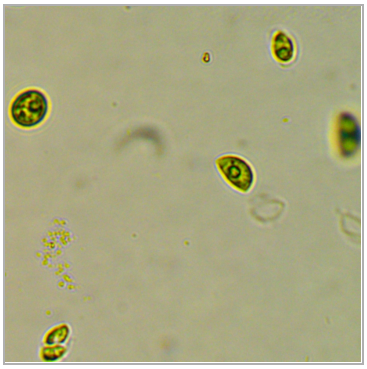

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CTAB/Chloroform-Isoamyl Alcohol DNA Extraction Protocol

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ABSTRACT

Protocol for extracting high quality DNA.

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

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MATERIALS

Appendix, reagents:
CTAB: for 1L of CTAB buffer
100 ml of 1 M Tris, pH 8.0
280 ml of 5 M NaCl
40 ml of 0.5 M EDTA
20 g of CTAB (Cetyl Trimethyl Ammonium Bromide)
to 1L with H20

1 M Tris, pH 8.0: for 1 L
121.1 g Tris
700 ml ddH2O
Dissolve tris and bring to 900 ml.
pH to 8.0 with concentrated HCl (will need ~50ml)
Bring to 1 L.

0.5 M EDTA pH 8.0: for 1 L
186.12 g of EDTA
750 ml ddH2O
Add about 20 g of NaOH pellets
Slowly add more NaOH until pH is 8.0, EDTA will not dissolve until the pH is near 8.0.

5 M NaCl: for 1 L
292.2 g of NaCl
700 ml ddH2O
Dissolve and bring to 1 L.

7.5 M Ammonium acetate: for 250 ml
144.5 g ammonium acetate
Bring to volume with ddH20

Clean the working area.

1 Routinely, we use ethanol and bleach to clean working areas and surfaces. Wipe, wipe, wipe.

Micro pestles need to be properly treated beforehand. DNA is thermostable, so it might not be completely degraded by autoclaving alone. The treatment involves using bleach (DNA is more unstable at low pH, leading to depurination, compared to high pH, which causes denaturation). Rinse multiple times in distilled water to remove any remnant of bleach.
Additionally, using high-intensity UV, such as that provided by a crosslinker UV machine, is also desired.

Prepare the CTAB buffer

2 Prepare a fresh extraction buffer mix in the hood, which will be used on the same day. In this case, combine CTAB and β-mercaptoethanol**, and mix the extractions by inverting. Please note that I am currently using a commercial CTAB, but you can find the recipe in the appendix if needed.

	A	CTAB	β-mercaptoethanol** (2-4%)
		490 ul	10ul (for 2%)
		4900 ul	100 ul
		9800 ul	200 ul

This extraction buffer is a standard CTAB. There are more complex extraction buffers available, containing additional compounds such as vitamin C (ascorbic acid) and PVP (polyvinylpyrrolidone). These different compounds can target specific metabolites in the samples, typically to prevent DNA degradation and ensure the stability of the extracted substances.

β-mercaptoethanol**
Strong reducing agent: This agent effectively removes tannins and other phenolic compounds from the solution. Additionally, it denatures proteins by breaking the disulfide bonds in cysteine residues. While you can add more of the reducing agent if necessary, it is advisable to keep the amount low unless specifically required.

Washing debris from the algal cultures

3 To remove cell wall debris and minimize bacteria contamination, we will wash the cells in culture several times using the following steps:

1. Allow the cells to settle in an Erlenmeyer flask.
2. Slowly pour off most of the medium (which should appear clear) into the bleach bucket.
3. Retain approximately 5-10 ml of the medium.
4. Gently resuspend the cell culture in the remaining medium.
5. Transfer 1.5 ml of the resuspended culture to an Eppendorf tube.
6. Prepare as many Eppendorf tubes as needed for the washing process.
7. Replenish the Erlenmeyer flask with algal medium (e.g. 150 ml of BBM/WH).
8. Always try to gently resuspend the cells using a pipette and remove the whitish interphase (where most debris is located).

Next, proceed with the following steps for the washing process:

1. Spin the algae briefly in the Eppendorf tubes to pellet the cells (5000 rpm for 1 minute).
2. Carefully remove the supernatant without disturbing the pellet.
3. Resuspend the pellet in 1 ml of fresh medium by briefly vortexing the tube.
4. Spin the tube for 30 seconds at 2500 rpm.
5. Again, remove the supernatant without disturbing the pellet.
6. Resuspend the pellet in 1 ml of fresh medium.

Finally, perform the last spin as follows:

1. Spin for 1 minute and 30 seconds at 5000 rpm.
2. Carefully remove the supernatant without disturbing the final pellet.

Following these steps will help to effectively remove cell wall debris and minimize bacteria contamination from the cell culture.

