FLOW CYTOMETRY BINDING ASSAY

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BACKGROUND

- This protocol provides a means of measuring the ligand binding affinity of soluble receptor proteins through a flow cytometry-based assay.
- In this protocol, bacterial cells are incubated with purified receptor. The level of receptor binding is assessed by probing with a receptor-specific antibody tagged with a fluorophore and measuring the level fluorescence using a flow cytometer.

NOTES

- This protocol is written specifically for the binding of the hMARCOI-SRCR domain heterologous construct to heat-killed *S.pneumoniae*, but may be adapted for other receptor-ligand pairs by adjusting the antibody identities to suit the receptor.
- If working with a larger amount of construct, note that larger amounts of antibody can be added to improve signaling
- Tubes will contain bacteria bound to construct, construct only, bacteria bound to construct bound to IgG isotype (instead of regular 1^{o} Ab), and bacteria only. All of these will have 1^{o} rabbit anti-human MARCOI SRCR 9805 Ab, with the exception of the isotype, which instead will have normal rabbit IgG. All tubes except the bacteria only tube should have at least 10µg of construct. Add 100µl of bacterial solution to each tube with the exception of the construct only tube. Ensure that the sum of the remainder of the volume of each tube totals 500µL of solution by evening the difference through the addition of Tris-HCl.
- During any of the nutating periods, it is safe to store the samples for an extended period of time in a 4^{o} refrigerator

EQUIPMENT AND MATERIALS

- Heat-killed *Streptococcus pneumonia* ($5x10^{8}$ CFU/mL)
- Primary rabbit anti-human hMARCOI SRCR 9805 IgG antibody
- Secondary goat anti-rabbit IgG antibody, Alexafluor633-conjugated
- Normal primary rabbit anti-human IgG isotype control
- Tris-HCl pH 8
- Nutator
- FACS wash (see Bowdish Lab FACS staining protocol)
- hMARCOI-SRCR construct 9805
- BD Biosciences FACSCanto II flow cytometer

PROTOCOL

A. Binding of Construct to Bacteria and Preparation of Bacteria Only and Construct Only Samples

1. Add >10 µg of construct to each 1.5mL tube except for the bacteria only tube
2. Add 100µL of bacteria to each tube except for the construct only tube
3. Add Tris-HCl buffer to each sample until a volume of 500µL is occupying each tube
4. Nutate samples for 1 hour
5. Centrifuge at 18,000 x g for 5 minutes
6. Carefully remove supernatant

B. Binding of Antibody to Samples

1. Resuspend samples in a solution containing 1:5000 1^{o} rabbit anti-human hMARCOI SRCR 9805 IgG Ab to FACS wash, except for the isotype tube; instead add normal rabbit IgG at a dilution of 1:100 to FACS wash
2. Nutate for 1 hour
3. Add a 1:1000 dilution of 2^{o} goat anti-rabbit IgG Ab (conjugated to Alexafluor633) to samples
4. Nutate for 1 hour
5. Centrifuge at 18,000 x g for 5 minutes
6. Carefully remove supernatant
7. Resuspend in 200µL FACS wash

C. Flow Cytometry

1. Measure APC fluorescent emission on the BD Bioscience FACSCanto II flow cytometer.
2. Analyze data using desired software