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🌐 CTAB-Beads: a combined protocol for HMW DNA Extraction using the MagAttract HMW DNA kit and the E.Z.N.A. Mollusc & Insect DNA Kit

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We use this protocol and it's working

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Disclaimer

The duration times presented are estimations only.

Abstract

This protocol was developed within the framework of the BGE project (<https://biodiversitygenomics.eu/>) for the extraction of HMW DNA from challenging invertebrate samples characterized by the presence of pigments of unknown chemical nature or by a suspected high content of mucopolysaccharides. Indeed, these compounds frequently co-purify with DNA, remaining tightly associated with it, and can adversely affect downstream PacBio or Oxford Nanopore sequencing.

The protocol integrates a CTAB-based lysis step with a subsequent magnetic bead-based purification. The lysis step follows the Omega Bio-Tek E.Z.N.A. Mollusc & Insect DNA kit protocol, which employs a CTAB-containing buffer and a chloroform–isoamyl alcohol extraction to remove polysaccharides and other inhibitory compounds. The downstream purification step is adapted from the Qiagen MagAttract HMW DNA kit protocol, leveraging its bead-based binding technology to maximize DNA recovery while minimizing mechanical shearing and preserving fragment length.

To enhance sample disruption efficiency, particularly for resistant specimens with a hard outer covering resistant to grind or mechanically disrupt, the pestle motor Diagnocine PowerMasher II is used in combination with dedicated Diagnocine BioMasher II disposable tubes and pestles.

The protocol was validated on flash frozen arthropods (i.e., *Typhloglomeris coeca* – Diplopoda; *Lacinius dentiger* – Opiliones) and on small colonial ascidians processed intact, without removal of the cellulose-containing tunic.

Acronyms:

BGE = Biodiversity Genomics Europe

HMW = High molecular weight

CTAB = cetyltrimethyl ammonium bromide

PWM = Pestle motor Diagnocine Powermasher II

BM tube / BM pestle = 1.5 ml disposable homogenizer BioMasher II, consisting of tube and pestle

EZNA_Moll_Ins= Omega Bio-Tek E.Z.N.A. Mollusc & Insect DNA kit

Materials

- Pestel motor Diagenode Powermasher II (Product ID/SKU: FNK-893002)
- Disposable homogenizer 1.5 ml for Powermasher II: Diagenode BioMasher II, EOG-sterilized (Product ID/SKU: FNK-893062)
- 2 ml and 1.5 ml DNA LoBind Eppendorf tubes (Cat n° 0030108051 and 0030108078)
- Omega BIO-TEK E.Z.N.A. Mollusc & Insect DNA Kit (D3373-01)
- Qiagen MagAttract HMW DNA kit (Cat no. 6756)
- Thermomixer
- Pipettes, standard and wide-bore pipette tips
- Chloroform:isoamyl alcohol (24:1, v/v)
- Vortexer
- Centrifuge
- Magnetic separation rack

Recommended for QC:

- Qubit 4 fluorometer (Cat. no. Q33238) or similar
- Qubit fluorometer 1X dsDNA high sensitivity (Cat. no. Q33230) or 1X dsDNA broad range (Cat. no. Q33266)

Assay Kits for Qubit 4 fluorometer

- QubitTM Assay Tubes (Cat. no. Q32856)
- Nanodrop 1000 spectrophotometer or other UV/VIS measurement
- Agilent FemtoPulse System (Cat. no M5330AA) or Fragment Analyzer System (Cat. no. M5310AA)
- Agilent Genomic DNA 165 kb kit for FemtoPulse System (Cat. no. FP-1002-0275)
- Agilent HS Large Fragment 50kb Kit, 500 for Fragment Analyzer System (Cat. no. DNF-464-0500)

Safety warnings

- ! The operator must wear a lab coat, powder-free nitrile gloves and work in a fume hood to perform the laboratory procedures in this protocol. In addition, chloroform-resistant gloves must be worn, and chloroform waste bottles should be stored in the fume hood.

Waste needs to be collected in a suitable container and disposed of in accordance with local regulations.

Liquid waste needs to be collected in a suitable container and disposed of in accordance with local regulations.

See best practices, thawing and handling instructions, and safety instructions for all the kit reagents, as well as instructions and safety data sheets for other reagents used in this protocol **before starting**.

