**SDS Extraction**

adapted from Hake Lab protocol (UC Berkeley) by Chodon Sass and Clarice Guan, last modified September 2022 for Nanopore sequencing by Jacob Landis and Gisel De La Cerda.

**Notes**:

Modifications from the original protocols specified for this lab procedure are **bolded**.

When doing repeat extractions, it is recommended that samples from the first extraction reach step 7 of the protocol, at which point tubes with ground tissue will return to step 4 (with 400 µL of added SDS extraction buffer). While re-extraction tubes sit in the hotblock, samples containing the first extraction may continue with the protocol until reaching IPA precipitation (step 12). While the first extractions precipitate, wait for the re-extractions to finish incubating on the hot block then proceed with the protocol until they, too, reach IPA precipitation. The first set of extractions may continue with the protocol once -20°C incubation is complete. If both sets of extractions remain in the freezer overnight, they can be washed and eluted together the next day.

1. Prior to extraction, turn on a heat block or oven and set to 65°C.
2. **Pour liquid nitrogen** **into a mortar** and allow it to completely evaporate. **Refill** the mortar and submerge fresh tissue (can be frozen in -80°C previously), adding liquid nitrogen as needed to keep everything cold. Using the chilled **mortar and pestle**, grind the tissue into a fine powder.
3. Divide the ground tissue among **24 tubes** (max), being careful not to put too much tissue in each tube. Ground tissue may fill to about **300 µL** in a tube without packing it down.
4. Add 700 μL of SDS extraction buffer to each sample. Incubate at 65°C for an hour, inverting the tubes manually every 15 minutes to mix sample tissue with the buffer.
5. Spin the samples for 2 minutes at a speed of 13,000 g.
6. With **wide bore tips**, transfer 400 µL of supernatant from tubes containing tissue into new, labeled 1.5 mL sample tubes.
7. Before proceeding to the next step, add 400 µL of SDS extraction buffer to each sample tube containing tissue and place the tubes back on the hot block for repeat extraction. Let the tubes sit for one hour.
8. To the newly labeled tubes containing supernatant from the first extraction, add in **400 µL of 24:1 Chloroform:IAA**, invert approximately 10 times to ensure samples are fully mixed, then spin for **10 minutes** at 13,000 g.
9. With a pipette set to 400 µL using **wide bore tips**, transfer the clear supernatant from each sample to a new, labeled tube. Be careful to only aspirate the top aqueous layer from the sample.
10. Add 173 μL of 5M NaCl to each new sample tube. Mix gently by inverting, then spin for 2 minutes at 13,000 g.
11. Using a P1000 pipette set to 580 μL, use **wide bore tips** to transfer as much supernatant as possible into newly labeled 1.5 mL sample tubes. Be careful not to disturb the pellet in the tube.
12. Add **400 μL of 100% IPA** to each sample tube. Mix gently by inversion, then leave at -20°C for at least one hour. Samples may be left in the freezer for as long as **overnight**. Longer times in the freezer can boost DNA recovery but at the cost of dirtier DNA. For Nanopore sequencing one hour is best.
13. Spin samples for 5 minutes at 13,000 g, then pour their supernatant out into a waste bin using one smooth pouring motion. Take care to only invert the tube one time so as to not lose the DNA pellet.
14. Add 500 μL of 70% EtOH to each sample tube, inverting until the pellet is suspended to ensure a thorough wash. Spin tubes for **3 minutes** at 13, 000 g, then pour out their supernatant into the waste bin using one smooth motion.
15. Repeat step 14 with **95% EtOH**. After you pour the supernatant out of each tube, keep the tube upside down and open as you set it on a paper towel.
16. Let the tubes dry for 30-45 min (until no EtOH remains).
17. Add **100 μL** **10 mM Tris pH 8** to each sample tube and keep **overnight** at 4°C to allow for full resuspension. Thereafter, samples can be stored at 4°C (short term) or -20°C (long term).

**SDS extraction buffer (makes 50mL):**

10mL 1M Tris (8.0 pH)

2.5mL 5M NaCl

2.5mL 0.5M EDTA

2.5mL 10% SDS

32.5mL H2O

It is recommended to aliquot all other reagents into the tube **before** adding water, so that one only needs to fill the tube up to the 50 mL mark rather than measure 32.5 mL in a separate tube to be poured.

**Cleaning beads**

adapted from Rowan et al., 2017, last modified September 2022 for Nanopore sequencing by Jacob Landis and Gisel De La Cerda.

**Note:** Before using cleaning beads, remove them from 4°C to allow equilibration to room temperature (**about 20 minutes**).

1. **Combine** five to 10 samples into a 2 mL tube. If the total volume is less than 1000 µL, **add** **10 mM Tris** to reach 1 mL.
2. Add **960 µL** (roughly 1x ratio) of cleaning beads so as to not overfill the tube.
3. Incubate the sample at room temperature for **10 minutes** to allow the DNA fragments to adhere to the beads. Carefully mix the sample by inversion every two minutes.
4. Transfer the tube to an appropriately sized magnetic rack and incubate for five minutes until all beads are pulled toward the magnet and the solution is clear.

