Georgia Ward

Eucheumatoid DNA Extraction

Required Consumables:

Liquid nitrogen	Sample homogenisation	150 – 200mL, depending on Dewar size
2 mL Cryovial (Sigma Aldrich V9380-100EA)	Sample homogenisation	1 per extraction
2.8 mm ceramic beads (Qiagen 13114-325)	Sample homogenisation	4 per extraction
Plastic pestle (VWR BELAF199230001)	Sample homogenisation	1 per extraction
EZNA homogeniser columns (VWR HCR003)	Protein elimination	1 per extraction
2 mL Eppendorf tubes	Multiple stages	2 per extraction
1.5 mL Eppendorf tubes	Multiple stages	1 per extraction

Filter tips (P10, P200, P1000) – all stages.

Required Equipment:

0.2 µm sterile filter modules and syringes, or autoclave	Solution sterilisation	Filter tips and tubes do not require autoclaving.
Cryogenic Dewar and ladle/storage canes	Sample homogenisation	Approx. capacity 1L (need to be able to place samples in vapour phase above liquid)
Frozen metal block, capable of holding cryotubes	Sample homogenisation	Placed in ice bath to retain low temperature for sample grinding
Homogeniser (PreCellys, FastPrep or similar)	Sample homogenisation	Capable of 5500rpm or 5m/s speeds – rotor must be compatible with cryovials
2x Thermomixer/oven with sample mixer	Digestion steps	Temperatures 37 ºC – 65 ºC
LEV	Chloroform phase separation	
Refrigerated centrifuge	Multiple stages	Temperature < 4 ºC, rotor compatible with cryotubes and Eppendorf tubes

Required Solutions (see appendix for recipes):

Tris-EDTA (10 mM Tris-HCl, 1mM EDTA), pH 8.0	Sample lysis, pellet resuspension	1 mL per extraction
10% SDS (sodium dodecyl-sulphate)	Sample lysis/enzymatic homogenisation	30 µL per extraction
5M NaCl (sodium chloride)	Phase separation	120 µL per extraction
10% CTAB (hexadecyltrimethyl-ammonium bromide)	Polysaccharide precipitation	96 µL per extraction
70% ethanol (filter-sterilised, or prepared with molecular-grade water) – **STORED AT -20 ºC**	DNA precipitation	250 µL per extraction
Chloroform:isoamyl alcohol (24:1)	Phase separation	2 mL per extraction
2-propanol (isopropanol)	DNA precipitation	250 µL per extraction
Linear polyacrylamide solution (LPA) (Sigma 5-6575)	DNA precipitation	1 µL per extraction
Lysozyme solution (50mg/mL) (Fisher Scientific 10249843)	Bacterial cell lysis	10 µL per extraction
Proteinase K solution (20 mg/mL) (Fisher Scientific AM2546)	Protein digestion	3 µL per extraction

Extraction Protocol

1. Homogenisation (1 – 2 h for 24 samples)

a) Remove samples from preservative (RNALater or ethanol). If tissue sections are larger than $2mm^3$, use a sterile scalpel or razorblade to cut into smaller sections. Transfer samples to empty, labelled cryotubes, and freeze at -80 ºC until ready to proceed with homogenisation (do not leave more than 12 hours)

b) Prepare a dewar with liquid nitrogen. The dewar should be full enough that your samples can sit in your ladle/sample sticks in the vapour phase and **not** in the liquid phase. The Dewar should be used within an LEV in case of accidental spillage

c) Loosen the lids on your sample tubes, and place the samples in the vapour phase within the Dewar for a minimum of 5 minutes. Remove tubes (4 samples at a time) and place in the frozen metal block. With a sterile disposable pestle, carefully grind the tissue into a coarse powder. As the samples thaw, the carrageenan content will cause them to become sticky. If necessary, return tubes to the Dewar to re-freeze and re-grind

d) If you are processing a larger number of samples, keep the tubes on ice after grinding, and freeze at -80 ºC between batches.

SAMPLES CANNOT BE FROZEN OR STORED FOR LONG PERIODS AT ANY STAGE BEYOND THIS – DO NOT CONTINUE UNLESS YOU HAVE TIME TO COMPLETE THE WHOLE PROTOCOL (6 – 7 HOURS)

e) When all samples have been ground, move the cryotubes to a room temperature Eppendorf rack and add 570 µL (room temperature) Tris-EDTA (TE) to each sample, followed by 4x 2.8 mm ceramic beads

f) Transfer samples to a homogeniser, ensuring the machine is not over-filled (max capacity 16 for NHM Precellys), and that tubes are balanced.

Homogenise samples for 1 minute at 5m/s, or 2 bursts of 40 seconds at 5500rpm, depending on the machine

g) Repeat this homogenisation step if intact tissue is still visible. Take care to check tube lids are still intact, and swap if necessary. Do not homogenise more than twice

h) If samples are especially viscous (i.e. cannot be easily inverted or pipetted), add a further 570µL TE, vortex thoroughly, and divide the sample into two tubes.

