

Extraction and purification from low-biomass rocks
Brazelton Lab
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Provenance: adapted from the Schrenk method for sulfides, the Brazelton 2010 method for carbonates (which was modified from Barton 2006), and informed by Lever et al. (2015) *Frontiers in Microbiology* doi: 10.3389/fmicb.2015.00476.

The primary author of this protocol is Shahrzad Motamedi, with contributions from August Longino and Mac Pierce. Steps involving the Aurora system are based on the Aurora manual (Boreal Genomics).

Sample preparation:

- Rock hammer
- Flame Sterilized pestle and mortar
- Whirlpack sterile sample collection bags
- 2mL sterile tubes
- Single use sterilized plastic spatula
- 50mL Falcon tubes

Lysis:

- Filter-sterilized and boiled DNA Extraction Buffer (DEB)
- Filter-sterilized and boiled 20% sodium pyrophosphate solution
- ThermoMixer at 54°C and 75°C, speed of 600 and 1500 rpm
- 2mL sterile tubes
- MiniSpin 5,000g (rcf)

Bead beating (for the samples with higher DNA concentration)

- 2mL sterile tubes
- Bead beater

Washing sample with VivaSpin column (100,000 MW)

- 70°C filter-sterilized and boiled TE and Ultra-Pure water
- Eppendorf Centrifuge 2,500 g (rcf)
- 2mL sterile tubes

Measuring conductivity:

- Distilled water
- Kimwipes
- Standard solution
- Moisture solution
- Laquatwin conductivity meter

Measuring DNA concentration:

- Invitrogen Qubit 2.0 fluorometer
- Qubit dsDNA (High-Sensitivity) Assay Kit
- 500 µL thin-walled PCR tubes

Aurora Purification/Optional Pre-Run:

- Agarose powder
- 0.25x TBE Buffer

- 50 and 15 mL falcon tube
- Ultra-pure water
- Milli-Q water
- Aurora reusable cartridge
- Cartridge dams
- 10% concentration bleach
- Laminar flow hood
- Incubator at 65°C
- 500 mL Polystyrene cell culture flask
- PCR tape
- Sealing tool
- Forcep
- Cleaning brush

Recipes:

Low Biomass DNA Extraction Buffer (different from the one uses for Sterivex)

for 45 mL:

| | |
|---|---------------------------|
| 0.03M Tris-HCl (pH 8) | 1.35 mL of 1.0 M |
| 0.01M EDTA | 0.135 g |
| 0.02M EGTA | 0.342 g |
| 0.1M KH ₂ PO ₄ (pH 8) | 0.54 g |
| 0.8M guanidine HCl | 3.44 g |
| 0.5% Triton-X 100 | 0.225 mL (225 µL) of 100% |

Add Ultrapure water to ~40 mL

Add NaOH to pH 10

Add Ultrapure water to 45 mL

Use 0.2 µm filter and a syringe to remove possible spores

Boil the buffer in microwave for 2.5 minutes with Med-Hi power in a 1000ml glass bottle, tight the lid in order to not losing any liquid during evaporation.

Aliquot into sterile microfuge tubes.

20% Sodium pyrophosphate solution: 2 g sodium pyrophosphate decahydrate, 10 mL Ultrapure water

For 10 mL:

Add the provided amount of sodium pyrophosphate decahydrate and water into the 15 mL falcon tube and vortex it pretty well and place it in the 70°C incubator for ~10 mins.

Vortex it occasionally during this time. After it dissolve completely, filter-sterilize and boil it along with the DEB buffer.

0.25x TBE Buffer: 1.25 mL 10x TBE Buffer, 48.75 mL Ultra-pure water

For 50 mL:

Add the provided amount of filtered buffer and water into a 1000ml glass bottle and boil it for 3 minutes with Med-Hi power in the microwave, then pour the buffer in a polystyrene cell culture flask and place it under the UV light under the lamina flow hood for couple of hours. You may need to rotate the flask after each 30 minutes in order to let the UV penetrate the buffer pretty well. This is the minimum required amount for a single Aurora run. So, if you plan to do a multiple run or you want to keep more buffer for later, you need to make more. This buffer is used for both making the agarose gel and running the Aurora.

