Dec 07, 2017

High quality DNA from Fungi for long read sequencing e.g. PacBio, Nanopore MinION V.4

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

Benjamin Schwessinger and Megan McDonald[1]

[1]Australian National University

3 Works for me dx.doi.org/10.17504/protocols.io.k6qczdw

Solomon Lab - Australian National University | High molecular weight DNA extraction from all kingdoms

view 1 more group

Megan Mcdonald

ABSTRACT

Extraction of high quality DNA for long read sequencing e.g. PacBio and MinIon
Optimized for DNA extraction from Bipolaris sorokiniana. Also tested on Parastaognospora nodorum, Zymoseptoria tritici, wheat stripe rust, barley stripe rust and Pyrenophora tritici-repentis
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.

GUIDELINES

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)

I am gratefull for critical suggestion from the following scientists.
Dr. Claire Anderson, Dr. Andril Gryganskyi, and Dr. David Hayward.

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.

Reagents required
BUFFER A: 0.35 M sorbitol
0.1 M Tris-HCl
5 mM EDTA, pH 8
autoclave to sterilize

BUFFER B: 0.2 M Tris-HCl
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
Polyvinylpyrrolidone (40000 MW) 10 % [w/v] (Sigma PVP40)
Polyvinylpyrrolidone (10000 MW) 10% [w/v] (Sigma PVP10)
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 A or T1 (1000 U/ml, Thermo Fisher EN0541)
Proteinase K (800U/ml, NEB P81072)

AMPure beads from Beckman

Lysis Buffer For 14 ml for 500 mg starting material
2.5 volume of Buffer A 5 mL
2.5 volume of Buffer B 5 mL
1.0 volume of Buffer C 2 mL
PVP 40 10% 1mL
PVP 10 10% 1mL

Extraction I

1 Make lysis buffer by mixing buffer A+B+C+PVP10+PVP40 in 50mL Falcon tubes.
All following steps are based on 14 mL lysis buffer as starting volume.

2 Add 10uL (10kU) RNAse A to lysis buffer

3 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 bursts adding liquid nitrogen after each 15 sec grinding burst.

4 Transfer powder to 50mL Falcon containing lysis buffer and RNAse, mix well by vortexing

5 Incubate at RT for 30 mins mixing by inversion every 5 mins
00:30:00

6 Add 200uL Proteinase K, incubate at RT for 30 mins mixing by inversion every 5 mins
00:30:00

7 Cool on ice for 5 mins
00:05:00

8 Add 2.8 mL (0.2 vol) of KAc 5M, mix by inversion, incubate on ice for max 5 mins
00:05:00

9 Spin at 4°C and 5000g for 12 mins

00:12:00

10 Transfer supernatant to fresh Falcon tube containing 17 mL (~1vol) (P/C/I) and mix by inversion for 2 mins.

Supernatant may/may not have a lipid layer on the top (depends on the fungus), I don't really see a difference if I take this layer or not into the phenol/choroform mix. I try to not take too much of it to make the next steps slightly easier.

00:02:00

11 Spin at 4 °C and 4000g for 10 mins

00:10:00

12 Transfer supernatant (might be milky but do not worry) to fresh Falcon tube containing 17 ml (~1vol) P/C/I and mix by inversion for 2 mins

00:02:00

13 Spin at 4 °C and 4000g for 10 mins

00:10:00

14 Transfer supernatant to fresh 50 mL Falcon tube. If solution remains milky, repeat P/C/I wash. If solution is clear proceed to DNA precipitation.

Usually I am able to transfer ~15 mL of the supernatant.

DNA Precipitation

15 Add 1.5mL (~0.1vol) NaAc and mix by inversion

16 Add 15mL (~1vol) RT isopropanol and mix by inversion

17 Incubate at RT for 5-10mins

00:05:00

18 Spin at 4 °C and 8000g for 30 mins

00:30:00

19 Carefully pour off supernatant.
DNA will form a mostly translucent to white film/pellet at the bottom of the tube (colour may vary depending on your fungus).

20 Wash pellet in 5mL of fresh 70% EtOH. Gently tap tube to dislodge the pellet from the side to get a thorough wash.

Spin 3000-5000g for 3-5 min to stick pellet back to the side of the falcon tube.

21 Pour off EtOH wash untill about 0.5 mL remaining. Gently dislodge pellet with 1mL pipette tip and pour pellet with remaining 70% EtOH into fresh 1.5mL eppendorf tube.

If DNA is not quite pure (as is the case for some fungal DNA extractions), pellet will be brittle and will break into small pieces. Try to recover as much as possible with 1mL pipette.
If the pellet breaks apart too much, add 1.5mL fresh 70% EtOH to the 50mL Faclon and spin for 5min at 4000g.
Remove 1mL and transfer the remaining volume and DNA pellet to same 2mL eppendorf tube.

