

Chloroplast Isolation Kit

Cat. No. CIF-319K **Lot. No.** (See product label)

SPECIFICATION

Product Overview	The Chloroplast Isolation Kit provides an efficient procedure for isolating intact chloroplasts from plant leaves. The Chloroplast Isolation Kit has been tested for use with spinach, pea, lettuce, cabbage, mangold, and tobacco.
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Applications	Chloroplast Isolation Kit may be used in chloroplast organelle research to isolate intact chloroplasts from plant leaves for structural and functional studies.
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Storage	Store the kit at 2 to 8 centigrade. The Filter Mesh 100 can be stored at room temperature. It can be reused and can be autoclaved. Sigma recommends preparing the BSA solution fresh for each preparation; however, a stock solution of BSA can be prepared with deionized water at a concentration of 50 mg/ml. This stock solution should be stored at -20 centigrade in aliquots.
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Kit Components	C 7236, Chloroplast Isolation Buffer 5x (CIB) 500 ml P 4937, Percoll 100 ml A 8022, Bovine Serum Albumin (BSA) 3 g F 6801, Filter Mesh 100 4 each
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Materials Required but Not Supplied	· Scissors · Centrifuge tubes (50 ml, 14 ml) · Centrifuge (Sorvall with SS-34 rotor or equivalent) · Funnel · Blender or homogenizer
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Separation Protocol

Isolation of Chloroplasts

- Perform all steps at 2 to 4 centigrade.
- Use pre-cooled buffers and equipment. All centrifugations should be performed at 2 to 4 centigrade with pre-cooled rotors.
- Mix all solutions thoroughly.
- For optimal yield of intact chloroplasts the plant material must be kept in the dark before and during the preparation in order to avoid high levels of starch accumulation. Starch grains can rupture the chloroplast envelope during centrifugation.
- For optimal yield of intact chloroplasts use actively growing, healthy plants. If previously harvested leaves are used, keep them in the cold and in the dark for no longer than one night before isolating the chloroplasts.
- The procedure is optimal for the isolation of spinach chloroplasts. For best results when using other plants, see the comments in procedure steps 5, 8, and 11 and in Table 1.
- The quantities used in this procedure are for chloroplast isolation from 30 grams of leaves.

1. Dilute the required volume of 5x Chloroplast Isolation Buffer (CIB) 5-fold with deionized water to prepare 1x CIB. Mix well. 135 ml of 1x CIB are required for 30 gm of leaves.
2. Prepare a 0.1% (w/v) BSA solution using the 1x CIB. (135 mg of BSA in 135 ml of 1x CIB). Alternatively, a 50 mg/ml stock solution of BSA can be made with deionized water (3 gm in 60 ml of water). The stock solution is mixed with the 1x CIB buffer to give a BSA concentration of 1 mg/ml (2.7 ml of 50 mg/ml BSA stock solution and 132.3 ml of 1x CIB).
3. Form the filter mesh into a funnel shape and place it inside a suitable funnel.
4. Wash 30 gm of leaves thoroughly with deionized water and remove the excess water. Remove the midrib veins with sharp scissors and cut the leaves into small (1-3 cm) pieces.
5. Add 120 ml of 1x CIB buffer with BSA (4 ml/gm of leaves). Optimal chloroplast isolation from other plants requires different ratios of 1x CIB buffer to gram of leaves (See Table 1).