1% Agarose Gel: 0.05 g Agarose powder, 5 mL 0.25x TBE (made in the previous step)

Each run uses approximately 4 ml of agarose. Preparing 5 ml is preferential in case extra agarose is needed (e.g. sealing the edge of each well that contact with the gel)

For 5 mL:

Weight 0.05 g of the dry agarose powder in a 15 mL falcon tube. Add 5 ml of the 0.25x TBE Buffer. Vortex to ensure no agarose is clumped together.

Microwave approximately 9-11 seconds with lid slightly loosened, until mix is about to boil.

Tighten the lid of the falcon tube, vortex and incubate the molten agarose at 90-95°C for 1 hour. This leads to improved DNA recovery and it is recommended that this step is not omitted.

After 1 hour, transfer the agarose to a water bath at 60-70°C and leave the agarose in the water bath until use.

****Shouldn't use the molten agarose that is stored for more than 3 days****

Extraction Protocol

Hammering, grinding, preparing samples

1. Remove sample from freezer. Use rock hammer to crush a small sized chunk of rock into chunks as small as possible under the hood. Use whirlpak bags to avoid contamination. Crush 15-20 grams of sample.
2. Use the flame sterile mortar and pestle to crush the sample into a fine powder. Do this for all 15-20 grams.
3. Pour crushed sample into 50 mL falcon tube, mix well and store at -80 C. This is your working sample.
4. When ready, measure out 0.5 g of sample into 2 mL sterile tubes.
5. Add 1000 µL DEB, 150 µL 20% sodium pyrophosphate solution, and 150 µL dATP to each tube.
6. Shaking them and vortexing them couple of times before put them in the fridge.
7. Store at 4°C overnight to allow sample to become immersed in the buffer. Occasional vortexing is recommended during this period.

Possible Stopping Point. Store at -20°C after the initial overnight period at 4°C

Lysis

1. Remove tubes from fridge, vortex them and invert them couple of times.
2. Put them in -80°C freezer for 30'.
3. Remove the tubes from the freezer and place them in Thermo Mixer at 54° C with 600 rpm speed for 45'-1 h.
4. Increase the temperature to 75° C and the speed to the maximum (1500 rpm) and let them stay in the thermomixer for 30'.

You can do beating step (40'') without bead for samples with higher DNA concentration or for targeting the G+ bacteria. However, it might increase the conductivity of the samples by let more salt free in the supernatant

5. Centrifuge them with MiniSpin for 3' at 6,100 g/rcf.
6. Transfer the supernatant (fluid) to a new sterile 2 mL tubes. (do this really carefully and make sure that your tip doesn't touch the muddy precipitate)

Possible Stopping Point. Store at -20°C

Washing sample with VivaSpin column (100,000 MW)

1. Before starting: filter sterilize TE with 0.2 µm syringe filter and boil it.
2. Keep the TE and ultra-pure water in incubator at 65°C.
3. Load the samples into the columns.
4. If the volume of your samples would be less than 2 mL, add 70°C TE to bring total volume up to 2 mL.
5. Assemble the columns as it is shown in the manual.
6. Spin for 35' at 2,500 g (not rpm).
7. Empty filtrate container and refill the columns with 2 mL 70°C TE.
8. Spin again for 35' at 2,500 g.
9. Empty the container again and refill the columns with 2 mL 70°C ultra-pure water.
10. Spin for 30' at 2,500 g.
11. Remove filtrate tube, invert the concentrator body and insert the recovery cap (the smaller one) into the tube.
12. Spin for 2,500 g for 5'.
13. The recovery caps can be sealed for the storage or you can transfer your clean sample into a new sterile microfuge tube.

Tip:

The column can be pre-rinsed with ultra-pure water before loading your sample.

Measuring conductivity:

1. Dilute the sample with ultra-pure water. The total amount of sample should be up to 5 mL for each Aurora run. So, you can add 4 mL or more of water to the sample. (usually after the washing step you will get something ~200 µL)
2. Open the protection lid of the conductivity meter. Pour some drops of the sample (~100 µL) on the sensor.
3. Press the MEAS button to enter the measurement mode.
4. When it lights up, the measurement is completed.
5. To lock the measured value, press the MEAS button again.
6. The sample conductivity needs to be ≤100 µS/cm for DNA clean-up run with Aurora.
7. After measurement, clean the sensor with DI water and dry it by tapping it gently on some kimwipes.
8. Turn off the power and close the protection cover before storage.