Liquid Nitrogen (LN) Grinding

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1. Add just enough medium (e.g., 100 ul) to recover the pellet and transfer it to the mortar. You can use these very dense cultures to recover the pellets from other replicas of the SAME culture (same flask!). Replicas (from the same flask) can be pooled together for grinding and then transferred back to three Eppendorf tubes.
2. Pre-cool the mortar and pestle by using liquid nitrogen. Grind the cells until they are damaged (check under the microscope). On 5th September 2018, perform 6 rounds of LN grinding followed by 5 freeze-thaw cycles in the Eppendorf tube.
3. Transfer the powdered tissue to a clean Eppendorf tube with 100 ul of extraction buffer mix as prepared above (adding a little extraction buffer mix to the tube helps with the transfer).

Note. other methods to break the cells.

• Pellet algal cells and add autoclaved commercial silica sand or zirconium beads (one scoop). Use a mini pestle to grind the mixture in the Eppendorf tube. You can add a little CTAB to help with the grinding process. I usually prefer a pellet size similar to that of a rice grain, whether frozen or fresh. I remove excess liquid because it makes grinding difficult (algae tend to float!). I check the sample under a microscope to assess the progress of grinding, as well as observe the greenness of the CTAB solution. Typically, I grind each sample for around a minute.

• Alternatively, you can use an automatic vortex adapter (e.g., MOBIO) to process multiple samples simultaneously. After pelleting, place the silica sand and cells in the tube with CTAB. Allow it to stand on the rack for 5 minutes, and then transfer it to a heat block for 20 minutes. If the CTAB buffer did not become green after this step, repeat the vortexing process.

A general rule, especially in chloroform-only extractions, is that adding more tissue can yield more DNA up to a certain point, but it also increases the amount of dirt or impurities in your sample. Residues in the DNA can oxidize your sample, leading to degradation, and they may interfere with enzymes used in downstream applications. Hence, it is essential to find the right balance between the amount of tissue used and the quality of DNA extracted to ensure reliable results in further experiments.

Extraction

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1. Add 500µl of extraction buffer for a total volume of 600µl. Previously, 100µl (refer to step 8) was added to aid in resuspending the cell powder after LN grinding.
2. Incubate the samples at 55-60°C for 1 hour.
3. Add 700µl of Chloroform:Phenol:Isoamyl alcohol and mix well to form an emulsion by pipetting up and down, shaking the tubes, and/or vortexing. In the case of extra dirty samples, they can be placed in a rack on an orbital shaker for 10-30 minutes to increase the contact of the two liquid phases. This will help further remove debris and pigments from the samples.
4. Centrifuge for 10 minutes at maximum speed (13-15,000 rpm). After centrifugation, you should have three layers: top - aqueous phase, middle - debris and proteins, bottom - chloroform. Proceed to the next step quickly to avoid remixing of the phases.
5. Pipette off the aqueous phase (top), taking care not to suck up any of the middle or chloroform phases.
6. Transfer the aqueous phase into a new labeled 1.5 ml tube.
7. Add 4µl of RNAse A (Quiagen or prepared from powder 1mg/ml - keep aliquots frozen until use. Do not re-freeze aliquots multiple times) and mix by inverting. Incubate at 37°C for 30 minutes.
8. Repeat steps 8-11 (second time, centrifuge for 5 minutes).
9. Estimate the volume of the aqueous phase, which should be approximately 450µl.
10. Add 0.1 volume of cold sodium Acetate 3M (final concentration 0.3M). For example, for 450µl, add 45µl.
11. Add 0.7-0.9 volumes (using the combined volume of aqueous phase and added NaAc) of cold molecular-grade isopropanol. This should be approximately 400µl for 500µl CTAB. Mix well.
12. Let it sit in the freezer for 45 minutes to an hour. Longer times (i.e., overnight) will tend to yield more DNA but also more contaminants.
13. Centrifuge for 5 minutes at maximum speed. Orient the tubes equally to facilitate subsequent removal of supernatant without disturbing the DNA pellet.
14. Pour or pipette off the liquid, being careful not to lose the DNA pellet, which is very loose and difficult to see at this stage.
15. Add 700µl of cold 70% Ethanol and invert once to mix.
16. Centrifuge for 1 minute at maximum speed.
17. Pipette off the liquid, being careful not to lose the DNA pellet.
18. Repeat steps 18-20.
19. Pour or pipette off the liquid, being careful not to lose the DNA pellet. If you can't remove all the ethanol, that is okay. It is better to leave some ethanol than risk sucking up your DNA!
20. Dry the pellet by inverting the tubes on a dry, clean paper towel.
21. Resuspend the samples with 45µl of TE. Allow them to resuspend for 1 hour at room temperature or overnight at 4°C before using. (I favor overnight resuspension. Usually, it won't require much pipetting to resuspend the pellet if it has been rehydrating overnight).