High quality DNA from Fungi for long read sequencing e.g. PacBio

Benjamin Schwessinger

Abstract

Extraction of high quality DNA for long read sequencing e.g. PacBio

Optimized for DNA extraction from wheat stripe rust spores and also tested on barley leaf rust.

Buffers are best when fresh and not older than 3-6 months. Buffered Phenol:Chloroform:Isoamylalcohol (25:24:1) should not be older than 3 months.

Critical steps to obtain high quality DNA:

- Do NOT heat samples during DNA extractions! Perform all steps at RT or 4oC as indicated.
- Do NOT incubate samples with KAc for prolonged time periods
- Perform two steps of buffered Phenol:Chloroform:Isoamylalcohol purification to reduce co-purifying metabolites.

DNA fragments were well above the 40kb mark based on Pippin Pulse Gels. The sequencing center performed a second AMPure purification step before library construction. Summary statistics of sequencing runs to follow.

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Protocol

Step 1.

Modified from protocols of Prof. Pietro Spanu (Imperial College, London) and T. M.

Fulton, J. Chunwongse, S. D. Tanksley, Pl Mol Biol Rep 13, 207 (1995)

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Reagents required

BUFFER A: 0.35 M sorbitol

0.1 M Tris-HCl, pH 9

5 mM EDTA, pH 8

autoclave to sterilize

BUFFER B: 0.2 M Tris-HCl, pH 9

50 mM EDTA, pH 8

2 M NaCl

2% CTAB

autoclave to sterilize

BUFFER C: 5% Sarkosyl (N-lauroylsarcosine sodium salt SIGMA L5125)

Filter-sterilize

Other solutions:

Potassium Acetate 5M (KAc precipitate polysaccharides) pH 7.5

PVP 1 %

Sodium Acetate (NaAc) 3M pH 5.2

Filter-sterilize

Isopropanol 100%

Ethanol 70%

Buffered Phenol:Chloroforme:Isoamylalcool P:C:I (25:24:1, Sigma P2069)

Autoclave acid washed Sand

Enzymes

RNAse T1 (1000 U/ml, Thermo Fisher EN0541)

Proteinase K (800U/ml, NEB P81072)

AMPure beads from Beckman

Lysis Buffer For 17.5 ml for 500 mg starting material

2.5 volume of Buffer A 6.5 ml

2.5 volume of Buffer B 6.5 ml

1.0 volume of Buffer C 2.6 ml

PVP 0.1 % 1.75 ml

Extraction I:

- Mix buffers lysis buffer by votexing and briefly head to 64oC. Let cool to room temperature for use in 50mL Falcon tubes.

- add 10uL (10kU) RNAse T1to lysis buffer

- Grind tissue/spores with liquid nitrogen in a mortar with sand, use 1g of sand per 100mg of starting material, grind for 2 mins in 4x 15 sec burst adding liquid nitrogen after each 15 sec grinding burst

- transfer powder to 50mL Falcon containing lysis buffer and RNAse, mix well by vortexing

- incubate at RT for 30 mins mixing by inversion every 5 mins

- add 200uL Proteinase K, incubate at RT for 30 mins mixing by inversion every 5 mins

- cool on ice for 5 mins

- add 3.5 mL of KAc 5M, mix by inversion, incubate on ice for max 5 mins

- spin at 4oC and 5000g for 12 mins

- transfer supernatant to fresh Falcon tube containing 18ml P/C/I and mix by inversion for 2 mins

- spin at 4oC and 4000g for 10 mins

- transfer supernatant (might be milky but do not worry) to fresh Falcon tube containing 18ml P/C/I and mix by inversion for 2 mins

- spin at 4oC and 4000g for 10 mins

- transfer supernatant (17mL) to fresh Falcon tube and add 5uL RNAse T1

- incubate for 20-30mins at RT

- add 1.8mL NaAc and mix by inversion

- add 18mL RT isopropanol nad mix by inversion

- incubate at RT for 5-10mins

- spin at 4oC and 10000g for 30 mins

- carefully pipette off supernatant till about 1-2 mL left, DNA will form a mostly translucent to white film/pellet at the bottom of the tube

- use 1mL pipette tip to transfer pellet and remaining liquid into fresh 1.7mL eppendorf tube

- spin in table top centrifuge for 5 mins at 13000g

- remove supernatant with pipette and wash with 1.5mL fresh 70% Ethanol, invert several times to dislodge pellet

- spin in table top centrifuge for 5 mins at 13000g

- remove supernatant with pipette and wash with 1.5mL fresh 70% Ethanol, invert several times to dislodge pellet

- spin in table top centrifuge for 5 mins at 13000g

- remove supernatant with pipette

- spin in table top centrifuge for 1 mins at 13000g

- remove remaining ethanol with pipette

- air-dry pellet for 7 mins

- add 200uL of 10mM Tris pH9 leave at RT for 3 hours

- flick tube slightly for mixing and add 200uL of TE buffer

- leave at RT over night

- next day add another 100uL TE buffer and incubate for 1h at 28oC with 1400rpm shaking

Measure dsDNA concentration using BR Qubit and measure absorbance with Nanodrop.

At this point Qubit to Nanodrop ratios were 1/1000

Extraction II:

- use AMPure beads for secondary clean up at beads 0.45 (Vol/Vol) following the PacBio protocol

- elute in 10mM Tris pH8

- measure dsDNA concentration using BR Qubit and measure absorbance with Nanodrop

- at this stage Qubit to Nanodrop ratios were 0.64, 260/280 1.87 and 260/230 1.37

These samples were submitted for PacBio sequencing using the 20kb library. DNA fragments were well above the 40kb mark based on Pippin Pulse Gels. The sequencing center performed a second AMPure purification step before library construction.