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## Collect and Extract Biomass Cyanobacteria (English)

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**Protocol status:** Working  
We use this protocol and it's working

**Created:** Jan 09, 2024

### ABSTRACT

It is crucial to pay meticulous attention to high-quality materials, including HPLC-grade solvents, pristine microtubes, and never-before-used Eppendorf Quality tips. Stringent quality controls encompass the 'Cultivation Blank' (culture medium without cyanobacterial inoculation), 'Extraction Blank' (extraction sample container without biomass), 'Combined Quality Control (QC-pool)' (containing aliquots from different cultures), and 'External Quality Control' (strain CCRM0280 cultured simultaneously).

These are defined as follows:

- Cultivation Blank: culture medium devoid of cyanobacterial inoculation, subjected to the same procedures as the samples with cultivated cyanobacteria.
- Extraction Blank: sample vial for extraction (2 mL microtube with screw cap) devoid of dry biomass, subjected to the same extraction procedures.
- Combined Quality Control (QC-pool): a representative sample of the study that faithfully represents an average of all extracted metabolites. It can also be used for optimization of technical analysis parameters.
- External Quality Control (QCExt): The sample containing strain CCRM0280 cultured in parallel will serve as a reference for comparative studies. After drying, the new CCRM0280 sample obtained in the current cultivation will be combined with the CCRM0280-QCExt stock for equitable distribution of inter-batch Quality Control. This protocol is described in a separate document.

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## GUIDELINES

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**HPLC and LCMS Solvents:** All solvents used in this laboratory must be of a quality compatible with HPLC and LCMS. Solvent purity is crucial to ensure reliable results in our experiments. Make sure that all acquired solvents meet these quality standards.

**New Glassware:** When using glassware, ensure that it is new and clean. Any residue or contamination present in glassware can negatively impact experiment results.

**High-Quality Plastic Materials:** It is recommended to use high-quality plastics, such as those labeled as 'Eppendorf Quality,' to prevent cross-contamination and ensure sample integrity during handling and storage. The quality of plastic materials plays a critical role in the accuracy of analyses.



### Methanol

**Toxicity:** Methanol is highly toxic when ingested, inhaled, or absorbed through the skin. Never drink or consume food in areas where methanol is handled. Avoid direct contact with the skin and eyes. Use appropriate protective equipment such as gloves and safety goggles.

**Ventilation:** Always work with methanol in a well-ventilated area, such as a fume hood, to minimize exposure to toxic vapors.

**Storage:** Store methanol in proper and labeled containers, away from heat sources or ignition. Keep it locked in an appropriate cabinet and out of reach of unauthorized individuals.

**Spills:** In case of a spill, immediately clean it up with suitable absorbent materials. Avoid inhaling vapors and dispose of cleaning materials in accordance with local regulations.

### Dichloromethane

**Toxicity:** Dichloromethane is a highly toxic substance that can cause irritation to the skin, eyes, and respiratory system. Avoid direct contact with the skin and eyes. Use appropriate protective equipment, including gloves, safety goggles, and, when necessary, respiratory protective mask.

**Ventilation:** Work with dichloromethane only in well-ventilated areas or under a fume hood. Avoid inhaling toxic vapors.

**Storage:** Store dichloromethane in proper and labeled containers, away from heat sources or ignition. Keep it locked in an appropriate cabinet and out of reach of unauthorized individuals.

**Spills:** In case of a spill, immediately clean it up with suitable absorbent materials. Avoid inhaling vapors and dispose of cleaning materials in accordance with local regulations.

**Handling:** Avoid unnecessary use of dichloromethane and strictly follow laboratory best practices when handling it.

- It is essential that all laboratory team members are aware of the risks associated with handling these solvents and strictly adhere to safety procedures. Additionally, it is advisable to have an action plan for emergency situations, such as accidental spills, and ensure that everyone knows how to respond in such circumstances.

## BEFORE START INSTRUCTIONS

### Important

- Before starting the procedure, it is imperative that the entire protocol be thoroughly read and understood. Additionally, a conversation with the responsible party is essential to clarify any doubts before proceeding with the steps. This will ensure that the procedure is conducted accurately and reliably."

