L929-CONDITIONED MEDIA (LCM) PRODUCTION

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Background

Macrophage colony stimulating factors (M-CSF) are secreted by L929 cells (ATCC) and promote bone marrow progenitors to differentiate into a heterogeneous population of bone marrow derived macrophages (BMDM) expressing various myeloid surface markers (CD11b, F4/80, BM8, CD31, CD68, CD11c, Ly6C, GR1)[1]

Protocol

DMEM-10 Production

DMEM-10 is the only media used throughout this protocol for washing and culturing L929 cells. As such, ~3.145 L of DMEM-10 will be required. Since a significant component of DMEM-10 is FBS, ensure there are sufficient stocks of FBS before initiating this protocol. To facilitate media production, consider creating aliquots of 50 mL FBS 50 mL, 5 mL penicillin/streptomycin (10,000 U/mL penicillin, 10 mg/mL streptomycin), and 5 mL L-glutamine (0.2 M) stored at -20°C until use.

1. Remove 60 mL of media from a pre-prepared bottle of DMEM for a total of 440 mL DMEM, discard the excess into liquid waste.
2. Add 50 mL FBS, 5 mL penicillin/streptomycin, and 5 mL L-glutamine to the 440 mL of DMEM, bringing it to a total volume of 500 mL of DMEM-10.
    a. 10% FBS, 1% pen-strep, 1% L-glutamine
3. Store DMEM-10 at 4°C until use.

L929 Culturing

Protocol Preface

This protocol relies on the method of cell culturing, which involves a series of cell splittings once the cells have reached a specified confluency. By splitting cells, or transferring cells from a smaller size or fewer number of flasks to a larger size or greater number of flasks, the amount of open surface area and media available to the growing cells is increased. Thus, splitting cells is a method to encourage cells to continually grow by remoting the limiters of spent media or lack of room. If cells are split too far above the specified confluency, they will over-grow in their new flasks rapidly; if cells are split too far below the specified confluency, they may die or grow more slowly due to a too-low density of cells. When splitting cells, it is important to keep track of the passage number, or the number of times the cells have been split. Always label flasks

with the passage number (n + x, where x is the known number of passages if the passage number for the original stock is unknown).

Protocol Overview: thaw L929 cells into a T150, split 1:4, then 1:3 (+1 buffer flask; i.e. 4 → 12 + 3), split these flasks 1:5 (to yield 60 flasks). Incubate these 60 flasks for 10 days, collect their cell culture media. Centrifuge the media to remove cell debris, filter-sterilize the media, and store the media at -20°C in 37.5 mL aliquots until use.

Day 1: Thawing L929 Fibroblasts

Before beginning this section of the protocol: warm at least 50 mL DMEM-10 in a 37°C degree water bath

1. Thaw L929 fibroblasts stored in -140°C in a 37°C degree water bath for 2 minutes.
2. Pipette thawed L929 gently into a 50 mL falcon tube containing 20 mL of warm DMEM-10.
3. Centrifuge the cell suspension at 500 g (1500 rpm on large centrifuge) for 5 minutes.
4. Resuspend the cell pellet in 25 mL DMEM-10 media and pipette into a T150 tissue culture flask.
5. Incubate at 37°C, 5% CO_2 for 2-3 days to grow to 70% Confluency.

Day 3-4: Splitting Cells (T150; 1:4)

Begin this section of the protocol when the L929 cells grow to at least 70% confluency.

