Extraction of *C. diff* genomic DNA and Minion Prep

This protocol describes the extraction of genomic DNA from a *C. difficile* culture. This method yields high-quality genomic DNA (Wren and Tabaqchali, 1987).

**Materials**

TE buffer (APPENDIX 2A)

Genomic DNA solution (see recipe)

Fresh lysozyme solution (see recipe)

20% Sarkosyl (see recipe)

10 mg/ml RNase A (see recipe)

10 mg/ml proteinase K (see recipe)

25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol

Chloroform/isoamyl alcohol, 24:1

3 M sodium acetate (see recipe)

75% and 95% ethanol

Phase-lock gel in 2 ml tubes

**Method**

**Inoculate and prepare culture**

1. Inoculate 50 ml BHIS with a colony of C. difficile using sterile techniques and incubate overnight in a 37°C anaerobic chamber.  .

**This step is completed by Elliot.**

1. Harvest by centrifuging 10 min at 4000 × g, 4°C. Discard the supernatant.
2. Resuspend pellet in 1 ml TE buffer and mix by vortexing.  Transfer to eppendorf tube.
3. Centrifuge 5 min at max speed on microcentrifuge. Discard the supernatant.
4. Freeze at -80C if not immediately proceeding to next step.

**Lyse bacteria**

1. Resuspend cell pellet in 200 µl of genomic DNA solution and add 50 µl lysozyme solution (50 mg/ml).
2. Incubate 2 hr at 37°C.
3. Add 100 µl of 20% Sarkosyl and 15 µl RNase A (10 mg/ml) and incubate at 37°C for 1 hr or until the solution clears (up to overnight). The solution should become viscous as the detergent lyses the bacterial cells.
4. Add 15 µl proteinase K (10 mg/ml) and incubate for 30 min at 37°C.
5. Use TE buffer to bring the volume up to 600 µl and transfer to a 2 ml phase-lock gel tube.

**Isolate DNA**

1. Add 600 µl of 25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol and mix by inversion until an emulsion forms. **DO NOT VORTEX**
2. Microcentrifuge 10 min at maximum speed, room temperature.
3. Decant aqueous phase into a new phase-lock gel tube.  Sometimes the gel remains at the top, if this happens break the seal with a pipet tip.
4. Back-extract by adding 600 ul TE to the original phase-lock gel tube.  Mix by inversion until an emulsion forms.
5. Microcentrifuge 10 min at maximum speed, room temperature.
6. Decant aqueous phase into the phase-lock gel tube from step 3.
7. Add 600 µl Chloroform/isoamyl alcohol, mix by inversion, and microcentrifuge 10 min at maximum speed, room temperature. This step will remove traces of phenol.
8. Decant the upper phase into a new clean eppendorf tube.

**Precipitate and wash DNA**

1. Precipitate DNA with 50 µl of 3 M sodium acetate, pH 5.2, and 3 vol of cold 95% ethanol.
2. First option, Spin to pellet DNA:
   1. Centrifuge DNA 5 min at maximum speed, room temperature.
   2. Remove ethanol by decanting in one continuous movement and then blotting rim on a paper towel.
   3. Wash the DNA pellet with 500 µl of 70% ethanol.
   4. Centrifuge DNA 5 min at maximum speed, room temperature.
   5. Remove ethanol by decanting in one continuous movement and then blotting rim on a paper towel.  Let the remaining ethanol evaporate by leaving on a 40°C heat block for 10 minutes.  DO NOT OVER DRY PELLET
3. Second Option, Spool DNA (this is the preferred option)
   1. Make a hook by melting a glass capillary in a blue flame.
   2. Hook out the web-like DNA in one-piece if possible. Lift up and allow the excess liquid to drop off.
   3. Submerge the DNA in an eppendorf tube containing 1 ml 70% ethanol.
   4. It should tighten up into an opaque pellet.
   5. Go back for the rest of the DNA if it broke apart and repeat.
   6. Spin down at max speed, room temperature.
   7. Remove ethanol by decanting in one continuous movement and then blotting rim on a paper towel.  Let the remaining ethanol evaporate by leaving on a 40°C heat block for 10 minutes.  DO NOT OVER DRY PELLET
4. Resuspend the DNA pellet in 50 to 100 µl EB at 4°C overnight.
5. Store DNA at 4°C.  **Do not freeze.**

**DNA QC**

1. Take 1 ul using a cut-off P10 pipette tip and dilute 1:10 in EB.
2. Quantify on the Qubit and NanoDrop.

The concentration of total DNA is evaluated using a spectrophotometer at 260 nm (1 OD at 260 nm = 50 ng/µl DNA). Pure preparations of DNA have a A260/280 ratio of 1.8 to 2. Clean DNA will usually have similar Qubit and NanoDrop readings.  If the extraction is not pure, repeat from Step 1 of Isolate DNA. For larger volumes of *C. difficile* culture, scale up volumes accordingly.

