High quality DNA from Fusarium oxysporum conidia suitable for library preparation and long read sequencing with PacBio Version 2

Sukhwinder Kaur, Quyen Anh Pham, and Lynn Epstein

Abstract

This protocol is for isolation of high quality, high molecular weight DNA (20 kb and larger) that is suitable for PacBio library preparation. This protocol has been tested on lyophilized conidia from multiple isolates of *Fusarium oxysporum,* including f. sp. *apii*, f. sp. *ciceris*, and f. sp. *lycopersici*.

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Guidelines

- When pipetting solutions with high molecular weight DNA, use low retention pipet tips with ca. 2 mm of the ends removed, i.e., wide-bore tips.
- Use low DNA-binding microfuge tubes.

Mixing is critical for purification, but avoid vigorous vortexing (which shears DNA)

We thank Martijn Rep, Benjamin Schwessinger, and Oanh Nguyen for advice. This protocol is modified from protocols from Dr. Martijn Rep, University of Amsterdam, and Dr. Benjamin Schwessinger, Australian National University.

Before start

Buffered Phenol Chloroform Isoamyl alcohol (P:C:I) ((25:24:1, saturated with 10 mM Tris, pH 8.0 and 1 mM EDTA) (Sigma P2069) [**Note: use stocks that are less than 3 months old]**

Protocol

Step 1.

Sterilize all dry supplies by autoclaving.

Preparation of conidia for DNA extraction.
Step 2.

Spread a Fusarium oxysporum conidial suspension onto 10 to 20, 100-mm-diam Petri dishes with potato dextrose agar.

NOTES
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The goal is to obtain ca. 0.5 ml packed conidial volume, which yields ca. 250 mg lyophilized conidia.

Preparation of conidia for DNA extraction.
Step 3.

Produce conidia by incubating dishes under fluorescent lights at ca. 25ºC for 6 to 7 days.

Preparation of conidia for DNA extraction.
Step 4.

To harvest the conidia, from each dish, pour 5 ml water on the culture, and gently rub the culture with a metal “hockey stick.”

AMOUNT
5 ml Additional info: water

Preparation of conidia for DNA extraction.
Step 5.

Pour the dislodged conidia through two layers of Miracloth into 50 ml tubes.

Preparation of conidia for DNA extraction.
Step 6.

Repeat the conidial collection from each dish with 5 ml water.

AMOUNT
5 ml Additional info: water

Preparation of conidia for DNA extraction.
Step 7.

Centrifuge the conidia at 4 °C at 3620 g for 15 min.

Preparation of conidia for DNA extraction.
Step 8.

Remove the supernatant (SN), resuspend and re-pellet.

Preparation of conidia for DNA extraction.
Step 9.

Suspend the pellet in 3 to 5 ml water.

Preparation of conidia for DNA extraction.
Step 10.

Transfer the conidial suspensions in 1 ml aliquots, each in a 2 ml microfuge tube.

AMOUNT
1 ml Additional info: aliquots

NOTES
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The goal is ca. 50 mg conidia dry wt/tube.

Preparation of conidia for DNA extraction.
Step 11.

Centrifuge at 3620 g for 15 min.

Preparation of conidia for DNA extraction.
Step 12.

Remove water, freeze the conidial pellet at -70 ºC overnight (ON) and lyophilize it dry.

NOTES
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Lyophilized conidia can be stored at -70 °C before grinding and extraction.

DNA extraction buffer for approx. 250 mg of lyophilized conidia.

Step 13.

Prepare Buffer -5X RNB

1M Tris-HCl pH 8.0

1.25 M NaCl

0.25 M EDTA

Autoclave and store at 4ºC

DNA extraction buffer for approx. 250 mg of lyophilized conidia.

Step 14.

Use 1 ml of freshly prepared extraction buffer with RNase per 50 mg of lyophilized *Fusarium oxysporum* conidia.

Prepare fresh just before extraction.

For 5 ml extraction buffer first, add 4 ml Milli-Q nuclease-free water.

AMOUNT

4 ml Additional info: Milli-Q nuclease-free water

NOTES

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Autoclave and store at 4 ºC.

DNA extraction buffer for approx. 250 mg of lyophilized conidia.

Step 15.

Add 1 ml 5xRNB.

AMOUNT

1 ml Additional info: 5xRNB

DNA extraction buffer for approx. 250 mg of lyophilized conidia.

Step 16.

Add 240 mg PAS (Sodium 4-aminosalicylate dihydrate).

AMOUNT

240 mg Additional info: PAS

DNA extraction buffer for approx. 250 mg of lyophilized conidia.

Step 17.

Mix and then vortex until dissolved.

DNA extraction buffer for approx. 250 mg of lyophilized conidia.

Step 18.

Then add 40 mg TIPS (Triisopropylnaphthalenesulfonic acid sodium).

AMOUNT

40 mg Additional info: TIPS

DNA extraction buffer for approx. 250 mg of lyophilized conidia.

Step 19.

Mix and then vortex until dissolved.

DNA extraction buffer for approx. 250 mg of lyophilized conidia.

Step 20.

Wait until the foam disappears.