*****Make sure to store the sensor without any moisture*****

Tips:

1. If you are going to use the conductivity meter after a long storage, you need to put some drops of the moistening solution. Let it sit for 10 mins then wash the sensor with DI water. After this treatment you can turn the meter on.
2. If you want to recalibrate the meter, you should pour the 1.41 mS/cm standard solution. Press the CAL button over 2 seconds. When the CAL and smiley face sign light up, the calibration is completed. Clean the sensor with DI water before loading your sample.

Measuring DNA concentration:

1. For preparing the Qubit Working Solution, the Qubit reagent need to be diluted 1:200 in Qubit buffer. 200 μ L of Working Solution is required for each sample.
2. Ensure all reagents are at room temperature.
3. Add 198 μ L of the Working Solution and 1 μ L of your sample to a 500 μ L thin-walled PCR tube.
4. Vortex the tube for 2-3 second.
5. Incubate the tube for 2 mins at room temperature.
6. Insert the tube in the Invitrogen Qubit 2.0 fluorometer, select “DNA” and “dsDNA high sensitivity” setting.
7. Select “no” when asked whether to read new standards.
8. Press “read next sample”.
9. Select “calculate stock concentration”.
10. Select “2 μ L” for stock volume.
11. Do the math for final volume of your sample for the total DNA concentration.

*****Make sure to store the dye in the dark all the time*****

Purification with Aurora instrument

1. Soak the cartridge and dams in 10% household bleach (5% sodium hypochlorite) for thirty minutes. Do not soak any items longer than 30 minutes; this can damage the equipment.
2. Wash the cartridge and the dams with Milli-Q water thoroughly.

******Do not use ethanol to clean cartridge; it will cause the cartridge to crack******

3. Place both cartridge and dams under the UV hood for 30 minutes, or until dry.
4. Insert dams into their proper locations (see “Aurora Reusable Cartridge Handling Manual”) and pour molten agarose into the designed gap between the dams as it is shown in the manual.
5. Be sure to check for, and correct, any abnormalities such as **bubbles** in the molten gel.
6. Expose the cartridge with molten agarose to UV for 15-20 minutes until agarose sets.
7. Once gel is ready, gently remove dams by slightly pulling out, and then up.
8. Check extraction well to ensure that no agarose is present, which could affect DNA yield and concentration.
9. Fill each buffer chamber with 5 ml of 0.25x TBE Buffer, except for the chamber to the left of the extraction well, which will be filled with 4 ml of buffer.
10. Place lid on cartridge.
11. Pipette 60 μ L of 0.25x TBE buffer into the extraction well, and immediately seal with PCR tape, using the extraction well sealing tool to ensure a strong seal.
12. Turn on Thermocube (next to the Aurora) and press “start”.
13. The Thermocube can take a while to set. Make sure the “*” on the cube turns into a “+” or “-” when the start button is pushed. The asterisk means the thermocube temperature control is not active.
14. Don’t forget to check the level of the water in the Thermocube’s tank. Add more water if it needs.
15. Open the cartridge drawer on the Aurora instrument.
16. Place approximately 1 ml of Milli-Q water on the cold plate of the Aurora to ensure good thermal contact between the cartridge and cold plate.
17. Place the cartridge onto the cold plate oriented so the concentration gel is on the left hand

side (see “Aurora Reusable Cartridge Handling Manual”).

18. Load the sample that passed the required amount of conductivity ($\leq 100 \mu\text{S}/\text{cm}$) into the sample chamber in the cartridge.

Tip:

***As an added precaution to reduce the risk of DNA contamination, cut a piece of PCR tape large enough to cover the sample chamber.

Remove the white backing from the adhesive film and carefully lay it over the sample chamber from left to right, while leaving a small air gap between the concentration gel and the sample chamber to relieve any pressure during the run. Use a scalpel blade and forceps to cut away and remove any tape that may be covering any of the electrodes.

