Protocol protein coupling – coupling control – assay

Materials:

- purified/synthesized tagged proteins (e.g. Strep or His_6) in appropriate buffer (CRITICAL! maximally 2.4 M urea for coupling, when working with His_6-tagged proteins keep the imidazole concentration as low as possible still yielding enough product since it quenches binding on the beads)

- xMAP® MagPlex® bead stocks at 12.5 million beads/mL (Luminex®) of multiple bead regions depending on the number of proteins to be included in the panel

- antibodies:
    - anti-His_5 antibody (Qiagen 34660, mouse) or anti-Strep antibody (Qiagen 1023944, mouse) depending on the protein purification tag used
    - anti-mouse antibody RPE-conjugated (Dianova 115-116-146)
    - anti-human IgG antibody RPE-conjugated (Dianova 109-116-098)

Chemicals:

- PBS (Sigma P5368)

- activation buffer (100 mM NaH_2PO_4 (Sigma S3139) in H_2O at pH 6.2)

- coupling buffer (50 mM 2-(N-Morpholino)ethanesulfonic acid (Sigma M2933) in H_2O at pH 5.0)

- wash buffer (0.05% Tween® 20 (Sigma P7949) in PBS)

- block store buffer (1% BSA (Sigma A7906) in PBS)

- bead buffer (50% block store buffer/50% low cross buffer (Candor 100 050))

- ProClin™ 300 (Sigma 48912-U)

- EDC (Thermo 22980)

- Sulfo-NHS (Thermo 24510)

- DMSO (Sigma 276855)

- xMAP® Sheath Fluid (Thermo 4050015)

Consumables:

- flat bottom 96-well plates for coupling of volumes up to 200 µl (Greiner bio-one 655095) and matching magnetic separator block for 96-well plates (Novex® A14179/Thermo)
- low binding reaction tubes for volumes above 200 µl (Sorenson 39640T) and matching magnetic separator rack for reaction tubes (DynaMag™-2 12321D/Thermo)
- flat bottom half-well 96-well plates (Greiner bio-one 675101)
- adhesive aluminum foils for 96-well plates (Sarstedt 95.1995)

Instrumentation:

- ultrasonic bath (e. g. Bandelin Sonorex Super RK 52)
- plate shaker with a 3 mm shaking orbit (e. g. Thermo 88880024)
- vortex mixer (e. g. Vortex Genie 2/Scientific Industries)
- incubation shaker (e. g. ThermoMixer® C/Eppendorf)
- read-out instrumentation (e. g. Flexmap 3D®)

Bead calculation:

As a starting point we suggest a coupling ratio of 25 µg protein to 100 µl bead stock solution containing approx. $1.25*10^6$ beads. It might be necessary to adapt the amount of protein depending on its coupling properties. The number of beads needed depends on the number of samples in the later experimental setup. We recommend using 0.1 µl of the original bead stock per well, so 100 µl of bead stock solution would allow a maximum of 1,000 wells for measurement including a calculated 1,250 beads per well. The actual bead count will be lower due to losses during the whole workflow (e. g. while washing) but still ensures a count of at least 100 beads per well. Make sure to include additional beads into the calculation required for the coupling control, at least two blanks on each assay plate, and about 5% auxiliary volume at the preparation of the multiplexing mix and its distribution to all wells.

Protein dilution:

Prepare a dilution of the protein stock solutions (e. g. from purification) to include the necessary amount of protein in a fixed volume depending on the selected setup. For the small-scale approach up to 200 µl of bead stock dilute the calculated amount of protein in a total volume of

150 µl using coupling buffer as diluent. When using the large-scale setup for volumes above 200 µl of bead stock include the calculated amount of protein in a volume of 1 ml also using coupling buffer as diluent.

Coupling workflow:

1. Resuspend the bead stock solutions after storage by shaking on a vortex mixer for at least 30 s and additional treatment in an ultrasonic bath for 3 min. Proceed quickly to avoid settling of the beads and shake the flasks again directly before transferring the calculated volume to a well in a flat-bottom 96-well plate for volumes up to 200 µl or a low binding reaction tube for volumes above 200 µl. (CRITICAL! carefully keep track which bead regions were used to enable unambiguous assignment of bead region to protein!)

2. Use the matching magnet system to separate the beads from the storage liquid, let the setup rest for 3 min to minimize bead losses in the following removal of the liquid. Discard the liquid by inverting the plate together with the magnet quickly, or, in the case of using reaction tubes, keep them in the magnetic separator rack and remove the liquid with a pipette trying not to disturb the microsphere pellet. Remove the magnet and add activation buffer to the beads, 150 µl per well or 1 ml per tube. Repeat this wash process twice. Keep the beads suspended in activation buffer while preparing the next solution in the following step.

