Standard Operating Procedure for the DK U54 Cooperative Center of Excellence in Hematology (CCEH) Hypoxia Core at the Indiana University School of Medicine

NOTE: The below methodology may require some changes in the future as we learn more from our work and the work of others.

Mouse Colony Assay: Examining BFU-E, CFU-GM, and CFU-GEMM (Burst Forming Unit-Erythroid, Colony Forming Unit-Granulocyte/Macrophage, and Colony Forming Unit-Granulocyte/Erythroid/Macrophage/Megakaryocyte)

Materials:

NOTE: Unless otherwise stated, ALL MATERIALS SHOULD BE STERILE. Our chambers are set to 3% O_2 unless otherwise directed. It is important to always include extra supplies other than just what you calculated needing. Additional material cannot be added into chamber at time of experiment as it will not have been acclimated to the lower O_2 levels. All hardware and reagents (e.g. culture media, antibodies, etc.) must be pre-equilibrated to hypoxia chamber at least 18 hours before the chamber is to be used for experiments. Be advised that very small volumes left to equilibrate overnight might result in loss of volume due to the evaporation caused by the moving gasses.

- Cells to be analyzed and concentration adjusted to > 10x final plating concentration
- Methylcellulose, 2.1%
    - Methylcellulose is especially thick. It is highly recommended that it is vortexed frequently over the 18 hours it is in the hypoxia chamber prior to use to become equilibrated to the lowered O_2.
- IMDM with pen/strep
- FBS
- Glutamine (200 mM)
- 2-Mercaptoethanol, $1x10^{-2}$ M (7*ul* + 10ml IMDM)
- Hemin, 4mM
- Erythropoietin-EPO (500 U/ml; AmGen, Epogen)
- Source of Growth Factors (PWMSCM (conditioned medium), recombinant, etc.)
- Petri dishes, 35 mm
- Assortment of disposable pipets
- 3-10 mL syringe(s) assorted
- 18ga needles

Procedure: All steps are performed aseptically.

1. Combine the following components, except the methylcellulose and mix well. Add methylcellulose and vortex well. Allow the tube to sit for several minutes to allow all the bubble to rise to the top.

Starting Volume of 5 ml		Final [ ]	
Methylcellulose	2.5 ml	1.0	%
FBS	1.5 ml	30.0	%
Glutamine	50 ul	2	mM
2ME	10 ul	$2x10^{-5}$	M
EPO	0.0-5 ul	1	u/ml
Growth Factors*			
SCF (50ug/ml)	5 ul	50	ng/ml
Other	____	____	
PWMSCM	0.25 ml	5%	v/v
Hemin	0.125 ml	0.1	mM
Cells	0.25 ml	$2.5\text{-}10x10^4$	cells/ml
IMDM	q.s. to 5 ml	_____	

NOTE: These amounts are for a 5 mL culture (plating 1 mL per plate for triplicates with extra volume just in case). However, by adjusting volumes proportionately, larger volumes can easily be obtained.

2. Add methylcellulose and vortex/mix well. Allow the tube to sit for several minutes to allow all the bubble to rise to the top.
3. Using a syringe fitted with 18 guage needle (or larger) pull 3-5 ml into syringe and quickly dispense back into tube. This will eliminate bubbles at the end of plunger.
4. Dispense 1 ml cell culture mix into each dish (at least 3 per point) and replace the lid. Since methylcellulose does not solidify, the entire experiment can be plated before each plate is dispersed.
5. Once all of your plates are dispensed, the culture mix must be evenly mixed and distributed in the plates. This is done by picking up an entire stack (or more) of plates and rocking them gently back and forth. Take care not to spill culture mix out of the plate.
6. AS QUICKLY AS POSSIBLE, transport culture plates to the hypoxic incubator. Culture plates in a humidified environment @ 37^0 C and 5% CO_2 / O_2 for 7-8 days for mouse.

Comments/Notes:

- Growth factors may include conditioned medium (PWMSCM) and/or recombinant factors such as IL-3, GM-CSF, SLF or others. If the volume of these factors comprise less than or more than the 0.25 ml designated, simply adjust the q.s volume with IMDM.
- Hemin can be made and stored in NaOH (protocol upon request) and made to working dilution as needed.
- The cell concentration is completely subjective baring two things in mind. One, you want to plate enough cells so you get enough growth to score and mean something statistically. Two, you do not want to plate too many cells and have to worry about accessory cell affects. If plating an enriched or depleted cell population, you will need to plate several concentrations.

In general: Mouse,	whole BM	@	$2.5\text{-}5.0 \times 10^4$ c/ml
	lin^-	@	500-5000 c/ml

References

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