**Note:** In this step, the beads may clump such that the solution remains cloudy beyond the normal incubation period. Keep the tube on the magnetic stand for at least **45 minutes** or until the supernatant can be removed and transferred to a separate tube without retaining any of the beads.

1. Remove and discard supernatant while tubes are sitting on the magnetic rack. Be careful not to disrupt the bead pellet.
2. Add **800 µL** of 80% EtOH. Wait 30 seconds, then remove the ethanol without disrupting the beads. Repeat this wash.
3. After removing the second ethanol wash, allow the beads to dry at room temperature until all the ethanol has evaporated (one to five minutes at most so as to not cause cracking of the pellet).
4. Remove the tube from the magnet and add **64 µL** of sterile water. Gently flick the tube to ensure beads are resuspended and thoroughly mixed with the water.
5. Incubate the tube at room temperature for 10 minutes to allow the DNA to come into solution.
6. Transfer the tube to the magnetic rack and incubate for about 5 minutes or until all the beads are pulled toward the magnet and the water appears clear.
7. With a **wide bore tip**, transfer **62 µL** of the eluted DNA to a new tube and proceed with DNA quantification, size selection, and cleanup.

**Size Selection**

For size selection, the Circulomics Short Read Eliminator XS kit (Circulomics Inc., Baltimore, MD, USA) manufacturer’s protocol was followed with the exception of the final elution steps (steps 9 & 10), which were specified to maximize DNA concentration per sample while ensuring complete elution.

9. After completing the second ethanol wash of the sample, add **50 µL** of Buffer EB to the tube.

10. Incubate at **4°C overnight** to allow full rehydration of the DNA.

**Note:** After SRE is complete and if samples are available, two tubes of the same accession can be combined to a total volume of **100 µL** to boost input concentration for the following cleaning step.

**Cleanup**

For cleanup, the DNeasy PowerClean ProCleanup Kit (Qiagen, Germantown, MD, USA) manufacturer’s protocol was followed with the exception of the final elution step (step 15) which was modified to boost DNA recovery per sample.

15. **Retain** the MB spin column and place it into a new 2 mL collection tube.

16. Add **50 µL** of Solution EB to the center of the filter membrane and incubate at room temperature for one minute.

17. **Centrifuge** at 10,000 g for 1 minute at room temperature.

18. Discard the MB Spin Column. There are now two sets of tubes for each sample that can be combined for library preparation.

**Library Preparation**

All cleaned and quantified DNA samples were used to generate libraries for sequencing using the Oxford Nanopore Genomic DNA by Ligation SQK-LSK110 kit (Oxford Nanopore Technologies, Oxford, UK) with the following modifications of the standard protocol:

**End Repair**

In a 0.2 ml thin-walled PCR tube, mix the following:

1 μL DNA CS

**48 µL of DNA that is between 25-50 ng/µL and cleaned with the ProClean kit**

3.5 μL NEBNext FFPE DNA Repair Buffer

2 μL NEBNext FFPE DNA Repair Mix

3.5 μL Ultra II End-prep reaction buffer

3 μL Ultra II End-prep enzyme mix

Mix gently by flicking the tube, and spin down

Using a thermal cycler, incubate at 20°C for **30 minutes** and 65°C for **30 minutes**.

**Bead cleanup**

Add 60 µL of resuspended AMPure beads to the end-prep reaction and mix.

Spin down the sample and pellet on a magnet until eluate is clear and colorless (**2 minutes**). Keep the tube on the

magnet, and pipette off the supernatant

Proceed with ethanol wash as stated in protocol.

**Adapter ligation and clean-up**

Proceed with adapter ligation as specified in the standard protocol. After incubation, add in 40 uL resuspended AMPure beads and incubate for 5 minutes.

Spin down the sample and pellet on a magnet for **2 minutes**. Keep the tube on the magnet, and pipette off the supernatant.

Wash the beads by adding either **200 μL** Long Fragment Buffer (LFB) or **200 µL** Short Fragment Buffer

(SFB). Flick the beads to resuspend, spin down, then return the tube to the magnetic rack for **3 minutes** and allow the beads to pellet. Remove the supernatant using a pipette and discard.

Remove the tube from the magnetic rack and resuspend the pellet in 15 μL Elution Buffer (EB). Spin down

and incubate for 10 minutes at 37°C to improve the recovery of long fragments.

Pellet the beads on a magnet for **2 minutes** until the eluate is clear and colorless.

Pipette **13 µL** directly into the loading mixture, and save the remaining sample for Qubit quantification.

**Library loading**

In a new tube, prepare the library for loading as follows:

37.5 μL Sequencing Buffer II (SBII)

25.5 μL Loading Beads II (LBII) mixed immediately before use, or Loading Solution (LS), if using

**13 μL** DNA library

After priming the flow cell with 800 μL of flush buffer and waiting 5 minutes, lift the SpotON sample port.

Load **300 μL** of the priming mix into the flow cell via the priming port (not the SpotON sample port), avoiding

the introduction of air bubbles.

Add 75 μL of sample (library, loading buffer, and loading beads) to the SpotON sample port in dropwise fashion.