# High quality DNA from Fusarium oxysporum conidia suitable for library preparation and long read sequencing with PacBio Version 4

**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.

ANNOTATIONS

**Lynn Epstein** 02 Aug 2017

**Reagents required**

Buffer -5X RNB

1M Tris-HCl pH 8.0

1.25 M NaCl

0.25 M EDTA

Autoclave and store at 4C

**Other materials**:

PAS-Sodium 4-aminosalicylate dehydrate, (Fluka Catalog # 09415)

TIPS -Triisopropylnaphthalenesulfonic acid sodium (Sigma Catalog # T8324)

Autoclaved, acid-washed sand (or J.T. Baker 3382-05 sand, washed and ignited for boats)

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

Chloroform:Isoamyl alcohol (24:1)

Sterile 4 M NaCl

Isopropanol (IPA) 100%

Ethanol 70% [Note: freshly prepared]

EB (Qiagen, Tris, pH 8.5)

Water refers to sterilized deionized water

AMPure XP magnetic beads (Beckman Coulter)

**Enzymes**

RNase A 10mg/ml, DNase and Protease-free (Thermo Scientific EN0531)

Proteinase K (800U/ml = 20 mg/ml), New England BioLabs P8107S)

Equipment:

A vortexer with low speed (200 rpm) and a holder for horizontal microfuge tubes.

Magna Rack Catalog # 15000 (Invitrogen)

**Jakob Riddle** 06 Feb 2018

It seems that TIPS has been discontinued and is no longer available from Sigma. Can you clarify the role this chemical plays in the extraction buffer or suggest a replacement?

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

**Lynn Epstein** 27 Jun 2017

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.” Repeat conidial collection with an additional 5 ml 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.**

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

Preparation of conidia for DNA extraction.

**Step 7.**

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

Preparation of conidia for DNA extraction.

**Step 8.**

Suspend the pellet in 3 to 5 ml water.

Preparation of conidia for DNA extraction.

**Step 9.**

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

NOTES

**Lynn Epstein** 27 Jun 2017

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

Preparation of conidia for DNA extraction.

**Step 10.**

Centrifuge at 3620 g for 15 min.

Preparation of conidia for DNA extraction.

**Step 11.**

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

NOTES

**Lynn Epstein** 27 Jun 2017

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

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

**Step 12.**

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

Prepare fresh just before extraction

For 5 ml extraction buffer add sequentially:

4 ml Milli-Q nuclease-free water

1 ml 5xRNB

240 mg PAS (Sodium 4-aminosalicylate dihydrate). Mix and then vortex until dissolved.

40 mg TIPS (Triisopropylnaphthalenesulfonic acid sodium) Mix and then vortex until dissolved.

Wait until the foam disappears. Add 5µl (50 µg) of RNase A (10mg/ml stock)

Conidial grinding.

**Step 13.**

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

Conidial grinding.

**Step 14.**

Add additional liquid $N_2$ to cool the sand.

ANNOTATIONS

**Mitchell Roth** 28 Nov 2017

250 mg of sand?

Conidial grinding.

**Step 15.**

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

Conidial grinding.

**Step 16.**

Then add more liquid $N_2$.

Conidial grinding.
**Step 17.**

Grind again.

Conidial grinding.
**Step 18.**

Add more liquid $N_2$.

Conidial grinding.
**Step 19.**

Grind again.

Conidial grinding.
**Step 20.**

Transfer the ground conidia and sand mixture to a 2 ml microfuge tube (T#1) containing 1 ml of extraction buffer with RNase A from step 12.

NOTES
**Lynn Epstein** 27 Jun 2017

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.]

**Lynn Epstein** 27 Jun 2017

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 21.**
Label 3 additional tubes (T#2, T#3, & T#4) per 50 mg conidia.

DNA extraction and purification.
**Step 22.**

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 23.**

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

DNA extraction and purification.

**Step 24.**

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

DNA extraction and purification.

**Step 25.**

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

DNA extraction and purification.