22 Spin in table top centrifuge for 5 mins at 13000g

00:05:00

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

24 Spin in table top centrifuge for 5 mins at 13000g. Repeat wash steps (21-24).

00:05:00

25 Pour off ethanol and remove remaining ethanol with pipette. Spin down briefly and remove any remaining ethanol with pipette.

26 Air-dry pellet for 2-3 mins

00:02:00

Re-Dissolve HMW DNA

27 Add 200uL (or desired final volume) of 10mM Tris pH8.5 leave at RT until dissolved

03:00:00

28 Flick tube slightly for mixing. DO NOT! vortex as it shears DNA.

Quality Control

29 Measure dsDNA concentration using BR Qubit and measure absorbance with Nanodrop.
This might be also a good step to assess DNA quality by runing a 0.8% TBE agarose gel with 500ng dsDNA and a lamda-Hind-III ladder as control.
If you have a Pulse Field Gel Electrophoresis around even better.

Qubit to Nanodrop ratio varies widely from fungal species to fungal species.
See next step below for comparisons before/after clean up with a silica column

30

Measure DNA concentration with the Qubit and Nanodrop.

The closer your nanodrop measurement is to your Qubit the better.
Typical values after my DNA extractions are:

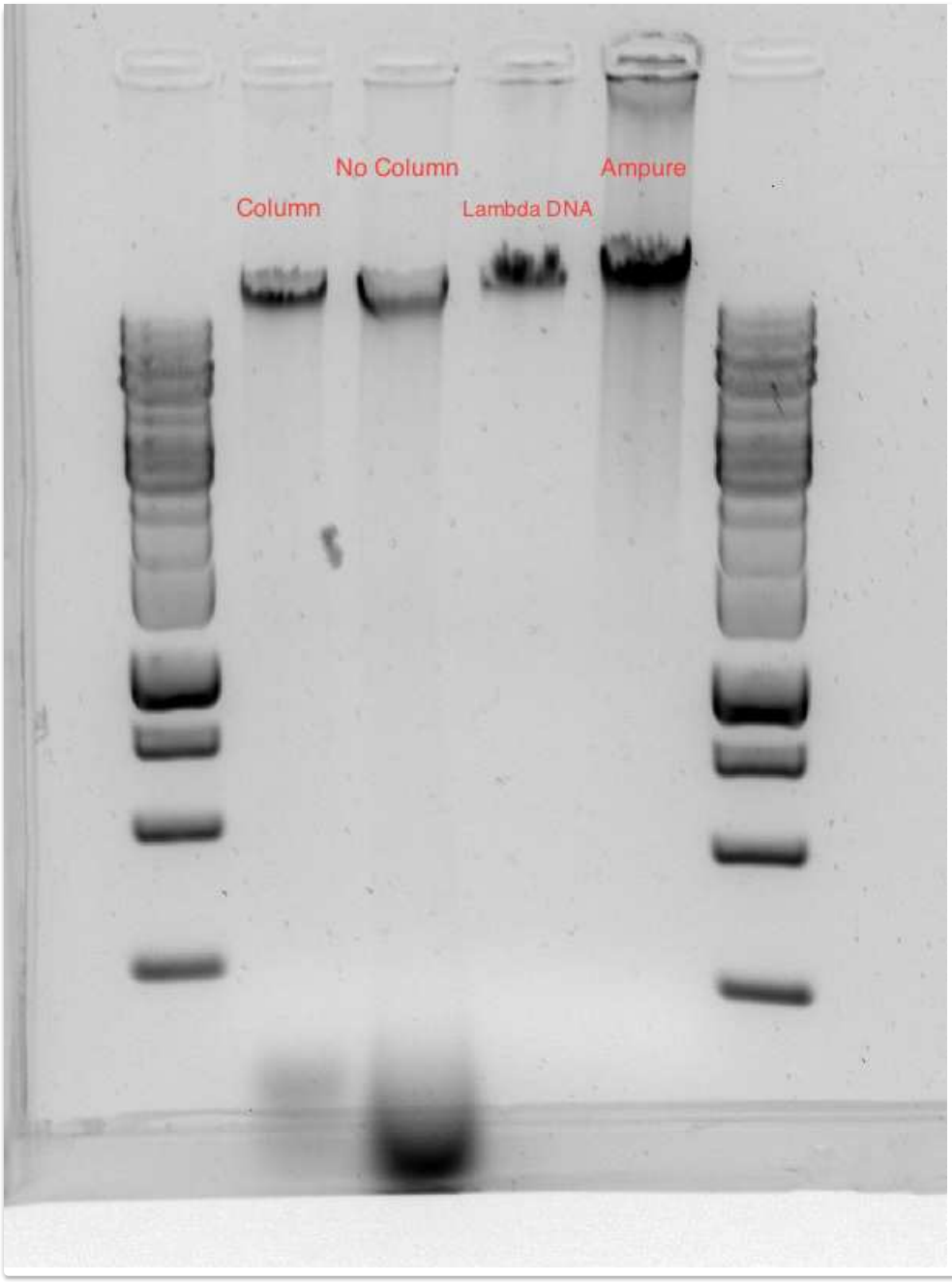
B. sorokiniana CS27

Sample	Volume	Qubit ng/uL	Nano ng/uL	260/230	260/280
CS27	200 uL	65	1389	1.94	1.92
CS27-2	200uL	84	1800	1.92.	1.93

Cleanup to remove small pieces

31 Use AMPure beads (or cheaper versions) for secondary clean up at beads 0.45 (Vol/Vol) following the PacBio protocol. Repeat AMPure bead clean-up at LEAST TWICE before starting MinIon Library Prep.

Run agarose gel to check for remaining RNA, small fragmets or smears. You want your sample to look like Lane 4 (Ampure) where there is NO visible smear on the lower parts of the gel.



I you have low amounts of DNA you should add a higher volume of beads to your sample to retain more DNA. **NOTE:** Adding higher volume will keep a lot more of the smaller fragments.

32 **For most fungal species I've worked with there is a contaminant that co-purifies with the AMPure beads and**

absorbs at 230 on the Nanodrop.
This means that my 260/230 ratios for the Nanodrop are always low (1.0-1.7). The more purification you do with AMPure the worse this ratio gets...haven't found a solution yet. MinIon or PacBio sequencing with a low 260/230 ratio still works well (you get long reads), though your total output data may be less than a sample with a better ratio.
Example of Nanodrop values decreasing with every AMPure clean up:

Sample	Volume	Qubit ng/uL	Nano ng/uL	260/230	260/280
