A Simplified Method to Recover Urinary Vesicles for Clinical Applications, and Sample Banking

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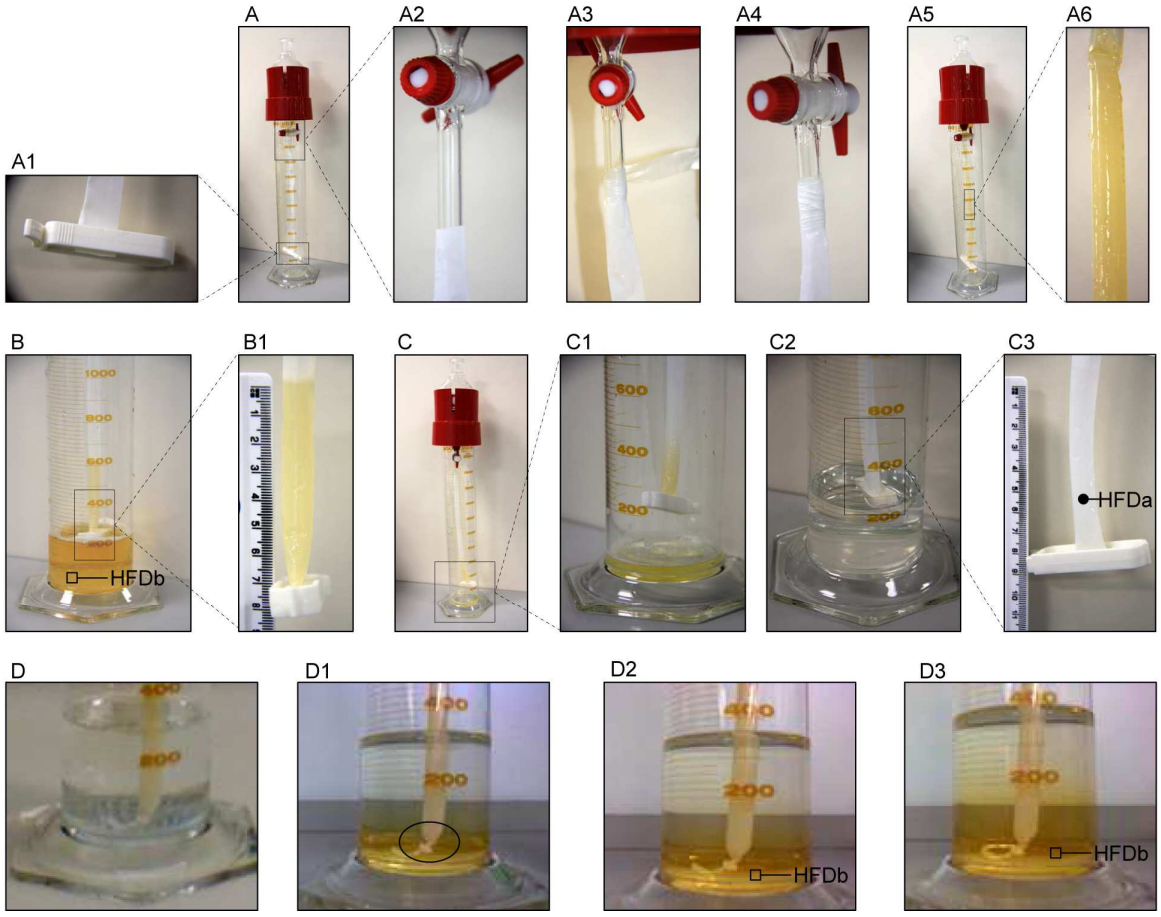
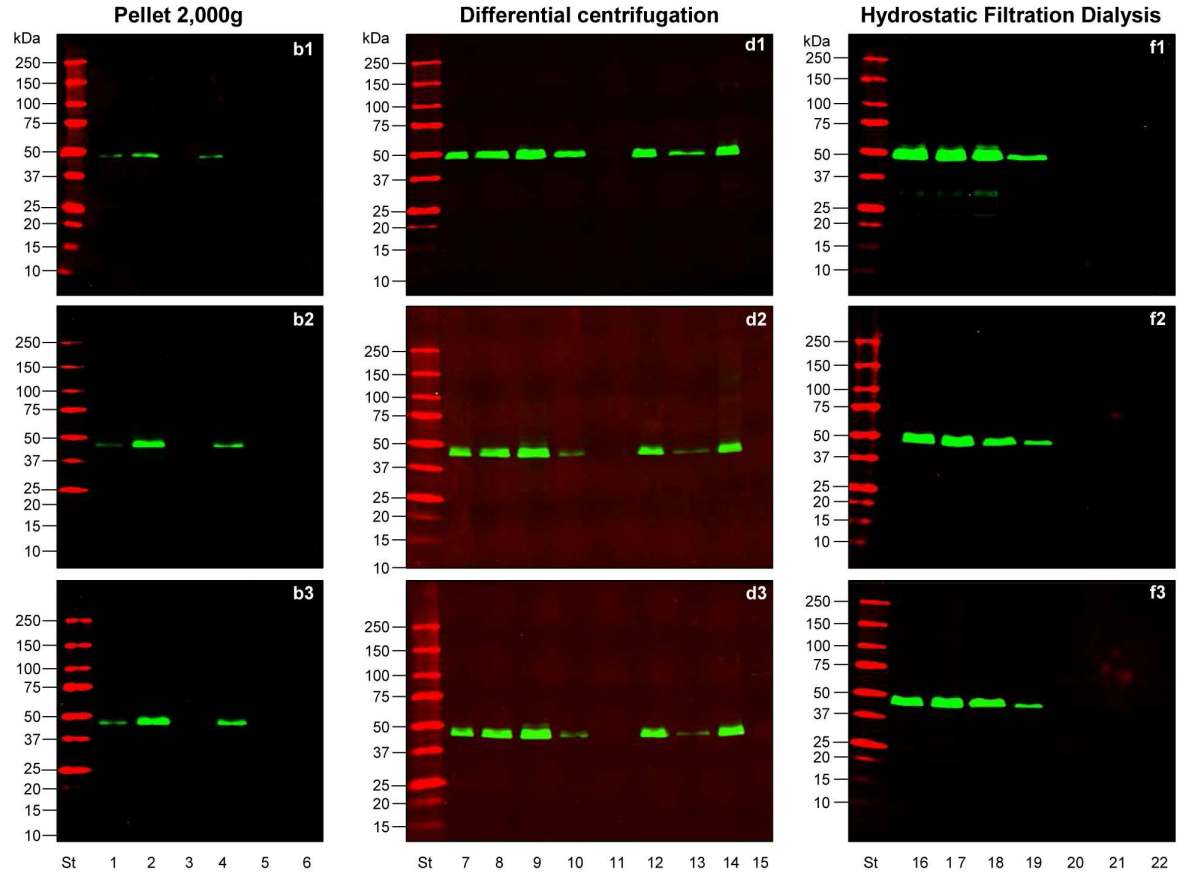