If the tissue sample is ground in cryogenic conditions, take care to follow appropriate safety measures.



Before start

1. Set a thermomixer for 2 mL tubes to 60 °C
2. Heat Qiagen AE buffer to 55°C
3. Prepare chloroform:isoamyl alcohol (24:1, v/v) solution
4. Pre-chill in dry ice the BM tube and pestle, and any utensils you need for grinding/homogenising the sample



Tissue lysis with Diagnocine PowerMasher II and E.Z.N.A.® Mollusc & Insect DNA Kit

3h 15m

- 1 Working in dry ice, transfer the sample into the sterile pre-chilled 1.5 mL BM tube:

2m

 - If the sample presents an exoskeleton or a hard external structure that makes grinding difficult, perform an initial sample-breaking step using pre-chilled, sterilized scissors or tweezers to break the external structure.
 - Grind the sample using a pre-chilled BM pestle, either manually or with the PWM. Apply short pulses of 2–4 seconds while visually monitoring the level of sample disruption. Position the sample in the gap between the tube wall and the pestle to facilitate efficient grinding.
 - If grinding remains very difficult or insufficient after repeated attempts, proceed to step 2, otherwise, continue to step 3.

Note

The sample quantity varies depending on the species, whether whole organisms or dissected tissues are used, and on the nature of the outer structure. For the analyzed arthropod samples, the weight ranged from 40 to 60 mg.

- 2 Move the tube on normal ice and wait 30 seconds – 1 minute to let the sample warm up.

1m
- 3 Add 350 µL ML1 Buffer and 25 µL Proteinase K Solution.

2m

Note

Caution: If the sample was not fully disrupted in the previous step and does not appear homogeneous, continue to grind the sample in the lysis buffer (ML1 and Proteinase K), either manually or using the PWM instrument, until it becomes homogeneous. Thawing the sample in the lysis buffer will facilitate disruption while simultaneously protecting the sample.

- 4 Move the sample into a 2ml tube with a wide-bore tip.

1m



5 Invert gently 20 times to mix thoroughly

1m

Note

The large round bottom shape of the 2ml tube improves the action of Proteinase K.

6 Incubate at 60°C, 300 rpm, for  03:00:00 , or until the entire sample is dissolved.

3h



Note

Actual incubation time varies and depends on the elasticity of the tissue. Most samples require a minimum of 30 mins, up to 4 hours.

Alternatively, an overnight incubation at 37°C will produce adequate results.

7 Add 350 µL chloroform:isoamyl alcohol (24:1, v/v). Invert gently 20 times to mix thoroughly.

1m



8 Centrifuge 10,000 x g for  00:02:00 at room temperature.

3m



9 Carefully transfer the upper aqueous phase to a clean 2 ml microcentrifuge tube using a wide-bore pipette tip. Avoid the milky interface containing contaminants and inhibitors.

4m

Note

This step will remove much of the polysaccharides and proteins from solution.

Caution: If a small upper aqueous phase is present after centrifugation:

1. Add 200 µL ML1 Buffer and Invert gently 20 times to mix thoroughly.
2. Repeat Step 8 (centrifugation)
3. Repeat Step 9 (transfer the upper aqueous phase).

Purification of HMW gDNA with MagAttract HMW DNA kit

2h

10 Add 4 µL RNase A (100 mg/ml), mix by flicking and incubate for  00:02:00 at room temperature (RT) or use a thermomixer at 25 °C.

3m



- 11 Resuspend MagAttract Suspension G by vortexing at max speed for 00:03:00 .
- 12 Add 150 μ l Buffer AL to the sample. Mix by pipetting up and down with a wide-bore tip because the Buffer AL is very viscous.
- 13 Add 280 μ l Buffer MB to the sample.
- 14 Add 40 μ l of MagAttract Suspension G (pre-vortexed for 3 min) to the sample.
- Note

Ensure that the magnetic particles are fully resuspended by gently tapping, don't invert.
- 15 Incubate the sample in a mixer at room temperature (or in a thermomixer at 25°C) for 00:03:00 at 1400 rpm.
- 16 Without spinning down, place the tube on the magnetic base, wait 2 mins or until bead separation has been completed and remove the supernatant. Avoid disturbing the magnetic bead pellet while aspirating the supernatant. Remove the supernatant completely.
- 17 Remove the tube from the magnetic base, add 700 μ l Buffer MW1 directly on the magnetic bead pellet of the sample and place the tube on the mixer. Incubate at RT for 00:02:00 at 1400 rpm.
- 18 Without spinning down, place the tube on the magnetic base, wait 2 min or until bead separation has been completed, and remove the supernatant.
- 19 Repeat steps 17 and 18 (i.e., Buffer MW1 wash).
- 20 Remove the tube from the magnetic base, add 700 μ l Buffer PE directly onto the magnetic bead pellet of the sample and place the tube on the mixer. Incubate at room temperature for 00:02:00 at 1400 rpm.