To seal the tape, use a PCR paddle or a flat object to seal the edges, take care to avoid any wrinkles or bubbles.***

19. Close the drawer and select the desired protocol.
20. Select the “106-0001-CA-D_AURORA_DNA_CLEAN-UP_PROTOCOL”. This protocol recovers DNA molecules 0.3-50 kb in length.
21. Create an experiment folder for the run if you want to save the logs.
22. The run will complete in 4 hours based on the selected protocol.
23. If you run the Aurora over night or the time that you won’t be around after the run to obtain your cleaned sample, you can choose the same protocol with “wait-time” option. This option allows you to determine the time that you will be available for obtaining your sample. It basically postpones the focus process (the last step that concentrates and leads the DNA into the extraction well).
24. The initial conductivity check done by the Aurora may also takes a while, in which errors can occur. It is ideal to wait around for a short amount of time to make sure the run continues properly.

Tip:

***When run is complete, extract purified DNA as soon as possible. DNA will begin to be reabsorbed by the gel if left too long.

If you’re late to extract the sample, a block of refocusing can be done to try and recover any DNA loss that may have occurred.

Normal focus block is done for about an hour and a half, but the Focus Block protocol can be adjusted to desired length. The longer the focus, the (theoretically) higher DNA yield.***

25. When run is complete, open the drawer.
26. Examine the concentration gel and ensure that there are no large bubbles present. Presence of large bubbles indicates that the gel overheated during the run, which will adversely affect the quality of the output.
27. Using forceps to remove the PCR tape covering the extraction well.
28. Transfer concentrated DNA from this well using a micropipette.
29. Typically, two pipetting steps are required in order to extract most of the liquid from the extraction well. Be sure to extract any sample that is suspended on the walls of the extraction well. **The expected output volume is 50-60 μl , but may vary from 40-70 μl depending on sample and run conditions.**
30. Decant and dispose of the buffer from the cartridge. Using the cleaning brush, gently break up and remove the gel from the cartridge and rinse it with Milli-Q water.

Tip:

****It is very important to take care **not to scratch the bottom layer of plastic** in the cartridge.

Do not use brushes or other cleaning implements with sharp edges or points. The layer of plastic separating the gel and buffer in a cartridge from the aluminum plate on the bottom of the cartridge is very thin, and if it is scratched it may be necessary to replace the cartridge.****

Please see the Aurora User Manual and Aurora Reusable Cartridge Handling Manual for troubleshooting information

*****Optional Pre-Run***:**

This protocol is for removing any possible contaminating DNA in the Aurora cartridges. It is recommended for any sample that is expected to have low levels of DNA, and is run **prior to loading your sample and running a subsequent Aurora protocol of your choice**. Since the pre-run concentrates any contaminating DNA molecules within the cartridge, it ensures that contaminating DNA is not co-purified with the DNA from the sample of interest in the subsequent Aurora protocol.

1. Prepare the Aurora reusable cartridge like before, cast the gel, and fill the buffer chambers and extraction chamber with the same amount of 0.25x TBE buffer.
2. Don't forget to seal the extraction well with the PCR tape.
3. Load the sample chamber with 5 mL of ultra-pure water.
4. Place the cartridge in the Aurora drawer and select the "106-0017-AA-D Aurora Pre-Run Protocol.SP" file.
5. The run will complete in 1 hour and 30 minutes.
6. When the run is complete, carefully peel off the tape on the extraction well and remove the buffer.
7. Rinse the extraction well with 100 µl fresh buffer and discard.
8. Refill the extraction well with 60 µl fresh buffer and reseal the well with a new piece of tape.
9. Remove the water from the sample chamber, and refill the chamber with the intended sample.
10. Continue by selecting the protocol most appropriate for the sample.

Tip:

It doesn't need to change the buffer in other chambers or recast the gel after this run.

*****Multiple Run***:**

1. Prepare the cartridge as normal, with fresh buffer in the extraction well.
2. Begin the run.
3. Pause the run after the Injection block as completed, near the beginning of the Wash Block.
4. Remove the buffer from the extraction well, replacing it with the extracted sample from previous run (bring the volume up to 60ul with buffer prior to adding it to the extraction well).
5. Seal the extraction well with a fresh piece of PCR tape and resume the run.
6. Recover your sample at the end of the run, as usual.

Tip:

It is important that you **do not** begin the run with your previous run extracted sample in the extraction well, because during the Injection block that DNA will run off the back side of the gel into the buffer chambers and be lost.