Protocol 8: **Preparation of unevenly coated beads**

8.1. Take 1.5 μl of a 1% suspension of 1 μm polystyrene streptavidin-coated beads. Wash them once at 4°C in PBS-BSA buffer (PBS supplemented with 2 mg/ml BSA and 2 mM DTT) by spinning 15 min at 6,000 rpm and resuspending in 60 μl of PBS-BSA.

8.2. Take one "centrifugal device" (PALL, Nanosep MF 0.2 μm). With a sharp razor blade, cut out the green plastic bottom part of the filter device, so that the filter membrane can be accessed from the bottom. Pre-wet the membrane by adding 20 μl of PBS-BSA on top of the membrane, then add the beads. Spin 2 min at 2,000 rpm at 4°C.

8.3. Add 2 μl of biotinylated anti-His5 antibodies (Qiagen) in 58 μl PBS-BSA. Resuspend beads by pipetting and incubate with gentle vortexing/shaking for 1 hr.

8.4. Add 0.1 mM biotin to block all remaining streptavidin binding sites, spin immediately, and wash twice with 400 μl of PBS-BSA by spinning as above. After the last wash, blot all remaining liquid with a filter paper, so that the membrane is moist but not wet.

8.5. Add 0.5-2 μl of unlabeled protein with His6 tag (0.1 mg/ml) directly to the bottom side of the filter and incubate for 5-10 min. When all conditions are right, the monolayer of the beads on the inner side of the filter will be exposed to unlabeled protein diffusing through the filter, so the beads will become coated unevenly by this protein. Wash twice as above.

8.6. Add 20 μl of fluorescently labeled protein with His6 tag (0.1 mg/ml) to the top of the membrane. Mix well with beads by pipetting and incubate with gentle vortexing for 1 hr at 4°C. All remaining sites for binding the polyhistidine tag to beads surface will now become saturated with fluorescent complexes, eventually providing even coating by protein but with asymmetric fluorescent mark. Wash twice with 400μl of PBS-BSA.