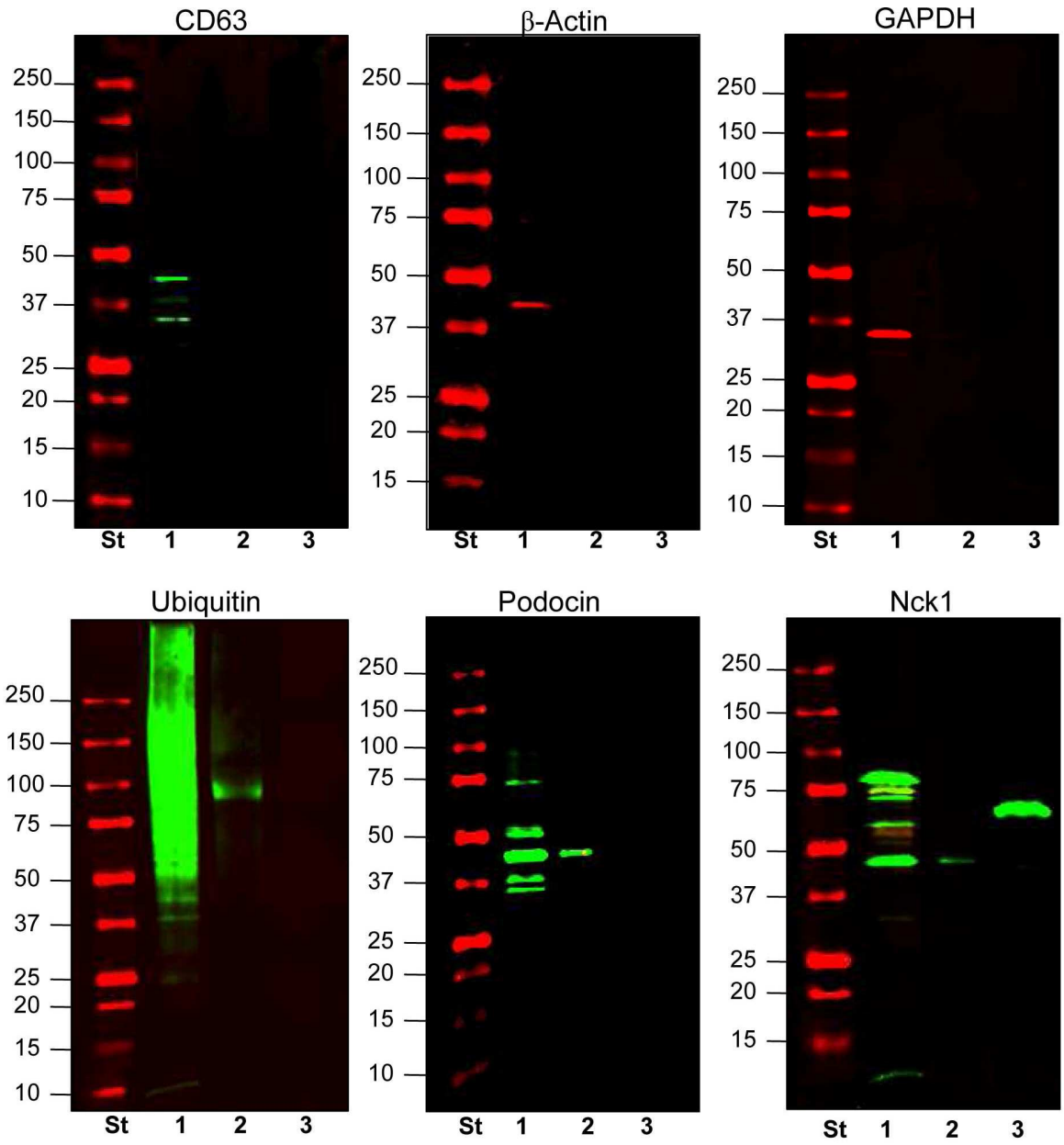
Supplemental Figure 1. Hydrostatic filtration dialysis system assembly and function. One end of the membrane dialysis tube (MWCO 1,000kDa) was inserted in the pipe of a 0.5l separating funnel and tightened with a strip of parafilm (**A2-A4**). The other end was sealed with a universal dialysis tube closure (**A1**). The end of the tube can be also closed with a double knot. However, material can be entrapped in the fold of the membrane in proximity of the knot circle (**D1**). The separating funnel was filled in with supernatant from 2,000g

