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HMW gDNA extraction from pure archaeal cultures for long-read sequencing

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Abstract

This protocol can be used to extract High Molecular Weight gDNA from bacterial and archaeal cultures, resulting in gDNA suitable for long-read sequencing. The original protocol “HMW gDNA extraction from prokaryotic cultures and cryo preservation stocks V.1” (Stöckl, 2023) was optimized for and tested with Thermococcales, Thermotogales, E. coli, and Desulfurococcales, whereas this version was optimized for Ignicoccus but also tested with Staphylothermus and Thermococcales. Cell lysis is based on a combination of enzymatic digestion, chemical disruption using sodium dodecyl sulfate (SDS), and gentle mechanical disruption via freeze-thaw cycles, reducing DNA fragmentation. The protocol includes two paths, one which was optimized for the archaeal clades mentioned above, and one for samples rich in complex polysaccharides and protein contaminants.

Related publication(s): Stöckl, 2023 (Previous version)

Background

Long-read sequencing methods such as nanopore sequencing require high molecular weight DNA for optimal results. Many traditional DNA extraction methods and commercially available kits either fail due to extensive fractionation of the DNA, or fail to produce sufficient yields, especially for archaeal samples.

This protocol represents an evolution of the protocol “HMW gDNA extraction from prokaryotic cultures and cryo preservation stocks V.1” (Stöckl, 2023), adapted for higher yields in Desulfurococcales (especially Ignicioccus species), and with improved RNA depletion. Additionally, more optional steps with recommendations have been added, to enable this protocol to be more adaptive to different samples. Unfortunately, this protocol takes more time than commercially available kits and uses hazardous phenol containing solutions, but it has produced much better results regarding HMW DNA from non-model organism samples.

To accommodate a wider variety of bacterial and archaeal species, this protocol includes some recommendations of parameters that could be tuned, and features two distinct paths:

- Path A is the standard procedure suitable for most samples it was tested with.
- Path B incorporates a Cetyltrimethylammonium bromide (CTAB) step. CTAB is highly effective at removing complex polysaccharides and protein contaminants commonly found in biofilms, soils, and hard-to-lyse environmental samples. However, it adds complexity and can introduce impurities to the final result and is only recommended if Path A results in high polysaccharide contamination of the final sample.

Materials

Product name	Brand	Manufacturer	Catalogue number	Notes
Proteinase K	Invitrogen	Thermo Fisher Scientific (US)	25530049	
RNase A	Monarch	New England BioLabs GmbH (DE)	T3018L	
Buffer EB	Qiagen	Qiagen N.V. (NL)	19086	Final elution
Cetyltrimethylammonium bromide (CTAB)	SERVA	SERVA Electrophoresis GmbH (DE)	16530.04	Only required for “Path B”
Sodium acetate trihydrate	ROTH	Carl Roth GmbH + Co. KG (DE)	3856.1	
Sodium dodecyl sulfate (SDS)	ROTH	Carl Roth GmbH + Co. KG (DE)	CN30.3	
Sodium chloride	ROTH	Carl Roth GmbH + Co. KG (DE)	9265.2	
ROTI Phenol/Chloroform/ Isoamyl alcohol	ROTH	Carl Roth GmbH + Co. KG (DE)	A156.1	
ROTI Chloroform/ Isoamyl alcohol	ROTH	Carl Roth GmbH + Co. KG (DE)	X984.1	
Nuclease-free Water	NEB	New England BioLabs GmbH (DE)	B1500S	Final elution
Ethanol, p.a.				Generic lab grade
Propan-2-ol, p.a.				Generic lab grade

Equipment

Use standard molecular biology lab-ware. Ensure all tubes are certified DNase- and RNase-free. Make sure to adhere to proper personal protection practices when working with solutions containing phenol.

Equipment name	Brand	Manufacturer	Catalogue number	Notes
DNALoBind Tubes, 1.5mL	Eppendorf AG (DE)	Eppendorf AG (DE)	0030108051	HMW DNA can stick to “normal” surfaces more easily, these are supposed to help
Centrifuge 5425 R	Eppendorf AG (DE)	Eppendorf AG (DE)	5406000313	Example for a centrifuge that can cool
NanoDrop One	ThermoScientific (US)	ThermoScientific (US)	ND-ONE-W	Optional for QC
Qubit 4 Flurometer	Invitrogen	ThermoScientific (US)	Q33226	Optional for QC

Protocol

Part 1 – Prepare Cell Pellet

1. Pellet a well-grown liquid culture (usually 1-20 mL) by centrifuging at 15,000 rcf for 15 min and 4°C.
2. Carefully remove the supernatant as much as possible without disturbing the pellet.
3. Resuspend the cell pellet in 50 µL TE buffer.
4. Transfer the suspension to a 1.5 mL reaction tube.

Part 2 - Cell Lysis

1. Add 510 µL TE buffer to your resuspended pellet.
    - OPTIONAL for bacterial samples:

      Only add 490 µL TE buffer, followed by 20 µL freshly prepared lysozyme solution (10 mg/mL, in

TE buffer) and vortex briefly. Incubate 30 min at 37°C. Continue as usual.

