

Nucleic acid isolation from moss protonemata

1. For all nucleic acid extractions, the following points are important in obtaining good results.

(i) Use protonemal tissue that has been subcultured relatively recently. We routinely isolate nucleic acids from cellophane-grown tissue that is no more than 1-week post-subculture in age. Use of older tissue, or tissue stored at low temperature for long periods results in co-extraction of unidentified crud (probably carbohydrate and phenolics) which is detrimental to subsequent enzyme reactions.

(ii) After harvesting the tissue (typically by scraping off the cellophane) you will have a mass of sloppy wet green material. It is important to remove as much extraneous liquid as possible. We do this by placing the glob of tissue on a sheet of thick filter paper (such as Whatman 3MM chromatography paper), and overlaying with another sheet. Then press down hard, squeezing liquid out of the tissue. Transfer the squeezed tissue to a dry part of the paper, and repeat this twice (*i.e.* three times in all). A lot of the crud is squeezed out of the tissue in this way.

(iii) Freeze the squeeze-dried mat of tissue in liquid nitrogen. This can then be stored for long periods at -80 C, or extracted immediately.

2. DNA extraction

Commercially available Plant DNA extraction kits can be used to isolate DNA from protonemal tissue with little difficulty - we used the "Nucleon Phytopure Plant DNA extraction kit" to isolate genomic DNA for the construction of our *Physcomitrella* genomic library.

However, we normally use a modified CTAB extraction method for DNA isolation, which is (i) reliable (ii) quick and (iii) cheap.

Extraction buffer:

0.1M Tris-Cl, pH 8.0

1.42M NaCl

2.0% CTAB

20mM Na₂EDTA

2% PVP-40

autoclave and store at room temperature.

Immediately prior to use, add 7 μ l β -mercaptoethanol, and 10mg ascorbic acid to 10 ml buffer stock. Prewarm at 65 C.

For small-scale (PCR) isolation

1-5mg tissue can be picked from a spot-inoculum, or from a cellophane culture, with a pair of forceps.

Squeeze-dry this and drop it into a 1.5 ml Eppendorf tube.

Freeze the tissue in liquid N₂ and immediately homogenise to a powder using a glass rod – the powder should not be allowed to thaw. This can be achieved by ensuring that the Eppendorf tube is suspended in liquid N₂ throughout, or is embedded in a container of dry ice.

This powder can be stored indefinitely at -80, so long as it is not thawed. When processing multiple samples, it is convenient to proceed to this stage for all the samples, before then adding extraction buffer.

Add **100 µl** extraction buffer and gently homogenise to produce an even slurry. Add **1 µl 10mg/ml Rnase A** (pre-boiled for 10 minutes to denature any contaminating DNase) and incubate at 65 C for 5 min.

Add **100 µl chloroform-*iso*-amyl alcohol** (24:1) and mix briefly but thoroughly: the aim should be to avoid mixing so violent as to shear high-molecular-weight DNA.

Separate the phases in a microcentrifuge for 10 min.

Transfer the upper phase to a fresh tube and add **70 µl *iso*-propanol**

Mix well and centrifuge immediately for 5 minutes.

Decant the supernatant and wash the pellet with **70% ethanol**. Drain and air-dry the pellet. Dissolve in **15 µl TE** buffer (10mMTris-Cl, pH8 - 1mM Na₂EDTA).

This provides good quality DNA readily amplifiable by PCR.

For large-scale (Southern Blot) isolation

The same basic-procedure is used, but scaled-up.

Harvest one plate of tissue (by scraping protonemata from the cellophane) and squeeze-dry.

Freeze the tissue in liquid N₂ and grind with a small mortar and pestle. (The liquid N₂ treatment facilitates effective rupture of the cells. It is not necessary to use a pre-chilled mortar). Add **1ml extraction buffer** and continue gentle mixing with the pestle to obtain a smooth paste.

Add a further **1ml** extraction buffer, stirring to obtain a uniform homogenate and transfer this to a suitable high-speed centrifuge tube (e.g. a 15ml Corex tube). Wash out residual homogenate into the tube with another **1ml** aliquot of buffer.

Add **30 µl 10mg/ml Rnase A** and incubate at 65 C for 5 min.