centrifugation (**A5**). The pressure of the liquid in funnel pushes the solvent trough the meshwork of dialysis membrane (filtration) and liquid below the MWCO (HFDb) accumulates on the external surface of the membrane (**A6**) and drips to the bottom of the cylinder forming the fraction called **HFDb** hydrostatic filtration dialysis below 1,000 kDa (open square).(**B**). When the urine solution within tube reaches 7-8ml (**B1**) the urine collected in the measuring cylinder is removed (**C**) and the funnel is re-filled with milli Q water which flushes away the retained solution (**C1** and **C2**) until the solution is concentrated to a desired final volume which was named **HFDa** hydrostatic filtration dialysis retained above 1,000 kDa (filled circle), (**C3**). When the tip of the dialysis tube is immersed in a small volume of water without agitation it is possible to observe the diffusion of urinary pigments from the tube and their precipitation on the bottom of the cylinder in the HFDb fraction (**D1-D3**).



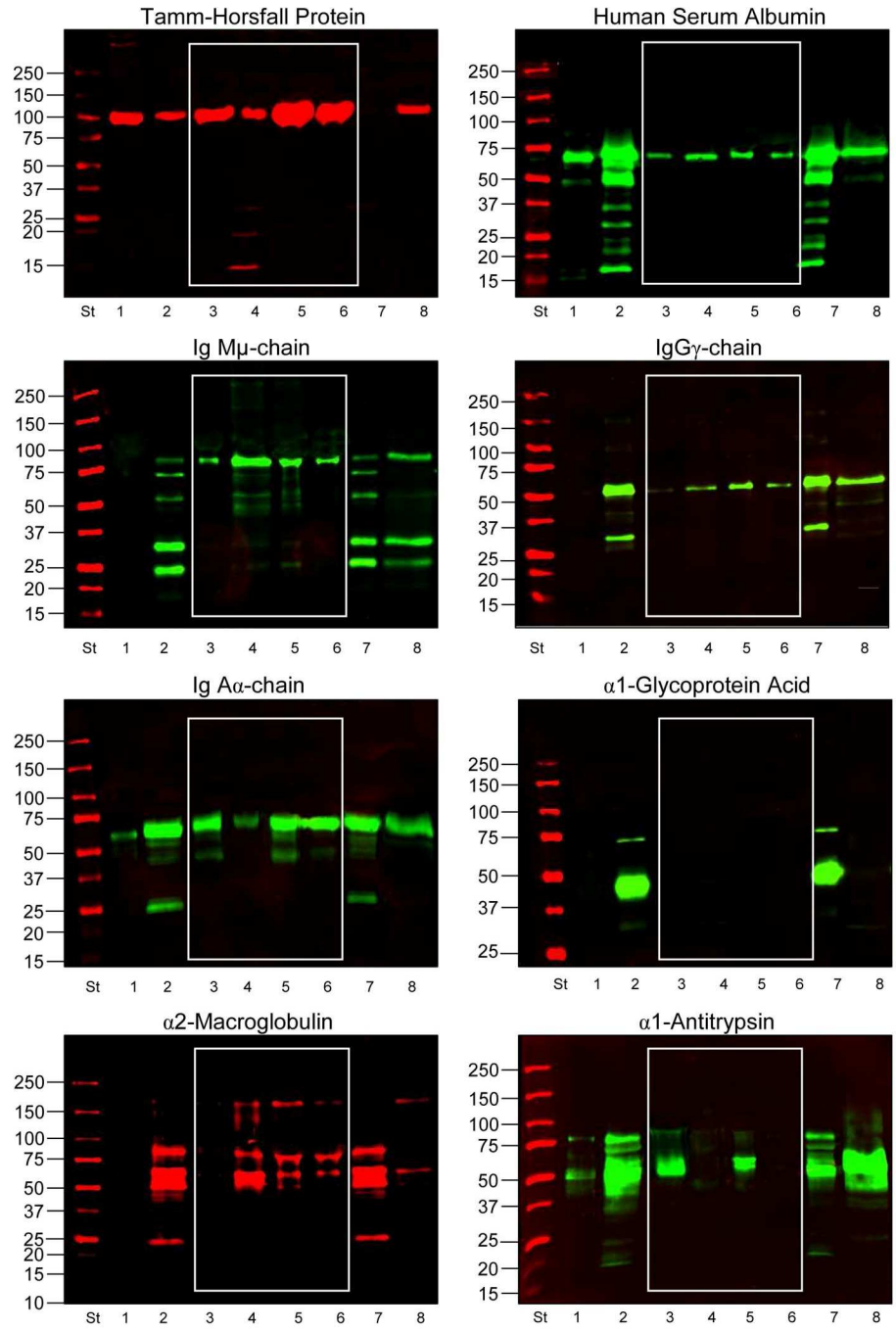
Supplemental Figure 2. TSG101 immunoblots signal detection for the estimation of exosomes recovery in HFD and differential centrifugation method.

