Diatom Chloroplast Isolation Steps Version 2

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Abstract

A protocol to separate chloroplasts from diatom cells using ammonium fluoride to permeate the silica frustrule and a percoll gradient to separate the plastid from other cellular components.

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Guidelines

All steps should be done with cold reagents and samples should be placed on ice between steps. Buffers and reagents can be mixed up ahead of time and stored cold (refrigerated). Percoll gradients (layering of 40% and 80% percoll solutions) should be done the same day of the extraction shortly before use.

Before start

Make sure all buffers are prepared according to the instructions in "Buffers for Chloroplast Isolation from Diatoms"

Protocol

Initial Diatom Growth/Filtration

Step 1.

- Grow diatom culture in autoclaved seawater media (e.g. f/2 media). [Protocol was developed with cells grown in f/2 medium to a density of $1x10^6$ cells/mL - optimization may be necessary for cells achieving a lower cell density]
- Gently filter culture through a 2 µm 47 mM filter.
- Periodically, as filtration slows due to growing biomass on the filter, place filters in 50 ml of media used for growth to gently was cells from filter. Repeat until all culture is filtered and cells resuspended.
- Remove filters from tubes and centrifuge @ 1200 x g for 5 minutes to pellet cells. Gently decant media.

Silica Frustrule Removal

Step 2.

- Resuspend pellet in 7ml 10M NH_4F
- Vortex periodically while incubating tube on ice for 10 minutes

- Centrifuge @1200 x g @ 4°C for 5 min and pour off NH_4F
- Resuspend and rinse pellet 3x with isolation buffer (5-7 mls), centrifuge @1200 x g @4°C in between rinses.
- Resuspend pellet in 1 ml cold isolation buffer.
- Sonicate @ 10W for 10 s

Percoll Gradient

Step 3.

- Prepare 2 two-step Percoll gradients (in 15 ml COREX tubes).
    1. Add 2 ml Bottom Layer Solution (80% Percoll) to 15 ml corex tube.
    2. Apply 10 µl of gel loading dye the Top Layer Solution (40% Percoll) and gently add 4 ml Top Layer to the 80% Percoll layer using a Pasteur Pipette.
- Load homogenate from step 9 (Silica Frustrule Removal) carefully onto the top of each two-step Percoll gradient with a Pasteur pipette.
- Centrifuge in swing-out rotor at 1500 x g for ten minutes brake off (@4°C)
- Recover intact chloroplasts (bottom layer) using a Pasteur Pipette. Add to sterile 15 ml corex tube.

Wash (2x)

Step 4.

Wash twice with cold isolation buffer (without added BSA) as below:

1. Add buffer
2. Centrifuge in a swing out rotor at 1000 x g @4°C for five minutes (brake on).
3. Discard Supernatant.

Final Resuspention of Plastids

Step 5.

- Use a soft paintbrush to carefully resuspend pelleted plastids in a small amount of cold isolation buffer (or alternate preservation buffer of your choice) [soft paintbrush is specified so that you do not disrupt the fragile exposed plastids]

Confirmation of Plastid Extraction

Step 6.

- Use phase contrast microscopy to visualize and confirm successful extraction of intact chloroplasts.
- Plastid enrichment can be followed by subsequent protein extraction and gel electrophoresis on SDS PAGE gels for relative enrichment of RuBisCO in the plastid fraction.

NOTES

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Plastids and whole cells were extracted by ice cold homogenation (A. Barkan, *Plant Cell* **5**, 389 (1993)). 5 µg of each sample was mixed with Invitrogen™ LDS buffer and β-me, denatured at 70°C and run on a 4-12% Bis-Tris mini gel with MOPS buffer. Gels were stained with Invitrogen™ SimplyBlue™ SafeStain and imaged on a Syngene Genius^2 gel documentation system. The order of samples on the example protein gel are:

1- T. pseudonana plastid +Fe

2- T. pseudonana plastid -Fe

3- T. weissflogii plastid +Fe

4- protein ladder

5- T. pseudonana whole cell +Fe

6- T. pseudonana whole cell -Fe

7- T. weissflogii whole cell +Fe

8 - Spinach

Warnings

Make sure all reagents are kept cold to prevent degradation of samples during extraction process.