Genomic DNA extraction from freshwater snail tissues

Protocol modified from original description in Winnepenninckx et al. (1993) Trends in Genetics 9(12): 407. DOI: 10.1016/0168-9525(93)90102-n

This protocol (version 2) has been adapted by Kaitlin Bonner (STFU), Stephanie Bollmann (OSU), Coenraad Adema (UNM), Lijun Lu (UNM) and Tom Pennance (WU), and reviewed by other members of the GSA Snail Vectors working group. This protocol is to help researchers working with freshwater gastropods related to the transmission of schistosomes, and particularly those looking at extracting high quality intact DNA for next generation sequencing purposes. This protocol offers a general outline that can be tailored to the contents, purposes and labs that use them, and may require further refinement to fit users' needs. the manufacturers of any products mentioned or referenced should be contacted for more information on products, and other/alternative products may be available.

Why use a CTAB DNA extraction protocol for freshwater snails?

Commercially available kits for the extraction of freshwater snail gDNA, namely non-CTAB based spin-column-based kits (such as the Qiagen Blood & Tissue kits, see below), have many advantages for their speed and ease of use in the lab, however there are also considered to be some downsides. These downsides include an often lower quantity and lower quality of gDNA due to carryover of buffers and/or pigments, or simply pose a problem due to blockages in the column membrane so that samples are difficult to process. We have noted that this can be true for samples that have been stored for long periods of time in ethanol.

For application in next generation sequencing (NGS), researchers may desire unfragmented (i.e. for long read sequencing such as PacBio), high gDNA yields (i.e. for full genome sequencing or multiple applications from same DNA pool) and minimal contamination (to not interfere with library preparation kits in NGS sequencing preparation). The CTAB DNA extraction method detailed here for extracting gDNA

from freshwater snails (tested primarily on *Biomphalaria* and *Bulinus* species) is considered by members of the GSA Snail Vectors working group to provide the preferred protocol (as of May 2024) for obtaining high yields and best possible quality gDNA for downstream applications.

We also would like to highlight the **OMEGA bio-tek E.Z.N.A. Mollusc & Insect DNA kit** (D3373), a commercially available CTAB-based spin column kit which has also shown favourable results for the extraction of snail gDNA for purposes of next generation sequencing (Schols & Huyse, RMCA). This product has recently been reformulated, and as of May 2024, has not been extensively tested by the Snail Vectors WG in comparison to the CTAB phenol-chloroform gDNA extraction protocol detailed below, however may prove to be a great alternative.

For further reading on the difficulties associated with recovery of DNA from molluscs, see: Adema (2021) Sticky problems: extraction of nucleic acids from molluscs. Phil. Trans. R. Soc. B **376:** 2020016. **http://doi.org/10.1098/rstb.2020.0162**

Could a commercial spin-column based kit (non-CTAB) work for me?

Spin-column based DNA purification kits, such as the Qiagen DNeasy Blood and Tissue Kits, have been a favourite for extracting genomic DNA from a wide range of organisms, including gastropods. As previously mentioned, a downside to these kits have been the lower quantity and quality of gDNA that can be achieved from some sample types (such as those stored in ethanol), however gDNA extraction from other sample types may work well in many other circumstances. The advantages of commercial spin-column kits available (for example Qiagen and Zymo branded products) are the speed, ease of use and the absence of harmful chemicals in the process. The Snail Vectors working group suggest that several spin-column based kits and methods can be used effectively, where small amounts of tissue (such as partial head foot) can be taken from fresh tissue, that avoids overloading and blocking spin-columns and avoids to much carryover of inhibitory substances (see Adema 2021). Also, for PCR based applications (or even amplicon panels) where lower quality and quantity of DNA is still suitable, these offer a great option. Note however, that genomic DNA from *Biomphalaria glabrata* snails extracted using Qiagen B&T spin-column kits have produced PacBio assemblies with excellent read length (Bollmann, OSU).

To maximise the performances of the Qiagen DNeasy Blood and Tissue kit using the Animal Tissue protocol for the extraction of gastropod gDNA we suggest:

- Use a smaller amount of starting tissue (e.g. half of a head-foot from average size adult *Biomphalaria* snail measuring shell diameter ~10mm) per column and lyse it overnight at 37C in a heat block / incubator, starting late afternoon. In the morning, vortex and continue incubating at 37C for a further 15 minutes,

then check for how well the tissue is dissolved, then repeat as necessary. As stated in the CTAB protocol (see below), it is not necessary for everything to be fully dissolved. Note, the temperature of lysis incubation is critical – when everything else the same but tissue is incubated for a shorter time at the recommended higher temperature (56C), the visible tissue lysis was good, but the gDNA we extracted was below standard.

