Method Sheet 42

Compound interference test for the L929 bioassay

Overview

This method sheet explains how to test whether hit natural compounds or extracts of interest may interfere with the measurement of TNF-α when using the L929 cell bioassay. This is a helpful test to perform when seeking new immunomodulatory drugs, as some natural products have the potential to non-specifically alter the rate of growth or viability of L929 cells, independent of TNF-α signalling. As TNF-α is measured in this assay via its capacity to reduce the viability of sensitised L929 cells, the compound interference test aims to establish if the natural compounds or extracts of interest also affect the viability of L929 cells.

Reagents

- A growing culture of the L929 mouse fibroblast cell-line
- Stock actinomycin-D (⚠ toxic, handlle with care) at 1 mg/ml in DMSO or ethanol (store at -20ºC)
- DMEM / 10% FCS (5% FCS works equally well at all stages of the assay)
- Crystal violet reagent (0.2% crystal violet in 20% methanol, see Notes section for how to prepare)
- 20% acetic acid in dH_2O (see Notes section for how to prepare)
- Optional if standard curve desired for modified experiments: 10,000 IU/ml TNF-α in PBS (store in small aliquots at -20ºC)

Equipment

- Sterile, clear plastic 96-well microplate(s) with lids
- Sterile pipette tips (compatible with each pipette)
- Sterile reservoir(s) to dispense sensitised L929 cells
- Multichannel pipette (8- or 12-channel) capable of dispensing 1 - 10 µl
- Multichannel pipette (8- or 12-channel) capable of dispensing 100 - 200 µl
- Tissue culture suite with a Class 2 biosafety cabinet and an incubator capable of maintaining a 5% CO_2 atmosphere at 37ºC
- Squeezable wash bottle dispensing distilled water (H_2O)
- A large plastic tray to collect waste runoff from plate washing
- Waste stream for halogenated chemicals
- Microplate reader capable of measuring absorbance of 96-well plates at 570 nm (or alternatively, any wavelength between 540 nm and 600 nm)

Day 1 (morning) - Seeding plate(s) with L929 cells

1) Stock L929 cells are cultured in DMEM supplemented with 10% FCS and penicillin / streptomycin.
2) At any time in the morning, split / passage L929 cells from the stock flask by adding trypsin / EDTA and incubating for 2 - 5 minutes at 37°C.
3) Note that cell clumping limits the performance of this assay, so it is helpful to ensure that trypsinisation proceeds for long enough to yield a suspension of single cells at this stage.
4) Pellet the cells by centrifugation (e.g. 300 g for 5 minutes).
5) Discard the supernatant and resuspend the cell pellet in 10 ml DMEM / 10%FCS.
6) If cell clumps remain visible by microscopy, pipetting up and down gently several times can help assist the process of disaggregating towards a suspension of single cells.
7) Use ~1 ml to seed a new stock flask, then count the remaining cells using a haemocytometer or an automated cell counter.
8) Prepare 12 ml of L929 cells at a concentration of 4 x 10^5 cells/ml in DMEM / 10%FCS (this is sufficient for one 96-well plate, multiply accordingly if preparing more than one plate).
9) Supplement the L929 cell suspension with 1:1,000 volume of 1 mg/ml stock actinomycin-D solution (e.g. add 12 µl actinomycin-D to 12 ml of cell suspension).
10) ⚠ Note that actinomycin-D is very toxic - it must be handled with care, ideally with double gloves.
11) Place the cap on the tube and mix well by gentle inversion several times.
12) Pour the L929 cell suspension into a sterile plastic reservoir.
13) Use a multichannel pipette to transfer 90 µl of this suspension into every well of a 96 well tissue culture plate.
14) Incubate the cells at 37°C for between 3 and 6 hours to allow the cells to adhere.