3. Prepare the EDC/Sulfo-NHS (ES)-mix freshly, calculate the necessary volumes depending on the number of bead regions to be coupled. When working with the plate setup 150 µl of ES-mix are needed for each well/bead region, for the tube setup 1 ml should be applied per tube/bead region. The final ES-mix contains 5 mg/ml EDC and 5 mg/ml Sulfo-NHS and is prepared as follows: the necessary volume is calculated and an auxiliary volume of about 10% is added to ensure a sufficient volume for all reactions (e. g. 10 bead regions are prepared for coupling in a plate setup, 1.5 ml of ES-mix are necessary, so 1.65 ml should be prepared). For 10% of the full volume an EDC stock solution needs to be prepared at 50 mg/ml in H_2O. The same volume is necessary of a Sulfo-NHS stock also at 50 mg/ml in DMSO. The final ES-mix is prepared by mixing activation buffer with the two stock solutions in a ratio of 80:10:10 (e. g. for a total of 1.65 ml ES-mix, 165 µl of EDC stock are required containing 8.25 mg EDC in H_2O, 165 µl of Sulfo-NHS stock containing 8.25 mg Sulfo-NHS in DMSO, both are added to 1.32 ml of activation buffer).

4. Separate the beads from the activation buffer and remove the liquid, then add the freshly prepared ES-mix to the beads and incubate for 20 min at room temperature with shaking at 900 rpm. Therefore, seal the wells with an adhesive aluminum foil if working with the plate setup.

5. Wash the beads three times using coupling buffer following the instructions in 2. (magnetic separation – discard the liquid – remove magnet – add coupling buffer).

6. Remove the coupling buffer after separating the beads magnetically from the liquid and add the protein dilution to the respective bead region. Seal the wells with adhesive aluminum foil and incubate this mix for 2 h at room temperature with shaking at 900 rpm.

7. Wash the beads three times using wash buffer following the instructions in 2. (magnetic separation – discard the liquid – remove magnet – add wash buffer).

8. Resuspend the protein coupled beads in block store buffer supplemented with 0.05% ProClin™ 300 using at least a fivefold volume compared to the starting volume of the used bead stock (e. g. when 50 µl of bead stock solution were used, at least 250 µl of block store buffer should be applied for resuspension to ensure sufficient blocking of remaining binding sites, carefully note the applied volume since the later volume per well depends on the dilution: at a fivefold dilution 0.5 µl are later needed per well). Split the resuspension volume in two to three parts to "wash out" the coupling reaction well or tube in multiple steps finally collecting the full volume in one storage tube for each bead region separately. Let the coupled beads rest over night at 4 °C for blocking.

Coupling control/bead loading estimation:

1. Prepare dilutions of the detection antibodies, use a primary antibody targeting the used protein tag (e. g. anti-His_5 or anti-Strep derived from mouse). 220 µl of the anti-tag detection antibody at a final concentration of 10 µg/ml are necessary including 50 µl for four wells plus auxiliary volume for pipetting. Dilute the antibody stock of a higher concentration in a matching amount of bead buffer to reach the desired concentration. The secondary antibody needs to be RPE-conjugated and match the applied primary antibody (e. g. anti-mouse RPE-conjugated). 420 µl of the secondary antibody should be prepared at a concentration of 5 µg/ml to grant enough volume for eight wells at each 50 µl.

2. The multiplex of the coupling control should be kept the same as it will be used for the actual assay. Resuspend all separately coupled bead regions which will be included in the multiplex by shaking them on a vortex mixer for at least 30 s and treatment in an ultrasonic bath for 3 min. Shake the tubes again directly before transferring the needed volume to the multiplexing mix.

3. A total of eight wells should be included in the coupling control, four wells as blank and four wells targeting the protein tag with the matching detection antibody. The multiplexing bead mix volume is therefore calculated for nine wells granting enough volume for the included wells. If the beads had been diluted in the fivefold block store buffer volume after coupling, 4.5 µl of each bead region are combined to the multiplexing mix containing all bead regions, the volume is filled up to a total of 450 µl using bead buffer to continue with a volume of 50 µl multiplexing mix per well (e. g. for a 20-plex the combination of the bead regions results in 20*4.5 µl making up 90 µl; 360 µl of bead buffer need to be added to reach a volume of 450 µl for the calculated nine wells). (CRITICAL! We suggest combining only the volume necessary

for the coupling control at first to allow the replacement of single bead regions in case of a bad protein coupling efficiency before combining the whole volume of beads for the actual assay.)

4. Keep the beads from settling in the multiplexing mix tube by shaking them well on a vortex mixer and transfer aliquots of each 50 µl to eight wells of a flat bottom half-well 96-well plate. Place the plate on the magnetic separator block to let the beads settle down for at least 3 min. Discard the storage liquid by inverting the plate together with the magnet and apply 50 µl of bead buffer as blank to the first four wells containing beads, add each 50 µl of the prepared primary detection antibody solution to the remaining four wells. Seal the wells with foil and shake the plate protected from light for 45 min at 1,200 rpm at room temperature.