- Follow RNase protocol (as described in detail below for CTAB tissue lysis) at this stage and continue incubating a further 15-30 minutes before.
- Proceed with the rest of the steps in the standard protocol as described by the kit. Adjust elution volume to between 110-200 µl to recovery desired final concentration of gDNA.

CTAB DNA extraction protocol from freshwater snail tissues

Safety/PPE

- Use double nitrile gloves (thick) to protect skin against harmful CTAB buffers and other reagents.
- Wear Tyvek disposable lab coats and protect sleeves/wrists.
- Wear safety glasses when handling CTAB buffer.
- Work in fume hood throughout.

Necessary equipment

- Media storage bottles
- 1.5 / 1.7 / 2.0 ml DNA-free Eppendorf's
- 10/20 µl, 200 µl, 1000 µl pipettes and filtered pipette tips
- Refrigerated centrifuge
- Kim wipes
- Heated magnetic stirring plate
- Shaking incubator

Preparing CTAB buffer (250 ml stock solution)

CTAB Buffer: (250ml) components	Final Concentrations
1.9g Tris Base	
1.5g Tris HCl	100mM Tris-HCl, pH 8.0

20.5g NaCl	1.4M NaCl
1.9g EDTA (Na_4-H_2O)	20mM EDTA
5.0g CTAB (cetyltrimethylammonium bromide)	2% CTAB

*Also required: Concentrated hydrochloric acid (HCL)

a. In fume hood - place 200ml distilled/purified H_2O in an appropriately sized media storage bottle. Add magnetic stirrer and place on heated stirring plate (37ºC) at a low stirring speed.
b. Weigh and add one-by-one to dissolve all compounds (listed above) in the stirring 200ml distilled/purified H_2O in fume hood. (solution may heat upon addition of components)
c. Adjust pH to 8.0 (at 37ºC) by adding HCL in drops using glass pipette.
d. Stir on a low speed to dissolve all components [*Note – CTAB doesn't fully dissolve without heat, but it is not completely necessary to have fully dissolved. Stirring overnight on a low speed and heating to 37ºC whilst attended will help dissolving, but follow caution that fumes are toxic and heating may pose an unnecessary risk. Do not leave CTAB solution on a hot plate unattended for safety reasons*].
e. Allow solution to cool to room temperature if necessary whilst stirring on low speed.
f. Check pH of solution and adjust pH to 8.0 again by adding concentrated HCL
g. Label and store final solution of the toxic CTAB buffer appropriately

Protocol for gDNA extraction from freshwater snail

Part A. Preparing sample and lysis

1. **Prepare CTAB buffer for number of samples**. Each sample/tube requires 200 µl CTAB Buffer (stock solution produced following protocol described above), 1µl Proteinase K [20mg/ml] and 0.4 µl BME (2-Mercaptoethanol). Make buffer in fume hood, lightly vortex in hood and distribute between 1.5ml centrifuge tubes labelled with extraction number OR to bead rupting tubes (e.g. OMNI) containing five ~2.8 mm ceramic beads.

2**. Prepare tissue**. Using forceps / scalpel / clean glass slide, cut whole / half / quarter of excised adult head-foot (around ~20mg of tissue) depending on size of snail – if destroying shell to access soft tissue, consider photographing shell shape and aperture first for later reference. If tissue is frozen in liquid nitrogen, use pestle to grind tissue with CTAB buffer in sterile tube. If extracting larger amounts of tissue from whole

snail (e.g. for pathogen diagnostic / detection purposes), if snail is large (e.g. >10 mm diameter for Biomphalaria or height for Bulinus), it may be necessary to split sample between two extraction tubes (A and B) to obtain optimal gDNA extraction quality, and then combine extracted gDNA at the end. If sample is preserved in ethanol, set snail tissue on Kim Wipe to remove excess ethanol briefly, but do not allow for complete drying out of tissue. Chop tissue using sterilized forceps and blade on clean glass slide before adding to CTAB buffer in Eppendorf and vortexing tissue / solution to thoroughly mix. If using ceramic beads and a ruptor to lyse tissue (recommended), add tissue to the prepared 2ml reinforced screw cap tube containing 2.8mm ceramic beads and insert into bead ruptor for 5-15 seconds at speed 6.0 (OMNI). Transfer samples immediately to incubator.