Add **3 ml chloroform/iso-amyl alcohol** and vortex to emulsify.

Separate the phases by centrifuging at **10,000rpm** for **10 min.** in a **swingout** rotor (e.g. Sorvall HB-4)

Transfer the upper (aqueous) phase to a fresh tube and precipitate the DNA by adding **2.1 ml iso-propanol**, mixing well and immediately centrifuging at **10,000 rpm** for **5 min** in a **swingout** rotor.

Wash the pellet with **70% ethanol** and air-dry. Dissolve the pellet in **2 x 100 μ l TE**, transferring it to a 1.5ml Eppendorf tube.

Centrifuge this for **2 min** at **12,000 x g** in a microcentrifuge. You may observe a translucent pellet (carbohydrate). Recover the supernatant carefully and transfer it to a clean tube for storage at -20 C.

Typically, this provides sufficient DNA for at least 5 Southern blots.

Digestion of DNA

Although the design of any particular experiment may require the use of a specific restriction enzyme for Southern blot analysis of *Physcomitrella* DNA, it should be noted that some enzymes cleave *Physcomitrella* DNA to a greater extent than others. This probably relates to the extent and distribution of methylation of the moss genome. - This has been discussed by Krogan & Ashton (1999) who demonstrated that enzymes with recognition sites subject to C-methylation at CG and CNG sequences were less effective in digesting *Physcomitrella* DNA. In our hands, *HindIII* routinely yields the most effective digests.

The most heavily methylated sequences in *Physcomitrella* may lie outside the coding sequences. We have noted that in screening a lambda genomic library for numerous *Physcomitrella* genes, that in every case the cloned sequence was located at the end of the inserted genomic fragment. This implies that the sites most accessible to the restriction enzyme used to generate the cloned fragments (a partial digest using *Sau3A*) were undermethylated sites within transcribed regions of the genome.

3. RNA extraction

The isolation of RNA from most biological materials is not difficult. Nevertheless, a body of myth has built up that leads some workers to take precautions that lie on the borders between paranoia and superstition. This includes the segregation of laboratory supplies "for RNA extraction only", the treatment of solutions with diethyl pyrocarbonate and the baking of glassware at temperatures rarely seen outside a thermonuclear explosion. These precautions are largely unnecessary, so long as simple common-sense informs your procedures.

1. There is **always** more Ribonuclease in the tissue you are extracting than there is ever likely to be in the solutions/chemicals/glassware you use.
2. SDS is **very** effective at eliminating any trace contamination by ribonuclease than may occur. – For example, I routinely use the same centrifuge tubes used for DNA isolation (during which procedure they contain a suspension supplemented with RNase A at a final concentration of 100 µg/ml) as I do for RNA extraction. – Thorough washing of the centrifuge tubes by soaking overnight in 0.1% SDS ensures that I have never suffered from RNase degradation of my samples.

Physcomitrella protonemal tissue presents no great difficulties for the extraction of RNA, so long as the general preliminary procedures used in DNA isolation are followed: namely the “squeeze-drying” of the tissue to remove residual liquid, prior to extraction.

There is little endogenous ribonuclease activity, and the tissue is readily amenable to RNA isolation using most commercially available kits. However, I typically use an aqueous-SDS/phenol extraction procedure that has served me well for the past 30 years.

Extraction buffer:

0.1M Tris-HCl, pH9

0.5% SDS

2% PVP-40

5mM Na₂EDTA

Autoclave and store at room temperature.

Immediately prior to use, add 7 µl β-mercaptoethanol to 10ml extraction buffer.

For small scale extractions:

Harvest the tissue by scraping from the cellophane, and squeeze-dry as described for DNA extraction. Tissue equivalent to ca. half a 9 cm plate can be homogenised in a 1.5 ml Eppendorf tube using a glass rod.

Drop the squeeze-dried plug of tissue (ca. 50 mg) into an Eppendorf tube, and freeze the sample by brief immersion in liquid N₂. Grind to a powder. Add 250 µl extraction buffer, and homogenise to produce a slurry.

Add 250 µl phenol-chloroform-iso-amyl alcohol (25:24:1), cap the tube and vortex thoroughly to produce an emulsion.