1m

2m

1m

1m

3m



3m

3m



3m

5m



3m





21 Without spinning down, place the tube onto the magnetic base, wait 2 minutes or until bead separation has been completed, and remove the supernatant.

3m

22 Repeat steps 20 and 21 (i.e., Buffer PE wash). Remove all the supernatant.

5m

Note

Use a small pipette tip to remove any traces of Buffer PE.



23 Rinse the beads with 700 µl distilled water while the tube is on the magnetic base and the beads are fixed to the wall of the sample tube. Incubate for exactly 1 min at room temperature and remove the supernatant.

2m

Note

Caution: Do not pipet water directly onto the bead pellet – pipet it into the sample tube against the side facing away from the bead pellet. All pipetting steps must be performed carefully to avoid disturbing the fixed bead pellet.

As a precaution, save the supernatant at 4°C until the quality check.

24 Repeat step 23.

2m

25 Remove the tube from its magnetic base and add 50–150 µl of Buffer AE pre-heated at 55°C. Place the tube on the mixer and incubate at room temperature at 1400 rpm for

 00:10:00 .

11m



26 Place the tube on its magnetic base, wait 2 minutes until bead separation has been completed, and transfer the supernatant with the HMW DNA to a new sample tube.

3m

27 Repeat step 25, but incubate at room temperature at 1400 rpm for

 00:30:00 .

31m



28 Repeat step 26. Collect the second eluate in a new tube or use the same tube of the first eluate, according to the expected yield.

3m



29 **Optional:** Perform a third elution in 50 µl of Buffer AE pre-heated at 55°C, incubating at room temperature at 1400 rpm for 10 min to recover any residual DNA bound to the beads.



30 Incubate the eluates at room temperature overnight to improve homogeneity.



31 After overnight incubation and immediately before the quality check, improve the homogeneity of the DNA preparations as follows:

32m



1. Gently pipette mix 5 times using a P200 pipette with a wide-bore tip.
2. Gently pipette mix 5 times using a P200 pipette with a standard tip.
3. Incubate at 37 °C for  00:30:00 .
4. Gently pipette mix 5 times using a P200 pipette with a standard tip

It is recommended that quality control (QC) be performed only after the DNA sample has been incubated at RT overnight. Prior to QC analysis, the sample must appear visually homogeneous and uniform consistency during pipetting

Recommended quality control procedures

2h 20m

32 Quantify DNA concentration using the Qubit fluorometer with either the High Sensitivity or Broad Range dsDNA Assays. Depending on the expected DNA concentration, use 1 µL of undiluted sample or a 1:5 dilution.

10m

33 Assess DNA purity by measuring the A260/A280 and A260/A230 absorbance ratios using a NanoDrop instrument.

10m

- The A260/A280 values should be in the range of 1.8 - 2.0
- The A260/A230 values should be in the range of 2.0 - 2.3

Note

The concentration measured with the NanoDrop should generally be similar to – or only slightly higher than – that measured with the Qubit. NanoDrop concentration values more than twice as high may indicate the presence of contaminants, very high molecular weight or uneven sample concentration

