Method Sheet 12

Plating macrophages for cytokine production assay

Overview

This method sheet explains how to seed 96-well plates with adherent macrophage cell-lines, such as RAW, J774 or THP-1, for the purpose of screening plant extracts for anti-inflammatory molecule discovery.

Reagents

- A growing culture of a mammalian monocytic or macrophage-like cell-line (e.g. RAW 264.7, J774, J774A.1, THP-1)
- Optional: If using THP-1 cells, phorbol 12-myristate 13-acetate (PMA) solution at 1 mM in ethanol or DMSO
- RPMI with L-glutamine, 10% FCS and antibiotics

Equipment

- Sterile, clear plastic 96-well microplate(s) with lids
- Multichannel pipette (8- or 12-channel) capable of dispensing 100 - 200 µl
- Sterile pipette tips (compatible with the multichannel pipette)
- Sterile reservoir(s) for cell suspensions to set up the plate
- Tissue culture suite with a Class 2 biosafety cabinet and an incubator capable of maintaining a 5% CO_2 atmosphere at 37ºC
- Waste container for used tips

Plating cells (RAW or J774 macrophages)

1) One day before challenge, suspend adherent RAW or J774 cells from a T75 flask using a sterile plastic scraper (trypsin / EDTA is not necessary for these cell-lines).
2) Pellet the cells by centrifugation (e.g. 300 g for 5 minutes).
3) Discard the supernatant and resuspend the cell pellet in 10 ml RPMI / 10% FCS.
4) Count the viable cells using a haemocytometer or automated cell counter.
5) Adjust the cell suspension to a concentration of 4 x 10^5 cells per ml in a total volume of 12 ml (enough for one 96-well plate, multiply if you intend to prepare more plates).
6) Pour the cell suspension into a suitable sterile plastic reservoir.
7) Use a multi-channel pipette to aliquot 99 µl of cell suspension into every well of a 96-well plate.
8) Incubate the cells at 37ºC in a 5% CO_2 atmosphere overnight (12-24 hours) before challenge.
9) This volumes given in this method are sufficient to prepare one 96-well plate, multiply appropriately if you intend to prepare more plates.

Plating cells (THP-1 macrophages)

1) Three days before challenge, suspend adherent RAW or J774 cells from a T75 flask using a sterile plastic scraper (trypsin / EDTA is not necessary for these cell-lines).
2) Pellet the cells by centrifugation (e.g. 300 g for 5 minutes).
3) Discard the supernatant and resuspend the cell pellet in 10 ml RPMI / 10% FCS.
4) Count the viable cells using a haemocytometer or automated cell counter.
5) Adjust the cell suspension to a concentration of 4 x 10^5 cells per ml in a total volume of 12 ml.
6) Pipette 199 µl of RPMI / 10% FCS into a separate, sterile 1.5 ml microtube and supplement with 1 µl of the 1 mM PMA stock to yield a 5 µM sub-stock.
7) Supplement the 12 ml of THP-1 cells with 120 µl of the 5 µM PMA solution and mix well (the final concentration of PMA in the cell suspension is now 50 nM).
8) Pour the cell suspension into a suitable sterile plastic reservoir.
9) Use a multi-channel pipette to aliquot 99 µl of cell suspension into every well of a 96-well plate.
10) Incubate the cells at 37°C in a 5% CO_2 atmosphere overnight for 3 days before challenge.
11) This volumes given in this method are sufficient to prepare one 96-well plate, multiply appropriately if you intend to prepare more plates.

Challenging cells with *Phytotitre* extracts and LPS

1) Perform this step 12-24 hours after plating J774 or RAW macrophages, or 3 days after plating THP-1 macrophages (follow Method Sheet 13).

Typical timetables for plating, challenging and harvesting tasks

Day	Task (J774 or RAW cells)	Task (THP-1 cells)
Mon	Split / passage stock culture and prepare 96-well plate(s)	Split / passage stock culture and prepare 96-well plate(s) with PMA
Tue	Challenge cells in 96-well plate(s) with extracts and LPS, collect and freeze supernatants 3 hours later	-
Wed	-	-
Thu	-	Challenge cells in 96-well plate(s) with extracts and LPS, collect and freeze supernatants 3 hours later
Fri	Split / passage stock culture	Split / passage stock culture

Notes

- The PMA step is necessary to promote differentiation of the THP-1 pro-monocytic cell-line from its typical suspension form into an adherent, macrophage-like form that is also more responsive to LPS.
- Remember to supplement the stock bottle of RPMI with L-glutamine if this is not present in your variant of RPMI medium.
- Never add PMA to the stock flask of growing THP-1 cells, this would halt their proliferation permanently.
- PMA treatment is not necessary for the J774 or RAW macrophage cell-lines as they are already adherent and sensitive to LPS treatment.
- PMA is barely soluble in water, so ethanol or DMSO is used as the solvent for the high concentration stock solution.
- It is possible to perform more than one experiment per week with the THP-1 cells, by plating on Friday and challenging on Monday.