Concentration of depleted plasma fraction and bound proteins

### Four concentration methods were used to try to concentrate depleted plasma fraction and the bound proteins in order to achieve the concentration more than 3.5 μg/μl.

### Vivaspin4 5 kDa MWCO concentrator

Low or high-abundance plasma protein fractions from two runs were combined separately and each was concentrated using Vivaspin4 concentrators with a fixed angle rotor to a final volume of 50 μl (depletion from Seppro column treated) and 120 μl (for both depletion and bound fractions from ProteoExtract and bound fraction from Seppro column treated). Samples were desalted using 50 mM Tris buffer (pH7.4).

#### 2) B.YM-3 Microcon—3 kDa MWCO concentrator

0.5 ml each of depleted protein fraction or non-specified bound protein fraction and 2 ml for bound protein were concentrated using YM-3 Microcon (3,000 Nominal Molecular Weight Limit, Millipore) 25°C, at 7000g.

#### 3) Trichloroacetic acid (TCA) precipitation

Five hundred microliters of protein collection from Seppro treatment was added with 500 μl of ice-cold 6.1 mol TCA (Sigma-aldrich) solution containing 80 mM DTT (sigma). The mixture was incubated for 1h at 4°C to allow the protein to precipitate completely. The extraction was then centrifuged at 10000×g for 10 min at 4°C. The supernatant was discarded, and the pellet was washed twice with 500 μl of acetone (Vsample:Vacetone=1:1) containing 20 mM DTT; the pellet was recovered in each step by centrifugation at 10000×g. Thereafter, the centrifuged pellet was dried by air evacuation. For 2-DE, the pellet was resolubilised in rabilloud buffer (7 M urea, 2M thiourea, 4% CHA.PS, 2.0% bio-lyte 3/10 ampholyte). The tube was pierced and put into liquid nitrogen and then stored at -80°C freezer.

ReadyPrep 2-D Clean up Kit

ReadyPrep 2-D Cleanup Kit （Sigma-Aldrich）was used to concentrate the solution. The protocol is shown as in the followings: Deplete high abundant plasma protein with

1. Transfer two 100 μl of sample1(1) & 3(1) into a 1.5 ml mirocentrifuge tube (marked ck1(1)1,ck1(2),ck3(1),ck3(2))
2. Add 300 μl precipitating agent 1 into the mixture of protein and mix well by vortexing. Incubate the samples on ice for 15 min.
3. Add 300 μl precipitating agent 2 to the mixture of protein and precipitating agent 1. Mix well by vortexing.
4. Centrifuge the tubes at 13000 rpm for 5 min to form a tight pellet. Remove the tube promptly once the centrifuge stops so that the pellet does not disperse. Without disturbing the pellet, remove and discard the supernatant using 1000 μl & 20 μl pipettes.
5. Position the tube in the centrifuge as before (protein pellet facing outward) and centrifuge at 13000 rpm for 30 sec to collect any residual liquid at the bottom of the tube. Use a pipette to carefully remove the remaining supernatant.
6. Add 40 μl of wash reagent 1 on top of the pellet. A precipitate may form along the tube wall. In these cases, vortex wash solution over the pellet several times to ensure entire pellet is thoroughly washed. Position the tube in the centrifuge as before and centrifuge at 13000 rpm for 5 min.
7. Add 25 μl of Milli-Q water on top of the pellet. Vortex the tube 20 sec. Protein pellet may disperse, but it will not dissolve in the water.
8. Add 1 ml of wash reagent 2 (stored at -20°C) and 5 μl of wash 2 additives. Vortex the tube for 1 min. The protein pellets will not dissolve in wash reagent 2.
9. Incubate the tube at -20 °C for 30 mins. Vortex the tube for 30 sec every 10 mins during this incubation period.
10. Centrifuge the tube at 13000 rpm for 5 mins to form a tight pellet. Remove the supernatant to a 1.5 ml tube marked ck1(1) A,ck1(2) A, ck3(1), ck3(2) (For RC-DC assay to measure the protein loss in this step later). Centrifuge the tube 30 sec and remove any remaining wash. The pellet appear white at this stage.
11. Air-dry the pellet at room temperature for no more than 5 mins.
12. Resuspend each pellet by adding 20 μl vercoe buffer to the pellet. Vortex 30 sec. The tube was pierced, put into liquid nitrogen and then -80°C freezer.