Product Information

# Chloroplast Isolation Kit

For isolation of intact chloroplasts from leaves

**CPISO**

## Product Description

The Chloroplast Isolation Kit provides a quick and efficient procedure to isolate intact chloroplasts from plant leaves. Intact chloroplasts are the best starting material for studies of chloroplastic processes such as carbon assimilation, electron flow and phosphorylation, metabolic transport, or protein targeting. The chloroplast fraction can be further extracted to obtain membrane, stroma, or thylakoid proteins as well as chloroplastic DNA and RNA.

The chloroplast isolation method includes mechanical cell wall and membrane breakage, removal of cell debris and unbroken leaf tissue by filtration, collection of total cell chloroplasts by centrifugation, and separation of intact from broken chloroplasts using a Percoll® layer or gradient.[1] The Chloroplast Isolation Kit has been tested for use with spinach, pea, lettuce, cabbage, mangold, and tobacco. Several publications[2,3] and dissertations[4-10] have cited use of product CPISO in their protocols.

CPISO provides sufficient reagents to perform the protocol 18 times as described, using 30 grams of spinach leaves. Leaves from other plants may require a higher ratio of Chloroplast Isolation Buffer to grams of leaves (see Procedure section), thus affecting the number of uses attainable with the kit.

## Precautions and Disclaimer

For R&D use only. Not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

## Components

- Chloroplast Isolation Buffer 5× (CIB, Component C7236): 500 mL
- Percoll® (Component P4937): 100 mL
- Bovine Serum Albumin (BSA, Component A8022): 3 g
- Filter Mesh 100 (Component F6801): 4 each

## Additional Reagents and Equipment Required for Chloroplast Isolation

(Not provided)

- Scissors
- Centrifuge tubes (50 mL, 14 mL)
- Centrifuge with SS-34 rotor or equivalent
- Funnel
- Blender or homogenizer

## Storage/Stability

Storage Temperature: 2-8 °C

The Filter Mesh 100 can be stored at room temperature. It can be reused and can be autoclaved.

We recommend preparing the BSA solution fresh for each preparation. However, a stock solution of BSA at 50 mg/mL can be prepared in deionized water. This stock solution should be stored at –20 °C in aliquots.

## Procedure

### Isolation of chloroplasts

- Perform all steps at 2-4 °C.
- Use pre-cooled buffers and equipment. All centrifugations should be performed at 2-4 °C 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, 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. For different amounts, scale the quantities accordingly.

1. Dilute the required volume of 5× Chloroplast Isolation Buffer (CIB) 5-fold with deionized water to prepare 1× CIB. Mix well. 135 mL of 1× CIB are required for 30 g of leaves.
2. Prepare a 0.1% (w/v) BSA solution (1 mg/mL) using the 1× CIB (135 mg of BSA in 135 mL of 1× CIB).
   - Alternatively, a 50 mg/mL stock solution of BSA can be made with deionized water (3 g in 60 mL of water). The stock solution is mixed with the 1× CIB buffer (2.7 mL of 50 mg/mL BSA stock solution and 132.3 mL of 1× CIB) to give a BSA concentration of 1 mg/mL.
3. Form the filter mesh into a funnel shape. Place it inside a suitable funnel.
4. Wash 30 g of leaves thoroughly with deionized water.
   4.1. Remove the excess water.
   4.2. Remove the midrib veins with sharp scissors.
   4.3. Cut the leaves into small (1-3 cm) pieces.
5. Add 120 mL of 1× CIB buffer with BSA (4 mL per gram of leaves).
   5.1. Optimal chloroplast isolation from other plants requires different ratios of 1× CIB buffer to gram of leaves. See Table 1 for guidelines.
6. Process the leaves with 2-4 blender strokes (within 5 seconds) to a coarse macerate with minimal frothing. Alternatively, 2-3 strokes of a homogenizer may be used.
7. Pass the macerate gradually through the filter mesh into 50 mL tubes.
   7.1. Squeeze the filter to collect all the liquid.
   7.2. Evenly divide the filtrate between four 50 mL tubes so that the fill volume will not exceed 2/3 of the tube total volume (~35 mL in each tube).
8. To remove unwanted whole cells and cell wall debris, centrifuge the tubes for 3 minutes at 200 × *g*. A white pellet is precipitated.
   8.1. See Table 1 for optimal centrifugation conditions for other plants.
9. Transfer the supernatant to fresh, chilled 50 mL tubes. Centrifuge for 7 minutes at 1,000 × *g* to sediment the chloroplasts as a green pellet.
10. Discard the supernatant.
    10.1. Gently break the pellet by finger tapping.
    10.2. Resuspend the pellets of each tube in 1-2 mL of 1× CIB with BSA by gently pipetting up and down. Avoid foaming.
    10.3. Pool the suspended pellets into one tube.
11. For purification of intact chloroplast, at this step, the intact chloroplasts can be separated from the broken chloroplasts by centrifugation either:

    (a) on top of a 40% Percoll® layer, **or**

    (b) on top of a 40%/80% Percoll® gradient.

    See Table 1 for the optimal method for different plants.

**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 1× 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®. Centrifuge for 6 minutes at 1,700 × g.
   - The broken chloroplasts will form a band on top of the Percoll® layer.
   - The intact chloroplasts will sediment to the bottom as a small green pellet.
3. Carefully remove the upper phases. Keep the pellet.
4. Resuspend the pellet in 0.5 mL of 1× 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 1× CIB with BSA.

2. Prepare 5 mL of 40% Percoll® by mixing 2 mL of Percoll® with 3 mL of 1× CIB with BSA.
   2.1. Carefully layer the 40% Percoll® on top of the 80% Percoll®.
3. Carefully apply the chloroplast suspension on top of the Percoll® gradient(s). Spin for 15 minutes at 3,200 × g.
   - The broken chloroplasts will form an upper band.
   - 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.
   4.1. Suspend in 3 volumes of 1× CIB **without BSA**.
   4.2. Centrifuge at 1,700 × g for 1 minute.
5. Resuspend the chloroplast pellet in 0.5 mL of 1× 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.

**Table 1. Optimal conditions for intact chloroplast isolation**

| Plant | Ratio: mL of 1× CIB to g of leaves | Centrifugation (Step 8) | Percoll® |
|---|---|---|---|
| Spinach | 4:1 | 200 × *g*, 3 minutes | 40% layer |
| Tobacco | 6:1 | Not required | 40%/80% gradient |
| Mangold | 4:1 | 400 × *g*, 1 minute | 40%/80% gradient |
| Cabbage | 4:1 | 200 × *g*, 3 minutes | 40% layer |
| Pea | 6:1 | Not required | 40%/80% gradient |
| Lettuce | 4:1 | 200 × *g*, 3 minutes | 40% layer |

**Note**: The following protocols are provided to help scientists determine the quality of the resulting chloroplast preparations. Reagents and equipment needed are indicated in the protocols.

## 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.[11]

1. Add 10 µL of the chloroplast suspension to 1 mL of an 80% acetone solution. Mix well.
2. Centrifuge for 2 minutes at 3,000 × g. Retain the supernatant.
3. Measure the absorbance of the supernatant at 652 nm ($A_{652}$). Use the 80% acetone solution as the reference blank.
4. Multiply the absorbance ($A_{652}$) 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:

   (mg chlorophyll / mL) = ($A_{652}$ × 100) / 36

## Estimation of the percent of intact chloroplasts[12]

### 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 $CO_2$ fixation, and $NH_4Cl$, 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 30-50 µg of chlorophyll in each of two parallel reactions:

**Reaction A: Without Osmotic Shock**

- Mix the chloroplasts with 2 mL of 1× 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).

**Reaction 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 2× CIB, 30 µL of 100 mM ferricyanide* (final concentration 1.5 mM) and 10 µL of 2 M D,L-glyceraldehyde (final ferricyanide concentration of 10 mM).

* Ferricyanide (usually potassium ferricyanide) is prepared freshly in deionized water.

1. Measure the oxygen evolution continuously using an oxygen electrode, while illuminating the samples using a slide projector or 150 W bulb, at 25 °C for 2 minutes.
2. After illumination for 1 minute, add 10 µL of 500 mM $NH_4Cl$ (final concentration 2.5 mM) to each sample, to increase the rate of oxygen evolution.

   **Note**: Readings are taken before and during illumination, as well as before and after the addition of $NH_4Cl$.

For the calculation of chloroplast intactness, it is best to compare the results, with and without osmotic shock, in the presence of $NH_4Cl$. The slope of the graph is used for the calculation of the $O_2$ evolution.