Day 1 (afternoon) - Challenging plate(s) with extract or compounds

1) At least 3 - 6 hours after plating the L929 cells, defrost the hit *Phytotitre* extract vial(s) you want to examine and a control vial of sterile DMSO alone.
2) Label 5 sterile 1.5 ml microtubes with the following DMSO concentrations: D0, D16, D32, D64 and D128.
3) Label 5 sterile 1.5 ml microtubes with the following extract concentrations: E0, E16, E32, E64 and E128 (repeat with a different letter for as many additional extracts you want to examine, up to a maximum of 3 extracts per plate).
4) Add 39 µl of RPMI into tubes 0 through to 64 in both rows (i.e. for both the DMSO and extract tubes).
5) Add 77 µl of RPMI into the D128 and E128 tubes.
6) Add 1 µl of DMSO into the D128 tube, and 1 µl of hit extract into the E128 tube, then mix both tubes well by gently pipetting up and down.

7) This gives a concentration of 128 µg/ml extract in the highest concentration tube of this dilution series, which is the same as the highest concentration of extract added to macrophages in the previous set of experiments.

8) That concentration will fall by a factor of ten when adding 10 µl of the suspension to 90 µl of each L929 cell culture, which also matches what was done when measuring TNF-α in the previous set of experiments (i.e. the final concentration of extract on the L929 cells will be 12.8 µg/ml, which is the same as in the previous experiments).

9) Now perform serial doubling dilutions in each row by transferring 39 µl of the suspension in the 128 tube to the 64 tube, mixing gently by pipetting several times, then doing the same to the next tube until reaching the D2 or E2 tube, but do not put any extract in the D0 or E0 tubes, these must remain RPMI only.

10) Once all of the serial dilutions have been prepared in the microtubes, remove the plate containing the L929 cells from the incubator to a Class 2 biosafety cabinet.

11) Use a single channel pipette to add 10 µl of each dilution to the middle wells of the L929 cell plate according to the following map:

	1	2	3	4	5	6	7	8	9	10	11	12
A				DMSO				Hit extract 1				
B		0 µg/ml	0.16 %	0.32 %	0.64 %	1.28 %	0 µg/ml	16 µg/ml	32 µg/ml	64 µg/ml	128 µg/ml	
C		0 µg/ml	0.16 %	0.32 %	0.64 %	1.28 %	0 µg/ml	16 µg/ml	32 µg/ml	64 µg/ml	128 µg/ml	
D		0 µg/ml	0.16 %	0.32 %	0.64 %	1.28 %	0 µg/ml	16 µg/ml	32 µg/ml	64 µg/ml	128 µg/ml	
E		0 µg/ml	16 µg/ml	32 µg/ml	64 µg/ml	128 µg/ml	0 µg/ml	16 µg/ml	32 µg/ml	64 µg/ml	128 µg/ml	
F		0 µg/ml	16 µg/ml	32 µg/ml	64 µg/ml	128 µg/ml	0 µg/ml	16 µg/ml	32 µg/ml	64 µg/ml	128 µg/ml	
G		0 µg/ml	16 µg/ml	32 µg/ml	64 µg/ml	128 µg/ml	0 µg/ml	16 µg/ml	32 µg/ml	64 µg/ml	128 µg/ml	
H				Hit extract 2				Hit extract 3				

12) It is vital that the monolayer of cells at the bottom of the well is not scratched by the pipette tip during this procedure, so do not push the tips too far below the surface of the liquid.

13) Return the plate containing L929 cells to the 37°C incubator and culture overnight.

Day 2 - Staining L929 cells with crystal violet dye

1) Cell culture methods preceding the staining step should be performed in a sterile environment with attention to sterile technique.

2) However, after the cells have been challenged, the staining part of the crystal violet assay does not need to be performed in a sterile environment - the following steps can be performed on an open bench in any laboratory with a suitable chemical waste stream..

3) Note that this assay can be messy, so it may be helpful to avoid stains from accidental spills by covering the bench surface with protective paper (such as Benchkote).

4) It is essential to wear appropriate PPE for this protocol, including lab coat, gloves and safety glasses.

5) Aliquot 6 ml crystal violet solution (see notes) per microplate to a suitable reservoir.

6) Remove all medium from the wells by inverting vigorously over the plastic tray, then patting dry with paper tissue.