Gel loading was based on the Bradford assay (10µg of protein per lane). Lane 1 crude urine, Lane 2 SN 2,000g Lane 3 Pellet 2,000g, Lane 4 P2,000g_DTT_P17,000g, Lane 5 P2,000g_DTT_P200,000g, Lane 6 P2,000g_DTT_SN 200,000g, Lane 7 P17,000g, Lane 8 P17,000g_DTT_ P17,000g, Lane 9 P17,000g_DTT_ P200,000g, Lane 10 P17,000g_DTT_ SN 200,000g HFDa, Lane 11 P17,000g_DTT_ SN 200,000g HFDb, Lane 12 P200,00g, Lane 13 SN200,000g, Lane 14 SN200,000g HFDa, Lane 15 SN200,000g HFDb, Lane 16 HFDa, Lane 17 HFDa P17.000g, Lane 18 HFDa P200,000g, Lane 19 HFDa SN 200,000g, Lane 20 HFDb, Lane 21 HFDb P 200,000g, Lane22 HFDb SN 200,000g. St – molecular weight markers (LI-COR Biosciences) are expressed in kilo Dalton. SN-Supernatant; P-Pellet;

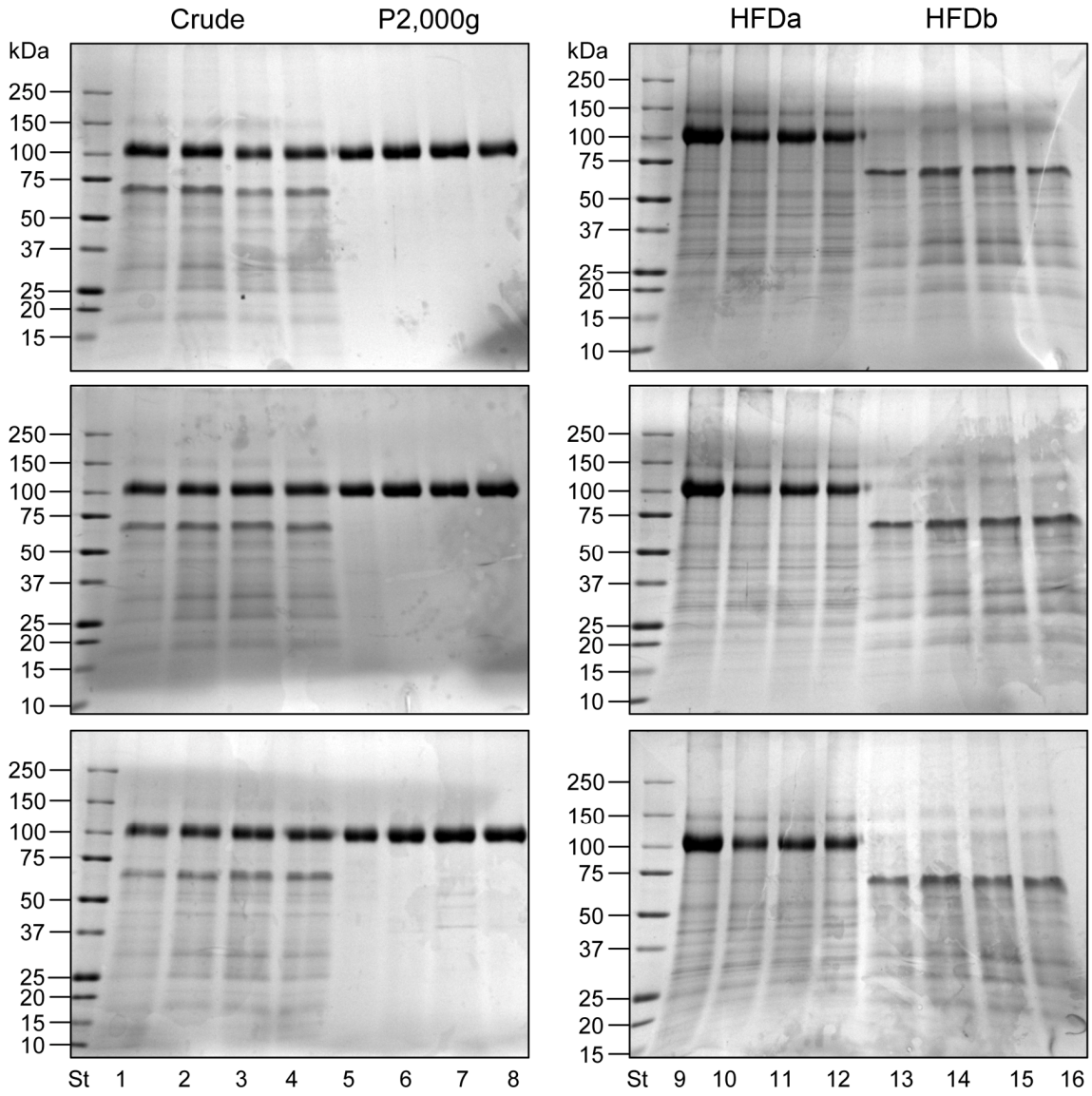


Supplemental Figure 3. Efficiency of hydrostatic filtration dialysis to retain vesicle proteins.

