Sanger Tree of Life Sample Homogenisation: Cryogenic Bead Beating of Samples with the FastPrep®-96

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Abstract

This protocol describes the cryogenic homogenisation of tissue samples for DNA, Hi-C, or RNA extraction, intended for long-read sequencing or RNA-seq, using the MP Biomedicals FastPrep®-96. This process is highly effective for the homogenisation of tissues from certain taxonomic groups in the Tree of Life Programme, with plants, bryophytes, fungi, chordates, hard corals and arthropods are currently suitable for this method. In comparison to the Covaris cryoPREP®, the FastPrep®-96 enables lower input requirements and a higher throughput of samples. The output of this protocol are homogenised samples that can be used in any of the Sanger Tree of Life DNA and RNA extraction protocols, or in Hi-C library preparation.

Health & Safety warnings

- Users should wear powder-free nitrile gloves and a lab coat at all times when performing this procedure.
- Glove liners are strongly recommended when handling cryogenic substances.
- In addition to all PPE listed above, users should wear eye protection and an oxygen depletion alarm at all times when handling or operating near liquid nitrogen.
- In addition to all PPE listed above, users should wear a face shield and cryogenic gloves when decanting liquid nitrogen
- Users should have training appropriate for the handling of all hazardous equipment used in this procedure (e.g. sharps, cryogenics).
- Waste needs to be collected in a suitable container (e.g. plastic screw-top jar) and disposed of in accordance with local regulations.
- Do not open the lid of the FastPrep®-96 whilst in operation.

Guidelines

- Tissues processed should be preserved via flash freezing and stored under constant cryogenic conditions thereafter. Tissues with alternative preservation methods can be processed, but disruption and downstream outcomes may vary.
- For plants, high-quality, young leaf material, which generally has less secondary metabolite accumulation and is most pliable, should be preferentially selected for optimal disruption and downstream outcomes.
    - Alternative pliable tissue types (e.g. petiole, soft stem or root) can be selected and should disrupt with little issue, but downstream outcomes may vary.

    - Additional disruption cycles or alternative bead types (e.g. 2 x 3 mm tungsten carbide) will be required if whole frozen tissue persists.
- High quality plant tissues with no observable damage, disease or other stressors present should be preferentially selected for optimal downstream outcomes. Lower quality plant tissues with signs of stress can be selected, but downstream outcomes may vary.
- A variety of fungal tissue can be homogenised with the FastPrep®-96 including the cap, stipe, spore bearing structures and mycelium.
- For chordates, mammalian or avian tissues are currently suitable for cryogenic disruption on the FastPrep®-96. Tissue types which particularly benefit from this method include skeletal muscle, cardiac muscle and skin, with improved yields upon HMW DNA extraction compared to powermashing or the cryoPREP®.
- For arthropods (excluding isopods, amphipods and decapods), cryogenic disruption is currently only recommended if samples are ≥ 25 mg, otherwise samples should undergo powermashing for disruption. Homogenisation on the FastPrep®-96 is performed as an alternative to that using the Covaris cryoPREP®.
- For bryophytes, different methods of cryogenic disruption using the FastPrep®-96 are recommended depending upon their downstream process – see Tables 1 and 2 for details on the different beads and tubes required.
- For hard corals (Scelaractinia), 50–200 mg of flash frozen tissue can be homogenised per tube. It is recommended to process a vial of tissue for each downstream process required, as reduced tissue mass per vial improves disruption efficiency.
    - The same process for bead beating hard corals can also be applied to non-stony corals, however these species should be individually assessed to determine the composition of polyps and therefore suitability.
- For isopods, amphipods and decapods, cryogenic disruption is currently only recommended for samples ≥ 10 mg, otherwise samples should undergo powermashing for disruption. It is also currently only for samples in which the downstream process will be HMW DNA or RNA extraction.
- Chordates and all arthropod species are not routinely bead beaten for HiC - this is due to the weight requirements for bead beaten tissue being > 50 mg, therefore it is more convenient for whole flash frozen tissue to be provided for this process. These samples however can be bead beaten for HiC if there is sufficient material.
- Recalcitrant sample types (e.g. tough, rigid, thick or fibrous tissues) can be selected, but should be dissected to < 5 mm^2 pieces and structures should be manually interrupted by crushing prior to disruption e.g. using a pestle and mortar. Disruption and downstream outcomes may vary.
- All dissection, weighing or general handling of tissues should be performed on sterilised surfaces over dry ice to avoid contamination and freeze/thaw cycles.
- Avoid freeze/thaw cycles following cryogenic disruption prior to the addition of a lysis buffer (or equivalent steps) to prevent degradation of nucleic acids.

