***Dispersion Buffer MCAV Extraction***

**Note:** We have found that this protocol works to extract high quantities and quality of DNA from *Montastraea cavernosa, Stephanocoenia intersepta, and Xestospongia muta* tissue preserved in 100% molecular grade ethanol. A brief soak of the tissue scrape in TRIzol reagent appears to get rid of inhibitors and greatly improves DNA quality and downstream PCR amplification. It also works well from *Porites astreoides* tissue samples preserved directly in TRIzol. This extraction protocol is relatively time consuming but may work well for people who are having trouble with their extractions (especially with pigmented pellets, poor downstream digestions or amplification.)

Pre-label tubes:

* Bead tubes with glass beads
* 3 sets of 2 mL tubes (4 sets if samples are not already stored in trizol)
* 1 set of Zymo tubes
* 1 set of 1.5 mL tubes
* 1 set of qubit tubes (plus two extra for running standards)

Pre-Steps:

* Set a refrigerated centrifuge to 4° C.
* Set heat block to 55° C.

1. Prepare dispersion buffer (recipe at the end of this protocol) which can be kept in the 4°C refrigerator in a foil wrapped tube protected from light for several days to weeks.

NOTE: If starting from EtOH preserved samples, start here; if preserved directly in TRIzol, skip to step #4

1. Scrape tissue from coral fragment (tissue from 1-2 polyps is plenty) and place into a 2mL add 500 uL of TRIzol reagent to the tube, let sit for at least 5- 10 minutes but can refrigerator or freeze the tissue in TRIzol as well if doing the extractions later.
   1. If the coral fragment is preserved in TRizol the tissue can be placed directly into prepped bead tube.
2. Centrifuge the tubes at 20,000 x g for 2 minutes. Carefully decant the TRIzol and leave tubes open and under the hood to evaporate some of the TRIzol, or transfer tissue to a new tube.
3. To each tube add .2mL (~.075 g) of 0.5mm glass beads.
   1. Tubes can be prepped before to adding tissue directly

If starting with tissue preserved directly in TRIzol, add small amount of tissue directly from TRIzol tube into bead tube here.

1. Make an extraction buffer master mix.

|  |  |  |
| --- | --- | --- |
| **Reagent** | **Volume (**µL**)** | **Master Mix for 24 samples (+10% error)** |
| Dispersion Buffer (Fridge) | 1000 | 24.4 mL |
| Proteinase K (-20) | 10 | 264 µL |
| RNAse A (-20) | 1 | 26.4 µL |
| **Total** | **1011** | **24.68 mL** |

1. Add 1000 µL of the extraction buffer master mix to each tube.
2. Wrap the top of each tube with parafilm to prevent leakage, unless using fast prep tubes. Invert to mix tubes.
3. Bead beat for 2 – 3 mins (6 M/s, three 45 sec intervals w/ 2 min cool down between) (Bead beater is upstairs in lab 216, code to door is \_\_\_\_).
4. Incubate at 55°C for 1.5 hours while mixing (can stay longer if necessary).
5. Spin the tubes at 20,000 x g for 2 min to pellet some of the proteins and skeletal fragments, transfer 800 uL to a clean 2 mL tube.
6. Add 800µL phenol:chloroform:isoamyl alcohol (pH 8, 25:24:1)(fridge). Do this in the hood and remember to pipette from the bottom layer if this is PCI that has been made in-house that has a layer of water at the surface. Invert tubes to mix, place on ice.
7. Vortex samples for a few seconds and leave on ice for 1 minute. Vortex samples again for 1-2 seconds. You want to make sure the two phases are homogenized, sometimes it is necessary to shake the tubes before vortexing.
8. Centrifuge at 20,000 x g for 15 mins at 4°C, remove carefully.
9. Transfer aqueous phase (top layer) to new tube (~600-700 µL), taking care not to disturb interphase layer. **Only get as much as you can w/o disturbing the interphase layer.**
   1. Take 200µL at a time, about 3 rounds
10. Add 600µL chloroform:isoamyl alcohol (24:1) (flammable cab), place samples on ice. Repeat step 12.
11. Mix and centrifuge at 20,000 x g for 15 mins at 4°C, remove carefully.
12. Transfer aqueous phase (top layer) to new tube (~500-600 µL), taking care not to disturb interphase layer which should be non-existent or much thinner. **Only get as much as you can w/o disturbing the interphase layer.**
13. Add 800µL 100% isopropanol (flammable cabinet), invert samples to mix 25-30 times.
    1. Precipitation step
14. Centrifuge at 20,000 x g for 30 mins at 4°C to pellet the DNA
    1. Make sure tubes are placed in the same direction in centrifuge (cap facing up)
15. Carefully pour off supernatant in sink or waste bucket.
16. Add 1000µL of 70% EtOH (flammable cabinet) @ room temperature. Gently wash EtOH around tube and invert to mix.
17. Centrifuge at 20,000 x g for 10 mins at 4°C.
18. Remove supernatant (carefully pour off, quick spin the samples, and pipette off the remaining fluid avoiding pellet).
    1. Can spin down in room temp centrifuge
    2. Use 200µL pipette
19. Dry for 15 mins upside down on a kimwipe at room temperatur.
20. Elute in 100 uL of NFW.
21. Incubate at 55°Cfor 10 min.
22. Purify DNA extractions using the adapted Zymo Clean and Concentrate Kit protocol (see ‘Using Zymo Clean and Concentrate Kit’ in appendix)