Centrifuge at **12,000 x g** for **2 minutes** (microcentrifuge), and recover the upper (aqueous) phase, transferring it to a fresh tube.

Add **25 μ l 3M Na-acetate (pH 5.2)** and **600 μ l ethanol**, and mix well. Plunge the tube into liquid nitrogen for a few seconds, then centrifuge at **12,000 X g** for **5 minutes**.

Discard the supernatant and wash the pellet by resuspension in **70% ethanol**, followed by immediate repelleting at **12,000 x g** for **2 minutes**.

Drain the pellet and allow it to air-dry briefly. The pellet contains DNA, RNA and carbohydrate.

Dissolve the pellet in **100 μ l sterile water**, and add **5 μ l 5M NaCl**. Mix well and place **on ice** for **20 minutes**.

Centrifuge at **12,000 x g** for **2 minutes**. This will precipitate the carbohydrate. Transfer the supernatant to a fresh tube for precipitation of the RNA.

High-molecular weight RNA (including mRNA) is recovered by selective precipitation with NaCl. At 2.5M NaCl, DNA and low molecular weight RNA remain in solution, high molecular weight RNA species precipitate.

To the supernatant, add **95 μ l 5M NaCl** and incubate **at 4 C** for at least **4 hr** (Overnight incubation is often more convenient)

To recover the RNA, centrifuge at **12,000 x g** for **10 minutes** at **4 C**. Carefully aspirate the supernatant using a micropipette. Care is required, since the (RNA) pellet can be relatively sloppy, having been centrifuged through a relatively viscous DNA-rich supernatant.

Resuspend the pellet by vortexing with **200 μ l 2.5M NaCl**, and recentrifuge for **5 minutes** at **12,000 x g**. This time, the pellet should pack tightly as residual DNA is washed out.

Discard the supernatant, and wash the pellet with **70% ethanol** by resuspension and repelleting at **12,000 x g** for **5 minutes**. Drain the pellet and allow it to air-dry thoroughly. Finally, the pellet can be dissolved in **30 μ l sterile water** and stored at **-20 C**.

Typically, approximately **30-50 μ g** RNA is obtained by this method. Agarose gel electrophoresis reveals it to be substantially composed of cytoplasmic and chloroplast rRNA species (the latter appearing as a ladder of fragments resulting from the "hidden breaks" within the molecules). Occasionally a trace of residual DNA is apparent in such preparations. If this is a problem, this can be removed by repeating the 2.5M NaCl wash step in the protocol.

Large-scale isolation.

The same basic protocol is followed, but scaled up appropriately.

For 1 or 2 plates of protonemal tissue (7 days post subculture), harvest the tissue and squeeze-dry thoroughly. At this stage, tissue can be placed in “envelopes” made by folding aluminium foil, and frozen in liquid N₂, for storage at -80 C indefinitely.

Use a small mortar and pestle. To aid in homogenisation, a small quantity (ca. 100mg) of glass beads (80-100mesh) or sand can be added to the mortar. – It is recommended to (i) acid-wash and (ii) autoclave this material in advance.

Pre-chill the mortar and pestle by pouring a little liquid N₂ into it, then add the lump of squeeze-dried, frozen tissue and grind to a powder.

As the powder thaws, add **1ml extraction buffer** and homogenise to a smooth slurry. Transfer this to a centrifuge tube (e.g. 15 ml Corex tube). Wash residual material from the mortar and pestle with a further **1ml** extraction buffer, and bulk this with the homogenate.

Add **2 ml phenol-chloroform-iso-amyl alcohol (25:24:1)** and vortex thoroughly, to produce an emulsion.

- Speed is the principal criterion to observe: each sample should take no longer than 2 minutes to process to the emulsion stage.

If multiple samples are being processed, this emulsion can be stored on ice prior to preceeding to the subsequent centrifugation step: I generally do not accumulate more than 4 such emulsions at one time.

Clarify the emulsion by centrifuging in a swingout rotor (e.g. Sorvall HB-4 rotor) at **10,000 rpm x 5 min**.

Transfer the upper (aqueous) phase to a clean centrifuge tube, and add **0.2 ml 3M Na-acetate, pH 5.2** and **4.5ml ethanol**. Mix well and place at -20 C for 30 minutes.