3. **Lyse tissue.** Incubate tissue in shaker at 37°C overnight (or for quicker lysis, 56-60C for 0.5-3hrs). It helps to vortex samples intermittently during the digestion. May be some non-digested tissue after this process; it is fine to proceed if there is tissue undigested as this will be removed following proceeding phase separation. Samples can be stored after lysis at -20°C after the digestion and prior to processing, although the impact on DNA has .

4. RNA removal: After lysing tissue, users can add appropriate amount of RNase (e.g. to final concentration of 100µg/ml – usually maximum recommended concentration) to the lysed tissue and digest further for 30 mins at 37°C, although this may not be absolutely necessary depending on application. 2ul of 10mg/ml RNase = 100ug/ml.

Part B. Phase separation and precipitation

4. **Transfer 1.** To new 1.5/1.7ml tube for each sample, add 300µl Phenol-Chloroform-Isoamyl (PCI) Alcohol (25:24:1) [*alternatively, Chloroform only may work just as well*]. Add lysed tissue / CTAB solution (i.e. ~200 µl tissue) to this new tube. Note, that rations are important, at least the same volume of PCI/Chloroform versus the sample volume are needed to achieve effective concentrations.

5. **Mix 1.** Close cap and invert samples gently for 2 minutes to mix Phenol-Chloroform-Isoamyl Alcohol and tissue / CTAB buffer.

6. **Phase separation 1.** Centrifuge samples at 15,000g for 5 minutes at room temperature (~17-18ºC). Carefully remove tubes from centrifuge (do not mix!) and remove (slow pipetting!) aqueous layer (top only), approximately 180µl, using a 200µl pipette and aspirate it into a new tube. Avoid precipitate and disturbing bottom layer! Dispose of tube containing phenol/chloroform (and same for other containing chloroform steps) appropriately (i.e. store in waste container in fume hood).

TIP: To help achieve maximum yield - tip tube carefully to 45-degree angle while pipetting top layer, to help target the concentrated aqueous layer. Be careful for a 'shift' in layers when top layer gets to a lower volume. If you do get some unavoidable carryover of precipitate, or bottom layer, continue but be sure to remove all during phase separation 2 step.

7. **Transfer 2.** To the ~180µl of aqueous phase from the previous step (in a new 1.7ml tube) add equal volume Chloroform (180-200µl) to this solution.

8. **Mix 2**. Close cap and invert samples gently for 2 minutes to mix aqueous phase and chloroform

9. **Phase separation 2.** Centrifuge samples at 15,000g for 5 minutes (room temp ~17-18ºC). Following same precautions in Phase separation 1, carefully remove tubes from centrifuge (do not mix!) and remove (slow pipetting!) aqueous layer (top only), approximately 150µl, using a 200µl pipette and aspirate it into a new tube.

TIP: avoid ALL precipitate at this stage, no carryover even if volumetric yield pf aqueous layer is lower (e.g.150µl)

After Phase separation 2. complete, change temperature of refrigerated centrifuge to 4ºC to allow time for machine to reach desired temperature.

10. **Transfer 3.** To the ~150µl of aqueous phase from the previous step (in a new 1.7ml tube) add two-thirds the volume of 100% Isopropanol to this e.g. if obtaining 150µl of aqueous phase from previous step – use 100µl of isopropanol. If adding less than 150 µl of aqueous phase from previous step, continue with 100 µl.

11. **Mix 3**. Close cap and invert / flick gently to precipitate DNA

12. **Precipitate gDNA.** Leave sample at room temperature for 30 minutes or can store sample at 4°C for longer*.

* First possible stopping point reached. One can add isopropanol and then store sample at 4°C

Part C. Concentrating, washing, drying and eluting gDNA

13. **Pellet gDNA.** Following precipitation time, centrifuge samples at a maximum speed (e.g. 13,300 - 14,000rpm) for 10 minutes in refrigerated centrifuge set to temperature 4ºC. Remove from centrifuge and carefully pour off supernatant, make sure you do not lose the pellet (white/grey substance) which should be attached rather securely to the inside of your tube!

TIP: Try and keep Eppendorf's in the same orientation (e.g. hinges facing up) for the remainder of the protocol, as this will help keep the gDNA pellet secure.

N.B. Colour of the gDNA pellet can vary considerably depending on the starting tissue used. Colour can range to an almost opaque/white to a grey/black, if heavily pigmented. Heavily pigmented samples may interfere with spectrophotometer readings (see Part D below).