Before beginning this section of the protocol: warm at least 4 mL trypsin, PBS, 120 mL DMEM-10

1. Discard the cell culture supernatant
2. Rinse the bottom of the T150 flasks (where the L929 cells are adhered) with warm PBS
    a. Rinsing is necessary as FBS contains α1-antitrypsin and proteinases that can inactivate trypsin
3. Pipette 4 mL trypsin into the T150 flask and swirl the trypsin solution to coat the bottom surface of the flask
4. Incubate the 37°C, 5% CO_2 for 10 minutes to lift cells
    a. While the L929 cells are incubating, add 20 mL DMEM-10 to a 50 mL Falcon tube
5. Confirm cell lifting by light microscopy
    a. Lifted L929 fibroblasts should appear spherical (not spindly) and should be move when the media is disturbed
6. Transfer the cell suspension from the T150 flask to the 50 mL Falcon tube prepared in step 4a
    a. Trypsin is toxic to cells, inactivation in DMEM-10 before separating the L929 cells from their media reduces any loss in viability due to prolonged trypsin exposure
7. Centrifuge the cell suspension at 500 g (1500 rpm on large centrifuge) for 5 minutes
    a. While the cell suspension is centrifuging, add 22 mL warm DMEM-10 to each of 4x new T150 flasks

8. Discard the cell-free supernatant
9. Resuspend the cell pellet in 12 mL warm DMEM-10
10. Transfer 3 mL the cell suspension to each of the 4x T150 flasks prepared in step 7a (for a total volume per flask of 25 mL)
11. Incubate at 37°C, 5% CO_2 for 2-3 days to grow to 70% confluency

Day 5-7: Splitting Cells (T150; 1:3+1)

Begin this section of the protocol when the L929 cells grow to at least 70% confluency.

Before beginning this section of the protocol: warm at least 16 mL trypsin, PBS, 425 mL DMEM-10

1. Discard the cell culture supernatant from each of the flasks
2. Rinse the bottom of each of the T150 flasks with warm PBS
3. Pipette 4 mL Trypsin into each of the T150 flasks and swirl the trypsin solution to coat the bottom surface of the flask
4. Incubate the 37°C, 5% CO_2 for 10 minutes to lift cells
    a. While the cells are incubating, add 20 mL warmed DMEM-10 to 4x 50 mL Falcon tubes (one for each T150 flask)
5. Confirm cell lifting by light microscopy
6. Transfer the cell suspension from each of the flasks into one of the 50 mL Falcon tubes prepared in step 4a
7. Centrifuge the cell suspensions at 500 g (1500 rpm on large centrifuge) for 5 minutes
    a. While the cell suspension is centrifuging, add 21 mL warm DMEM-10 to each of 13x new T150 flasks
8. Discard the cell-free supernatant from each Falcon tube
9. Resuspend each cell pellet in 10 mL warm DMEM-10
10. Pool all four cell suspensions into one 50 mL Falcon tube
11. Transfer 3 mL the cell suspension to each of the 13x T150 flasks prepared in step 7a (for a total volume per flask of 25 mL)
12. Incubate at 37°C, 5% CO_2 for 2-3 days to grow to 70% confluency

Day 7-10: Splitting Cells for Final 10-Day Incubation (T150 1:5)

Begin this section of the protocol when the L929 cells grow to at least 70% confluency.

Before beginning this section of the protocol: warm at least 52 mL trypsin, PBS, 2.6 L DMEM-10. Ensure sufficient stocks of T150 flasks are available.

1. Discard the cell culture supernatant from each of the flasks
2. Rinse the bottom of each of the T150 flasks with warm PBS
3. Pipette 4 mL Trypsin into each of the T150 flasks and swirl the trypsin solution to coat the bottom surface of the flask
4. Incubate the 37°C, 5% CO_2 for 10 minutes to lift cells