CS27	200 uL	65	1389	1.94	1.92
CS27-amp1	100 uL	114	291	1.74	1.09
CS27-amp2	50 uL	102	305	1.68	0.92

NOTE^3:Ampure beads sometimes don't always collect nicely on the side of the tube. If this happens I remove the supernatant as best I can (SAVE IT!). Then BREIFLY spin the tube to help the beads pellet. (The more you spin the more you will shear your DNA). Remove as much as the supernatant as you can. Then proceed to next step. During 70% EtOH wash with beads, gently rotate the 1.5mL tube 360º on the magnet. The beads will slowly follow as you rotate. This helps get a better wash.

If your final ellution doesn't contain DNA, you can recover it from the SAVED supernate from the very first step. To recover add equal volume of beads to this saved supernatant and proceed with normal protocol.

COMMENTS

Caio Leal-Dutra May 30, 2018
Aberystwyth University
Hi Megan,

Do you have any update in this 230nm contamination issue?

Thanks,
Caio

> **Megan Mcdonald** May 30, 2018
> Hi Caio,
>
> Thanks for your interest. Unfortunately no I haven't really improved upon this issue.
>
> @Benjaminschwessinger and his PhD student Ram have made some improvements I think but I haven't tried out that protocol yet. I just spoke with Ram and he said he needs to update the protocol again, I think it's this one (dx.doi.org/10.17504/protocols.io.n5ydg7w). He said he gets really good 230 ratios but the sequencing data wasn't good from this protocol. He has made some improvements though and will update the site soon. (Probably faster if you ask him a question :))
>
> I can say from my part I'm sequencing with bad 230 ratios and I get on average 3-4Gb of basecalled data that's usable.
>
> Cheers,
> Megan
>
> > **Caio Leal-Dutra** Jun 6, 2018
> > Aberystwyth University
> > Hi Megan,
> > Thank you very much for your reply.
> >
> > I was having this problem with the ampure beads making the 230 ratios worse. But using the homemade ampure. In my cas,e I removed the EDTA from the beads buffer and it worked.
> >
> > But I am still having some problems with the extraction. I will refer to Benjamin's protocol and see if it improves my DNA quality.
> >
> > Kind regards,
> > Caio

Clean Library Ready DNA

33 Elute in 50 uL 10mM Tris pH8, and measure dsDNA concentration using BR Qubit and measure absorbance with Nanodrop

MinION 1D library

34 Stats shown below are for sample **CS27-amp2**
Used 5 ug of starting DNA for 1D Nanopore MinION Library prep.
Remaining DNA after FFPE repair: ~3.75 ug
Remaining DNA after daTailing ~2.7 ug
Remaining DNA after Adapter Lig ~1 ug

35 MinIon Results after 40 hrs