\*Good stopping point for the day, store extractions in fridge

**Cleaning genomic DNA with Zymo DNA Clean &Concentrator-5 Kit**

After extracting genomic DNA, Zymo DNA Clean & Concentrator-5 (D4014) is used to clean DNA and remove inhibitors prior to running PCR, this protocol has a few slight modifications from the manufacturer’s protocol.

1. Set NFW for elution step in heat block at 65° C

2. Add to your eluted DNA a 2:1 volume of Binding Buffer:DNA (in this case 200µL) and vortex thoroughly, spin down.

3. Transfer the entire mixture (~300 µL) to a provided Zymo-Spin Column in a collection tube.

4. Centrifuge 16,000 x g for 1-2 minutes at room temperature. Discard flow through. Check to make sure all of the solution has passed through the filter, if not then spin the filter column again. Issues with getting binding buffer to pass through the filter suggests that there may be too much DNA for the filter and it is getting clogged. If this is happening for a lot of your samples, consider scraping less tissue in the beginning of the protocol, or consider switching to a larger filter column set-up, which Zymo has available.

5. Add 200 μL DNA Wash Buffer (make sure ethanol has been added) to the column. Centrifuge at 16,000 x g for 1 min at room temperature. Repeat.

6. Transfer the column to a new labeled 1.5 mL tube. Elute by adding 15-20 µL (half if re-cleaning) of heated NFW directly to the column matrix and incubate at room temperature for 3–5 min. Centrifuge for 2 min to elute DNA. Ensure the DNA has completely eluted before discarding the column, if there is too much DNA you may have to spin the column twice to ensure you have all of the sample.

7. Nanodrop cleaned DNA, if 260/280 values are <1.8 and 260/230 values are below 2.0 then re-clean and elute in a smaller volume (8 µL).

**Nanodrop**: a. Clean with ethanol

b. Run NFW as blank

c. Run NFW name “BLANK” make sure DNA reading is ~0, if not then re-blank

d. Begin with samples

8. Quantify via picogreen or Qubit.

**Protocol: Making Dispersion Buffer**

\*Handle all reagents under fume hood

* Materials: Guanadine thiocyanate (light sensitive), sodium citrate dihydrate, beta-mercaptoethanol (stored in flame cabinet), milliQ or DEPC water.
* Equipment: Plate with stirring rod, beaker for mixing, glass bottle for storage.

**Buffer Contents:**

|  |  |  |
| --- | --- | --- |
| **Reagent** | **Target Concentration** | **Molecular Mass** |
| Guanadine thiocyanate | 4 M | 118.16 |
| Sodium Citrate dihydrate | 30 mM | 294.10 |
| Β-mercaptoethanol | 30 mM | Stock concentration = 14.3 M |

**Recipe for making 50 ml of buffer:**

Guanadine thiocyanate: 23.632 g

Sodium citrate dihydrate: 0.441 g

β-mercaptoethanol: 105 μL

1. Set up stirring plate under hood
2. Set 100 mL beaker with 50 ml of milli Q water stirring
3. Add reagents slowly to stirring liquid
4. Transfer dispersion buffer to labeled storage buffer tube.
5. Store at 4°C protected from light