# Materials and Equipment

* Carboxylated microspheres or beads
* EDC coupling reagent (Pierce Biotechnology)
* Sulfo-NHS (Pierce Biotechnology)
* Streptavidin R-phycoerythrin, 1 mg/ml
* Biotin labeled Goat anti-human IgG (H+L) 2 mg/ml
* Activation Buffer (0.1M Sodium Phosphate pH 6.2).
* Coupling Buffer (MES 50mM pH 5)
* Wash Buffer (PBS pH 7.4, 0.05% Tween 20)
* Storage or blocking buffer (PBS, 1% BSA, pH 7.4, 0.05% sodium azide)
* Stop solution (0.5 % formaldehyde in PBS)
* Vortex
* Sonicator
* Microcentrifuge
* Aluminium foil
* Pipettes and tips
* 1.5 ml Eppendorf tubes
* Antigens

# Procedure

## Antigen (Ag) coupling

1. Label 200 µl (2.5 X 106) of beads for each Ag concentration.
2. The optimum working concentrations of the various antigens for coupling as determined in prior optimization assays (described below, Section B) are as follows:

Ag Amount of Ag Ag concentraion (µg/ml)

 (µg per 200 ul beads)

EBA175RII 10 25

AMA1 variants 4 10

BSA 40 100

Coupling is performed as follows:

1. Allow all reagents to warm at room temperature (RT, ~25˚C).
2. Centrifuge each bead stock for 5 min at 310xg. Disperse beads pellet by sonication and vortex for at least 20 sec.
3. Dispense 200 µl (2.5x106 beads) of each bead stock (1.25 x 107 beads/ml). Centrifuge the bead aliquots for 2 min at 11,300xg and aspirate the supernatant.
4. Wash twice with 80 µl of activation Buffer; sonicate & vortex for at least 20 sec between washes.
5. After the last washing step, aspirate supernatant, add 80 µl of activation buffer, sonicate and vortex.
6. Add 10 µl of 50 mg/ml Sulfo NHS, followed quickly by 10 µl of 50 mg/ml EDC to the beads and mix by gentle vortex.
7. Incubate beads suspension for 20 min (+/- 2 min) in the dark (cover with foil) at RT with gentle mixing by vortexing after 10 min.
8. Centrifuge the activated beads for 2 min at 11,300xg; aspirate the supernatant and resuspend the activated beads in 250 µl of coupling buffer (50 mM MES, pH 5.0), vortex and sonicate for 20 sec. Repeat steps 10 and 11 for a total of two washes with coupling buffer.
9. Resuspend the activated and washed beads in 100 µl of coupling buffer by vortex and sonication for 20 sec.
10. Add 400 µl of antigen at the appropriate concentration in coupling buffer to the beads. Mix coupling reaction by gentle vortex and Incubate for 2 h with rotational mixing at RT.
11. After 2 h of incubation, centrifuge the coupled beads for 2 min at 11,300xg; aspirate the supernatant.
12. Add 1000 µl of wash buffer (PBS pH 7.4, 0.05% Tween 20) and resuspend by vortex and sonication for 20 sec.
13. Centrifuge the coupled beads for 2 min at 11,300xg; aspirate the supernatant. Resuspend the beads in 1 ml of storage buffer (PBS, 1% BSA, pH 7.4, 0.05% sodium Azide) by vortex and sonication for approximately 20 sec.
14. Centrifuge the coupled beads for 2 min at 11,300xg; aspirate the supernatant. Resuspend the coupled and washed beads in 1 ml of storage buffer.
15. Count beads diluted 1:10 using a haemocytometer and estimate the bead concentration (beads/ml). Adjust the bead concentration if necessary and store coupled beads at 2-8˚C until use; protect from light.

## Determination of optimum antigen concentration for coating

To determine the optimum concentration of Ag to be used in the assay, 2.5-fold serial dilutions of the various antigens (25 µg/400 µl to 0.64 µg/400 µl) are coupled to 100 µl (1.25 X 106) of beads with different signatures. The procedure for coupling is similar to that described above, but with few modifications as indicated below.

* step 4 above, dispense 5 aliquots of 100 µl (1.25x106 beads) from each bead stock (1.25 x 107 beads/ml). This is to test the same Ag at the five different dilutions.
* In steps 18 and 20 above, 500 µl instead of 1 ml of the appropriate buffer is added to the coupled beads.

Beads coupled with the similar concentrations of the different antigens were mixed and used for a multiplex assay to determine the optimal saturating concentration of each antigen to use in subsequent multiplex assays.

## Multiplexed assay

1. Thaw plasma samples on ice and centrifuge to remove debris.
2. Dilute test plasma as well as positive and negative control reagents as appropriate. For our assays, test plasma samples and negative plasma sample (from malaria-naïve individuals) were diluted 1:400, positive plasma samples (pooled hyperimmune plasma samples) was diluted 1:10,000).
3. Add 50 µl aliquots of diluted plasma samples into duplicate wells of a 96-well filtered plate.
4. Prepare the working solution of labeled beads. Thoroughly resuspend each coupled bead set by vortexing/sonication and aliquot the appropriate volume of each bead set (based on the estimated concentration) into a 15 ml tube. Approximately 1,250 beads per coupled bead set are required in the final mixture.
5. Subtract the volume of the mixed beads suspension from the total volume required (approximately 6 ml for one 96 well plate). This difference is the volume of buffer to add to the bead suspension (see table below) to obtain the expected bead concentration. Thoroughly vortex the diluted mixed beads suspension and aliquot 50 µl/well into the filtered 96 well plate with diluted plasma samples using a multi-channel pipette.
6. Incubate for 1 h at 4oC. Protect from light by covering plate with aluminum foil.
7. After sample incubation, wash beads with 250 µl/well of wash buffer using the pressure suction system. Repeat wash step 2 more times for a total of 3 washes.
8. Gently vortex the plate to resuspend the beads. Add 25 µl/well of 5 µg/ml detection antibody (1:200 dilution of stock) to the 96-well plate.
9. Gently vortex the plate again to ensure good mixing and incubate for 1 h at 4oC. Protect from light by covering plate with aluminum foil.
10. Wash 3 times as described above, and gently vortex plate to resuspend the beads.
11. Add 25 µl/well of 1:50 diluted SA-PE (substrate solution) to the plate.
12. Gently vortex the plate again to ensure good mixing.
13. Incubate for 30 min at 4oC. Protect from light by covering plate with aluminum foil.
14. After this incubation period, add 25 µl/well of Stop solution to the plate.
15. Acquire data using the Luminex 200 system.

## Quality control/quality management of Multiplexed assays

1. For quality control purposes, a pool of plasma from semi-immune adults with high levels of IgG to multiple malaria antigens is added at a specified dilution on each plate as a positive control.
2. The BSA antigen included in the assay also serves as a control to account for non-specific antibody binding in the assay.