7) Adjust a multichannel pipette to dispense 50 µl.
8) Firmly press the pipette into a row of sterile pipette tips (8 or 12, depending on the type of pipette), ensuring all are securely attached.
9) If the tips are loose or fall off during use, use gloved finger and thumb to pull up and seat firmly each tip individually, being careful to not get dye on your glove.
10) Push the plunger on the pipette down to the first stop (not all the way to the second stop).
11) Insert the tips into the liquid in the reservoir, ensuring all tips are below the surface of the liquid.
12) Slowly release your thumb to allow the plunger to return to the top position.
13) Look carefully across all the tips to ensure the level of liquid is the same in each tip, if not, dispense the suspension back into the reservoir and try again.
14) Likewise, if there are any large air bubbles in any of the tips, dispense back into the reservoir and try again.
15) Move the pipette to the plate containing cells and dispense all the liquid into the wells of the next available column, pipetting past the first stop all the way to the second stop of the plunger this time.
16) Repeat this process until every well in the plate has received crystal violet reagent.
17) Replace the lid on the plate and move the plate gently backwards and forwards to ensure the reagent covers completely the bottom of every well.
18) Incubate the plate at room temperature for **15 minutes**.
19) Gently wash all crystal violet solution from every well using distilled water from a squeezable wash bottle (tap water is also acceptable for this step).
20) Invert the plate over a large plastic waste tray until all liquid has been expelled.
21) Pat the plate dry after each wash by inverting the plate onto tissue.
22) Repeat the process of filling the wells gently with water, inverting the plate to remove the water and then drying with paper tissue, three more times.
23) The plate is correctly washed when little or no more blue reagent appears on the tissue.
24) Use a multichannel pipette to add 100 µl of 20% acetic acid solution to every well.
25) Pat the plate gently at the side to solubilise the cells until the colour in each well reaches visible homogeneity (about 30 seconds).
26) Use a microplate reader to measure absorbance of every well at 570 nm (filters between 550 and 600 nm will also work well for this purpose).
27) Retrieve the results from the microplate reader software for later analysis.
28) Empty the liquid contents of the plate into the waste tray, and discard the waste plastic to a suitable dry waste stream.
29) Discard the contents of the waste tray into a waste bottle that has been designated for the disposal of chlorinated chemical waste (often found in a fume cabinet).
30) Wipe the waste tray dry with tissue for re-use.
31) Clean up any spills by first wiping up any excess with tissue, then spraying the stain with 20% acetic acid, wiping with tissue, then spraying with 100% H_2O, then wiping again, repeat until all stain is removed.

32) Proceed to analysis of the crystal violet staining data, aiming to establish if any of the compounds alter L929 cell viability (proportional to the mean A600 absorbance values of the three replicate wells for each treatment) in comparison to the DMSO control at the same concentration.

Notes

- ⚠ Actinomycin-D is very toxic - it must be handled with care, ideally with double gloves.
- It is essential to wear appropriate PPE for this protocol, including lab coat, gloves and safety glasses.
- Be very careful not to get this stain on the bench, your skin or clothes - the stain can be messy and is very difficult to remove.
- If you do spill crystal violet stain on a surface, wipe up the excess with tissue, then spray with 20% acetic acid, wipe up with tissue, then with 100% H_2O, wipe again with tissue, repeat until all stain is removed.
- The methanol in the crystal violet reagent is the fixative that hardens the adherent cells.
- Methanol is toxic, volatile and flammable - prepare the working solution in a fume cupboard.
- Dead cells are washed away during the washing steps as they are non-adherent.
- The acetic acid in the resuspension reagent solubilises the crystal violet dye, allowing a uniform colour to be measured by a microplate spectrophotometer.
- Be careful to not wash the cells too vigorously - point the nozzle of the wash bottle at an angle away from the bottom of the wells by holding the plate at a 45° angle from vertical, and squeeze gently.
- Excessive pressure during the washing step will result in loss of adherent cells and poor reproducibility.
- [1] Method reference: Delahooke DM, Barclay GR, Poxton IR. Tumor necrosis factor induction by an aqueous phenol-extracted lipopolysaccharide complex from Bacteroides species. Infect Immun 63:840-6 (1995)