

The Fleischman Lab

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Title	Hematopoietic Stem Cell Differentiation Assay					
Introduction	While myeloid cells are relatively easy to obtain <i>in vitro</i> , lymphoid cell development from hematopoietic stem or progenitor cells requires culturing the cells in the presence of bone marrow stroma. This assay utilizes the mouse stromal cell line OP9 to support the differentiation of stem or progenitor cells to both lymphoid and myeloid lineages. This assay is particularly useful to investigate the effects of certain mutations on the differentiation of HSCs. The culture conditions defined in this protocol may be altered to address other questions as well. As lymphoid development can be difficult, it is important to optimize the FBS and cytokines.					
Materials	 OP-9 cells RPMI αMEM20 (MEM-α without nucleosides + 20% FBS + penicillin/streptomycin/L-glutamine) 0.05% trypsin-EDTA I10 (IMDM + 10% FBS [we use Sigma lot# 164382 for lymphoid colony development] + penicillin/streptomycin/L-glutamine) 2x cytokine mixture in I10 (the cytokines used will depend on your experiment) Mouse bone marrow, stained for LKS or progenitor cells 60 mm cell culture dishes 15 cm cell culture dishes 50 ml conical tubes 96-well cell culture plates microFACS tubes Serological pipettes and pipet-aid Micropipettes, sterile pipette tips, multichannel pipette, sterile reagent reservoir Cell sorter Flow cytometer 37°C water bath Swinging bucket centrifuge 37°C 5% CO₂ incubator with ≥95% humidity It is crucial to always maintain good aseptic technique. 					
Protocol	A. Culture and plate OP9 cells	Notes				
1.	Warm up 30 ml of RPMI in a 50 ml conical tube. Remove cells from freezer and thaw. Pour cells into pre-warmed RPMI and wash cryovial with RPMI to remove remaining cells. Centrifuge for 10 minutes at 1200 rpm (400g).	Thaw cells by swirling in a 37°C water bath until just thawed enough to pour the frozen pellet into the RPMI. Dry tube and sterilize with 70% EtOH.				
2.	Discard supernatant and resuspend in 3 ml α MEM20. Transfer to a 60 mm dish and place in the incubator. Monitor cells daily to prevent the cells from becoming overcrowded.	It is very important to prevent OP9 cells from overcrowding! Confluent OP9 cultures transform from stroma cells to fat cells, evidenced by large vacuoles in the cytoplasm. Discard cells and take fresh aliquot if cells become overcrowded.				