## Material

- 1
  - 2 mL screw-cap microtubes (e.g., SSIbio) for FastPrep, new (never used)
  - 2 mL Eppendorf-type microtubes (Eppendorf Quality, Eppendorf, Hamburg, Germany), new (never used)
  - P1000 pipette tips (Eppendorf Quality, Eppendorf, Hamburg, Germany), new (never used)
  - 1 mm Zirconia beads (e.g., BioSpec), new (never used)

Tip: Use a "ruler" to measure a single, consistent volume of beads for each sample.



- HPLC grade Methanol (Tedia, Fairfield, OH, USA | Honeywell, Charlotte, NC, USA)\*
- HPLC grade Dichloromethane (Tedia, Fairfield, OH, USA | Honeywell, Charlotte, NC, USA)\*
- Ultrapure water (e.g., Milli-Q)

\*or another of comparable quality.

- FastPrep (FastPrep-24, MP Biomedicals LLC., Santa Ana, CA, USA) at Platform for Expression, Purification, and Analysis of Biomolecules (PEPAB) - Bioficia - UFRJ
- Ultrasonic Bath (Cristofoli, Brazil)
- Vortex (model 772, Fisatom)
- Refrigerated Benchtop Centrifuge (Hettich Model 320R, Tuttlingen, Germany)
- Automatic P1000 pipette (Eppendorf, Hamburg, Germany)
- SpeedVac (Christ model RVC 2-25, Osterode am Harz, Germany) at LabMeta - Chemistry Institute - UFRJ



## Biomass Collection

- 2 After 28 days, extract ~80-90% of biomass from each Erlenmeyer flask (quantity determined visually only)
  - Allow the specimens to grow until a good amount of biomass is formed, with a tentative empirical limit of four months per specimen.
  - Each specimen will be allowed to continue growing until it becomes necessary to interrupt the cultivation for some reason (excess biomass, cell death, etc.).
  - Initially, a limit of four months will be established for each specimen.
- 3
  1. Transfer the culture medium from each Erlenmeyer flask with the biomass to a 50 mL Falcon-type conical centrifuge tube, properly labeled (corresponding to the reference code in the sample list).
  2. Centrifuge: 3011 xg (4500 rpm) at 4°C for 15 minutes.
  3. Discard the supernatant.
  4. Continue transferring the contents of each Erlenmeyer flask, centrifuging and discarding the supernatant until all aqueous content is completely eliminated.
  - Record the volume of biomass obtained (photo for file).
- 4 5. If possible, filter and dry the previously obtained biomass.  
This may not be feasible in some cases.
- 5 6. Subject each properly identified sample to a lyophilization process until dry.
  - Each sample should be separately identified in clean, semi-screwed Falcon tubes (to prevent cross-contamination of the samples).
  - Insert pertinent details and observations in the Metadata.
  - Always store in a freezer at -20°C.

## Prepare the material for the extraction step.

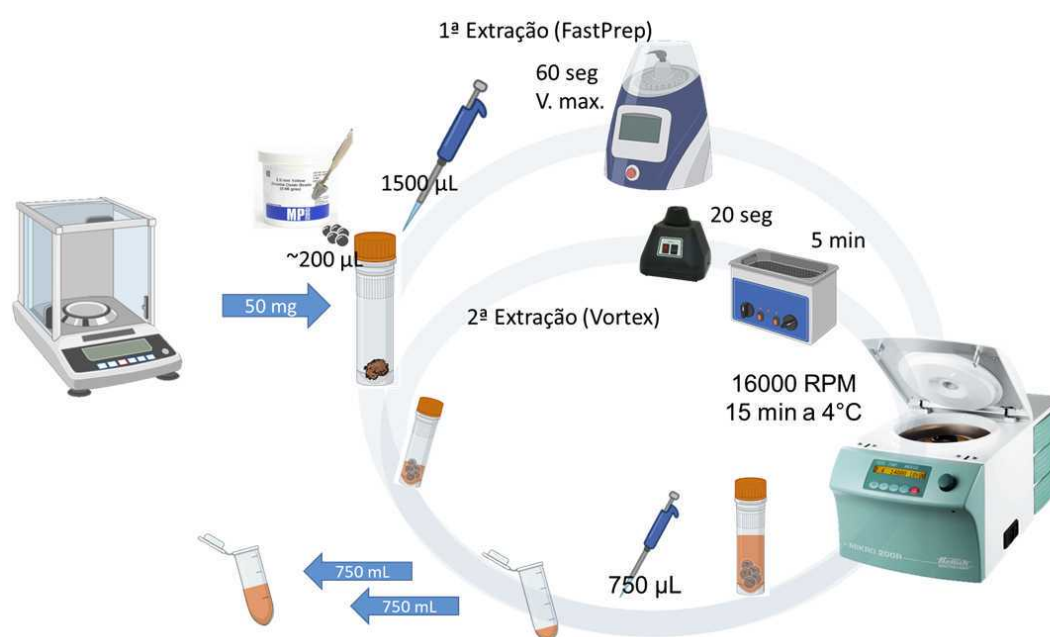
- 6
  - Verify the codes on the labels and the list of dried biomass samples.