2. Add 20 µL proteinase K (20 mg/mL) and 15 µL of 20% (w/v) SDS, then mix thoroughly.
3. Incubate 1–4 h at 56°C with gentle shaking.
    - Note: For some challenging species, increasing incubation time up to 8 h can be beneficial.
4. Freeze the sample either a) at -80°C for 30 min or b) in liquid nitrogen for 2 min. Thaw at 60°C for 10 min using a block heater.
5. Repeat step 5 two additional times (for a total of three freeze-thaw cycles).
    - Note: **Possible stopping point:** Last freezing step at -80°C overnight.
    - Note: From this point forward, **do not vortex** the samples to prevent shearing of the HMW gDNA. Mix by gentle, slow inversion or use wide-bore pipette tips for transfers.

Part 3 – Extraction

Decide your purification path based on sample type. Choose EITHER A or B:

Path A: Standard Extraction (recommended for most)

1. Add NaCl to a final concentration of 0.3 M (38 µL from 5 M stock solution) to improve phase separation.
2. Add one volume phenol:chloroform:isoamyl alcohol (25:24:1) and shake vigorously.
3. Centrifuge 2 min at 16,000 rcf and transfer the top (aqueous) phase to a new tube.
4. Add 5 µL RNAse A (20 mg/mL).
5. Incubate 0.5–1 h at 56°C with gentle shaking.
6. Add an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), centrifuge for 2 min at 16,000 rcf, and transfer the top (aqueous) phase to a new tube.
7. Mix the aqueous phase with an equal volume of chloroform:isoamyl alcohol (24:1), centrifuge as before, and transfer the top (aqueous) phase to a new DNA LoBind tube.
    - Note: A second chloroform:isoamyl alcohol wash can be performed here for extra purity.
8. Proceed to **Part 4: Precipitation and Elution**.

Path B: CTAB Extraction

1. Add 100 µL of 5 M NaCl and mix very well (final concentration ~0.7 M).
    - Note: **This step is crucial**. A CTAB–nucleic acid precipitate will form if the salt concentration drops below approximately 0.5 M at room temperature and the CTAB concentration used.
2. Add 80 µL of freshly prepared CTAB solution (10% CTAB in 0.7 M NaCl).
3. Incubate at 65°C for 30 min.
4. Add one volume of chloroform:isoamyl alcohol (24:1), shake vigorously by inversion, centrifuge 2 min at 16,000 rcf, and transfer the top (aqueous) phase to a new tube.
5. Add 5 µL RNAse A (20 mg/mL).
6. Incubate 0.5–1 h at 56°C with gentle shaking.
7. Mix the aqueous phase with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1), centrifuge

for 2 min at 16,000 rcf, and transfer the top (aqueous) phase to a new tube.

8. Mix the aqueous phase with an equal volume of chloroform:isoamyl alcohol (24:1), centrifuge for 2 min at 16,000 rcf, and transfer the top (aqueous) phase to a new DNA LoBind tube.
    - Note: A second chloroform:isoamyl alcohol wash can be performed here for extra purity.
9. Proceed to **Part 4: Precipitation and Elution**.

Part 4 - Precipitation and Elution

1. Add 0.6 volumes (relative to the transferred aqueous phase) of cold 100% isopropanol + 0.06 volumes of sodium acetate solution (3 M sodium acetate pH 5.2). Invert gently to precipitate the DNA.
2. Incubate at -20°C for > 5 min.
    - Note: **Possible stopping point:** Incubate at -20°C overnight.
3. Centrifuge at 16,000 rcf for 10 min, discard the supernatant, and air dry the pellet briefly.
4. Add 0.6 volumes of cold 70% ethanol (using the same volume as the 100% isopropanol added previously). Let the pellet soak for 1 min at room temperature to dissolve excess salts.
5. Centrifuge at 16,000 rcf for 2 min. Remove the supernatant without disturbing the pellet.
6. Repeat the ethanol wash and centrifugation, then air dry the pellet briefly.
7. Resuspend the pellet in 50 µL of nuclease-free water (preferred option), or Buffer EB (QIAGEN) or similar.
    - Note: **To avoid shearing the HMW DNA, d**o not pipette to mix. Ideally, let the DNA rest at 4°C for several hours or overnight to properly homogenize.
8. Quantify the DNA. See Data analysis section for recommendations.

Data analysis

Accurate measurement of HMW gDNA concentration and purity is critical. In high concentrations, HMW DNA solutions are highly viscous and non-homogenous. Always take the sample for measurement from different parts of the tube. Thorough resting at 4°C prior to measurement (e.g. over night) can help “relax” the HMW DNA and increase its solubility.

Use a DNA-specific fluorescent-based assay such as a Qubit Fluorometer to measure your baseline DNA concentration. The Qubit utilizes fluorescent dyes specific to double-stranded DNA, meaning it should not falsely elevate readings due to RNA, free nucleotides, or other impurities.

