Quantitative Dot Blot of Siglec Counter-Receptors

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Materials & Reagents:

- Tris-Buffered Saline (TBS): 20 mM Tris base, 500 mM NaCl adjusted to pH 7.5 with HCl
- Phosphate-buffered saline/0.1% Tween 20 (PBST): 137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.1% Tween 20
- Blocking Solution: 5% w/v Blotto (nonfat dry milk) in PBST
- Guanidinium Buffer: 6 M guanidinium hydrochloride, 5 mM EDTA, 10 mM monosodium phosphate, adjusted to pH 6.5 with NaOH. May be stored at ambient temperature (RT); cool to 4˚C prior to use.
- Dot-blot apparatus (e.g. BioRad Bio-Dot, Fig. 1)
- Siglec-Fc chimeras
- HRP goat anti-human IgG-Fc specific (Sigma A0170)

Method:

1. Place the gasket support plate into position in the vacuum manifold (Fig. 1). Place the sealing gasket on top of the gasket support plate. Inspect the gasket to ensure the holes are aligned. As needed, pull lightly at the corners until aligned.
2. Use forceps or gloves when handling membranes. Prewet nitrocellulose membrane (PVDF is not recommended) by slowly sliding it at a 45° angle into TBS. Soak for 10 min. Remove touch the corner to filter paper to remove excess buffer. Lay the membrane on the gasket in the apparatus so that it covers all of the holes but does not extend beyond the edge of the gasket. Take care to avoid any trapped air bubbles between the membrane and the gasket.
3. Place the sample template on top of the membrane. Finger-tighten the four screws in a diagonal crossing pattern to ensure uniform pressure (Fig. 2).
4. Set the 3-way valve to Setting 1 (full vacuum, Fig. 3) and start vacuum. Repeat the tightening process using the diagonal crossing pattern.
5. Set the 3-way valve to Setting 2 (air pressure only, Fig. 3). Add 100 µl TBS to all of the 96 sample wells.
6. Prepare samples starting with “mArG” “mBrG” and “mHrG” and “hTrG” and “hPcyG”. In 1.5-mL Eppendorf, prepare initial sample dilution 100 µl (Sample) + 200 µl (Guanidine-HCl buffer) for each extract.
7. Working 96-well plate: Add 125 µl tissue extract into Column #1 (Row B-“mArG”, Row C-“mBrG”, Row D-“mHrG”, Row E-“hTrG”, Row F-“hPcyG”) and Column #2. For the same row, to the column #’s 2-12 add 125 µL of 4 M Guanidine-HCl buffer. We are serially diluting the samples in 96-well cell culture plates (e.g. Falcon 353072) Column #3 – 12, using the appropriate dilution buffer (e.g. Guanidinium Buffer) such that each well contains 125 µl and Column #12 will carry 250 µl.
8. Set the 3-way valve to Setting 3 (gentle vacuum, Fig. 3). As soon as the buffer solution drains from all the wells, set the 3-way valve to Setting 2 (air pressure only) and disconnect the

vacuum.

9. Add 30 µl of sample to empty wells on the DOT Blot carrying the membrane. Add 30 µl of the same dilution buffer (e.g. Guanidinium-HCl Buffer) to any unused wells Row's A, G and H.
10. Allow to wells to drain by gravity (~1 h).
11. Add 100 µl TBS to every well. Set the 3-way valve to Setting 3 (gentle vacuum, Fig. 3). When the buffer solution drains from all the wells, set the 3-way valve to Setting 1 (full vacuum, Fig. 3), dismantle the apparatus, and recover the membrane.
12. Place membrane in Blocking Solution for 30 min.
13. Precomplex Siglecs (concurrent with step 11, volumes are per blot)
    a. **For Siglec-8**, add 6 µg of Siglec-8-Fc to 300 µl containing 4.4 µg (equivalent to 1:500 dilution) of HRP-human IgG-Fc (Sigma A0170) in a 1.5-ml low-bind Eppendorf tube; **For Siglec-9** add 1.25 µg Siglec-9-Fc to 60 µl containing 0.89 µg (equivalent to 1:500 dilution) of the above secondary antibody; For **Siglec-F** add 3 µg Siglec-F-Fc to 134 µl containing 0.89 µg (equivalent to 1:500 dilution) of the above secondary antibody
    b. Incubate on ice for 30 min
    c. Dilute to 6 ml with PBST
14. Wash membrane with PBST and then overlay with 6 ml/blot of precomplexed Siglec Fc
15. Incubate 40 min at RT.
16. Wash with PBST twice for 5 min each
17. Overlay with 1.6 ml of freshly prepared ECL Prime Detection Reagent (GE Healthcare RPN2232).

NOTES:

- Check the wells after sample has been applied to insure that there are no air bubbles in the wells. Air bubbles will prevent the sample from binding to the membrane. Air bubbles may be removed by pipetting the liquid in the well up and down.
- Apply samples directly to the center of each well to ensure that it evenly contacts the nitrocellulose membrane.

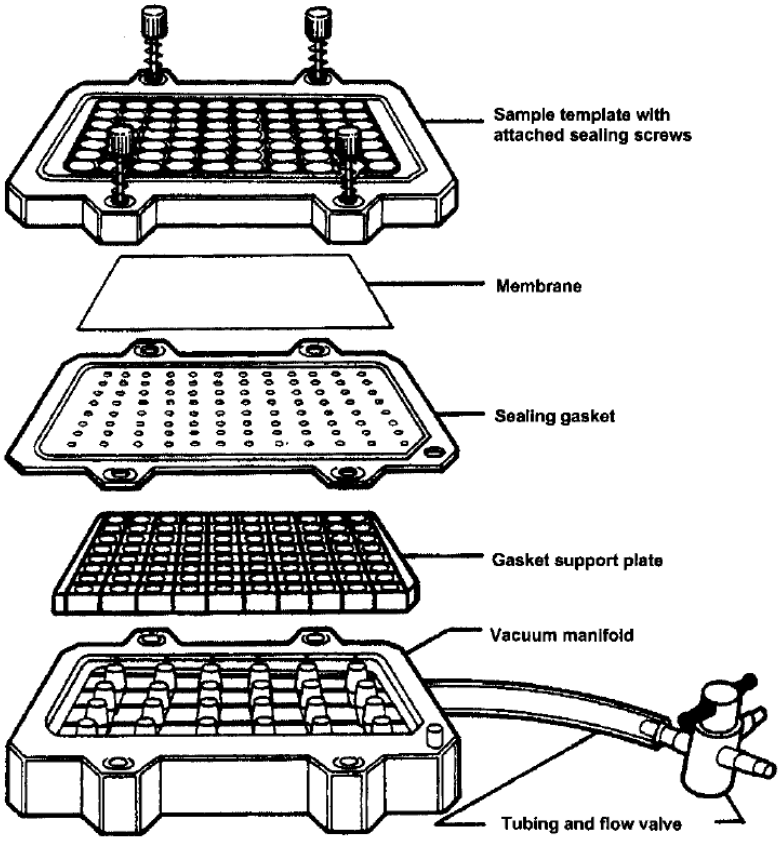

Fig. 1. Diagram of proper Bio-Dot apparatus assembly.