14. **Wash pellet**. Add 250µl ice-cold (from -20ºC freezer) 70% ethanol, close cap and invert gently a few times to rinse the inside of the sample tube and pellet with the 70% ethanol and proceed to next step.

15. **Remove ethanol.** Centrifuge samples at a maximum speed (e.g. 13,300 - 14,000rpm) for 10 minutes in refrigerated centrifuge set to temperature 4ºC. Pour off ethanol supernatant, make sure you do not lose the pellet! Can pipette off the liquid to be safe.

TIP: If obtaining carryover from CTAB/Phenol/Chloroform in your final extracted gDNA (determined by 260/230 values), you may consider repeating step 14 (Wash pellet) and 15 (Remove ethanol) before proceeding to drying and eluting.

16. **Dry pellet.** Dry samples by placing tubes inverted on paper towels for ~15 minutes. If you have access to a vacuum in a fume hood (or speed-vac), remove excess ethanol from inside of tube by attaching a tube and filtered tip, carefully vacuuming ethanol excess from around the inside of the tube through suction, be careful to not disturb pellet. Alternatively, after pouring off ethanol in previous step (15. Remove ethanol), you can spin sample briefly again to concentrate excess ethanol and remove using a pipette before moving to this step (16. Dry pellet).

TIP: Be careful not to 'over dry' pellet by leaving for too long, this can create more difficulty in resuspending gDNA in chosen elution buffer during the next step.

17. **Elute gDNA**. Elute DNA pellets in 50-100µl (or more as desired) of preferred elution buffer (Water / TE / EB etc.).

TIP: If you are planning on using the gDNA for next generation sequencing applications, library preparations can be impacted by components of different elution buffers, so check these protocols to determine best elution buffer. Using sterile water is generally recommended as avoids any downstream issues with kit interference.

TIP: If using template gDNA for end point PCR, dilute template DNA to 1:10 in sterile water for use in PCR to decrease concentration of inhibitors that are often present in snail DNA extracts.

Part D. Quantifying gDNA and checking for signs of contamination / carryover

18. **Check quality and quantity of gDNA.** Measuring absorbance peaks on a spectrophotometer such as a Nanodrop, is recommended to check for DNA purity and quality. From a small piece of head/foot tissue (i.e. ~20mg from a 8mm *Biomphalaria* snail), you might expect 500 – 1000 ng/µl of gDNA if eluted in 50µl of water. The 260/230 should be > 1.8, and 260/280 ideally = 1.8 – 2.0.

Running samples using gel electrophoresis is another convenient way to determine quality of your sample.

TIP: If you are having issues with low 260/230 – sodium acetate clean up (below).

Part E. Ethanol precipitation clean-up (ONLY IF NECESSARY!)

To be performed if excess carry over in final gDNA elution – e.g. if low 260/230.

TIP: This same protocol can be used to clean-up RNA samples.

** To make **3M Sodium acetate** (pH 5.2):

a) Prepare 800 mL of distilled water in a suitable container.
b) Add 246.1 g of Sodium Acetate to the solution on stirrer.
c) Adjust the pH to 5.2 with glacial acetic acid. Allow the solution to cool overnight on magnetic stirrer. Adjust the pH once more to 5.2 with glacial acetic acid.
d) Add distilled water until the volume is 1.
e) Filter-sterilize the solution.

Protocol

1. To eluted gDNA, add:
    a. 0.1 volume of 3M Sodium acetate (pH 5.2) ** to sample (i.e. 5µl in 50 µl gDNA elute)
    b. 2.5-3 times volumes of ice cold 100% ethanol (i.e. 150 µl)
2. Mix well and store for 1 hour at -20C to precipitate DNA (go for longer if gDNA has been stored for a while). Finger flick to mix intermittently.
3. Centrifuge at full speed in refrigerated microcentrifuge at 4ºC for 30 minutes to 1 hour.
4. Remove supernatant (observe DNA pellet, do not lose), pour off.
5. Wash: Add 300ul of ice cold 70% ethanol, centrifuge at full speed in refrigerated microcentrifuge at 4ºC for 10 mins, remove EtOH leaving pellet and repeat step.
6. Remove all ethanol including traces (can centrifuge again briefly / use vacuum to remove trace ethanol)
7. Resuspend in volume of buffer (e.g. TE or AE) or sterile water (NOTE: some buffers can have potential impact on downstream library preparations – i.e. AE

is a TE buffer with half conc of EDTA and therefore higher pH [9.0] vs. TE pH [8.0])