2. Enzymatic Lysis (1.5 – 2h for 24 samples)

a) Pre-heat a thermomixer or oven to 37 ºC. Add 10 µL lysozyme stock to each sample and mix by inversion. Incubate at 37 ºC for 30 minutes with mixing (approx. 600rpm). If a thermomixer is not available, use a standard (static) heatblock, and invert samples thoroughly regularly – at least every 5 minutes

b) Increase the temperature of the thermomixer to 55 ºC. Add 30 µL 10% SDS to each sample, followed by 3µL Proteinase K. Incubate for at least 1 hour with mixing (if mixing manually, invert samples every 10-15 minutes)

c) Preheat a second oven or thermomixer to 65 ºC, and pre-warm sufficient volumes of 5M NaCl (120 µL/extraction) and 10% CTAB (96µL/extraction) – remember to account for any split samples. **NB – CTAB normally precipitates when stored at low room temperature, so you may need to heat the whole bottle!**

d) After 1 hour, add 120 L pre-warmed NaCl to each tube. Mix well by inversion

e) Add 96 µL pre-warmed CTAB to each tube and mix by inversion. You should see a large precipitate form

f) Incubate samples at 65 ºC for 15 minutes, mixing by inversion halfway through. During this time, pre-warm the chloroform:IAA to room temperature, and pre-cool the refrigerated centrifuge to 4 ºC

g) Prepare a labelled homogeniser column and 2 mL collection tube per sample (2 for each split sample). Carefully transfer samples to the columns, avoiding the precipitate (use a P200 set to approx. 150µL, and pipette slowly)

h) Centrifuge the homogeniser columns at maximum speed for 1 minute

i) Avoiding the particulate matter forming a film at the top of the solution, transfer the liquid to a clean, labelled 2 mL Eppendorf tube.

3. Chloroform:isoamyl alcohol phase separation (1 – 1.5h for 24 samples, plus 2h incubation)

a) In an LEV hood, add 800 µL room temperature chloroform:IAA to each sample, secure the cap of the tube and vortex thoroughly for 5 seconds

b) Centrifuge for 5 minutes at maximum speed in a pre-chilled centrifuge. From this point forward, keep the tubes chilled until the alcohol precipitation stage

c) After centrifugation, remove the tubes to an ice bath. Transfer the top aqueous layer (max. 650 µL) to a clean, labelled 2 mL Eppendorf tube. Add 700 µL chloroform:IAA, and thoroughly vortex for 5 seconds

d) Centrifuge for 5 minutes at max speed in a refrigerated centrifuge

e) After this, the interphase layer should appear minimal and the aqueous phase should be clear. If there is still particulate matter, repeat steps c – d

f) With a P100 or P200 pipette set to 100 µL, transfer the top aqueous layer to a clean, labelled 1.5 mL Eppendorf tube. Record the total volume transferred per sample, and take care not to transfer chloroform or disturb the interphase.

g) Add 1 µL LPA to each sample and vortex briefly

h) Add 0.7 volumes isopropanol to each sample, and mix well by inversion

i) Incubate samples at room temperature for a minimum of 2 hours (do not leave overnight – this increases carry-over of inhibitors)

4. Alcohol Precipitation and Sample Resuspension (1-1.5h for 24 samples)

a) After at least 2 hours incubation, Centrifuge samples at room temperature for 30 minutes at maximum speed. Where total sample volumes are unequal, ensure the rotor is properly balanced

b) Remove the supernatant by pipetting, taking care not to disturb or discard the pellet

c) Wash the pellets with 250 µL ice-cold 70% ethanol (from the freezer). Rotate the tubes carefully so that the ethanol washes all surfaces, and keep tubes on ice until all samples are ready for centrifugation

d) Centrifuge at 4 ºC for 10 minutes at maximum speed

e) Carefully remove the ethanol by pipetting, and air-dry the pellets (take care not to over-dry, and avoid using a heat-block)

f) Add 50 µL TE to each sample to resuspend

g) Gently vortex and then spin down each sample and leave overnight in the fridge at 4 ºC to resuspend.

Appendix: Solution Recipes

Tris-EDTA, pH 8.0 (10 mM Tris-HCl, 1 mM EDTA) – if Sigma product code 93283 is unavailable

1M Tris-HCl, pH 8.0 (121.14 g in 1 L H_2O), filter-sterilised or autoclaved
0.5M EDTA, pH 8.0 (Sigma product code 03690-100ML)

- 1 mL Tris HCl
- 200 µL EDTA
- make up to 100 mL with molecular-grade water (if using dH_2O, filter/autoclave to sterilise)

makes sufficient buffer for 100 samples

10% (w/v) sodium dodecyl-sulphate (SDS)

- 5g SDS
- make up to 50 mL with molecular-grade water (if using dH2O, filter/autoclave to sterilise)

makes sufficient buffer for approx. 1500 samples – aliquot into 2 mL batches to avoid cross-contamination

10% (w/v) hexadecyltrimethylammonium bromide (CTAB)

- 5g CTAB
- make up to 50 mL with molecular-grade water (if using dH2O, filter/autoclave to sterilise)

makes sufficient buffer for approx. 500 samples – aliquot into 15 mL or 5 mL batches to avoid cross-contamination

5M sodium chloride (NaCl)

- 14.61g NaCl
- make up to 50 mL with molecular-grade water (if using dH2O, filter/autoclave to sterilise)

makes sufficient buffer for approx. 400 samples – aliquot into 15 mL or 5 mL batches to avoid cross-contamination

70% Ethanol

- 70 mL ethanol
- 30 mL molecular-grade water (if using dH2O, filter-sterilise (cannot autoclave ethanol)

store at -20 ºC

makes sufficient buffer for approx. 200 samples – aliquot into 15 mL batches to avoid cross-contamination