Enrichment of extracellular matrix from tissue samples

1. Cut tissue onto small pieces (roughly $1mm^3$) in a petri dish on ice using a scalpel blade and place in a 1.5 mL size Eppendorf tube with 1mL PBS and mix end-over-end for 5 minutes at 4°C. Centrifuge at 10,000*g*, 4°C for 2 minutes. Discard the supernatant. Repeat twice, for a total of three washes.
2. If sieving glomeruli from kidney cortex, cut cortex into $1mm^3$ pieces push through species appropriate sieves using a 20ml syringe plunger. Wash fragments through to the lower sieves with PBS. Glomeruli are collected from the lowest sieve and the wash 3 times by resuspending in 20ml sterile PBS and centrifuging at 1000rpm for 5min.
3. Re-suspend the pellets from step 1 or 2 into **buffer 1** (5:1 buffer volume to tissue weight). Aspirate the samples with a 1ml syringe and a 21-gauge needle (green) to homogenise. Incubate the samples end-over-end for 1 hour at 4°C under gentle agitation/rotation.
4. Centrifuge the sample at 14,000*g* for 10 minutes at 4°C. Remove the supernatant and store at -80C. This is **fraction 1**. Measure the protein concentration before adding sample buffer to the sample, using the Direct detect spectrometer, with the buffer used as the blank.
5. Resuspend the pellet in **buffer 2** (5:1 buffer volume to tissue weight). Aspirate the sample with a 1ml syringe and 21-gauge needle (green). Incubate the samples for 1h at 4°C under gentle agitation/rotation.
6. Centrifuge the sample at 14,000*g* for 10 minutes at 4°C. Remove supernatant and store at -80C. This is **fraction 2**. Measure the protein concentration before adding sample buffer to the sample, using the Direct detect spectrometer, with the buffer used as the blank.
7. Resuspend the pellet in 100ul of **buffer 3** (benzonase nuclease buffer to degrade all forms of DNA and RNA) and incubate for 30 mins at room temperature. Then incubate at 65°C for 20 mins.
8. Centrifuge the sample at 14,000*g* for 10 minutes at 4°C. Remove supernatant and store at -80C. This is **fraction 3**. Measure the protein concentration before adding sample buffer to the sample, using the Direct detect spectrometer, with the buffer used as the blank.
9. Wash the pellet with ice cold PBS
10. Centrifuge at 14,000xg for 10 mins at 4°C to remove PBS
11. Resuspend the pellet in **5x sample buffer (buffer 4)**. The volume will depend on the size of the pellet. Usually consider 5-times the volume of the pellet. Aspirate the sample with a 1ml syringe and 21-gauge needle (green). If you are using isolated glomeruli, the final pellet should solubilise completely and this is then **fraction 4**. If you are using the whole tissue, it will be more difficult to completely solubilise the pellet: in this case heat **fraction 4** to 70°C for 20-30 mins to help solubilise the pellet. Any insoluble material for can also be digested directly with trypsin in situ overnight at 37°C.
12. Heat all fractions at 95°C for 10 mins.
13. Protein extracted from whole tissue can be used for validation by western blotting.
14. Store samples at -20°C until preparation for in gel trypsin digestion.

PBS Phosphate buffered saline without calcium and magnesium

Buffer 1: 10 mM Tris pH8.0, 150 mM NaCl, 25 mM EDTA pH8.0, 1% (vol/vol) Triton X-100 and 1x Roche complete protease inhibitor cocktail tablet without EDTA.

Buffer 2: 20mM NH4OH, 0.5%Triton in PBS-. For 5ml 25ul triton X and 11.6ul NH_4OH

Buffer 3: Benzonase nuclease buffer: benzonase nuclease (E1014) 0.4 µl + 2.4 ml PBS

Buffer 4: 5x Sample buffer: 100mM Tris pH6.8, 25% glycerol, 10% SDS, 10% β-mercaptoethanol, 0.1% Bromophenol blue

Cell-derived matrices for mass spectrometry:

Culture cells in dishes for 10-14 days to allow matrix accumulation. Prepare 1x 10 cm plate for each replicate. For collagen IV studies supplement with ascorbic acid (50ug/ml) every 48 hours, to enable collagen fibril formation. This protocol is optimised for human immortalised podocytes and glomerular endothelial cells).