- Organize the microtubes for the addition of dried biomass (2 mL screw-cap microtubes) and for the storage of extracts (2 mL Eppendorf-type microtubes - Eppendorf Quality).
- Label all of them appropriately according to the sample table. All materials (Eppendorf microtubes, Eppendorf tips, vials, etc.) must be new. Never reuse these materials for this purpose.

## Extraction of Biomass - for obtaining low to medium-polarity ...

7. Weigh 50 mg of dried biomass and transfer it to a 2 mL screw-cap microtube.
  - Include Extraction Blanks: empty vials added at the beginning and end of the sample sequence, and at intervals of 10 samples.
8. Add approximately 200  $\mu$ L of Zirconia Beads to each biomass sample contained in the 2 mL screw-cap microtubes (use the template for transferring beads mentioned above).
  - Always treat the analytical blanks as if they were real samples.
9. Add 1.5 mL of the chosen extraction solvent (in this case: dichloromethane-methanol, in a 2:1, v/v ratio) to all screw-cap microtubes.
10. Homogenize all samples for 60 seconds at the highest speed (6 m/s) of the FastPrep homogenizer.
11. Centrifuge all samples at 24900 xg (16000 rpm) for 15 minutes at 4°C.
12. Collect 750  $\mu$ L (0.750 mL) of the supernatant from all samples in new 2 mL Eppendorf-type microtubes (Eppendorf Quality), properly labeled.
13. REPEAT EXTRACTION:
  13. Add an additional 750  $\mu$ L (0.750 mL) of the same chosen extraction solvent (in this case: dichloromethane-methanol, 2:1, v/v) to all the vials.

14. Homogenize the samples for 20 seconds on a Vortex-type shaker, followed by an ultrasonic bath for 5 minutes.
15. Centrifuge all samples at 24900 xg (16000 rpm) for 15 minutes at 4°C.
16. Collect 750 µL (0.750 mL) of the supernatant from all samples into the same Eppendorf-type 2 mL microtubes (Eppendorf Quality) used earlier.



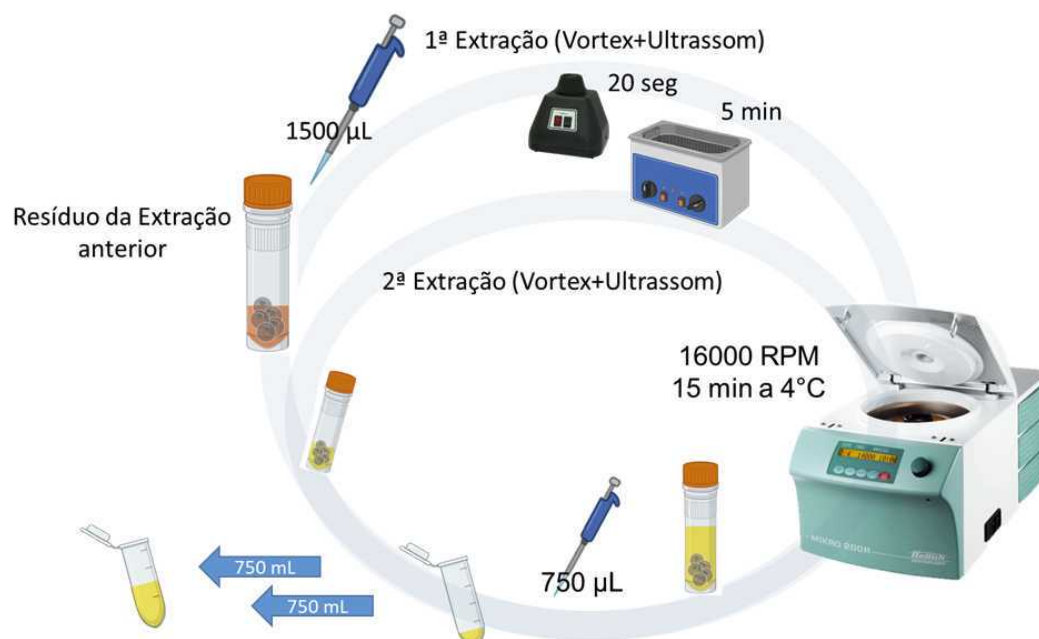
17. Completely evaporate all samples using the SpeedVac until dry.
- Store all samples in a freezer at -80°C until they are prepared for analysis.