Recover the ethanol-precipitate by centrifugation in the swingout rotor at **10,000 rpm x 5 min**. Discard the supernatant. Typically, you will observe a substantial translucent pellet in the bottom of the centrifuge tube. – This contains a substantial quantity of carbohydrate which must be removed.

Drain the pellets well, and resuspend in **200µl sterile water** by vigorous vortexing. Keep the tube on ice at this stage. This produces a very viscous solution which may still contain lumps of pellet. Using an automatic pipette, remove the liquid and transfer it into an Eppendorf tube, held on ice. Add a further **200µl sterile water** to the centrifuge tube, to recover the remaining pelleted material. After vigorous vortexing, the residual material should completely dissolve. Recover this and add it to the first aliquot in the Eppendorf tube. Vortex this vigorously to ensure the pellet has completely

dissolved. At this stage, you should have a viscous solution with a volume of approximately 500 μ l.

To this, add **25 μ l 5M NaCl**, mix well and incubate on ice for 20 minutes. Centrifuge in a microcentrifuge (**12,000 x g , 5 min**) to pellet the carbohydrate, which forms a substantial, compact gelatinous pellet.

Transfer the supernatant to a clean Eppendorf tube, and add **75 mg NaCl**. Vortex thoroughly to dissolve the NaCl, and incubate overnight at 4 C. – It is often convenient to prepare a series of tubes containing 75mg aliquots of solid NaCl to which the supernatants can be added.

Pellet RNA by centrifugation in a microcentrifuge at **12,000 x g, 10 min, 4 C**. Remove the supernatant and wash the pellet by vigorous resuspension in **300 μ l 2.5M NaCl**. Immediately centrifuge at **12,000 x g, 10 min, 4 C**.

Finally, wash the pellet by vigorous resuspension in **200 μ l 70% ethanol**, and centrifugation in the microcentrifuge. Drain the pellet (carefully – it may not adhere firmly to the wall of the centrifuge tube!!!) and air-dry until all traces of ethanol have evaporated. The pellet can be dissolved in an appropriate volume (ca. 50 μ l) of sterile water for storage at -20 C.

RNA obtained by this procedure can be used directly for Northern blot analysis, or for the subsequent enrichment of poly(A)-containing mRNA, by oligo-dT affinity chromatography.

We routinely use RNA prepared in this way for translation, *in vitro*, for the synthesis of cDNA in the construction of cDNA libraries and the labelling of probes for hybridisation with microarray chips.

Quality control

The yield and integrity of the RNA isolated can be determined by measuring the absorbance at 260nm, and by agarose gel electrophoresis. RNA can be electrophoretically analysed in the same way as DNA, in a 1.4% agarose gel buffered with Tris-borate-EDTA, followed by staining with ethidium bromide.

Typically, you should observe a number of discrete bands that correspond to the principal cytosolic rRNA species and the 16S chloroplast rRNA. You may also observe denaturation products of 23S chloroplast rRNA (Note: this chloroplast rRNA naturally contains “hidden breaks” – specific sites in the chain are cleaved, *in vivo*. The secondary structure of rRNA maintains the integrity of the molecule in the ribosome, but these breaks become apparent upon extraction of the RNA. These smaller bands are therefore NOT indicative of degradation during the extraction process). Degraded RNA typically appears as a background “smear” along the length of the gel. If you observe such a smear, you should determine whether it results from degradation during extraction, or degradation during electrophoresis: the commonest use of agarose minigel tanks is to analyse plasmid DNA

isolated from bacterial cultures. Most plasmid DNA isolation procedures utilise a ribonuclease digestion stage, and it is not uncommon for RNase to contaminate gel tanks and trays. This is one instance where it **is** important to ensure that your equipment is RNase-free, since purified RNA is very susceptible to degradation. Simple washing of the gel tank in 0.1% SDS is sufficient to ensure that RNase degradation during electrophoresis does not occur.

Example of RNA electropherogram:

Each well contains 1/15 of the RNA isolated from a half-plate of protonemal tissue. Note the 5th lane in the top tier of samples. This RNA sample appears degraded relative to the others, with weaker staining of the principal bands, and a diffuse, but obvious, lower molecular weight smear.