    a. While the cells are incubating, add 20 mL warmed DMEM-10 to 13x 50 mL Falcon tubes (one for each T150 flask)
5. Confirm cell lifting by light microscopy
6. Transfer the cell suspension from each of the flasks into one of the 50 mL Falcon tubes prepared in step 4a
7. Centrifuge the cell suspensions at 500 g (1500 rpm on large centrifuge) for 5 minutes
    a. While the cell suspension is centrifuging, begin adding 36 mL warm DMEM-10 to each of 60 new T150 flasks
8. Discard the cell-free supernatant from each Falcon tube
9. Resuspend each cell pellet in 10 mL warm DMEM-10
10. Pool all four cell suspensions into one T175 flask
11. Add 50 mL warm DMEM-10 to the pooled cell suspensions for a total volume of 180 mL
12. Finish adding 36 mL DMEM-10 to each of the flasks described in step 7a
13. Mix the pooled cell suspensions by swirling and by pipetting up and down with a serological pipette
14. Measure the concentration of the pooled cell suspension using the hemocytometer
    a. See macrophage killing assay protocol for a description of hemocytometer use
    b. The concentration of the pooled cell suspension should be around 9.6×10^6 cells/mL
15. Add 3 mL pooled cell suspension to each of the T150 flasks prepared in step 14 (for a total volume per flask of 39 mL)
16. Incubate at 37°C, 5% CO_2 for 10 days

LCM Harvesting

Before beginning this section of the protocol: confirm growth of the L929 cells by light microscopy. Ensure enough 0.45 µm filters are available. Autoclave a 4 L flask by capping its mouth with aluminum foil. Prepare a triple-lined biohazardous waste box beside your workstation to directly discard solid waste into.

This section of the protocol is best done with two technicians. While one technician is centrifuging the cell culture media, the other should be filter sterilizing. Aliquoting LCM should be done in parallel by both technicians.

1. Collect cell culture media in 50mL falcon tubes and centrifuge at 3000 rpm, 4°C for 5 minutes to remove any cell debris from solution
    a. Place 4 empty cuvettes in each centrifuge bucket, add water to empty cuvettes to balance loads opposite one another
    b. While cell culture media is centrifuging, the flasks with adherent cells can be discarded
2. Drain the supernatant into an autoclaved 4L flask, pool all supernatants
3. Discard the cell pellet
4. Filter the media containing M-CSF through a 0.45 µm filter into sterile, filter-compatible bottles.

    a. If switching filters is necessary while one filter still contains cell culture media, media can be poured from the old filter to the new one (since the contents will be filter-sterilized anyways)
5. Pool the filter-sterilized LCM (filtered cell culture media containing M-CSF) into the 4 L flask (to ensure homogeneity within the batch)
6. Carefully pour the LCM from the 4 L flask into sterile 500 mL bottles for easy access with a serological pipette.
7. Aliquot the LCM into 37.5 mL aliquots into 50 mL falcon tubes
8. Freeze at -20°C until use.

Protocol Technical Notes

Cell culture involves a pre-set series of flask sizes. Different flask sizes are typically associated with specific volumes of media. The rationale behind the amount of media associated with a specific flask size is to ensure sufficient media for the cells deplete/acidify until the next passage while ensuring there is sufficient surface area relative to volume to allow for adequate gas exchange. This relationship is disregarded when splitting cells for the final 10-day incubation so as to ensure maximal recovery of LCM from the cultured L929 cells. Common flask sizes and their associated volumes are available below, if modification of the LCM production protocol is desired:

Flask Size	Volume
T25	5 mL
T75	15 mL
T150	25 mL
T175	28 mL

This protocol also calls for many cycles of resuspending L929 cells after centrifugations. L929 cells should always be resuspended in a minimum of 10 mL DMEM-10 per T150 flask. L929 cells can clump at high concentrations, which can result in unequal distribution and poor adherence of cells to flasks.

Finally, culture enough L929 to aim for 192,000 cells/cm^2 of final flask surface area when designing the final split before the final 10-day incubation if seeking modification of this protocol.

Links and References

1. Bender AT, Ostenson CL, Giordano D, Beavo JA. Differentiation of human monocytes in vitro with granulocyte-macrophage colony-stimulating factor and macrophage colony-stimulating factor produces distinct changes in cGMP phosphodiesterase expression. *Cell Signal*. 2004;16(3):365-374.