## Extraction of Biomass - for obtaining high polarity metabolites

18. Add 1.5 mL of the more polar extraction solvent chosen (in this case: methanol-water, 1:1, v/v) to all screw-capped microtubes containing the previously extracted biomass residue.

- 19** 19. Homogenize the samples for 20 seconds on a Vortex shaker, followed by an ultrasonic bath for 5 minutes.
- 20** 20. Centrifuge all samples at 24900 xg (16000 rpm) for 15 minutes at 4°C.
- 21** 21. Collect 750 µL (0.750 mL) of the supernatant from all samples into new 2 mL Eppendorf-type microtubes, properly labeled with tags.
- 22** 22. Repeat the extraction:  
  
22. Add another 750 µL (0.750 mL) of the same more polar extraction solvent chosen (in this case: methanol-water, 1:1, v/v) to all the vials.
- 23** 23. Homogenize the samples for 20 seconds on a vortex shaker, followed by an ultrasonic bath for 5 minutes.
- 24** 24. Centrifuge all samples at 24900 xg (16000 rpm) for 15 minutes at 4°C.
- 25** 25. Collect 750 µL (0.750 mL) of the supernatant from all samples into the same 2 mL Eppendorf-type microtubes (Eppendorf Quality) used previously.





26. Completely evaporate all samples using the SpeedVac until dry.

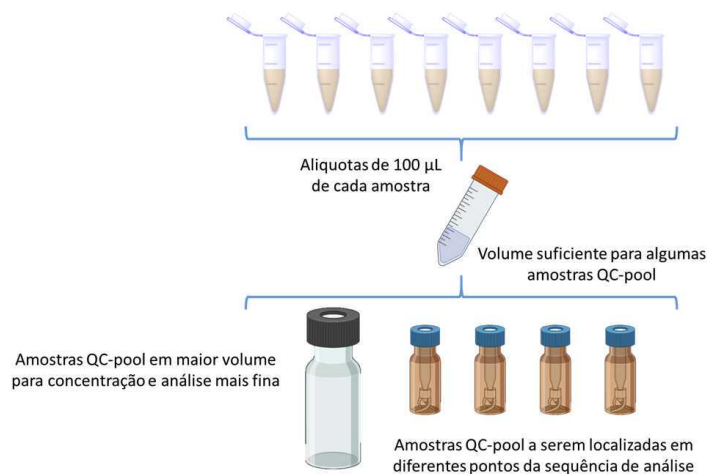
- Store all samples in a freezer at -80°C until they are ready for analysis.

## Pooled quality control sample

27. To prepare the combined Quality Control (QC-pool) samples from approximately 10% of the volume (50 µL) of each sample, follow these steps:

- Take an aliquot of 50 µL from each individual sample.
- Transfer these aliquots into a new 20 mL flask
- Aliquote this pooled quality control sample into four new labeled analytical vials.

Make sure to label the QC-pool samples accurately for identification. Store the remaining quantity for future evaluation or analysis, such as 2D NMR analysis.



## Expected Results

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- At the end of the protocol, it is expected to obtain extracts of low to medium polarity and high polarity for each produced sample, in addition to the Quality Control samples (Extraction Blank and QC-pool), allowing for a comprehensive and comparative analysis of the metabolites being produced by different cyanobacteria, with inter-batch quality control.