

Glycolipid Elisa using Siglec-8-Fc & Siglec-9-Fc

- Steve Fernandes

Materials & Reagents:

- 96-well plain flat-bottomed polystyrene plates (Corning/Costar #9017)
- Glass dishes and plastic bins for plate washing
- Bulk Butanol
- Bulk 95% ethanol
- Distilled 100% ethanol
- Glycolipids [e.g. GM1, GT1b, Mouse Brain extract (M.B), 6'-Su-SiLacNac, 6-Su-SiLacNac]
- Glass screw-capped vials with Teflon-lined caps (e.g. National Scientific MSCERT4000-35W)
- Glass screw-capped tubes with Teflon-lined screw caps
- Lectins (e.g. Fc-chimeras)
- 2° antibody (e.g. AP- goat α -Human IgG, Fc γ -specific, Jackson Immunoresearch 109-055-008)
- Dulbecco's PBS
- Bovine serum albumin (BSA, e.g. Sigma A7030)
- Developing buffer: 100 mM Tris base, 100 mM NaCl, 5 mM MgCl₂•6H₂O, adjust to pH 9.5
- *p*-Nitrophenylphosphate (PNPP)
- 96-well plate reader

Method:

1. Batch-wise, dip 96-well plates into a shallow dish containing 100% butanol and allow to incubate ≥ 15 min. Drain each plate and immerse in three sequential dishes of ethanol (95%), draining between transfers. Drain and allow to air dry. Store sealed in plastic bags.
2. Prepare a concentrated stock solution containing 100 μ M phosphatidylcholine (PC) and 400 μ M cholesterol in ethanol. Avanti Polar Lipids brain PC (#840053) is shipped as a solution (3.3 mM in chloroform) and cholesterol (#700000) as a powder. Make up a stock of 40 mM cholesterol in chloroform. To 4.8 ml of 100% ethanol, add 152 μ l of PC (as shipped, 3.3 mM in chloroform) and 50 μ l of the 40 mM stock of cholesterol. Store tightly capped at -20°C .
3. Dilute the concentrated stock 1:100 (e.g. 30 μ L to 3 ml) in 100% ethanol.
4. Calculate the total amount (pmol) of each glycolipid you want to adsorb to the wells and then 20% additional to account for handling losses.
5. Place the total amount of each glycolipid to be used into a glass vial or tube, evaporate to dryness (using a stream of dry nitrogen or in a SpeedVac), then dissolve in PC/Chol solution (from step 3) to provide the desired amount (pmol/well) in a volume of 25 μ l per well. Sonicate and vortex.

6. Add an equal volume of water, vortex, then distribute at 50 μ l per well.
7. Place the 96-well plate(s) in a fume hood at for 90 min to allow partial evaporation – DO NOT COVER
8. Shake out any remaining solution, then wash the plate by immersing in water to fill the wells and shaking out. Repeat the wash a total of 3 times, leaving the wells filled. Water-filled plates can be stored up to 24 h.
9. Shake out the water and add PBS containing BSA (NOTE: 1 mg/ml for Siglec-8. –E, or –F; 10 mg/ml for Siglec-9). Incubate covered 30 min at 37°C.
10. Prepare Siglec-Fc precomplexed with anti-Fc (example in Table 1) by adding the two components (1° and 2°) to the same PBS/BSA solution. Simultaneously along with blocking the plate, pre-incubate the pre-complex for 30 min at 37°C.

Table 1 – Precomplex Siglec-Fc chimera with anti-Human Fc-AP (for 10-wells)

<u>1° Siglec-8 Fc</u>		<u>2° anti-Human-Fc-AP</u>	
20	ng/ μ l preincubation	10	ng/ μ l preincubation
153	ng/ μ l stock	300	ng/ μ l stock
6.5	μ l added to PBS/BSA	1.67	μ l added in PBS/BSA
Add both (1° and 2°) above to 42 μ l PBS/BSA (50 μ l total)			
Incubate at 37°C for 30 min			
Add 450 μ l PBS/BSA; distribute 50 μ l/well into glycolipid-adsorbed wells (below)			

11. Wash plates three times with PBS
12. Add 50 μ l/well precomplexed Siglec-Fc (e.g. from Table 1), incubate 60 min at ambient temperature (RT)
13. Wash plates TWICE with PBS and ONCE with water
14. Add 100 μ l/well of 2 mg/ml PNPP in developing buffer
15. Read absorbance at 405 nm

NOTES:

- The butanol pre-wash removes “mold release agents” used in plastics stamping, which may include fatty acids.
- Plate readings should be kinetic with the rate determined in each well. Alternatively, take readings at multiple times and calculate the rate. This self-corrects for background.