The Fleischman Lab

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Preparation of WEHI-Conditioned Media

Introduction

The WEHI-3 cell line produces murine IL-3, providing an alternate source to recombinant IL-3. To ensure that adequate amounts of IL-3 are present in the conditioned media, cells are grown well past confluency, indicated by a yellow or orange color change in the media. The conditioned media is then centrifuged and filtered over a 0.45µm membrane to remove cells and cell debris. The WEHI-CM is then frozen to prevent degradation of the IL-3. Because the absolute concentration of IL-3 in WEHI-CM is unknown, each batch must be titered to determine the optimal concentration for growing Ba/F3 and FL5.12 cells. Generally, 5-15% WEHI-CM is sufficient for both cell lines.

Materials

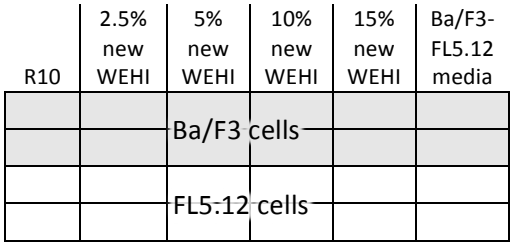
1. WEHI cells
2. R10 (RPMI + 10% FBS + penicillin/streptomycin/L-glutamine)
3. 0.05% Trypsin-EDTA
4. T75 and T175 flasks
5. 1000ml filter units, 0.45µm pore size
6. 50ml conical tubes
7. Serological pipettes and pipette-aid
8. Ba/F3 and FL5.12 cells
9. 24-well tissue culture plates
10. MicroFACS tubes
11. Cytometer (BD Accuri) for counting cells
12. 37°C 5% CO_2 incubator with ≥95% humidity

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

Protocol

A. Generation of WEHI-CM

		Notes
1.	Warm up 30ml of RPMI in a 50ml conical tube. Remove cells from freezer and thaw. Pour cells into the 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 20ml R10. Transfer to a T75 flask and place in the incubator.	
3.	Once the T75 flask is confluent, trypsinize and expand into two T175 flasks: 1. Aspirate media. 2. Add 10ml serum-free media, such as DPBS or RPMI and wash flask by gently tilting back and forth to cover the entire cell surface with media. 3. Aspirate media. 4. Add 4ml trypsin-EDTA and gently tilt flask back and forth to cover the entire cell surface with trypsin. 5. Place flask in the incubator until all of the cells have lifted, about 5-10 minutes. 6. Add 6ml R10 and pipette up and down vigorously to obtain a single-cell suspension. 7. Transfer 5ml to each T175 and add 45ml R10. Return cells to incubator.	*Trypsin is inactivated by FBS. Do not add trypsin to flasks without first washing with serum-free media.* *Check flasks on the microscope frequently to ensure that cells have adequately lifted. If trypsinization is difficult, tap flasks on the bench to dislodge cells.* *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.*

4.	Once the two T175 flasks become confluent, trypsinize and expand into three more T175 flasks for a total of 5 T175s with 50ml each. Return cells to incubator.	*Use 6-8ml trypsin for each T175 flask. Pool cells together to ensure that all flasks have an equal number of cells.*
5.	Check on flasks daily. When media begins to turn orange, add 50ml R10 to each flask and return to incubator.	
6.	Once media turns orange again, add 100ml R10 and return to incubator. Harvest WEHI-CM once the media turns yellow and cells begin to detach.	
	B. Harvesting WEHI-CM	
1.	Transfer media to 50ml conical tubes. Centrifuge at 4°C for 20 minutes at 3500 rpm.	*Use the floor centrifuge.*
2.	Pour media into a 1000ml 0.45µm vacuum filter and discard the pellets.	
3.	Transfer filtered media into fresh 50ml conical tubes and store at -20°C. Reserve one tube for testing.	*Store 40-45ml per tube to leave room for expansion during freezing.*
	C. Titering WEHI-CM	
1.	Thaw one vial each of Ba/F3 and FL5.12 cells as described above. Centrifuge for 10 minutes at 1200 rpm (400g).	*Only use parental or MIG cell lines.*
2.	Resuspend cells in 10ml Ba/F3-FL5.12 media and transfer to a T25 flask. Place cells in incubator. Maintain cell lines until each culture is over 90% viable by FSC/SSC.	
3.	Once each culture is healthy, plate 20k cells/ml in a 24-well plate according to the table below: 	*Wash cells 2x with RPMI to remove residual IL-3.*
9.	Count cells daily for 3-5 days by transferring 25µl to a microFACs tube containing 250µl PBS. Run on the Accuri on medium speed with a limit of 20µl. Graph the results and determine which WEHI-CM concentration encourages the best growth for each cell line.	*Mix each well with a p1000 prior to counting.* *Cells should grow in the logarithmic phase.*