Method Sheet 43

Quantitation of TNF-α using the L929 cell bioassay

Overview

This method sheet explains how to measure the concentration of tumour necrosis-factor (TNF)-α in a biological sample using a modification of the L929 cell bioassay. Briefly, a standard curve is prepared using recombinant TNF-α with an established bioactivity per microgramme, and the absorbance values elicited by the samples are compared against those of the TNF-α standard curve. This assay can be a more cost-effective alternative to ELISA for quantitation of TNF-α production, although it requires high technical proficiency to achieve reproducible results.

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)
- Recombinant TNF-α at 10,000 IU/ml in PBS (store in small aliquots at -80ºC)
- 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)

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)

Reconstitution of recombinant TNF-α

1) Recombinant TNF-α protein is available from numerous Life Science reagent suppliers.
2) Both human and mouse TNF-α work well in the L929 cell bioassay, but if you have been using mouse macrophages (e.g. J774 or RAW cell-lines), it will be better to purchase mouse TNF-α.
3) Recombinant TNF-α is typically supplied in a lyophilised (dry powder) format.
4) Follow the manufacturer's instructions to resuspend the TNF-α to the concentration and in the buffer they recommend.
5) Read the product specification sheet to find out how many International Units (IU) their specific protein product contains per microgramme.
6) Adjust the concentration of the stock to 10,000 IU/ml by adding the correct amount of buffer.
7) Aliquot the stock TNF-α into many small tubes (e.g. 10 µl per tube).
8) Store these aliquots at -80°C before use, and avoid reuse after thawing.

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) - Preparing the TNF-α dilution series

1) At least 3 - 6 hours after plating the L929 cells, defrost the samples containing TNF-α (e.g. from the replication experiment in Method Sheet 41), and one aliquot of recombinant TNF-α, prepared as described above to a concentration of 10,000 IU/ml.
2) Label a series of 10 sterile microtubes with the following concentrations: 1,000, 320, 100, 32, 10, 3.2, 1, 0.32, 0.1, and 0.032.
3) Add 34.2 µl of DMEM to all of these tubes except the first tube (labelled 1,000), which should receive 45 µl of DMEM.
4) Add 5 µl of recombinant TNF-α to the first tube and mix gently by pipetting (this 1:10 dilution yields a starting concentration of 1,000 IU/ml).
5) Pipette 15.8 µl of the suspension from the first (1,000) tube and transfer it into the second (320) tube, then mix up and down several times by pipetting gently.
6) Repeat this process until the entire series of dilutions has been prepared.
7) Note that these ratios are different from a more common 2-fold dilution between standards in the series, this is because we are using a 3.16-fold dilution since this is the square root of 10.
8) By using a 3.16-fold dilution between each standard, we achieve a wider dynamic range than achievable with a 2-fold dilution series, and each step of two tubes in the series gives a 10-fold change in concentration that is easier to plot on a chart.

Adding samples and standards to the L929 cells

1) Remove the plate containing the L929 cells from the incubator to a Class 2 biosafety cabinet.
2) Use a single channel pipette to carefully add 10 µl of supernatant from each of samples you want to analyse according to the plate map shown below (up to 12 different samples can be measured in quadruplicate on one plate).
3) In the lower two rows of the inner wells of the plate, add 10 µl of the dilution series of standards into the respective wells, as shown on the plate map below.
4) Return the plate containing L929 cells to the 37°C incubator and culture overnight.

	1	2	3	4	5	6	7	8	9	10	11	12
A					Up to 12 different test samples							
B		S1	S2	S3	S4	S5	....	....	....	....	S12	
C		S1	S2	S3	S4	S5	....	....	....	....	S12	
D		S1	S2	S3	S4	S5	....	....	....	....	S12	
E		S1	S2	S3	S4	S5	....	....	....	....	S12	
F		0.032 IU/ml	0.1 IU/ml	0.32 IU/ml	1 IU/ml	3.2 IU/ml	10 IU/ml	32 IU/ml	100 IU/ml	320 IU/ml	1,000 IU/ml	
G		0.032 IU/ml	0.1 IU/ml	0.32 IU/ml	1 IU/ml	3.2 IU/ml	10 IU/ml	32 IU/ml	100 IU/ml	320 IU/ml	1,000 IU/ml	
H						TNF-α standards						

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.

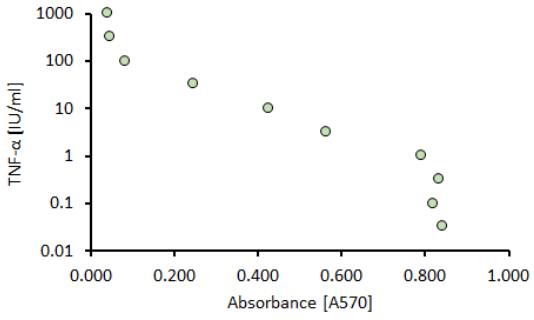
23) The plate is correctly washed when little or no more blue reagent appears on the tissue.
24) Use a multichannel pipette to add 100 µl of 20% acetic acid solution to every well.
25) Pat the plate gently at the side to solubilise the cells until the colour in each well reaches visible homogeneity (about 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.

