



Modified HMW DNA Isolation from Stramenopiles with Agar Plugs

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Protist Research to Optimize Tools in Genetics (PROT-G)



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ABSTRACT

This protocol is the adaptation of previously published methods for use in isolating intact high molecular weight DNA from stramenopiles for long-read sequencing. The advantages here are minimal amount of centrifugation steps on isolated DNA, as well as the long-term storage of the DNA while in plugs to allow experimental flexibility.

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Adapted from Karas, B. J., B. Molparia, J. Jablanovic, W. J. Hermann, Y.-C. Lin, C. L. Dupont, C. Tagwerker, I. T. Yonemoto, V. N. Noskov, R.-Y. Chuang, A. E. Allen, J. I. Glass, C. A. Hutchison, H. O. Smith, J. C. Venter and P. D. Weyman (2013). "Assembly of eukaryotic algal chromosomes in yeast." *Journal of Biological Engineering* 7(1): 30.



gDNA Agar Plug
Extractions 2018.docx



Karas et al 2013.pdf

PROTOCOL STATUS

Working

We use this protocol in our group and it is working

GUIDELINES

This protocol can be scaled accordingly depending on how much starting material is available. The volumes described below are based on a larger (1.5 g) amount of biomass.

MATERIALS TEXT

Sorbitol
Na₂HPO₄·7H₂O
NaH₂PO₄·1H₂O
Zymolase-T100
EDTA solution, pH 8.0
Tris solution, pH 7.5
Glacial Acetic Acid
Proteinase K
Pronase
Low-melting point agarose
50-well disposable plug mold
Sodium deoxycholate
Sodium dodecyl sulfate
Lysozyme
Beta-mercaptoethanol
Beta-agarase and buffer
Buffered phenol, pH 8.0
Sodium acetate

Bis-Tris-HCl
Table-top centrifuge
Microcentrifuge
50°C incubator
37°C water bath
EtOH
1.5 mL Eppendorf tubes
50 mL conical vial
End-over-end rocker
CaCl₂

Buffers:

1M sorbitol (filter sterilized)

SPEM solution (1 M sorbitol, 10 mM EDTA pH 7.5, Na₂HPO₄·7H₂O (2.08g L⁻¹), NaH₂PO₄·1H₂O (0.32g L⁻¹))

Protoplasting solution (4.56 mL SPEM, 200 µL Zymolase-T100 (50 mg/mL dissolved in H₂O), 200 µL lysozyme (25 mg/mL), 40 µL β-mercaptoethanol)

1x TAE (40 mM Tris, 20 mM acetic acid, 1mM EDTA, sterilized by autoclaving)

Wash buffer (20 mM Tris, 50 mM EDTA, pH 8.0)

Proteinase K solution (100 mM EDTA (pH 8.0), 0.2% sodium deoxycholate, 1% sodium dodecyl sulfate, 1 mg mL⁻¹ Proteinase K)

Pronase solution (50 mM EDTA (pH 8.0), 0.5% sodium dodecyl sulfate, 500 µg mL⁻¹ Pronase)

SAFETY WARNINGS

Low-melting point agar can bubble easily when mixed after heating, so make sure to cover your flask and wear gloves to prevent burns.

Care should be exercised when using phenol for extractions, wear proper PPE and safety glasses and avoid contact with the skin. Be sure to dispose of used phenol after experiment in proper waste containers.

BEFORE STARTING

Try to ensure that your cultures for extraction are as clean as possible to minimize contaminating DNAs from other organisms. Often, we will treat our diatom cultures with antibiotics during culture growth (*Phaeodactylum* is refractory to Kan, Amp and Tet), but this may vary in usefulness depending on your organisms.

The "protoplasting" solution and length of treatment described in the protocol is malleable and may be changed to reflect the nature of your chosen organism. We have also carried out this protocol using Driselease, Chitinase, Lytic Enzymes, and other cell wall treatments before molding the plugs.

- 1 Add 0.5 g of LMP agarose to 25 mL TAE (2% final)
- 2 Microwave for 30 secs or until solution begins to bubble
- 3 Repeat heating steps as necessary (2-3x)
- 4 Move flask to 50°C air incubator, swirling a few times to bring agarose into solution
- 5 Thaw 50 mL conical vial with cell pellets on ice (cell pellet ~1.5g for this expt)