- Following cryogenic disruption, ensure prepped tissues are completely disrupted into a fine powder. Additional disruption cycles or alternative bead types (e.g. 2 x 3 mm tungsten carbide) becomes essential if whole frozen tissue persists.
    - Complete disruption is crucial to ensure optimal extraction yields and nucleic acid integrity; poorly disrupted tissue drastically decreases downstream procedure efficiencies and can impact all quantifiable outcomes.
- The Tree of Life project operates using Tri-coded FluidX tubes for sample tracking purposes. For this procedure, 2 mL reinforced tubes (SPEX PN 2310) can be used in place of 1.9 mL Tri-coded FluidX tubes during disruption.
- The 4 mL polycarbonate vials must be used in place of 1.9 mL FluidX tubes for hard coral disruption, otherwise the zirconium oxide grinding satellite will shatter the FluidX tube and lid, resulting in loss of sample and damage to the FastPrep®-96.

Table 1: Suggested settings for disruption of samples using FastPrep®-96

Tissue Type	FluidX/Tube required	Bead(s) required	FastPrep Speed (rpm)	FastPrep Time (s)	Repetitions
Plants, Fungi, Chordates, Arthropods*, Bryophytes (RNA)	1.9 mL FluidX	3 x 3 mm stainless steel	1600	30	3
Bryophytes (DNA)	1.0 mL FluidX	1 x 5/32” zirconium oxide	1400	15	1
Hard Corals	4.0 mL polycarbonate vial	1 x 6 mm zirconium oxide grinding satellite	1800	30	3
Isopods, Amphipods, Decapods	1.9 mL FluidX	2 x 3 mm tungsten carbide	1600	30	1-2

*excluding isopods, amphipods & decapods

Table 2: Tissue weights required for FastPrep®-96 depending upon the downstream process

Downstream process	Tissue weight required before FastPrep®-96
DNA Extraction	Plants (MagAttract): 60–70 mg Plants (POE/SoPOE): 80–90 mg Fungi: 40–60 mg Chordates: 20–40 mg Arthropods*: 25–40 mg Bryophytes: 0.1–30 mg

	Hard Corals: 70–80 mg Isopods/Amphipods/Decapods: 10–35 mg
Hi-C	Plants/Fungi: 50–60 mg Bryophytes: Do not beadbeat Hard Corals: 60–70 mg
RNA Extraction	Plants/Fungi/Chordates/Arthropods*: 15–25 mg Bryophytes: 0.1–15 mg Hard Corals: 15–25 mg Isopods/Amphipods/Decapods: 10–25 mg

*excluding isopods, amphipods & decapods

Laboratory Protocol

1. Prepare all necessary equipment prior to starting and place any applicable items (e.g. cold blocks, tools) onto dry ice.
2. Add the required number of the specific type of beads required for the sample type (see Table 1 in the Guidelines section for details) to the required type and number of tubes, then place the tubes into a cold block on dry ice.
   Alternatively, beads can be added after the sample as long as they have been pre-chilled.
3. Weigh the required amount of the selected whole-frozen tissue on dry ice, dissecting into < 1 cm^2 pieces if required, and transfer into the pre-chilled tube containing beads.
   - Whole-frozen tissue can be dissected to size with scissors or a scalpel, or fragmented by crushing with an appropriate tool.
   - A cold sterile pestle and mortar may be required to apply enough force to break hard corals into smaller pieces.
   - Ensure tissues remain under cryogenic conditions at all times to avoid freeze/thaw cycles.
   - Recalcitrant tissue types (see Guidelines section for details), should be dissected into < 5 mm^2 pieces and structures should be manually interrupted by crushing.
4. Repeat step 3 for each sample, and then transfer all tubes to either a FluidX 48-rack or a FluidX 24-rack on dry ice, ensuring that the lid of the FluidX rack is securely fitted.
   - Up to 96 samples in 1.9 mL or 1.0 mL FluidX tubes can be processed simultaneously over two FluidX 48-racks.
   - Up to 48 samples in 4 mL vials can be processed simultaneously over two FluidX 24-racks.

- If only one FluidX rack is used, a second rack containing an equal number of empty FluidX tubes should be prepared to balance the FastPrep®-96 during operation.

5. Submerge the FluidX rack(s) containing samples in liquid nitrogen until temperature equilibrates (bubbling stops, ~15 seconds).
    - Ensure the appropriate PPE is worn when handling, operating near or decanting liquid nitrogen, see ‘Health & Safety warnings’.