The rate of oxygen evolution (µmole of $O_2$ per mg chlorophyll per hour) is proportional to the rate of ferricyanide reduction.

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

  [(B – A) / B] × 100% = % intact chloroplasts

**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 µg chlorophyll.

**Reaction A: Without Osmotic Shock**

- Mix chloroplasts with 4 mL of 1× CIB.
- Add 60 µL of 100 mM ferricyanide* (final concentration 1.5 mM).

**Reaction 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 2× CIB and 60 µL of 100 mM ferricyanide* (final ferricyanide concentration of 1.5 mM).

* Ferricyanide (usually potassium ferricyanide) is 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.
   2.1. Take a 1 mL sample before illumination, and then one every 2 minutes after illumination.
   2.2. Measure the absorbance at 410 nm ($A_{410}$) using a spectrophotometer.
   2.3. Continue illumination for 6 minutes.
3. Photoreduction of ferricyanide results in a decrease of the $A_{410}$:
   3.1. Plot the $A_{410}$ versus the time. The rate of decrease in absorbance of each sample is the slope ($\Delta A_{410}$/minute) of the graph.
   3.2. Calculate the slope for each reaction (A and B).
4. To calculate the percent of intact chloroplasts, use the following formula:

   [(B – A) / B] × 100% = % intact chloroplasts

## References


1. Robinson, S.P., *Photosynth. Res.*, **4(3)**, 281-287 (1983).
2. Dewi, H.A. *et al.*, *RSC Adv.*, **4**, 48815 (2014).
3. Diacci, C. *et al.*, *Adv. Mater. Technol.*, **5(3)**, 1900262 (2020).
4. Conte, Sarah Schorr, "The MAR1 Transporter of *Arabidopsis thaliana* Has Roles in Aminoglycoside Antibiotic Transport and Iron Homeostasis". University of Texas at Austin, Ph.D. dissertation, p. 83 (2009).
5. Crumpton-Taylor, Matilda, "Starch granule number and size in *Arabidopsis thaliana* leaves". University of East Anglia, Ph.D. dissertation, pp. 52, 78 (2010).
6. Dempewolf, Hannes, "Patterns of domestication in the Compositae and beyond". University of British Columbia, Ph.D. dissertation, p. 35 (2011).
7. Rueschhoff, Elizabeth Ellen, "Vitamin $B_6$ Metabolism in *Arabidopsis thaliana*". North Carolina State University, Ph.D. dissertation, p. 107 (2011).
8. Jun, Ji Hyun, "Development of high-spatial and high-mass resolution mass spectrometric imaging (MSI) and its application to the study of small metabolites and endogenous molecules of plants". Iowa State University, Ph.D. dissertation, pp. 117, 118 (2012).
9. Smith, Rachel Ann-Selina, "Detection of Protein Interactions at Cellular Membranes Using Total Internal Reflection Ellipsometry". Sheffield Hallam University, Ph.D. dissertation, p. 49 (2015).
10. Wang, Ching-Chieh Russell, "Chlorophyll and Silk-based Oxygen Producing Biomaterials for Tissue Engineering". Tufts University, Ph.D. dissertation, p. 12 (2020).
11. Arnon, D.I., *Plant Physiol.*, **24(1)**, 1-15 (1949).
12. Lilley, R.M. *et al.*, *New Phytol.*, **75(1)**, 1-10 (1975).


## Notice

We provide information and advice to our customers on application technologies and regulatory matters to the best of our knowledge and ability, but without obligation or liability. Existing laws and regulations are to be observed in all cases by our customers. This also applies in respect to any rights of third parties. Our information and advice do not relieve our customers of their own responsibility for checking the suitability of our products for the envisaged purpose.

The information in this document is subject to change without notice and should not be construed as a commitment by the manufacturing or selling entity, or an affiliate. We assume no responsibility for any errors that may appear in this document.

### Technical Assistance

Visit the tech service page at SigmaAldrich.com/techservice.

### Terms and Conditions of Sale

Warranty, use restrictions, and other conditions of sale may be found at SigmaAldrich.com/terms.

### Contact Information

For the location of the office nearest you, go to SigmaAldrich.com/offices.




CPISOpis Rev 08/22 EB,AC,GCY