Denuding cells:

1. Place the plates on ice.
2. Wash cells once with cold *PBS- then discard.
3. Tilt the plate for 1 min (on ice) so the PBS accumulates at the bottom of the dish then remove the residual PBS.
4. Gently add pre-warmed Buffer 1. Ensure coverage of cells with 10mls per 10cm plate. Leave for 5 minutes. Cell lysis is instantaneous and cells detach in 2-5 min.
5. Aspirate the buffer and wash twice with PBS+.
6. Digest residual DNA with 10ug/ml (= 1:1000 dilution of 10mg/ml stock) DNAse1 (Roche) in PBS+.
7. Incubate at 37^{o}C for 30 min.
8. Aspirate DNAse and wash twice with PBS+.
9. Aspirate as much of the PBS as possible.
10. For matrix extraction, use 150ul of **buffer 2** (5x sample buffer) per plate. If using multiple plates for the same condition, add 150ul sample buffer to the first plate then scrape, take this sample and put into the second plate and scrape again. Repeat this for the 3rd plate. The aim is to maintain the total extraction volume less than 500ul/condition.
11. Consider adding more bromophenol blue to aid loading of samples.
12. Solubilise matrix at 70^{o}C for 30 min before running SDS PAGE.
13. Store samples at -20^{0}C until preparation for in gel trypsin digestion.

*PBS-/+ : Phosphate buffered saline without (-) or with (+) calcium and magnesium

Buffer 1: (Alkaline detergent buffer): 20 mM NH_4OH and 0.5% Triton X-100 in PBS

Buffer 2: 5x Sample buffer: 100mM Tris pH6.8, 25% glycerol, 10% SDS, 10% β-mercaptoethanol, 0.1% Bromophenol blue

Co-culture matrix extraction

1. Prepare 1x 10 cm plate for each replicate.
2. (Optional for collagen IV studies): Supplement with ascorbic acid (50ug/ml) every 48 hours.
3. Place the plate on ice.
4. Wash cells once with cold *PBS- then discard.
5. Tilt the plate for 1 min (on ice) so the PBS accumulates at the bottom of the dish then remove the residual PBS.
6. Scrape cells and matrix from the plate into 1ml of **buffer 1** to solubilise cellular proteins and incubate for 30 minutes on ice.
7. Centrifuge at 14,000xg for 10 mins at 4°C. Keep supernatant referred as **Fraction 1**. This is the cell lysate part of cellular fraction.
8. Resuspend the resulting pellet in 0.5ml of **buffer 2** for 5 mins. Pipette up and down to break up the pellet.
9. Centrifuge at 14,000xg for 10 mins at 4°C. Keep supernatant referred as **Fraction 2**. This is also the cell lysate.
10. Combine **Fraction 1 and 2** for cellular fraction.
11. **Resuspend the pellet in 100ul of buffer 3 (benzonase nuclease buffer to degrade all forms of DNA and RNA) and incubate for 30 mins at room temperature. Then incubate at 65°C for 20 mins.**
12. **Centrifuge the sample at 14,000*g* for 10 minutes at 4°C. Remove supernatant and store at -80C. This is fraction 3.**
13. Resuspend the pellet in 100ul of in buffer 4 (5x reducing sample buffer). This is **Fraction 4** the ECM enriched fraction.
14. Heat denature the samples at 70°C for 20 mins.
15. Store samples at -20°C until preparation for in gel trypsin digestion.

*PBS-/+ : Phosphate buffered saline with**out (-) or with (+) calcium and magnesium**

Buffer 1 (extraction buffer)10 mM Tris, 150 mM NaCl, 1% Triton X-100, 25mM EDTA, protease inhibitor

Buffer 2: (Alkaline detergent buffer): 20 mM NH_4OH and 0.5% Triton X-100 in PBS

Buffer 3: Benzonase nuclease buffer: benzonase nuclease (E1014) 0.4 µl + 2.4 ml PBS

Buffer 4: 5x Sample buffer: 100mM Tris pH6.8, 25% glycerol, 10% SDS, 10% β-mercaptoethanol, 0.1% Bromophenol blue