

ELISPOT ASSAY - PART I

CELL PREPARATION



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Used by the laboratory of William C. Gause, Ph.D.

1. PREPARE SINGLE CELL SUSPENSION FROM MESENTERIC LYMPH NODES (MLN):

- Remove MLN from each mouse.
- Place in culture dish with 5 ml cold RPMI + 5 % FCS, on ice (each mouse's MLN in separate dish).
- Keep tissue cold throughout these procedures.
- Gently grind with each of the plunger against wire mesh, submerged in cold media.
- Filter through plastic mesh into orange capped 15 ml tube.
- Spin at 1200 rpm for 5 minutes
- Pour supernatant down sink quickly, and blot mouth of tube against paper towel
- "Rack" to resuspend, no extra media added (draw along plastic rack to dislodge pellet).
- Add 5 ml cold RPMI + 5 % FCS to pellet.
- Filter again through plastic mesh into new tube (any sterile small one will do).

2. DETERMINE NUMBER OF CELLS PER MILLILITER:

A. Coulter Counter Method:

- On counting machine, don't change anything except "power" and "reset/count".
- Always turn "reset/count" clockwise
- Vials are in drawer beneath counter
- Fill vial with blank counting media--press bar two times to dispense
- To count: blank, press button under support to move platform, make sure circle is in focus in the middle of the display, turn to reset, turn to count (usually count 5-40 μ l cell suspension added to media. Number on display is millions of cells in original suspension if volume counted is 40 μ l, if 5 μ l is counted, multiply that number by 8.)
- Wash counter with blank media when done.

B. Trypan Blue Method:

- Use trypan blue diluted 1:10
- Dilute cells in trypan blue as appropriate, usually between 1:10 and 1:50
- Count the number of cells in five large boxes in hemocytometer (#)----->>> (#) (2) (dilution factor)(1000)=number of cells per ml.

3. PREPARE CELLS FOR ELISPOT:

- Spin cells at 1200 RPM for 5 minutes. Discard supernatant.
- Dilute cells in cold RPMI + 5% FCS to 5 million per ml in tiny attached tubes.
- Serially dilute these cells 1:5 (1 million per ml). Use 100 μ L of each dilution, in duplicate.

ELISPOT ASSAY – Part II



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1. Coat Immulon II plates with the first antibody at (10 $\mu\text{g}/\text{ml}$) in PBS, 100 μl per well.
2. Plates are left overnight at 4°C.
3. Next day wash plates 3 times with PBS-Tween 20 (.05%), followed by 3 washes with PBS.
4. Block plates with 200 μl per well of RPMI + 5% FCS for a minimum of 1 hour at 37°C (i.e. while you prepare spleen cells, usually for more than 1 hour).
5. Prepare single cell suspension as indicated in Part I. Count the cells and adjust to starting concentrations of $5 \times 10^6/\text{ml}$. Do 5-fold serial dilutions four places out.
6. Aspirate the Immulon II plates and add 100 μl /well of cell dilutions. Remember to add just media to your background wells and don't forget your positive control (ie. ConA).
7. Put plates in 37°C incubator for 3 hours. DO NOT MOVE OR SHAKE PLATES.
8. Wash plates 3 times with PBS followed by 3 washes with PBS-Tween 20 (.05%).
9. Add biotinylated developing antibody at 4 $\mu\text{g}/\text{ml}$ in PBS-Tween 20 (.05%) +5% FCS, 100 μl /well. Put plates in 37°C incubator for 1 hour, or leave overnight at 4°C.
10. Wash plates 3 times with PBS followed by 3 washes with PBS-Tween 20.
11. Add Streptavidin alkaline phosphatase (Jackson Immuno Research) diluted 1:2000 in PBS-Tween 20 +5% FCS, 100 μl /well. Put plates in 37°C for one hour
12. Start to prepare the last reagent at this point. make 0.1 M 2-Amino-2-Methyl-1 propanol (2A2M1P) buffer (Sigma has a 1.5M solution that I dilute with ddH₂O) then pH it to 10.5 and then add SeaPlaque agarose (FMC Bioproducts) to a final concentration of 0.6% agarose. Heat solution until agarose is dissolved then place at 45°C until ready for last step.
13. Wash plates 5 times with PBS. Add BCIP (5-Bromo-4-chlor-3-indoly phosphate) at 1 mg/ml in 0.1 M 2A2M1P buffer pH 10.5 containing 0.6% agarose, 100 μl /well. DO NOT MOVE THE PLATES.
14. Score the results the next day using a dissecting microscope.