6. Process the leaves with 2 to 4 blender strokes (within 5 seconds) to a coarse macerate with minimal frothing. Alternatively, 2 to 3 strokes of a homogenizer may be used.
7. Pass the macerate gradually through the filter mesh into 50 ml tubes and squeeze the filter to collect all of the liquid. Evenly divide the filtrate between four 50 ml tubes so that the fill volume will not exceed 2/3 of the tube total volume (approximately 35 ml in each tube).
8. To remove unwanted whole cells and cell wall debris, centrifuge the tubes for 3 minutes at 200 x g. A white pellet is precipitated. See Table 1 for optimal centrifugation conditions for other plants.
9. Transfer the supernatant to fresh, chilled 50 ml tubes and centrifuge for 7 minutes at 1,000 x g to sediment the chloroplasts as a green pellet.
10. Discard the supernatant and gently break the pellet by finger tapping. Resuspend the pellets of each tube in 1 to 2 ml of 1x CIB with BSA by gently pipetting up and down. Avoid foaming. Pool the suspended pellets into one tube.
11. Purification of intact chloroplast. At this step the intact chloroplasts can be separated from the broken chloroplasts by centrifugation on top of a 40% percoll layer or on top of a 40/80% Percoll gradient.

Preparation of 40% Percoll layer

1. Prepare 10 ml of 40% Percoll in a 50 ml tube by mixing 4 ml Percoll with 6 ml of 1x CIB with BSA. Use 10 ml of 40% Percoll for every 6 ml of chloroplast suspension.
2. Carefully overlay the chloroplast suspension on top of the 40% Percoll and centrifuge for 6 minutes at 1,700 x g. The broken chloroplasts will form a band on top of the Percoll layer and the intact chloroplasts will sediment to the bottom as a small green pellet.
3. Carefully remove the upper phases and keep the pellet.
4. Resuspend the pellet in 0.5 ml of 1x CIB without BSA.
5. The chloroplast suspension should be kept in the dark, on ice, until further use. For functional detection of chloroplasts, the intact chloroplasts should be used as soon as possible because activity is lost rapidly.

Preparation of 40/80% Percoll gradient

These instructions prepare a gradient for use with 4 ml of chloroplast suspension. For other volumes scale the amounts accordingly.

1. Prepare 2.5 ml of 80% Percoll in a 14 ml tube by mixing 2 ml Percoll with 0.5 ml 1x CIB with BSA.
2. Prepare 5 ml of 40% Percoll by mixing 2 ml of Percoll with 3 ml of 1x CIB with BSA. Carefully layer the 40% Percoll on top of the 80% Percoll.
3. Carefully apply the chloroplast suspension on top of the Percoll gradient(s) and spin for 15 minutes at 3,200 x g. The broken chloroplasts will form an upper band and the intact chloroplasts will form a band at the interface between the 40% and 80% Percoll layers.
4. Collect the band at the interface with a Pasteur pipette, suspend in 3 volumes of 1x CIB without BSA, and centrifuge at 1,700 x g for 1 minute.
5. Resuspend the chloroplast pellet in 0.5 ml of 1x CIB without BSA. The chloroplast suspension should be kept in the dark, on ice, until further use. For functional detection of chloroplasts, the intact chloroplasts should be used as soon as possible because activity is lost rapidly.

Estimation of chlorophyll concentration The yield of isolated chloroplasts is usually expressed on a unit chlorophyll basis (mg of chlorophyll). This entails the extraction of the chlorophyll from the chloroplast suspension with an organic solvent. 3

1. Add 10 µl of the chloroplast suspension to 1 ml of an 80% acetone solution and mix well.
2. Centrifuge for 2 minutes at 3,000 x g. Retain the supernatant.
3. Measure the absorbance of the supernatant at 652 nm. Use the 80% acetone solution as the reference blank.
4. Multiply the absorbance by the dilution factor (100) and divide by the extinction coefficient of 36 to obtain the mg of chlorophyll per ml of the chloroplast suspension.

$$\text{Chlorophyll (mg/ml)} = (A_{652} \times 100)/36$$

Estimation of the percent of intact chloroplasts

Method 1: Ferricyanide dependent oxygen evolution

The principle of the assay is based upon the inability of ferricyanide (an artificial

electron acceptor) to cross the chloroplast envelope and react with the electron transport system within the intact thylakoid membranes. Electron transport from water to ferricyanide results in oxygen release, which can be measured by an oxygen electrode. D, L-glyceraldehyde which inhibits CO₂ fixation and NH₄Cl which uncouples the electron flow from the proton gradient, are added to the reaction in order to increase the rate of oxygen evolution. The level of oxygen released by the chloroplast preparation in isotonic medium is proportional to the fraction of ruptured chloroplasts within the preparation. The level of oxygen released by the same chloroplast preparation after osmotic shock represents the total chloroplast content. The percent of intact chloroplasts is determined by comparing the rates of oxygen evolution upon illumination before and after osmotic shock of the chloroplasts.

