The Fleischman Lab

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Title: Colony Forming Cell (CFC) Assay using Methylcellulose

Introduction

Methylcellulose is a semi-solid medium that allows individual progenitors called colony-forming cells (CFCs) to proliferate and differentiate to form discrete cell clusters or colonies. Colonies may then be classified according to morphological characteristics as belonging to erythroid (E), granulocyte-macrophage (GM), or granulocyte-erythroid-macrophage-megakaryocyte (GEMM) lineages. CFC assays are a powerful tool for evaluating the effects of different environments on hematopoietic progenitors as the methylcellulose formula is easily customized to contain specific drugs, growth factors, and other cytokines. Furthermore, the ability of methylcellulose to separate progenitor cells into distinct colonies allows screening for the $JAK2^{V617F}$ mutation (see our "Colony PCR – $JAK2^{V617F}$ Screening" protocol for more details).

Materials

1. Mouse or human MethoCult (Stem Cell Technologies) – we use M3231 and H4230
2. IMDM
3. RPMI
4. Sterile water
5. Cytokines (e.g. EPO, SCF, TPO, IL-3) – the specific profile depends on your experiment.
6. Serological pipettes and pipette-aid
7. Micropipettors and barrier tips
8. 3 ml syringes (for plating methylcellulose)
9. 5 ml syringes (for aliquoting methylcellulose)
10. 16 gauge 1.5" needles
11. Sharps container (to dispose of needles)
12. 35 mm cell culture dishes
13. 15 cm cell culture dishes
14. 60 mm gridded scoring dish
15. 1.5 ml Eppendorf tubes
16. 15 ml and 50 ml conical tubes
17. BD Accuri and microFACS tubes (for counting cells)
18. 37°C water bath
19. Vortex
20. Inverted light microscope for colony counting
21. Cell counter
22. Swinging bucket centrifuge
23. 37°C 5% CO_2 incubator with ≥95% humidity
24. 96 well plate

It is crucial to always maintain good aseptic technique.
Work in the hood, wear a lab coat and sterilize often!

Protocol	A. Methylcellulose Preparation	*Notes*
1.	Thaw methylcellulose either at room-temperature or in 37°C water bath.	
2.	To one bottle of methylcellulose (80ml), add 20ml IMDM. Vortex to mix.	

<table>
<tr><td>3.</td><td>Add cytokines as required for your experiment. A common recipe is as follows:
H4230 MethoCult + SIE
<table><tr><th>Cytokine</th><th>Final Concentration</th><th>Volume to Add</th></tr><tr><td>hEPO</td><td>3KU/ml</td><td>100µl</td></tr><tr><td>hIL-3</td><td>100µg/ml</td><td>100µl</td></tr><tr><td>hSCF</td><td>100µg/ml</td><td>50µl</td></tr></table></td><td>hEPO is frozen at a stock concentration of 3KU/µl.
hIL-3 is frozen at a stock concentration of 100µg/µl.
hSCF is frozen at a stock concentration of 200µg/µl.</td></tr>
<tr><td>4.</td><td>Vortex to mix. Using a 5ml syringe and a 16 gauge needle, aliquot 4ml of methylcellulose per 15ml conical tube. Use immediately or freeze at -20°.</td><td>Certain experiments may require alternate recipes or that additional cytokines be added on the day of plating.</td></tr>
<tr><td></td><td>B. Plating Cells</td><td></td></tr>
<tr><td>1.</td><td>Prepare methylcellulose as above or allow frozen aliquots to thaw. Frozen aliquots may be thawed at room temperature or in a 37°C water bath.</td><td>If necessary, add additional cytokines at this step. Vortex to mix.
Label methylcellulose tubes.</td></tr>
<tr><td>2.</td><td>Remove frozen cells from the freezer. Warm up 10ml of RPMI in a 15ml conical tube (or 45ml in a 50ml tube) per sample. Once cells have thawed place into 10 ml of warmed media.</td><td>Thaw cells by swirling in a 37°C water bath until just thawed enough to pour the frozen pellet into the RPMI. Wash sample tube with RPMI to remove remaining cells.</td></tr>
<tr><td>3.</td><td>Transfer 25µl to a microFACS tube containing 225µl PBS and count on the Accuri by running 20µl on medium. Gate out any dead cells or debris. Multiply the events/µl by (10,000*total volume in ml) to get the total number of cells.</td><td>Cell preparation methods depend on your experiment.</td></tr>
<tr><td>4.</td><td>Centrifuge for 5-10 minutes at 1200 rpm.</td><td></td></tr>
<tr><td>5.</td><td>Discard supernatant and resuspend in RPMI media. (see notes)</td><td>Resuspend in 100µl* (how many different conditions you are testing). If you are testing 8 conditions, resuspend in 800µl of media.</td></tr>
<tr><td>6.</td><td>Add 100µl of cells to each methylcellulose tube, vortex briefly, then set aside. Repeat for all conditions.</td><td></td></tr>
<tr><td>7.</td><td>Label the 15cm cell culture dish with the date. Label 35mm dishes with your conditions. Fill one 35mm dish with sterile water and place in center of 15cm dish.</td><td>Each 15cm dish holds 1 hydration dish and 6 sample dishes (i.e. 2 samples with 3 replicates each).</td></tr>
<tr><td>8.</td><td>Use a 3ml syringe with 16 gauge needle to draw up 3ml of methylcellulose and distribute 1ml to appropriately labeled dishes. Use the same syringe to draw up the remaining methylcellulose and distribute ~0.1ml per plate.</td><td>Rotate plates to ensure even coverage of the methylcellulose.</td></tr>
<tr><td>9.</td><td>Dispose of syringe in Sharps container. Place dish inside 15cm dish.</td><td></td></tr>
<tr><td>10.</td><td>Repeat steps #8-9 for all conditions.</td><td></td></tr>
<tr><td>11.</td><td>When done, place in incubator and wait 12 days.</td><td>Periodically check for contamination or cell colony formation.</td></tr>
</table>

	C. Counting Colonies	
1.	After 12 days, take out plates. Take plate to microscope along with cell counter and 60mm gridded scoring dish.	
2.	Place methylcellulose dish inside 60mm gridded scoring dish and count the number of E, GM, and GEMM colonies (see poster on the -20°C freezer for examples).	*This is easiest when starting at top left grid and continuing to count right to left, row by row.*
	D. Picking Colonies	
1.	Get a plate diagram and label with different conditions being tested.	
2.	Fill 96 well plate with 100µl of nuclease-free water.	*Colonies can be picked into PCR tube strips for low colony counts.*
3.	Locate a colony to pick. Use a 20µl pipet set to 10µl and plunge to the first stop. Place the tip into the methylcellulose by looking for the shadow of the tip and lowering it onto the colony. Draw up the colony and expel into 96 well plate. Note the type of colony on the plate diagram.	*Be careful not to draw up additional cells that are not part of the colony!* *Use a fresh box of tips to help keep track of your position on the plate.*
4.	Repeat until enough colonies are selected. Once done, attach microseal to 96 well plate.	*Rub the plate on the underside of the bench to ensure each well is sealed completely.*
5.	Incubate in thermal cycler at 97°C for 15 minutes with heated lid on to lyse the cells. Store plates at -20°C.	*Use 2µl of cell lysate per 25µl nested PCR reaction.*