- 6 Add 50 mL 1M sorbitol and resuspend pellet gently
- 7 Let cells sit on ice 1 min
- 8 Harvest pellet with 10 min spin at 3000 x g at 4°C
- 9 Remove sorbitol wash supernatant
- 10 Resuspend cells in 5 mL protoplasting solution
- 11 Incubate cell suspension at 37°C for 10 min, then remove from water bath
- 12 Keeping agarose warm, mix 5 mL of 2% agarose with cell suspension
- 13 Pipette 100 µL of cell-agar solution into disposable plug molds (Biorad #170-3713)
Note: Wide-bore tips can make it easier to dispense agar-cell solution into the molds if regular tips keep clogging
- 14 Place molds at 4°C for 15-30 min to solidify
- 15 Once solid, transfer plugs to new 50 mL conical vial
Note: The plug molds come with a comb for pushing the plugs out into the new conical vial
- 16 Wash plugs with 25 mL of wash buffer (30 min at RT with soft shaking), the tube can be taped on its side on an orbital rocker and mixed slowly
- 17 Add 10-20 mL of proteinase K solution
Note: This enzyme can be expensive, so scale volumes accordingly. You really just need enough to cover the plugs.
- 18 Incubate for 24 hrs at 50°C
Note: At this point you should see cell lysis and your solution change colors. For diatoms, the solution becomes green within 30 minutes, then changes to brown over the 24 hrs.
- 19 Wash plugs 2x with 25 mL of wash buffer (30 min at RT)
- 20 Add 10-20 mL of Pronase solution to plugs
Note: This step can be omitted if you are confident that the Proteinase K treatment is sufficient for your organism, but will not harm your DNA if left in.

- 21 Incubate plugs in Pronase solution for 4 hrs at 37°C
- 22 Wash plugs 2x with 25 mL wash buffer (30 min at RT)
- 23 Store at 4°C or proceed to next step

Note: Plugs can be left in wash buffer after this step for up to 1 month at 4°C
- 24 For agarose digestion, we often process a small amount of the total plugs to first determine DNA yield and often this small number of plugs (~5) can yield enough DNA for a Nanopore run
- 25 To digest, remove 3-4 plugs (or more) from conical vial and transfer into new 1.5 mL Eppendorf tube
- 26 Wash 2x with 500 uL of 1x beta-agarase wash buffer (NEB) on ice for 30 min each cycle

Note: the NEB buffer is composed of 10 mM Bis-Tris-HCl, 1 mM EDTA, pH 6.5@25°C if you want to make it in-house
- 27 Remove excess wash buffer from tube with pipette
- 28 Heat tube at 65°C for 10 min in heating block
- 29 Cool block and solution to 42°C

Note: Leave to cool at least 10 min, as agarase gets inactivated over 45°C
- 30 Add 1U Beta-agarase per 100 uL melted agarose volume
- 31 Incubate for 1-2 hr at 42°C

Note: Tubes can be gently shaken during treatment, but leaving on bench and mixing every 30 min should work as well
- 32 Allow tube to cool to RT
- 33 Store at 4°C overnight or move on to extraction (below)
- 34 Phenol extraction for HMW DNA

Note: Wear proper PPE and work with phenol in a fume hood as much as possible
- 35 Mix 250 uL of liquefied agar solution with 250 uL of Tris-buffered phenol
- 36 Rock end over end slowly for 10 min at RT

Note: Extractions can go for up to an hour at RT if greater DNA yield is desired

37 Separate phases by centrifuging for 10 min at 16,100 x g

38 Transfer aqueous layer (around 250 ul) to new tube

39 Add 25 ul of 3M NaOAc to tube

Note: We've noticed that addition of the 3M NaOAc causes the aqueous layer to become turbid and opaque, so if you see this it's not unusual

40 Invert tube slowly 10 times until mixed

41 Add 825 uL of 100% EtOH

42 Invert tube slowly 10 times until mixed

43 Allow to sit at -20°C for 1 hr minimum, overnight precipitations may increase DNA yield

44 Centrifuge for 20 min at 16,100 x g at RT

Note: make sure to have the centrifuge set to lowest acceleration/deceleration speed possible if you want to avoid shearing

45 Remove supernatant gently with pipet

46 Wash pellet quickly in 500 uL 70% EtOH + 50mM CaCl₂

Note: We have found adding calcium can help precipitate out carbohydrates or lipids often associated with the gDNA

47 Centrifuge for 5 min at 16,100 x g at RT

48 Repeat 70% EtOH + 50 mM CaCl₂ wash

49 Centrifuge for 5 min at 16,100 x g at RT

50 Remove wash solution with pipette

51 Allow pellet to air dry for 5-10 min at RT

Resuspend pellet gently in 50-100 uL TE, pH 8.0 - DO NOT MIX WITH PIPETTE

52

Note: If you have trouble resuspending your pellet, you can either leave it at 4°C overnight or heat the tube to 37°C for about 10 minutes to help solubilize DNA

53

Analyze 5 ul of resuspended pellet on 0.8% agarose gel to determine DNA quality, using wide-bore tips to transfer DNA solution to gel while preventing shearing

Note: We currently use Qubit quantitation for measuring the concentration of HMW DNA



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