Data analysis and calculation of TNF-α content of samples

1) Use Microsoft Excel to prepare a data table of two rows of values, label the first cell of the first row ‘Absorbance’ and the first cell of the second row ‘Concentration’.
2) Calculate the mean absorbance value for each of the standard curve concentrations (i.e. the average value of the two wells for each concentration) and use them to populate the first row of the data table.
3) Type the values of the concentrations of the standards in the same order that they appear on the plate (i.e. 0.032, 0.1, 0.32 etc. all the way up to 1,000, from left to right) in the second row of the table, it should look something like this:

3	Absorbance	0.841	0.820	0.835	0.792	0.563	0.425	0.245	0.082	0.045	0.040
4	Concentration	0.032	0.1	0.32	1	3.2	10	32	100	320	1000

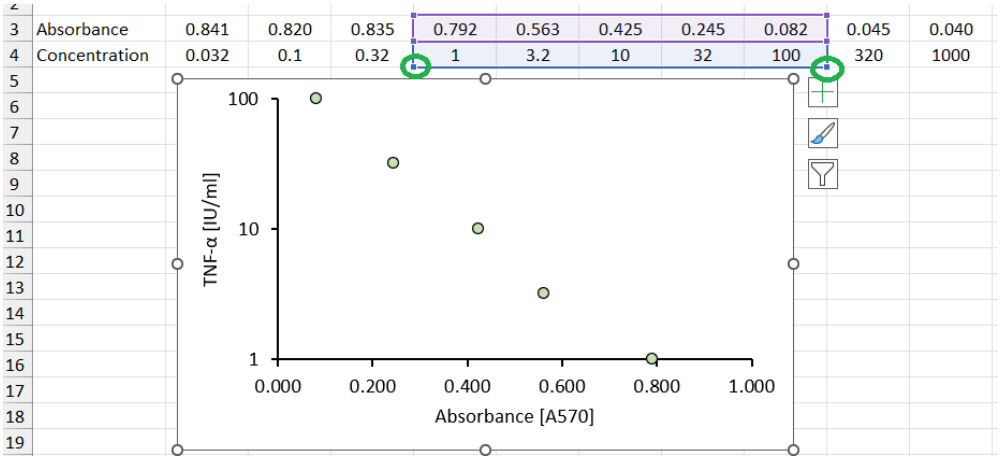
4) Now highlight both rows of data, and use Excel to create a ‘Scatter plot’ chart with dots only and without lines.
5) Double click on the y-axis, and in the ‘Format axis’ menu that appears at the right of the screen, select the ‘Logarithmic’ option.
6) Your standard curve chart should now look something like this:



7) Note that we are plotting this chart with the Concentration values on the y-axis and the Absorbance values on the x-axis - this is the opposite of how we would normally plot a chart if we were going to show it in a report.

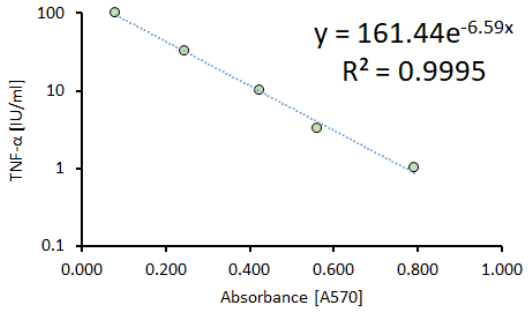
8) The rationale for this is that if we plot the curve on the opposite axes to normal, the Excel line fit option will give an equation to calculate the TNF-α values in the samples more easily when done this way.

9) Click on the chart and highlight the small blue square at the bottom right of the lower right cell of the selected data table, then drag it left until the dots all line up as closely as possible to a straight line, do the same thing with the left hand lower blue cell handle, excluding any points that form a plateau, it should look like this:



10) Now click on ‘Chart Design’ at the top ribbon, then ‘Add Chart Element’ then select ‘Trendline’ and ‘Exponential’.

11) Double click on the trendline that appears and select the ‘Display equation on chart’ and ‘Display R-squared value on chart’ options, it should now look like this:



12) The equation shown is what we will use to calculate the TNF-α concentrations in the unknown samples.

13) The R^2 value is a measure of how reliable the assay was - the closer R^2 is to 1, the less error and variability there was in the assay, and the greater confidence we can have in the data.

14) Type a formula similar to the one shown below into a cell below your main plate absorbance values:

```
=161.44*EXP(-6.59*C20)
```

15) Replace the first value, which appears just before the multiply `(*)` and `EXP` functions, with the first number shown by Excel on the equation on your chart.
16) Replace the second value, which appears just before the multiply function `(*)` within the brackets, with the second number shown by Excel on the equation on your chart, being careful to include a minus sign if it is a negative number.
17) Replace the cell reference `C20` with the reference for the first (top left) cell in your raw plate absorbance values corresponding to the first replicate of sample 1.
18) Drag to copy and fill this equation down and along to automatically calculate the TNF-α concentrations in all the other sample wells.
19) Take the average of the quadruplicate values to obtain your final TNF-α concentrations in International Units per ml (IU/ml) for all the samples.
20) Note that if the absorbance values of your samples fall beyond the range of absorbance data values for the standard points you selected to create the straight line on the chart, you will have to repeat the assay with diluted samples.

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.
- Remember that recombinant TNF-α is very unstable - it must be aliquoted and stored at -80°C, and a fresh aliquot used to prepare the standard curve each time.
- Using a standard that has been previously thawed and refrozen will result in a standard curve that underestimates the actual cytokine concentration in the samples.
- If you cannot find the relative bioactivity (in IU/µg) of the TNF-α you have bought, you can still complete the assay in the same way, but your calibration curve and the resultant measurements for your samples will be based on mass units per ml (e.g. pg/ml) instead of IU/ml (which is preferred but not essential for this type of assay).
- 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.
- 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 45º angle from vertical, and squeeze gently.
- Excessive pressure during the washing step will result in loss of adherent cells and poor reproducibility.