

Procedure Title: Testing of Monoclonal Antibody Coated Beads - Direct and Indirect Methods

Procedure #: HSC.D451.01

1. Reagents, Supplies, Equipment

- A. Pipets 10 ml
- B. Pasteur pipets
- C. Conical tubes 15 ml
- D. Pipets 1 ml
- E. Pipets 5 ml
- F. L15- tissue culture medium
- G. Fetal Calf Serum
- H. Conical tubes 5 ml
- I. Snap cap tubes 5 ml
- J. Cryovials 1- 2 ml
- K. Magnetic separator
- L. Centrifuge

2. Procedure

A. Beads

- 1. Do steps 1-8 in a laminar flow hood, using good sterile technique:
- 2. Dilute the beads saved for testing as in step A.3.- A.5. Count beads at a four hundred fold dilution.
- 3. Add 50ul of beads to 950ul of Turks solution for a 1:20 dilution
- 4. Remove 50ul of beads from the first tube (the 1:20 dilution) and add it to 950 ul of Turks solution in a second tube for a final dilution of 1:400
- 5. Count the beads in the 25 squares (0.10mm^3) in the center of the hemocytometer in both chambers.
The average number of beads/ 0.1mm^3 = total number beads counted in

$$0.2\text{mm}^3 \div 2 \text{ beads/ ml} = \text{average number beads} / 0.1\text{mm}^3 \times \text{dilution factor} \\ \times 1 \times 10^4$$

Store the remaining beads at 4⁰C for retesting or experiments.

B. PROCEDURE: NEUROBLASTOMA TUMOR CELL COUNT

1. Aspirate off old media and then gently wash the cells with a small amount of sterile L15 (sterile 0.02% EDTA if the cell line is LAN5).
2. Aspirate off the wash and add L15 to wash the cells off the bottom of the flask.
3. If EDTA is used, add enough to cover the monolayer. Let sit 5-10 minutes until cells loosen and can be washed off. Gently rock the flask containing cells and EDTA. Cells will glide off the bottom cleanly.
4. Pipet the cells into a 15 ml conical tube; rinse the bottom of the EDTA flask with more EDTA to remove all the cells and add L15/HSA to the 15 ml tube to a volume of 15 ml.
5. Pellet cells by centrifugation at 400xg for 10 minutes.
6. Aspirate off all the supernatant and break up any clumps by vortexing or disperse pellet by flicking the bottom of the tube with the forefinger.
7. Resuspend cells into L15 **without FCS** and count in trypan blue for the viability cell count.
8. Count LA-N-1 or LA-N-5 cell line in 0.2% trypan blue at a ten fold dilution (i.e. 450 ul of trypan blue and 50 ul of cells). Count the viable (non blue) and non viable (blue) cells in the four large outside squares (16 smaller squares = 1 large square or 0.1mm³) for each hemocytometer chamber. Calculate the cell concentration and the percent viability.

$$\text{The average number of cells/ } 0.10\text{mm}^3 = \text{total number of cells counted in} \\ 0.4\text{mm}^3 \div 4$$

$$\text{cells/ml} = \text{average number of viable cells/} 0.1\text{mm}^3 \times \text{dilution factor (10)} \times \\ 10^4$$

$$\% \text{ viability} = \text{total viable cells counted} \div (\text{total viable and non viable cells})$$

counted)*100

The viability of the cells must be $\geq 60\%$ to be used for the bead test. The cells may be ficolled to increase the viability.

C. PROCEDURE: DIRECT METHOD

1. In a 2 ml cryovial or snap cap tube, incubate 2×10^5 tumor cells, 20×10^6 beads, and L15/FCS +gent in a total volume of 1 ml on a rotator for 30 minutes at room temperature. Also incubate a control sample which contains only 2×10^5 cells in L15/FCS +gent in a total volume of one ml.
2. Place the cryovial in the magnetic separator, wait 40 seconds and remove cells and media to a snap cap tube.
3. Place the snap cap in the separator as in step 2 above and remove the cells and the media to a 3 ml conical tube that has been previously scored at 50 and 100 μl . Centrifuge at $\sim 400 \times g$ for 2-5 minutes.
4. Remove media down to a volume of 50 μl . Add an equal volume of trypan blue and resuspend the cells.
5. Count all the non blue cells (viable) in the trypan blue aliquot; count all the squares (18 total) in both hemocytometer chambers; count until all the volume is used up (usually 4-5 hemocytometer's both chambers).
6. Count the negative control (two 0.1mm^3 squares are sufficient- count the number of cells in the center square in both hemocytometer chambers) and calculate the average number of cells/ 0.1mm^3 .

$$\text{average number of cells } / 0.1 \text{mm}^3 = \text{total cells counted in } 0.2 \text{mm}^3 \div 2$$

$$\text{cells/ml} = \text{average number of viable cells} / 0.1 \text{mm}^3 \times \text{dilution factor (10)} \times 10^4$$
$$\% \text{ viability} = \frac{\text{total viable cells counted}}{\text{total viable and non viable cells counted}} \times 100$$

7. The average number of tumor cells remaining per 0.1mm^3 is the total number of tumor cells counted (the nine large squares in one hemocytometer chamber 0.9mm^3 - 18 squares total) divided by the total number of 0.1mm^3 squares counted.

i.e. the average number of tumor cells remaining for tumor cells counted

per hemocytometer $38/18, 10/18, 3/18, 3/18 = 0.75/0.1\text{mm}^3$

8. The average number of tumor cells remaining divided by the average number of cells in the controls subtracted from 1 and multiplied by 100 gives the percent removal of tumor cells.

i.e. %Removal = $1 - (0.75/127) * 100 = 99.4\%$ Removal. Beads must remove >95% of tumor to pass test.

D. PROCEDURE: INDIRECT METHOD

1. Incubate 2×10^5 tumor cells in a 2 ml cryovial with monoclonal antibodies at a concentration 10.0 ug/ml for each antibody; incubate tumor cells only (control) in a total volume of 1 ml in L15/FCS +gent on a rotator at room temperature for 15 minutes. For routine clinical testing use all seven (7) monoclonal antibodies (126.4, 390, 459, BA-1, HSAN 1.2, Leu7, NKH1A).
2. Centrifuge samples in a microfuge for one minute or as in step B.5. and discard the supernatant.
3. Wash each sample 2x with PBS-1X using the microfuge and resuspend to 0.5 ml.
4. Add 20×10^6 GAM only coated beads to each sample and incubate as in step C.1. in a total volume of 1 ml.
5. Follow steps C.2. - C.8. to complete the test.