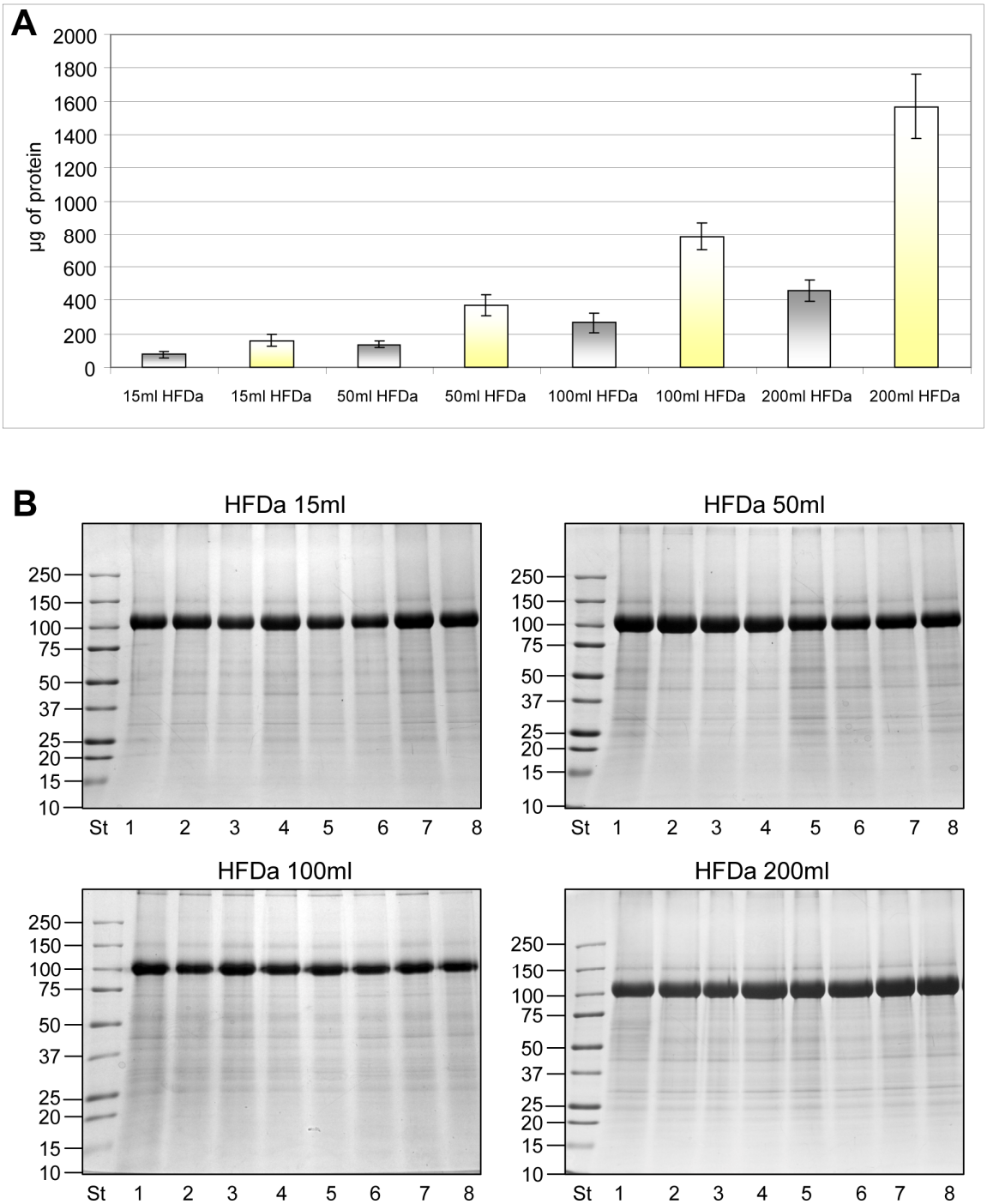
Gel loading was based on the Bradford assay (10µg of protein per lane). Lane 1, HFDa Pellet 40,000g; Lane 2, HFDa Pellet 200,000g; Lane 3, HFDa SN 200,000g; St – molecular weight markers are expressed in kiloDalton. SN-Supernatant; P-Pellet; GAPDH Glyceraldehyde-3-phosphate dehydrogenase



Supplemental Figure 4. Efficiency of hydrostatic filtration dialysis to remove soluble proteins. Protein loading, based on the Bradford assay with 10µg of protein per lane. Lane 1, Pellet 2,000g; Lane 2, SN 2,000g; Lane 3, HFDa; Lane 4, HFDa Pellet 40,000g; Lane 5, HFDa Pellet 200,000g; Lane 6, HFDa SN 200,000g; Lane 7, flow through HFDb; Lane 8, SDS elution of dialysis tube (30µg BCA assay); St – molecular weight markers (LI-COR Biosciences) expressed in kilo Dalton. SN- Supernatant; P-Pellet



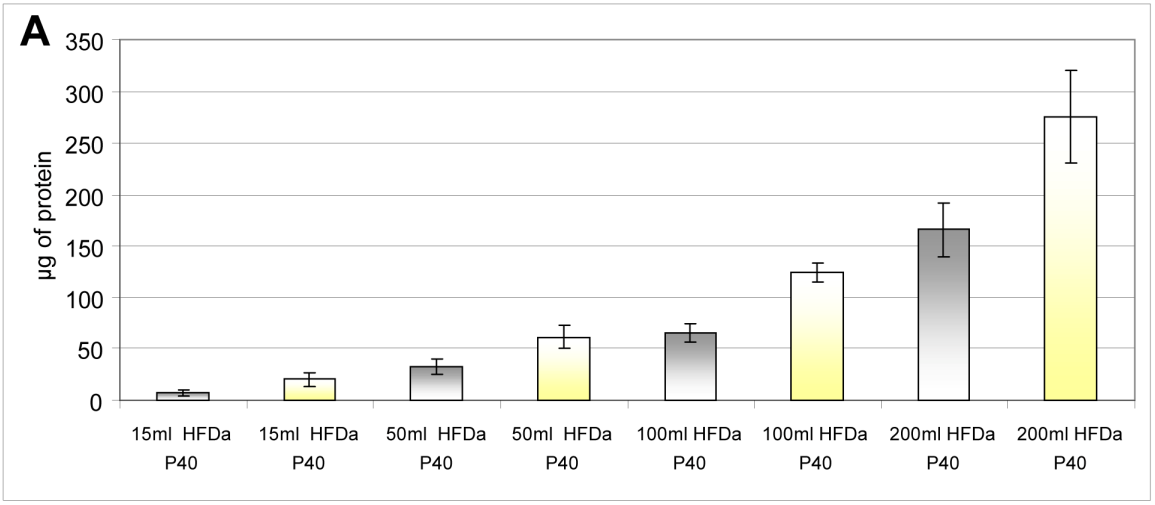
Supplemental Figure 5. Protein pattern of 3 independent gels of crude urine, pellet 2,000g, retained solution above 1,000kDa (HFDa) and flow through solution below 1,000kDa (HFDb). Protein loading was based on the volume: 50µl for crude urine, equivalent of 100µl for P2,000g, equivalent of 1.5 ml for HFDa and 100µl for HFDb. St – molecular weight markers (LI-COR Biosciences) expressed in kiloDalton. SN- Supernatant; P-Pellet. 1,5,9 and 13 fractions of day 1; 2,6,10 and 14 fractions of day 2; 3,7,11 and15 fractions of day 3; 4,8,12,16 fractions for day 4.

