Biological Activity Assay for Gibco™
Recombinant Proteins – Cell Proliferation Assay

A proliferation assay is the first choice for many cytokine bioassays. The following protocol describes a typical proliferation assay using murine B-cell hybridoma cell line B9 cells. The B9 cells require IL-6 for survival and proliferation in vitro. This method describes the measurement of B9 cell growth in response to IL-6. The growth response of B9 cells to IL-6 is determined by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt). MTS (Owen's reagent) is a dye that is reduced by cells into a colored product that is soluble in tissue culture medium. MTS can then be measured in a microplate reader at 490 nm. The quantity of colored product measured at 490 nm is directly proportional to the number of living cells in culture.

Procedure

Day 1: Cell culture

1. Two days before the assay, freshly feed one flask (T-75) of stock B9 cells (maintained cells in RPMI-1640 plus 10% FBS and 300 pg/mL murine IL-6).

2. Return the flask to the incubator for 2 days.

Day 3: Preparation of standard and test sample(s), and cells

3. Thaw a tube of the standard IL-6 (at 0.1 mg/mL).

4. Prepare the appropriate dilutions of the test sample(s) in medium to achieve a final concentration of 0.1 μg/mL in a final volume of 1 mL. At least one intermediate dilution must be made, and it should be in a volume of at least 1.0 mL so that it can be sterile filtered using a 3 mL sterile syringe with 0.2 micron Acrodisc™ filter into a sterile tube. This is the starting dilution of the dose response.

5. Prepare dose response dilution tubes (1.5 mL Eppendorf™ tubes) by adding 400 μL medium to each of 11 tubes.

6. Transfer 400 μL of the 0.1 μg/mL starting dilution of the standard into the first dilution tube and mix several times by up/down pipetting. (A serial 2-fold dilution).

7. From this tube, transfer 100 μL to the next tube and mix as before. (A serial 5-fold dilution).

8. Change pipet tips and transfer 100 μL to the next tube and mix as before.

9. Repeat step 8, moving 100 μL from the previous dilution tube to the next until all 11 tubes have been completed.

10. Repeat steps 5–9 for each test sample. Be sure to change pipet tips between each tube.

11. Label a sterile 96-well microtiter plate (tissue culture grade) along the left margin as to which rows will be encompassed by standard or sample.
12. Using the P200 pipette, begin transferring 100 μL from the last (tube 12) tube of the standard dilutions into well #12 of the appropriate, duplicate rows.

13. Repeat step 12 with the next tube (#11), transferring 100 μL into wells #11. The same pipet tip can be used since loading of sample is progressing from lower concentrations to increasingly higher, thus no carry-over.

14. Repeat step 13 until all dilutions have been completed.

15. Change pipet tips and repeat steps 12–14 for each test sample. The last two rows (G and H) will be used for controls.

16. Using a reagent reservoir (containing ~2 mL medium) and multichannel pipette, place 100 μL of medium alone into row(s) G. This is devoid of IL-6 and is the negative control.

17. Prepare enough complete culture media (RPMI-1640 plus 10% FBS with murine IL-6) to load 100 μL/well into row(s) H of the 96-well plate(s). This simulates normal culture conditions and thus is the positive growth control.

18. Place the plate(s) into the incubator (37°C with 5% CO2) and allow them to equilibrate for 10 minutes.

19. Meanwhile, begin washing the 2-day post-feeding B9 cells by transferring the flask contents to a sterile, 50 mL conical tube and add medium up to the 50 mL mark and centrifuge (1,500 rpm, 10 minutes).

20. Discard the supernatant, resuspend the pellet in 10 mL medium and add medium up to the 30 mL mark and centrifuge as before.


22. Discard the supernatant and resuspend the final pellet in 10 mL of medium, being sure to pipet up/down several times to assure homogeneity of the suspension.

23. Prepare a trypsin blue dilution with 200 μL trypsin blue and 200 μL medium in a sterile 12 x 75 tube.

24. Add 100 μL of the B9 cell suspension to the trypsin blue solution and mix.

25. Clean the hemacytometer and cover slip with isopropanol and dry.

26. Using the pipette, slowly load a drop of the cell–trypan blue solution into one side of the chamber.

27. Count the number of viable and nonviable cells and in turn determine the cell (viable) count per mL.

28. Dilute the cells to 1 x 10^5/mL using medium. Prepare enough to allow for loading of 100 μL cells/well in the plate(s).

29. Remove the 96-well plate(s) containing the standard/sample dilutions from the incubator.

30. From the cell suspension of step 28 transfer 10 mL to a sterile reagent reservoir, and using the multichannel pipette add 100 μL/well to all wells of the 96-well plate(s). Be sure to change pipet tips between standard, sample(s) and controls.

31. Return the plate(s) to the incubator for 48–60 hours (2–2.5 days).

32. Thaw the MTS/PES stock solution by placing into the 37°C water bath for 20–30 minutes.
33. Remove the 96-well plate(s) from the incubator and using the multichannel pipette, gently mix all wells 3 times to disrupt any cell clumps. Change pipet tips between samples, standard and controls.

34. Add 40 μL/well of the stain.

35. Return the plate(s) to the incubator for 2–2.5 hours (check the plate after one hour to determine a color change, if overdevelopment of the color is apparent, read immediately as in step 36).

36. Using the multichannel pipette, gently mix (do not bubble the contents) all wells 3 times to ensure homogeneity of color, and read the plate(s) on an ELISA plate reader at 490 nm.

![Typical cellular proliferation curve obtained using recombinant IL-6](image.png)