Minute™ Thylakoid Enrichment and Chloroplast Fractionation Kit

Catalog# CF-055

Description

A chloroplast, delimited by a two-membrane envelope, is a type of plastid that serves as the site of photosynthesis. The photosynthetic function is based on developing an operational extensive internal membrane network, called the thylakoids, and on enzymatic processes in the chloroplast matrix, called the stroma. Thylakoid membrane is structurally different from the chloroplast envelope, and their biogenesis depends on biosynthetic and transporting activities specific to the chloroplast envelope. This kit is designed to fractionate chloroplasts into three parts **(thylakoid membrane, envelope membrane, and stroma)** using a specialized filter cartridge and differential centrifugation (without ultracentrifugation). Isolated thylakoids are in native forms and can be used for functional studies and the studies of subcellular localization of proteins using Western blotting/Mass spectrometry. The buffers in the kit are detergent and EDTA-free. The protocol can be completed in about one hour.

Kit Components (20 preps):

1.	Buffer A	25 ml
2.	Buffer B	30 ml
3.	Filter Cartridge/2.0 ml Collection tube	40
4.	Pestles for 1.5 ml tube	2
5.	Protein Extraction Powder	5g

Additional Materials Required but Not Provided

- Table-top micro centrifuge (***Perform all centrifugation steps at 4-8^{o}C)***
- 1.5ml Eppendorf tubes.

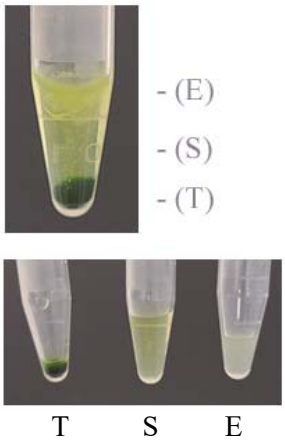
Shipping and Storage: Ship at ambient temperature and store at -20^{o}C upon arrival.

Protocol

Note: Read the protocol carefully before starting. It is recommended to add proteinase inhibitors to aliquot of buffer A and B prior to use. Thaw buffers at RT, invert buffer A a few times and chill buffers on ice prior to use.

1. Insert 400-500 mg fresh young green plant leaf into a 1.5 ml microfuge tube and push it down to the bottom using the flat end of the pestle, and place it on a rack for the Eppendorf tubes. Add 100 µl buffer A and punch the sample repeatedly (about 250-300 times) with a 1 ml pipette tip to release chloroplasts from cells. Add an additional 400 µl buffer A and grind the sample about 100 times using the pestle (the pestle is reusable, rinse with water and dry with a paper towel). Place a filter cartridge in a 2.0 ml collection tube and scrap the sample slurry into the filter using a 1 ml pipette tip until the filter is full. Discard extra slurry, if any.

2. Cap the filter and centrifuge at 2,000 X g for 5 min. Decant the supernatant and resuspend the green pellet (isolated chloroplasts) in 500 µl buffer A by pipetting up and down 20-30 times. Transfer resuspended chloroplasts into a fresh 1.5 ml microfuge tube and centrifuge at 1,500 X g for 5 min, and remove the supernatant completely.
3. Resuspend the green pellet in 100 µl buffer A by repeat pipetting, followed by adding 200 mg protein extraction powder. Grind the chloroplasts with the pestle by twisting back and forth 250-300 times. Add 500 µl buffer B to the tube while the pestle is still in the tube and continue to grind about 50 times.
4. Centrifuge at 600 X g for 5 min. Transfer all supernatant to a fresh microfuge tube (the **"Super-1"**) and place on ice. Grind the pellet again as described in step 3, and transfer **Super-1** back to the tube. Cap and invert a few times.
5. Centrifuge at 600 X g for 5 min. Transfer 0.5 ml supernatant to a fresh 1.5 ml microfuge tube and centrifuge at 12,000 X g for 20 min. Three fractions can be observed after centrifugation: a dark green pellet **(thylakoid)**, a light green top layer **(insoluble envelope membrane)**, and a clear green middle layer **(stroma)**.



T=Thylakoid; S=Stroma; E=Envelope Membrane

6. The stroma fraction can be collected by inserting a 200 µl pipette under the envelope layer and carefully withdrawing 150-200 µl of the middle layer without disturbing the thylakoid pellet and envelope layer.
7. Resuspend the top and middle layers by gently pipetting against the tube wall to detach the insoluble envelope membranes attached to the wall. Do not disturb the pellet. Transfer the suspension (cloudy due the insoluble envelope membranes) to a filter cartridge in a 2.0 ml collection tube, and centrifuge at 200 X g for 2-3 min. The envelope membranes are retained on the filter. Discard the passthrough and resuspend the envelope fraction with a method compatible with downstream experiments (see tech notes below).
8. Resuspend the thylakoid pellet from step 5 in 0.8 ml buffer B by pipetting, and centrifuge at 12,000 X g for 10 min. Decent all supernatant and save the pellet (this is the thylakoid fraction).

Tech notes

1. The thylakoid and envelope membrane fractions are water-insoluble. They can be dissolved in a detergent-containing buffer (see table below). The protein concentration can be determined by BCA assay (Pierce).
2. There are two ways to collect envelope membrane fraction from step 7. **A**. Rinse the filter by adding 100-150 µl of a detergent-containing buffer to the filter and pipetting up and down to solubilize the membranes. Place the filter cartridge in a 1.5 ml microfuge tube and incubate at RT for 5 min. Centrifuge at 10,000 X g for 10 seconds. The passthrough contains dissolved envelope membrane proteins. **B.** If detergent is not desired, resuspend the insoluble membrane on the filter with 100-150 µl of a detergent-free buffer. ***Block the small opening of the filter cartridge (with any objects, such as the index finger with the glove on) to prevent dripping.***
3. The protein concentrations vary significantly among the three fractions. Most of the proteins are in the thylakoid fraction. The envelope membrane fraction only contains a meager amount of protein.

Following reagents are recommended for solubilization of isolated fractions

Product Name	Cat. No.	Applications
Minute™ Denaturing Protein Solubilization Reagent	WA-009	SDS-PAGE electrophoresis and Western blotting, trypsin digestion, purification of proteins with biotin labeling or histidine labeling, etc.
Minute™ Non-Denatured Protein Solubilization Reagent	WA-010	ELISA, immunoprecipitation/Co-IP, enzymatic activity determination and other applications.
Minute™ Protein Solubilization Reagent for MS	WA-011	Trypsin digestion and subsequent mass spectrometry analysis.

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