**QC flow cell and update software on minION host computer**

1. Open MinKNOW from the desktop icon and follow the on-screen instructions to complete the automatic updates.
2. Open Metrichor Desktop Agent from the desktop icon and follow the on-screen instructions to complete the automatic updates.
3. Assemble MinION with the flow cell you will use for your run. The pins on the bottom of the flow cell should be closest to the hinge of the minION device.
4. Plug in minION device into USB port on the computer. You will see a light on the device and hear a fan.
5. Open MinKNOW from the desktop icon and establish a local connection.
6. Enter the SampleID for the run and FlowcellID (found on the white sticker on top of the flow cell). Click “Submit” in the Label Experiment dialogue box.
7. Select the Platform QC script under Choose Operation.
8. Run platform QC on the flow cell by clicking the “Execute” button at the bottom of the page. This is used to assess the number of nanopores that are available. This will change between uses. Actives pores are reported in four groups (“mux”).
9. Once Platform QC is complete, the software will return to the Connection page. To see the active pore report, check Notifications (the bell icon on the top right of the page). Record number of pores available in each group in “working\_platform\_QC\_log.xlsx” in the microbiome\_lab shared drive (ressmb01.research.chop.edu\projects\nanopore\flow\_cell\_QC).

**Library preparation with RAD002**

1. Pipette 15 µl DNA into a 0.2 ml PCR tube using a cut-off P20 pipette tip.  Retain the tip for later.
2. Add 5 µl FRM (if using a Rapid kit, e.g. SQK-RBK001, this mix includes the barcode and tubes are labeled RB01, RB02,...RB11, etc.).
3. Using a P20 set to 12 µl and the tip from before mix up and down as slowly as possible 10 times, retain the tip.  Take care not to produce bubbles as they are hard to remove.
4. Incubate at 30°C for 1 minute followed by 75°C for 1 minute on a thermocycler.
5. If multiplexing samples, quantify the now barcoded libraries by nanodrop and pool in the desired ratios in a total volume of 20 µl.
6. Add 1 µl RAD.
7. Add 1 µl Blunt/TA ligase.
8. Using a P20 set to 14 µl and the tip from before mix up and down as slowly as possible 10 times.
9. Incubate at room temperature for 30 minutes.
10. During incubation, move on to **Prime flow cell**.

**Prime flow cell**

1. Add 480 µl RBF to a new Eppendorf tube.
2. Add 520 µl nuclease-free water.
3. Vortex briefly and spin down.  This is the prime mix.
4. Using a P1000 remove a little storage buffer from the inlet port using the volume adjustment screw.
5. Load 800 µl prime mix via the inlet port slowly using the plunger.
6. Wait 5 minutes.
7. Lift the SpotON cover.
8. Load remaining 200 µl prime mix via the inlet port slowly using the plunger, try to dispense at a speed where a bead of liquid becomes visible over the SpotON port which then gets siphoned back in.

**Load library**

1. Add 25.5 µl RBF to a new Eppendorf tube.
2. Add 27.5 µl nuclease-free water.
3. Mix well by vortexing.
4. Using a P100 pipette set to 53 µl add the contents of the Eppendorf to the library tube.
5. Using a P100 set to 75 µl with a cut-off tip mix up and down as slowly as possible 5 times.
6. Slowly pipette the diluted library onto the SpotON port as it gets siphoned in, this can take much longer than usual due to the viscosity. If it gets blocked, use the volume adjustment screw to push a little air into the inlet port then back it out again

**Recipes**

**TE**

Make a 1X solution by adding 500 ul stock to 49.5 ml lab water.

**Genomic DNA solution**

Dissolve 17.12 g of sucrose in TE buffer to a final volume of 50 ml. Filter sterilize using a 0.45-µm filter and store indefinitely at room temperature.

**Lysozyme solution**

Dissolve 50 mg of lysozyme per 1 ml water. Prepare fresh for each use.

**Proteinase K, 10 mg/ml**

Dissolve 10 mg of proteinase K per 1 ml of water. Store for 6 months at −20°C.

**RNase A, 10 mg/ml**

Dissolve 10 mg of RNase A per 1 ml of water. Store for 1 year at −20°C.

**Sarkosyl, 20% (w/v)**

Dissolve 2 g of sarkosyl (N-lauroylsarcosine) per 10 ml of water. Store for 1 year at room temperature.

**Sodium acetate, 3 M (pH 5.2)**

Dissolve 24.6 g of sodium acetate per 100 ml of water. Adjust pH to 5.2 with acetic acid. Store indefinitely at room temperature.