DNA extraction buffer for approx. 250 mg of lyophilized conidia.

Step 21.

Add 5µl (250 µg) of RNaseA (10mg/ml stock).

AMOUNT

5 µl Additional info: RNaseA

Conidial grinding.

Step 22.

Cool a mortar (9 cm upper diam) with liquid N_2.

Conidial grinding.

Step 23.

After the N_2 evaporates, add 250 mg sand.

AMOUNT

250 mg Additional info: sand

Conidial grinding.

Step 24.

Add additional liquid N_2 to cool the sand.

Conidial grinding.

Step 25.

Then add 50 mg lyophilized conidia.

AMOUNT

50 mg Additional info: lyophilized conidia

Conidial grinding.

Step 26.

Add 20 to 25 ml liquid N_2. (1/3)

AMOUNT

25 µl Additional info: liquid N2

Conidial grinding.

Step 27.

Grind quickly with force for 15 to 20 sec. (1/3)

Conidial grinding.

Step 28.

Then add more liquid N_2. (2/3)

Conidial grinding.

Step 29.

Grind again. (2/3)

Conidial grinding.

Step 30.

Add more liquid N_2. (3/3)

Conidial grinding.

Step 31.

Grind again. (3/3)

Conidial grinding.

Step 32.

Transfer the ground conidia and sand mixture to a 2 ml microfuge tube (T#1) containing 1 ml of extraction buffer with RNase A.

NOTES

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To optimize your grinding procedure, examine ground lyophilized conidia microscopically (Fig. 1); you need to grind sufficiently so that most of the conidia are cracked open. Any more grinding than that only shears the DNA.]

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We typically prepare 4 tubes, each with 50 mg lyophilized conidia per preparation. The tubes are pooled at the end of the preparation.

DNA extraction and purification.

Step 33.

Label 3 additional tubes (T#2, T#3, & T#4) per 50 mg conidia.

DNA extraction and purification.

Step 34.

Mix the T#1 tube from step 31 on a horizontal low speed (200 rpm) vortex mixer for 2 min.

DNA extraction and purification.

Step 35.

Incubate at room temperature (RT) for 30 min, with mixing by inverting tubes every 5 min.

DNA extraction and purification.

Step 36.

Add 6 µl (120 µg) of proteinase K to T#1.

AMOUNT

6 µl Additional info: proteinase K

DNA extraction and purification.

Step 37.

Mix well on the low speed horizontal vortex mixer for 20 min at RT.

DNA extraction and purification.

Step 38.

Cool tubes on ice for 5 min.

DNA extraction and purification.

Step 39.

Add an equal volume of ice-cold, buffered P:C:I (25:24:1)

DNA extraction and purification.

Step 40.

Mix on the horizontal vortex mixer for 15 sec.

DNA extraction and purification.

Step 41.

Centrifuge at 15000g for 6 min at 4ºC.

NOTES

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The buffer is on top. Only use the lower PCI, here and in the subsequent step.

DNA extraction and purification.

Step 42.

Transfer supernatant (SN) to a new 2 ml microfuge tube (T#2).

DNA extraction and purification.

Step 43.

Add an equal volume of ice-cold, buffered P:C:I.

DNA extraction and purification.

Step 44.

Mix on the horizontal vortex mixer for 2 min at 4ºC.

NOTES

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Always avoid transferring any of the interfaces.

DNA extraction and purification.

Step 45.

Centrifuge at 15000g for 10 min at 4ºC.

DNA extraction and purification.

Step 46.

Transfer SN (Vol = 800 to 900µl) to a new 2 ml tube (T#3).

DNA extraction and purification.

Step 47.

Add an equal volume of ice-cold chloroform:isoamyl alcohol (C:I) (24:1).

DNA extraction and purification.

Step 48.

Mix on the horizontal vortex mixer for 15 sec at 4ºC.

DNA extraction and purification.

Step 49.

Centrifuge at 15000g for 5 min at 4°C.

DNA extraction and purification.

Step 50.

Very carefully, remove the SN without disturbing the interface to a new 2 ml tube (T#4).

DNA extraction and purification.

Step 51.

Add 0.1 volume of ice-cold 4M NaCl to T#4 and mix by inverting the tube several times. DNA strings should be visible.

DNA extraction and purification.

Step 52.

Add 1 volume of ice-cold isopropyl alcohol (IPA).

DNA extraction and purification.

Step 53.

Mix well by inverting tubes gently at least 20 times.

AMpure Bead Cleanup.

Step 54.