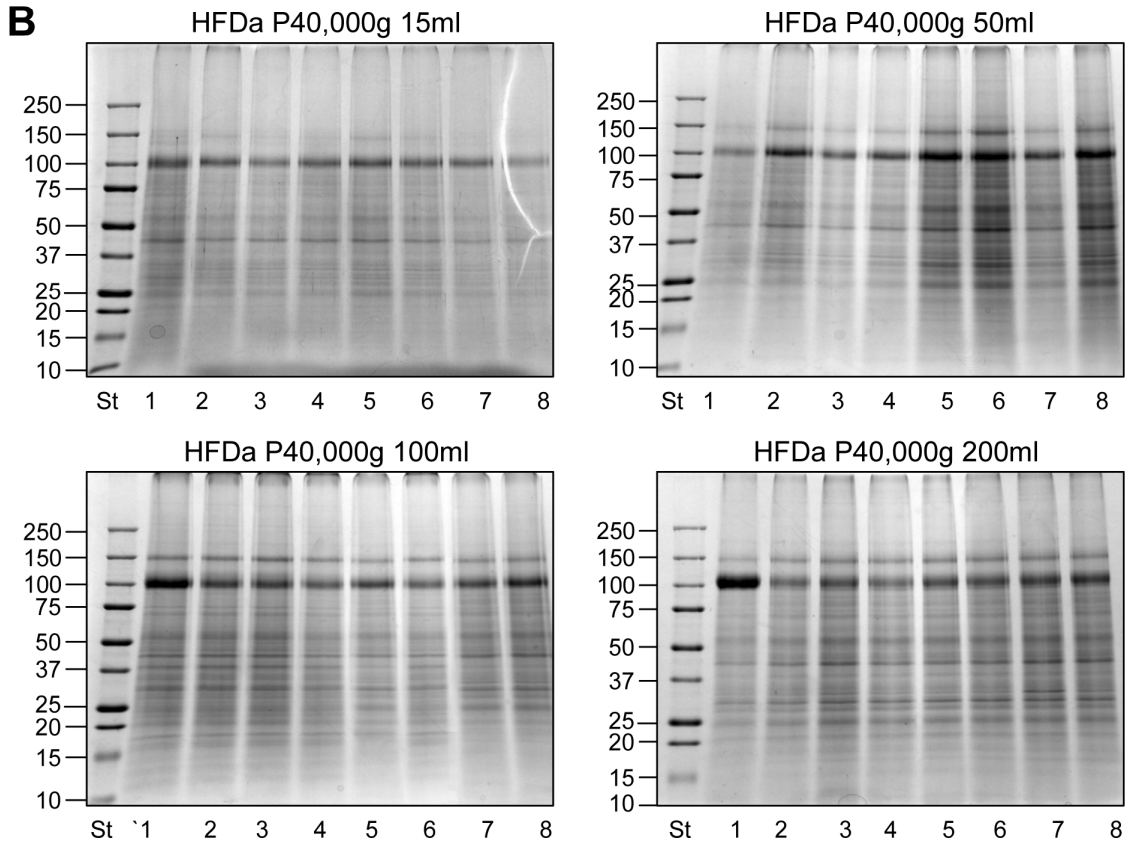


Supplemental Figure 6. Protein recovery and pattern in retained solution above 1,000kDa (HFDa).

(**a**) The histogram bars summarizes the amount of total protein recovered in HFDa estimate by Bradford (grey bars) and BCA (yellow bars) protein assay. Fifteen ml urine (Bradford: 51.57-103.5µg, 75.39 ± 17.34µg CV 23.0%; BCA: 107.71-222.86µg, 161.58 ± 38.92 CV

