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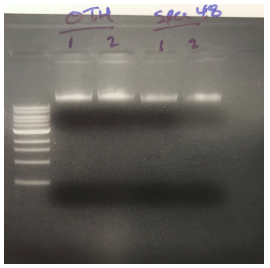
Modified HMW DNA Isolation from Stramenopiles with Agar Plugs



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Protocol status: Working
We use this protocol and it's working

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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.

Attachments

gDNA Agar Plug Extra... 18KB

Karas et al 2013.pdf 1.2MB

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

Sorbitol
Na_2HPO_4-7H20
NaH_2PO_4-1$H_2$0
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^{o}C incubator
37^{o}C water bath
EtOH
1.5 mL Eppendorf tubes
50 mL conical vial
End-over-end rocker
$CaCl_2$

Buffers:
1M sorbitol (filter sterilized)
SPEM solution (1 M sorbitol, 10 mM EDTA pH 7.5, Na_2HPO_4-7H20 (2.08gL^{-1}), NaH_2PO_4-1$H_2$0 (0.32gL^{-1}))
Protoplasting solution (4.56 mL SPEM, 200 uL Zymolase-T100 (50 mg/mL dissolved in $H_2$0), 200 uL lysozyme (25 mg/mL), 40 uL B-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 ug 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 start

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^{O}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^{O}C

9 Remove sorbitol wash supernatant

10 Resuspend cells in 5 mL protoplasting solution

11 Incubate cell suspension at 37^{O}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 uL 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^{O}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^{O}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^{O}C

22 Wash plugs 2x with 25 mL wash buffer (30 min at RT)

23 Store at 4^{O}C or proceed to next step

Note: Plugs can be left in wash buffer after this step for up to 1 month at 4^{O}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^{O}C for 10 min in heating block

29 Cool block and solution to 42$_{o}$C

Note: Leave to cool at least 10 min, as agarase gets inactivated over 45^{O}C

30 Add 1U Beta-agarase per 100 uL melted agarose volume

31 Incubate for 1-2 hr at 42^{O}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^{O}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^{O}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 $CaCl_2$

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 $CaCl_2$ 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

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

Note: If you have trouble resuspending your pellet, you can either leave it at 4^{o}C overnight or heat the tube to 37^{o}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