



**Title:** *Elispot Protocol for CX3CR1*

No: RTLP-GL-EL10

Location:  
*Old CCRC Tripp Lab*

Approval Date:  
10 September 2004

Supersedes Date:

**Materials:**

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•Lab coat	•Phosphate Buffered	•Aluminum foil	•Pipettes
•Gloves	Saline (PBS)	•Plastic wrap	•Pipetteman
• NaHCO <sub>3</sub> /100 ml H <sub>2</sub> O (1 M)	•Dulbecco's Modified	•Analytical balance	•Pipette Aid
• Na <sub>2</sub> CO <sub>3</sub> /100 ml H <sub>2</sub> O (1 M)	Essential Medium (DMEM)	• IP sterile plate	•Pipetteman tips
•Centrifuge	•BSA	•α-IFN $\gamma$	•4° cold storage
•37°C incubator	•pH Meter	•dH <sub>2</sub> O	•A2-Infected & un-infected
	•Tween-20	•Mitomycin-C	P815 cells
		•Mouse spleen cells	

**Buffers & Solutions:**

Bicarbonate Buffer:

A: 8.4g NaHCO<sub>3</sub>/100 ml H<sub>2</sub>O (1 M)

B: 10.6g Na<sub>2</sub>CO<sub>3</sub>/100 ml H<sub>2</sub>O (1 M)

Mix 45.3 ml A with 18.2 ml B, and bring up to 1 L with dH<sub>2</sub>O. Adjust to pH 9.6 as needed with A or B. Store at room temp.

Blocking Buffer:

5% BSA in DMEM

PBS-Tween Buffer

PBS + 0.05% Tween-20

PBS + 0.05% Tween-20 + 1% BSA

Mitomycin C

Dilute mitomycin C to a stock solution of 200 mg/mL in serum-free DMEM

Vector Blue Substrate Solution Kit

- Add 2 drops of reagent 1 to 5 ml of 100 mM Tris-HCl, pH 8.2, and mix well
- Add 2 drops of reagent 2 and mix well
- Add 2 drops of reagent 3 and mix well

**Procedure:**

1. **Two days prior** to assay, coat millipore mult-screen IP sterile plate with 100  $\mu$ L/well of capture antibody, diluted to 4  $\mu$ g/ml, in 0.1M bicarbonate buffer pH 9.6. Cover the plate with plastic wrap and aluminum foil and incubate over night (~16 hours) at room temperature.
2. The following day, wash plates 4 times with PBS, then add 200 $\mu$ L of blocking solution and block for 2 hours at room temperature. Be sure to wrap plate in plastic wrap and aluminum foil during blocking. Wash plates 3 times in PBS and store wrapped in plastic wrap and aluminum foil in 4°C storage until use.
3. The day of the assay, mitomycin C-inactivate infected P815 cells by re-suspending cells in 10 mL of serum-free DMEM and adding 0.5 mL of mitomycin C solution to for a 1:20 dilution. Incubate 2-3 hours at 37°C. Following incubation, wash cells 3 times in serum-free DMEM.

*NOTE: Begin mitomycin C inactivation prior to harvesting mouse spleen cells.*

4. Dilute spleen cells in DMEM +10% FBS and add 100  $\mu$ L/well to **achieve  $10^4$  or  $10^3$  cells/well.**
5. Add uninfected or A2-infected mytomycin C-inactivated P815 cells as “effector” cells, at a ration of 2:1 (stimulator : effector).

*NOTE: Be sure to reserve wells for the following negative controls: DMEM-10% alone, un-infected P815 cells and A2-infected P815 cells alone, and spleen cells alone.*

6. Incubate plates at 37°C for 48 hours.
7. Wash plate 1 time with PBS followed by 5 washes with PBS-Tween 20 buffer.
8. Add 100  $\mu$ L/well of biotinylated rat  $\alpha$ -mouse IFN $\gamma$  at a concentration of 2mg/mL.
9. Incubate the plates for 1 hour at room temperature.
10. Following incubation, wash plates 6 times with PBS then add 100  $\mu$ L/well of streptavidin-alkaline phosphatase diluted in 1:3000 PBS-Tween +BSA.
11. Incubate plates for 1 hour at room temperature, wrapped in aluminum foil.

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12. To visualize the assay, add 100 mL/well of vector blue substrate solution, and incubate at room temperature for 20 minutes.
13. Wash plates 6 times with PBS-Tween. To stop the reaction, flood the wells with water.
14. Count spots.

Author	Management Approval/Date	Effective Date