5. Wash the beads three times using each 100 µl wash buffer per well (magnetic separation – discard the liquid – remove magnet – add wash buffer). After discarding the third round of wash buffer add each 50 µl of the secondary antibody dilution to every well. Seal the wells with foil and shake the plate protected from light for 30 min at 1,200 rpm/room temperature.

6. Wash the beads three times using each 100 µl wash buffer per well; afterwards resuspend the beads in 100 µl sheath fluid per well and shake the plate for at least 1 min at 1,200 rpm/room temperature before subjecting the plate to measurement. Make sure that all blank wells are measured consecutively followed by all coupling control wells. Include as many beads as possible per well by setting the target bead count value higher than possible (e. g. set the target to 1,500 beads per region; from the original calculation a maximum of about 1,250 beads were included per well, the actual number will be much lower due to losses along the coupling procedure and can vary depending on the degree of bead settlement when preparing the multiplexing mix).

Assay workflow:

1. Prepare the multiplexing mix for all necessary wells of the assay including about 5% auxiliary volume since the liquid will form foam when it is shaken. Make sure that all protein coupled bead regions are very well resuspended before transferring volume to the multiplexing mix by shaking them at least 30 s on a vortex mixer, ultrasound treatment in an ultrasonic bath for 3 min, and shortly shaking them again on a vortex mixer directly before taking out the needed volume. The multiplexing mix includes all bead regions and is filled up with bead buffer to the calculated full volume. After preparation, transfer equal amounts (e. g. 50 µl) to the calculated number of wells in flat bottom half-well 96-well plates, always mix the multiplexing mix before taking out volume for aliquoting to ensure a consistent distribution of the beads over all wells. The beads remain in the multiplexing mix liquid until they are used for the actual assay, seal the wells with adhesive aluminum foil and store the plates at 4 °C in the dark.
   Assay calculation example: 20-plex measurement of 30 serum samples each represented by seven dilution stages (adding up to a total of 210 wells for all dilutions of all samples); to

include all samples three plates are necessary adding a total of six blank wells (two per plate); add auxiliary volume to make sure the prepared volume will be sufficient for all calculated wells of about 5% (216 * 0.05 = 10.8, round up to a total of 230 wells); with the beads diluted in a fivefold volume of block store buffer 0.5 µl are necessary per bead region per well, so 115 µl of each of the 20 bead regions are included making up 2.3 ml; the total volume of the multiplexing mix should include 50 µl of aliquot volume per well (i. e. 11.5 ml) and is therefore filled up with 9.2 ml of bead buffer

2. Prepare the detection antibody for all sample wells and blanks including some additional volume. We suggest applying 50 µl of detection antibody per well at a concentration of 5 µg/ml. This is highly dependent on the affinity of the antibody and the distribution of antibody (sub)classes in the given sample set and might need optimization. Use bead buffer as diluent for the antibody stock solution.

3. Prepare a serial dilution for each sample using bead buffer. 50 µl of each dilution is later on transferred to one well containing beads. We suggest a seven stage dilution series ranging from 1:50 up to about 1:200,000 as a starting point for the detection of total IgG against specific antigens (e.g. 1:50; 1:200; 1:800; 1:3,200; 1:12,800; 1:51,200; 1:200,000). Since the actual antibody levels are highly sample- as well as antigen- and targeted (sub)class-dependent, we also suggest using a pool of the included samples for testing the dilution series settings prior to measuring all samples separately. Complete all dilution series before transferring them to the bead mix containing plates to keep incubation times as equal as possible for all samples.

4. Place the plate containing the multiplexing bead mix on the magnet, let the beads settle down for at least 3 min and discard the storage liquid. Transfer 50 µl of each sample/dilution to one well, make sure the order of dilutions is kept the same for all samples (since there is some bead carry-over from well to well at measurement the order should be consistent). Use 50 µl of bead buffer as blank for the two blank wells per plate. Incubate the plate over night at 4 °C under constant shaking at 1,200 rpm.

5. Wash the beads three times using each 100 µl wash buffer per well (magnetic separation – discard the liquid – remove magnet – add wash buffer). After discarding the third round of wash buffer add each 50 µl of the detection antibody dilution to every well. Seal the wells with foil and shake the plate protected from light for 60-90 min at 1,200 rpm/room temperature. Keep the incubation time the exact same for all plates of one project to allow comparability.

6. Wash the beads three times using each 100 µl wash buffer per well; afterwards resuspend the beads in 100 µl sheath fluid per well and shake the plate for at least 1 min at 1,200 rpm/room temperature before subjecting the plate to measurement. Select 100 beads per region as target value.

Data analysis:

We suggest to use the xMAPr app (https://github.com/stemicha/xMAPr_app) for the data analysis. Therefore, a special sample naming is crucial for a seamlessly working analysis.

Use: "SampleID_replicate_xfold-Diluation" for sample naming (separated with an underscore), when performing the measurement

Example: sampleID = Patient12; Replicate = 2; xfold-Dilution = 10000
→ Patient12_2_10000