells.

Note: The low oxygen also helps promote hemoglobinization of erythroid cells.

Note: To minimize nitrogen utilization, the incubator should only be gassed after plates have been placed inside. Also the incubator should only be opened briefly to retrieve plates on the day(s) that they are counted.

Table 3. Reading Colonies

	Mix	GM-CFC	G-CFC	BFU-E	CFU-E
Day	7-12	7	7	5-6	2
Appearance	> 50 cells	> 50 cells	> 50 cells	200-10,000 cells in 3-8 clusters	6-60

Associated Products

- CSF3 (G-CSF), Human (GoldBio Catalog # 1120-02)
- EPO-α, Human (GoldBio Catalog # 1120-04)
- IL3, Murine (GoldBio Catalog # 1310-03)
- CSF2 (GM-CSF), Murine (GoldBio Catalog # 1320-03)

References

Fero Lab. Hematopoietic colony forming cell assays. Fred Hutchinson Cancer Research Center. Seattle, WA.

Fero, M. L., et al. (1996). A Syndrome of Multiorgan Hyperplasia with Features of Gigantism, Tumorigenesis, and Female Sterility in p27 Kip1-Deficient Mice. Cell, 85(5), 733-744.

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