1. Notes: all steps are at RT. Prepare fresh 70% EtOH.
2. Bring AMpure beads suspension to RT. Pipet out the beads slowly. Add 0.45X or 0.5X volume of the AMpure beads to the DNA.
3. Mix the beads in the DNA solution thoroughly by flicking the tube. Do not vortex.
4. Collect but do not pellet the beads at 7,000 *g* for 2 to 3 sec.
5. Allow the DNA to bind to the beads by shaking at 200 rpm on a horizontal vortex mixer for 10 min.
6. Incubate the tubes on a rotating wheel at 22 rpm for 20 min.
7. Centrifuge tubes at 7000 g for 10 sec to pellet the beads.
8. Place the tubes in a magnetic rack to hold the pellet on the side of the tube.
9. Slowly pipette off the SN. As a precautionary measure, save the SN in a new tube, but only as a backup–your DNA should be bound to the beads. Avoid disturbing the bead pellet.
10. With the beads in the magnetic rack, add 70% ethanol to the side of tube, i.e., avoid the pellet. Fill the 70% ethanol to the rim of the tube.
11. Pipette off the ethanol and wash again with 70% ethanol as above. Remove all the ethanol without disturbing the bead pellet.
12. Remove tubes from the magnetic rack and centrifuge briefly at 7000 g for 30 sec to collect the residual ethanol. Place tubes on the magnetic rack and remove the ethanol with a fine tip Pipette (P20 or P10).
13. Check for any ethanol droplets. If present, repeat the previous step.
14. Remove the tubes from the magnetic rack. Open the caps. Add the amount of EB for >100ng/ul DNA (by Qubit). Mix the EB and beads gently by flicking until the suspension is homogenous. Centrifuge the tubes briefly at 7000 *g* for 30 sec. Place the tubes in the magnetic rack and let the DNA elute off the beads for 5 to 10 minutes. When all the brown beads are on the side with the magnet, carefully and slowly remove the gDNA into a clean 1.5 ml microfuge tube. Do not pipet out any of the magnetic beads.
15. Quantify DNA concentration and quality on Nanodrop and Qubit. For sufficient quality, with Nanodrop, the 260/280 ratio should be between 1.7 to 1.9 and the 260/230 ratio should be higher than 2.0. The Qubit/Nanodrop concentration ratio should be > 0.7X.

Fragments should be size-selected with Blue Pippen before library preparation for PacBio.

AMpure Bead Cleanup.
Step 55.

Note: all the following steps are at RT.

AMpure Bead Cleanup.
Step 56.

Prepare fresh 70% EtOH.

AMpure Bead Cleanup.
Step 57.
Bring AMpure beads suspension to RT.

AMpure Bead Cleanup.
Step 58.
Pipet out the beads slowly.

AMpure Bead Cleanup.
Step 59.
Add 0.45X or 0.5X volume of the AMpure beads to the DNA.

AMpure Bead Cleanup.
Step 60.

Mix the beads in the DNA solution thoroughly by flicking the tube. **Do not vortex.**

AMpure Bead Cleanup.
Step 61.
Collect but do not pellet the beads at 7,000 g for 2 to 3 sec.

AMpure Bead Cleanup.
Step 62.
Allow the DNA to bind to the beads by shaking at 200 rpm on a horizontal vortex mixer for 10 min.

AMpure Bead Cleanup.
Step 63.
Incubate the tubes on a rotating wheel at 22 rpm for 20 min.

AMpure Bead Cleanup.
Step 64.
Centrifuge tubes at 7000 g for 10 sec to pellet the beads.

AMpure Bead Cleanup.
Step 65.
Place the tubes in a magnetic rack to hold the pellet on the side of the tube.

AMpure Bead Cleanup.
Step 66.
Slowly pipette off the SN.

AMpure Bead Cleanup.
Step 67.
As a precautionary measure, save the SN in a new tube, but only as a backup–your DNA should be bound to the beads. Avoid disturbing the bead pellet.

AMpure Bead Cleanup.

Step 68.

With the beads in the magnetic rack, add 70% ethanol to the side of tube, i.e., avoid the pellet. Fill the 70% ethanol to the rim of the tube.

AMpure Bead Cleanup.

Step 69.

Pipette off the ethanol and wash again with 70% ethanol as above.

AMpure Bead Cleanup.

Step 70.

Remove all the ethanol without disturbing the bead pellet.

AMpure Bead Cleanup.

Step 71.

Remove tubes from the magnetic rack.

AMpure Bead Cleanup.

Step 72.

Centrifuge briefly at 7000 g for 30 sec to collect the residual ethanol.

AMpure Bead Cleanup.

Step 73.

Place tubes on the magnetic rack and remove the ethanol with a fine tip Pipette (P20 or P10).

AMpure Bead Cleanup.

Step 74.

Check for any ethanol droplets. If present, repeat the previous step.

AMpure Bead Cleanup.

Step 75.

Remove the tubes from the magnetic rack.

AMpure Bead Cleanup.

Step 76.

Open the caps.

AMpure Bead Cleanup.

Step 77.

Add the amount of EB for >100ng/µl DNA (by Qubit).

AMpure Bead Cleanup.

Step 78.

Mix the EB and beads gently by flicking until the suspension is homogenous.

AMpure Bead Cleanup.

Step 79.

Centrifuge the tubes briefly at 7000 g for 30 sec.

AMpure Bead Cleanup.

Step 80.

Place the tubes in the magnetic rack and let the DNA elute off the beads for 5 to 10 minutes.

AMpure Bead Cleanup.

Step 81.

When all the brown beads are on the side with the magnet, carefully and slowly remove the gDNA into a clean 1.5 ml microfuge tube. Do not pipet out any of the magnetic beads.