 Once the 60 mm dish is ~80% confluent, trypsinize and expand into a 15 cm dish: Aspirate media. Add 3 ml RPMI and wash by gently tilting dish back and forth to cover the entire cell surface with media. Aspirate media. Add 0.5 ml trypsin-EDTA and gently tilt dish back and forth to cover the entire cell surface with trypsin. Place dish in the incubator until all of the cells have lifted, about 5 minutes. Add 2 ml αMEM20 and pipette up and down vigorously to obtain a single-cell suspension. 				Trypsin is inactivated by FBS. Do not add trypsin without first washing with serum-free media. Check cells on the microscope frequently to ensure that cells have lifted. Trypsin can be toxic to cells. Do not incubate for more than 10 minutes. If cells still do not lift, remove trypsin, wash, and add fresh trypsin.	
Return to inc	ubator. Mor				
			Scale the volumes of media and trypsin up accordingly.		
Centrifuge cells for 10 minutes at 1200 rpm (400g). Discard supernatant and resuspend in 30 ml I10.					
multichannel	pipette to p	olate 70 µl of o	Each 15 cm dish should fill 4x 96-well plates.		
B. Add mouse stem or progenitor cells					
One day after plating OP9 cells, harvest bone marrow and stain for the population(s) of interest (e.g. LKS, CMP, GMP, MEP, etc.).				See our Isolation of Bone Marrow from the Major Leg Bones and Cell Surface Staining protocols.	
cells. The nur	nber of cells	per well will	For LKS, sorting 1 cell per well will yield about 20-40% positive wells.		
		mixture. Belov	The cytokine formulation and serum used must be optimized for different		
Cytokine	Myeloid	Lymphoid	Myeloid + Lymphoid	conditions.	
SCF	50 ng/ml	50 ng/ml	50 ng/ml	For lymphoid colony development, it	
Flt3 ligand	10 ng/ml	10 ng/ml	10 ng/ml	is important to use Sigma brand FBS,	
IL-7		20 ng/ml	20 ng/ml	lot# 164382. If lymphocytes are not desired, other sera may be used.	
I 	5 ng/ml	400 1		desired, other sera may be used.	
β-IVIE		100 μΜ	100 μΜ		
Pour the 2x cytokine mixture into a sterile reagent reservoir. Use a multichannel pipette to add 70 µl to each well.					
Return the plates to the incubator. This is day 0. Monitor cell growth by examining at each well under the microscope daily. Add more I10 media as needed.					
	expand into a 1. Aspirate 2. Add 3 m forth to 3. Aspirate 4. Add 0.5 forth to 5. Place distiffed, as 6. Add 2 m to obtain Transfer cells Return to inc from becomi Once the 15 and transfer Centrifuge ce supernatant Pour cells int multichannel a 96-well plate B. Add One day afte stain for the MEP, etc.). Sort cells dire cells. The nur experimental Prepare the 2 formulations Cytokine SCF Flt3 ligand IL-7 IL-3 β-ME Pour the 2x of Use a multich Return the pl growth by ex	expand into a 15 cm dish 1. Aspirate media. 2. Add 3 ml RPMI and forth to cover the er 3. Aspirate media. 4. Add 0.5 ml trypsin-E forth to cover the er 5. Place dish in the inculifted, about 5 minur 6. Add 2 ml αMEM20 ato obtain a single-ce. Transfer cells to a 15 cm Return to incubator. Mor from becoming overcrow. Once the 15 cm dish is ~8 and transfer cells for 10 mi supernatant and resuspe. Pour cells into a sterile remultichannel pipette to pa 96-well plate. Place in the growth by examining a formulations: Prepare the 2x cytokine reformulations: Cytokine Myeloid SCF 50 ng/ml Flt3 ligand 10 ng/ml IL-7 IL-3 5 ng/ml β-ME Pour the 2x cytokine mix Use a multichannel pipette to the growth by examining at each daily. Add more I10 medical more in the growth by examining at each daily. Add more I10 medical more in the growth by examining at each daily. Add more I10 medical more into more into medical more into medical more into more into more into medical more into mor	expand into a 15 cm dish: 1. Aspirate media. 2. Add 3 ml RPMI and wash by genth forth to cover the entire cell surfa. 3. Aspirate media. 4. Add 0.5 ml trypsin-EDTA and gent forth to cover the entire cell surfa. 5. Place dish in the incubator until allifted, about 5 minutes. 6. Add 2 ml αMEM20 and pipette up to obtain a single-cell suspension. Transfer cells to a 15 cm dish and add a Return to incubator. Monitor cells daily from becoming overcrowded. Once the 15 cm dish is ~80% confluent and transfer cells to a 50 ml conical tult. Centrifuge cells for 10 minutes at 1200 supernatant and resuspend in 30 ml I1 Pour cells into a sterile reagent reserve multichannel pipette to plate 70 μl of α a 96-well plate. Place in the incubator. B. Add mouse stem or progen One day after plating OP9 cells, harves stain for the population(s) of interest (MEP, etc.). Sort cells directly into the 96-well plate cells. The number of cells per well will experimental conditions and the type of the conditions and the typ	expand into a 15 cm dish: 1. Aspirate media. 2. Add 3 ml RPMI and wash by gently tilting dish back and forth to cover the entire cell surface with media. 3. Aspirate media. 4. Add 0.5 ml trypsin-EDTA and gently tilt dish back and forth to cover the entire cell surface with trypsin. 5. Place dish in the incubator until all of the cells have lifted, about 5 minutes. 6. Add 2 ml αΜΕΜ20 and pipette up and down vigorously to obtain a single-cell suspension. Transfer cells to a 15 cm dish and add 15 ml of αΜΕΜ20. Return to incubator. Monitor cells daily to prevent the cells from becoming overcrowded. Once the 15 cm dish is ~80% confluent, trypsinize as above and transfer cells to a 50 ml conical tube. Centrifuge cells for 10 minutes at 1200 rpm (400g). Discard supernatant and resuspend in 30 ml I10. Pour cells into a sterile reagent reservoir. Use a multichannel pipette to plate 70 μl of cells into each well of a 96-well plate. Place in the incubator. B. Add mouse stem or progenitor cells One day after plating OP9 cells, harvest bone marrow and stain for the population(s) of interest (e.g. LKS, CMP, GMP, MEP, etc.). Sort cells directly into the 96-well plate containing the OP9 cells. The number of cells per well will depend on your experimental conditions and the type of cell. Prepare the 2x cytokine mixture. Below are some common formulations: Cytokine Myeloid Lymphoid Myeloid + Lymphoid SCF 50 ng/ml 50 ng/ml 50 ng/ml Flt3 ligand 10 ng/ml 10 ng/ml 10 ng/ml 10 ng/ml 117 - 20 ng/ml 50 ng/ml Flt3 ligand 10 ng/ml 10 ng/ml 10 ng/ml 10 ng/ml Pour the 2x cytokine mixture into a sterile reagent reservoir. Use a multichannel pipette to add 70 μl to each well. Return the plates to the incubator. This is day 0. Monitor cell growth by examining at each well under the microscope daily. Add more I10 media as needed.	

	C. Analyze colonies	
1.	Monitor cell growth daily. By day 4-5, colonies can be observed with the microscope. Positive wells are best identified on days 5-6.	
2.	On day 10-11, harvest cells from positive wells into microFACS tubes.	
3.	Stain cells with myeloid and lymphoid markers (e.g. Gr-1, NK1.1, CD11b, B220) and analyze by flow cytometry.	See our Cell Surface Staining protocol.
4.	Analyze the percent of positive wells in each experimental condition. Of the positive wells, determine the frequency with which each lineage is represented.	