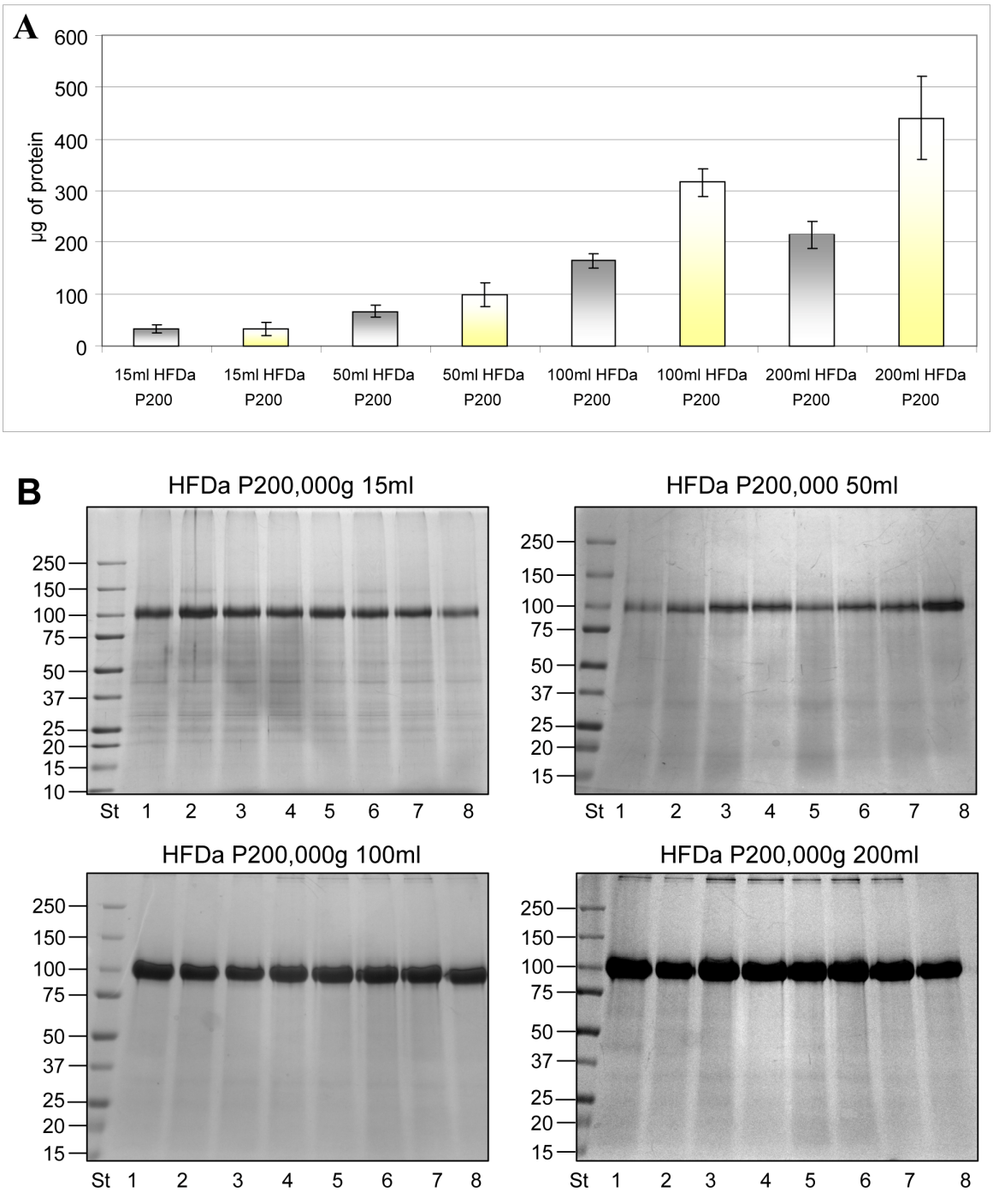
24.1%); 50ml urine (Bradford: 90.00-192.50µg, 138.22 ± 31.51µg CV 22.8%; BCA: 251.00-363.53µg, 371.79 ± 59.93 CV 16.12%); 100ml urine (Bradford: 172.50-363.53µg, 266.02 ± 56.62µg CV 21.3%; BCA: 633.90-894.29µg, 785.10 ± 81.74 CV 10.4%): 200ml urine (Bradford: 406.58-571.25µg, 460.10 ± 62.01µg CV 13.5%; BCA: 1112.20-1770.49µg, 1567.53 ± 194.80 CV 12.4%). (**b**) Colloidal Coomassie protein patterns. Gel loading was based on the Bradford assay 3µg of protein per lane. Each lane represents one HFDa single replica of 8 performed. Molecular weight markers are expressed in kiloDaltons.





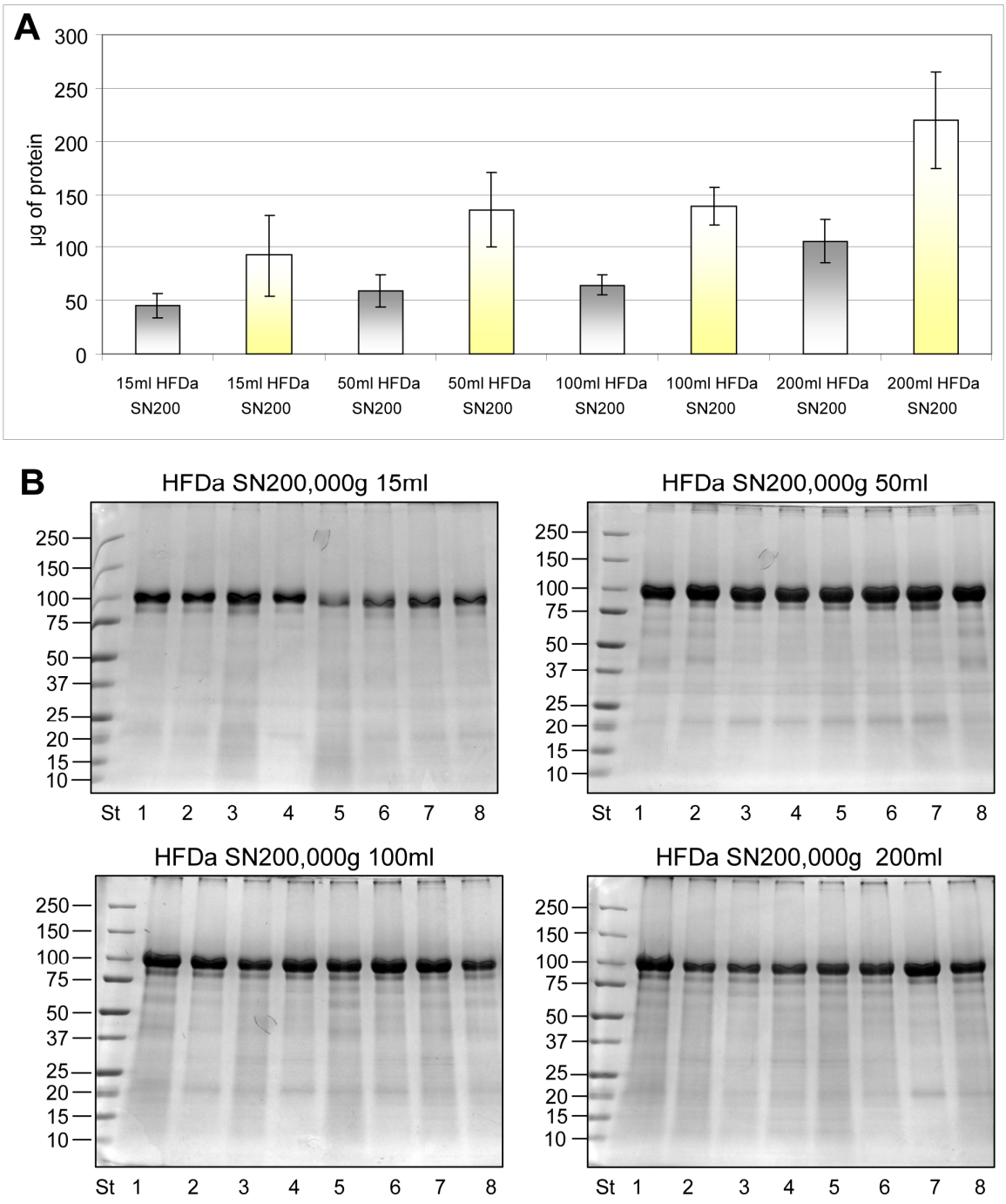
Supplemental Figure 7. Protein recovery and patterns of retained solution above 1,000kDa (HFDa) pellet after 40,000g centrifugation (P40). (**a**) The histogram bars summarise the amount of total protein recovered in the pellet 40,000g obtained from the retained solution above 1,000kDa. Protein concentration was estimate by Bradford (grey bars) and BCA (yellow bars) protein assay. Fifteen ml urine (Bradford: 3.85-11.50µg, 7.41 ±