**Step 26.**

Cool tubes on ice for 5 min.

DNA extraction and purification.

**Step 27.**

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

DNA extraction and purification.

**Step 28.**

Mix on the horizontal vortex mixer for 15 sec.

DNA extraction and purification.

**Step 29.**

Centrifuge at 15000g for 6 min at 4ºC.

NOTES

**Lynn Epstein** 27 Jun 2017

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

DNA extraction and purification.

**Step 30.**

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

DNA extraction and purification.

**Step 31.**

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

DNA extraction and purification.

**Step 32.**

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

NOTES

**Lynn Epstein** 27 Jun 2017

Always avoid transferring any of the interfaces.

DNA extraction and purification.

**Step 33.**

Centrifuge at 15000g for 10 min at 4ºC.

DNA extraction and purification.

**Step 34.**

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

DNA extraction and purification.

**Step 35.**

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

DNA extraction and purification.

**Step 36.**

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

DNA extraction and purification.

**Step 37.**

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

DNA extraction and purification.

**Step 38.**

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

DNA extraction and purification.

**Step 39.**

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 40.**

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

DNA extraction and purification.

**Step 41.**

Mix well by inverting tubes gently at least 20 times.

DNA extraction and purification.

**Step 42.**

Incubate on ice for 10 min.

DNA extraction and purification.

**Step 43.**

Centrifuge at 10,000g for 30 min at 4ºC.

**Step 44.**

Discard the SN. Wash the pellet with 1.5 ml ice-cold 70% ethanol. Centrifuge at 13000*g* for 5 min at RT.

**Step 45.**

Discard the SN. Wash the pellet with 1.5 ml ice-cold 70% ethanol. Centrifuge at 13000*g* for 5 min at RT.

Discard SN and centrifuge for 1 min at 13000*g* at RT. Remove residual ethanol with a fine Pippettor tip (e.g. P10 or P20).

**Step 46.**
Air dry the pellet for 15 to 20 min until there is no visible ethanol residue. Add 100 ul of EB (10mM Tris pH 8.5, Qiagen). Incubate at RT overnight. Next morning, incubate at 28 C on a platform orbit shaker at 150 rpm for 1 h.

**Step 47.**
Determine the DNA concentration with a Nanodrop (ND) and by Qubit. The Nanodrop 260/280 ratio should be 1.8-1.9 and the 260/230 ratio should be >2. Based on Qubit, the DNA quantity should be >200ng/ul. At this point, the concentrations of Qubit /ND is often ca. 0.1.

[Note: further cleanup is required until the Qubit/ND ratio $\geq$0.7.]

## AMpure Bead Cleanup

**Step 48.**

Perform an AMpure bead cleanup using either a 0.45X or 0.5X volume of beads as indicated below. Elute in 50 to100 ul EB.

**Step 49.**
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.

**Step 50.**
Mix the beads in the DNA solution thoroughly by flicking the tube. Do not vortex.

**Step 51.**
Collect but do not pellet the beads at 7,000 g for 2 to 3 sec.

**Step 52.**
Allow the DNA to bind to the beads by shaking at 200 rpm on a horizontal vortex mixer for 10 min.

**Step 53.**
Incubate the tubes on a rotating wheel at 22 rpm for 20 min.

**Step 54.**
Centrifuge tubes at 7000 g for 10 sec to pellet the beads.

**Step 55.**
Place the tubes in a magnetic rack to hold the pellet on the side of the tube.

**Step 56.**
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.

**Step 57.**
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.10. Pipette off the ethanol and wash again with 70% ethanol as above. Remove all the ethanol without disturbing the bead pellet.

**Step 58.**
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).

**Step 59.**
Check for any ethanol droplets. If present, repeat the previous step.

**Step 60.**

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.

**Step 61.**
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.

**Step 62.**

Based on Qubit, the DNA concentration should be >100ng/ul.

The size of the DNA should be checked by gel electrophoresis. We typically yield 80 ug DNA with mol wt. >48 kb per 250 mg lyophilized conidia.