Use a volume equivalent to 30 to 50 g of chlorophyll in each of two parallel reactions:

A. Without Osmotic Shock: Mix the chloroplasts with 2 ml of 1x CIB. Add 30 µl of 100 mM ferricyanide* (final concentration 1.5 mM) and 10 µl of 2 M D,L-glyceraldehyde (final concentration 10 mM).

B. With Osmotic Shock: Mix chloroplasts with 1 ml of water. Incubate for at least 15 seconds to allow for osmotic shock. Add 1 ml of 2x CIB, 30 µl of 100 mM ferricyanide* (final concentration 1.5 mM) and 10 µl of 2 M D,L-glyceraldehyde (final concentration 10 mM).

* Prepared freshly in deionized water

Measure the oxygen evolution continuously using an oxygen electrode, while illuminating the samples using a slide projector or 150 W bulb, at 25 centigrade for 2 minutes. After illumination for 1 minute, add 10 µl of 500 mM NH₄Cl (final concentration 2.5 mM) to each sample in order to increase the rate of oxygen evolution. Note: Readings are taken before and during illumination as well as before and after the addition of NH₄Cl

For the calculation of chloroplast intactness it is best to compare the results, with and without osmotic shock, in the presence of NH₄Cl. The slope of the graph is used for the calculation of the O₂ evolution. The rate of oxygen evolution (µmole of O₂ per mg chlorophyll per hour) is proportional to the rate of ferricyanide reduction. Reaction A

represents the fraction of ruptured chloroplasts within the preparation, while Reaction B represents the total chloroplast content. To calculate the percent of intact chloroplasts, use the following formula:

$$\% \text{ intact chloroplasts} = (B - A) \times 100\% / B$$

Method 2: Ferricyanide photoreduction

This simple procedure is also based upon the inability of the ferricyanide to cross the chloroplast envelope and react with the electron transport system in the thylakoid membranes. Ferricyanide reduction, as indicated by the decrease in the absorbance at 410 nm, occurs only when ruptured chloroplasts are present in the preparation. The percent of intact chloroplasts of the preparation is assessed by comparing the rates of ferricyanide photoreduction with and without osmotic shock of the chloroplasts.

For each of the following reactions use a volume of chloroplasts equivalent to 100 μ g chlorophyll.

A. Without Osmotic Shock: Mix chloroplasts with 4 ml of 1x CIB. Add 60 μ l of 100 mM ferricyanide* (final concentration 1.5 mM).

B. With Osmotic Shock: Mix chloroplasts with 2 ml of water. Incubate for at least 15 seconds to allow for osmotic shock. Add 2 ml of 2x CIB and 60 μ l of 100 mM ferricyanide* (final concentration 1.5 mM).

* Prepared freshly in deionized water

1. Place the tubes in a glass beaker filled with ice water.
2. Illuminate with a closely positioned 40 W bulb. Take a 1 ml sample before illumination, and then one every 2 minutes after illumination. Measure the absorbance at 410 nm using a spectrophotometer. Continue illumination for 6 minutes.
3. Photoreduction of ferricyanide results in a decrease of the absorbance at 410 nm. Plot the absorbance at 410 nm versus the time. The rate of decrease in absorbance of each sample is the slope ($\Delta A_{410}/\text{minute}$) of the graph. Calculate the slope for each reaction (A and B).
4. To calculate the percent of intact chloroplasts, use the following formula:

$$\% \text{ intact chloroplasts} = (B - A \times 100\%) / B$$

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