2.32µg CV 31.3%; BCA: 10.50-27.02µg, 20.36 ± 6.42 CV 31.5%); 50ml urine (Bradford: 50.31-92.69µg, 32.93 ± 8.01µg CV 24.3%; BCA: 44.32-93.21µg, 61.78 ± 10.89 CV 17.6%); 100ml urine (Bradford: 52.59-85.51µg, 65.92 ± 8.66µg CV 13.3%; BCA: 106.40-140.01µg, 124.64 ± 9.81 CV 7.87%): 200ml urine (Bradford: 129.21-195.62µg, 166.49 ± 26.08µg CV 15.67%; BCA: 222.25-339.19µg, 275.98± 44.44 CV 16.1%). (**b**) Colloidal Coomassie protein patterns. Gel loading was based on the Bradford assay 3 µg of protein per lane. Each lane represents one HFDa P40,000g single replica of 8 performed. Molecular weight markers are expressed in kiloDaltons



Supplemental Figure 8. Protein recovery and pattern in retained solution above 1,000kDa (HFDa) pellet after 200,000g centrifugation (P200). (**a**) The histogram bars summarise the amount of total protein recovered in the pellet 200,000g obtained from the retained solution above 1,000kDa after the centrifugation at 40,000g. Protein concentration was estimate by Bradford (grey bars) and BCA (yellow bars) protein assay. Fifteen ml urine

(Bradford: 20.83-51.53µg, 32.74 ± 8.54µg CV 26.1%; BCA: 21.47-62.3µg, 32.86 ± 13.57 CV 41.3%); 50ml urine (Bradford: 51.25-80.56µg, 66.69 ± 11.21µg CV 16.8%; BCA: 70.33-134.67µg, 99.05 ± 23.87 CV 24.1%); 100ml urine (Bradford: 145.28-186.11µg, 165.2 ± 14.03µg CV 8.5%; BCA: 271.17-366.50µg, 316.81 ± 27.47 CV 8.7%): 200ml urine (Bradford: 180.14-257.78µg, 215.30 ± 25.92µg CV 12.0%; BCA: 413.17-530.00µg, 440.29 ± 80.24 CV 18.2%). (**b**) Colloidal Coomassie protein patterns. Gel loading was based on the Bradford assay 3µg of protein per lane. Each lane represents one HFDa P200,000g single replica of 8 performed. Molecular weight markers are expressed in kiloDaltons.



Supplemental Figure 9. Protein recovery and patters in retained solution above 1,000kDa (HFDa) supernatant after 200,000g centrifugation (SN200). (**a**) The histogram bars summarise the amount of total protein recovered in the supernatant 200,000g obtained from the serial centrifugation of the retained solution above 1,000kDa. Protein concentration was estimated by Bradford (grey bars) and BCA (yellow bars) protein assay. Fifteen ml urine (Bradford: 23.79-62.36µg, 44.96 ± 11.04µg CV 23.4%; BCA: 35.70-161.70µg, 92.53 ± 38.01

CV 41.2%); 50ml urine (Bradford: 39.50-83.79µg, 58.99 ± 14.57µg CV 24.7%; BCA: 87.70-173.70µg, 135.58 ± 34.78 CV 25.6%); 100ml urine (Bradford: 53.79-83.07µg, 64.60 ± 9.77µg CV 15.1%; BCA: 107.70-168.70µg, 139.58 ± 17.55 CV 12.6%): 200ml urine (Bradford: 82.36-140.93µg, 105.93 ± 20.69µg CV 19.5%; BCA: 1611.70-308.70µg, 219.56 ± 45.55 CV 20.8%). (**b**) Colloidal Coomassie protein patterns. Gel loading was based on the Bradford assay 3µg of protein per lane. Each lane represents one HFDa SN200,000g single replica of 8 performed. Molecular weight markers are expressed in kiloDaltons.