6. Remove the FluidX rack(s) containing samples from the liquid nitrogen with tongs, allow any remaining liquid nitrogen to drain, and then place samples in the rack back onto the dry ice. Remove the lid from the FluidX rack and then place the rack onto the FastPrep®-96.
    - Samples should be handled efficiently once removed from the liquid nitrogen to limit exposure to ambient temperature.
    - Fit the top adapter directly on top of the sample tubes and fasten tightly.

7. Homogenise the frozen samples on the FastPrep®-96 for the time and speed directed for the sample type (see Table 1 in the Guidelines section for details).

8. Remove the rack(s) containing samples from the FastPrep®-96, place on dry ice and securely fit the FluidX rack lid. Resubmerge in liquid nitrogen until temperature equilibrates (bubbling stop, ~15 seconds).

9. Repeat steps 5 to 8 as required for the number of disruptions required by your sample type, until tissues are completely disrupted into a fine powder.
    - Manual inspection of samples following disruptions is recommended for novel specimens.
    - Manually inspect isopod, amphipod and decapod samples following the first disruption to determine whether the second disruption is required.
    - For plant samples, if any whole frozen tissue remains, steps 6 to 8 can be repeated up to a total of 6 times. After 6 disruptions, using an alternative specimen and/or tissue type or alternative bead type (e.g. 2 x 3 mm tungsten carbide beads) is recommended if the sample has not disrupted.
    - For hard corals, if the sample will not disrupt:
        - Empty the vial onto the sterile, cold weighing boat on dry ice - there will likely be a mix of fine coral powder and larger pieces.
        - Using clean sterile forceps, place the chunks of undisrupted tissue back into the 4 mL vial and repeat bead beating process.
    - Liquid nitrogen submersion is not necessary following the final disruption, but may be performed to avoid samples thawing.

10. Place the FluidX rack(s) containing samples onto dry ice for > 3 minutes to ensure all disrupted tissue remains frozen.
11. For plants, fungi, chordates and arthropods (including isopods, amphipods and decapods), ensure that the stainless steel or tungsten carbide beads are removed prior to their downstream processing.
12. For bryophytes being processed for DNA extraction, the 5/32” zirconium oxide bead can remain in the tube to ensure as much tissue as possible is retained, as these beads will not dissolve when exposed to solvents.
    For bryophytes being processed for RNA extraction, the stainless steel beads must be removed from the tubes prior to their downstream processing.
13. For hard corals:
    a. Decant disrupted tissue from the 4 mL vials onto clean cold weighing boats on dry ice using sterile tools. Working quickly is essential to be able to remove as much tissue from vials as possible. If needed, recool the tube before continuing to remove tissue into the weighing boat.
    b. Reweigh the disrupted coral tissue to ensure adequate weight for the downstream process and transfer into a new tube for storage e.g. FluidX tube or microcentrifuge tube. For tissues designated for either DNA or RNA extraction, the zirconium oxide bead can also be transferred to retain as much tissue as possible, as these beads will not dissolve when exposed to solvents.
14. Proceed to the required downstream procedure, or alternatively, disrupted tissues can now be stored long term at –70°C with few detrimental effects observed.
    Disrupted tissues in cryogenic storage should not be thawed at any time prior to its immediate use in downstream applications.

Materials

- Cotton glove liners (recommended)
- Dry ice
- Cryogenic gloves
- Safety spectacles
- Liquid nitrogen

Equipment

- FastPrep®-96 (Cat. no. 116010500)
- Corning® CoolRack CF45 (Cat. no. 432051) or equivalent
- Dewar flask
- Tongs or equivalent
- Ice bucket(s)
- FluidX 24-rack, or equivalent
- FluidX 48-rack, or equivalent
- 1.9 mL Tri-coded FluidX tubes, or equivalent
- 1.0 mL Tri-coded FluidX tubes, or equivalent

- 4.0 mL polycarbonate vial (OPS Diagnostics Cat. no. PCRV 04-240-09 or 04-240-02)
- 3 mm sterile stainless steel beads (OPS Diagnostics Cat. no. GBSS 118-2500-09)
- 5/32” zirconium oxide beads (OPS Diagnostics Cat. no. GBZO 156-1000-04)
- 3 mm tungsten carbide beads (Qiagen Cat. no. 69997)
- 6 mm zirconium oxide Ceria stabilised grinding satellite (OPS Diagnostics Cat. no. GBZO 006-1000-05)