Use a spectrophotometric-based assay such as a NanoDrop Spectrophotometer to assess purity. Pure gDNA should exhibit an A260/280 ratio of ~1.8 and an A260/230 ratio of 2.0–2.2. A lower A260/280 indicates protein or phenol contamination, while a low A260/230 indicates salt (e.g., sodium acetate) or polysaccharide carryover. For detailed information on these ratios, we highly recommend the guide by Koetsier and Cantor (2019).

Fragment size is easiest analyzed on a traditional agarose gel (e.g. 0.5%, 50 min, 95 V) with a suitable size standard (e.g. the Thermo Scientific “GeneRuler 1 kb Plus DNA Ladder” #SM1331 has a 20 kb and a 10 kb band; the NEB “Lambda DNA HindIII Digest” #N3012S has a 23 kb and 9.4 kb band), though more detailed insights can be generated with automated electrophoresis solutions such as the Agilent TapeStation or similar.

Recipes

- TE Buffer (10 mM Tris, 1 mM EDTA, pH 8.0)
- 20 % (w/v) SDS
- 5 M NaCl
- 10 % (w/v) CTAB in 0.7 M NaCl; prepare fresh
- 3 M Sodium Acetate pH 5.2
- 70 % (v/v) EtOH; prepare fresh

Additional Notes

- Input: We found the ideal input for maximum yield often varies between taxa. For example, we found a strong increase in yields when input was scaled from 1 mL up to 10 mL of well-grown *Pyrococcus furiosus* cultures (~1E+9 to ~1E+10 cells), but not beyond. For Ignicoccus species, we found the minimum input to be ~1E+10 cells for best yields. Limited testing also suggests that yield is increased when *Pyrococcus furiosus* cells in the stationary phase are used, whereas other taxa gave better results in late-exponential phases.
- Pellet: We found some taxa to require different centrifugation parameters for optimal pellets (e.g. up to 30 min at 15,000 rcf).
- Lysis: This is probably the most critical part of any gDNA Extraction protocol. The approach presented in this protocol is designed to use three different methods (“enzymatic”, “chemical disruption”, “mechanical disruption”) to increase the chances of successfully breaking the cell envelope compared to using only one mechanism, while utilizing a gentle version for each method to reduce fragmentation of the DNA. If the sample allows, removing one or more of these steps might increase yield of very HMW DNA, at the risk of less complete lysis. On the other hand, some samples might require additional or more extreme lysis steps. For example, one may increase Proteinase K concentrations or incubation time, use additional lysis enzymes (e.g. ”MetaPolyzyme” Sigma-Aldrich #MAC4L), use additional or stronger detergents (e.g. Sodium N-Lauroylsarcosine), or use more disruptive mechanical lysis (e.g. more aggressive freeze-thaw cycles or bead beating).
- Prevent Shearing: A common mistake during HMW DNA extraction is over-pipetting. Always pipette slowly in a controlled manner and ideally use wide-bore tips (you can snip the ends off standard tips with sterile scissors) and avoid vortexing the sample, especially after the lysis/freeze-thaw steps.
- Phase Separations: When recovering the aqueous phase after phenol-chloroform centrifugations, it is better to leave a small amount of the aqueous phase behind than to risk aspirating the proteinaceous white

interphase. The addition of NaCl before the phenol-chloroform steps is supposed to improve phase separation.

- "DNA LoBind Tubes": HMW DNA can stick to surfaces more easily than shorter DNA fragments. These specialized tubes are supposed to help, we use them for every step after Part 3: Extraction.
- CTAB: Extractions with CTAB are commonly used in plants, where complex polysaccharides and protein contaminants are found, but these contaminants can also be present in microbial samples. Unfortunately, this step also increases experimental complexity, as CTAB concentration, temperature, and salt concentration can strongly influence what is precipitated with CTAB. For example, at room temperature, nucleic acids co-precipitate with CTAB at salt concentrations below 0.7 M (Murray and Thompson, 1980), which would cause them to be removed during the following steps of the protocol. Also note, that in Path B of this protocol (using CTAB), the first phase separation is done using chloroform:isoamyl alcohol, NOT using phenol:chloroform:isoamyl alcohol, as described by Wilson (2001).
- Precipitation and Elution: Propan-2-ol is more efficient at precipitating HMW DNA, but you may also try Ethanol if other nucleic acids co-precipitate (Green and Sambrook, 2017). Precipitation with room-temperature Propan-2-ol can reduce co-precipitation of salts (Green and Sambrook, 2017). Sodium acetate pH 5.2 is added to aid with precipitation but may be omitted if other nucleic acids co-precipitate.
- Elution: Most downstream applications will be compatible with DNA eluted in nuclease-free water, however some applications recommend TE buffer (e.g. for nanopore sequencing) or similar (e.g. Buffer EB).

Competing interests

The authors declare that they have no conflict of interest.

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