34 Assess the DNA fragment distribution profile using the Agilent Femto Pulse or Fragment Analyzer instruments.

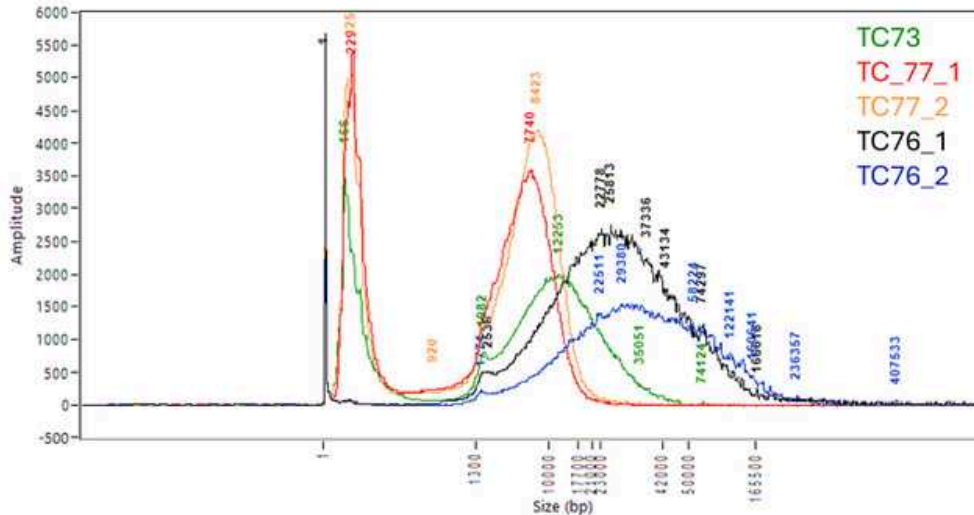
2h

- Use Femto Pulse when most DNA fragments are expected to be >40–50 kb, since it provides high-resolution analysis up to 165 kb.

- Use Fragment Analyzer when most DNA fragments are expected to be <50 kb and longer fragments are present in low amounts.
- Use the *ProSize Data Analysis Software*, available for both Agilent instruments, to perform a smear analysis. This quantifies DNA fragments within a desired size range, calculating total concentration, average size, and percentage of sample within that range. It also generates quality metrics for assessing DNA integrity. These results enable better planning of downstream treatments before proceeding with PacBio or Oxford Nanopore sequencing library preparation

Comparison Between the CTAB-Beads and the EZNA_Moll_Ins Protocols

- 35 DNA was extracted from three flash-frozen specimens of *Typhloglomeris coeca* (Diplopoda), which were obtained and preserved using the same handling procedure. The following protocols were applied:
- the unmodified EZNA_Moll_Ins protocol, performing a single DNA elution step for sample TC73;
 - the unmodified EZNA_Moll_Ins protocol, performing two DNA elution steps for sample TC77 (the first and second elution were designated TC77_1 e TC77_2, respectively);
 - the combined CTAB-Beads protocol described here, performing two DNA elution steps for sample TC76 (the first and second elution were designated TC76_1 e TC76_2, respectively).



The image shows the Femto Pulse fragment size distribution profile using the Agilent Genomic DNA 165 kb kit

Result

- The TC73, TC77_1, and TC77_2 DNA eluates, obtained using the EZNA_Moll_Ins protocol, showed NanoDrop concentrations more than twice those measured with Qubit, indicating the presence of contaminants. The Femto Pulse fragment size

distribution profiles (in green, orange, and red for TC73, TC77_1, and TC77_2, respectively, in the image above) showed two peaks: one at approximately 150–250 bp and the second at approximately 8 and 12 kb, depending on the sample, with no fragments detected above 23–35 kb, depending on the sample.

- The TC76_1 and TC76_2 DNA eluates, obtained using the combined CTAB–beads protocol, showed a Femto Pulse fragment profile with a single peak at 25 or 37 kb, significantly higher than those observed in the TC73, TC77_1, and TC77_2 preparations obtained with the EZNA_Moll_Ins protocol. Fragments up to approximately 150 kb were also detected. TC76_1 and TC76_2 were successfully sequenced using Oxford Nanopore technology after removal of the shortest fragments (< 5–10 kb) using the **"Modified Size Selection Protocol for SRE XS Kit (PacBio)"**.
- For both TC76 and TC77, the second elution showed a slightly higher peak, with a greater abundance of long fragments, compared to the first elution. The DNA obtained from the second elution step appeared to be enriched in longest fragments.

Protocol references

Sources for the creation of this protocol:

- Qiagen MagAttract HMW DNA Handbook 03/2020: manufacturer's manual for the Qiagen MagAttract HMW DNA kit <https://www.qiagen.com/it/products/discovery-and-translational-research/dna-rna-purification/dna-purification/genomic-dna/magattract-hmw-dna-kit-48?catno=67563>
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