EDVOTEK®

The Biotechnology Education Company ®

Revised and Updated

EDVO-Kit

910

Isolation of Plant Mitochondria & Chloroplasts

See Page 3 for storage instructions.

EXPERIMENT OBJECTIVE:

In this experiment, students will learn to isolate and detect specific markers in mitochondria and chloroplasts from plant tissue.

Table of Contents

Safety Data Sheets can be found on our website:
www.edvotek.com/safety-data-sheets

Each module is designed for 6 groups.

Storage:
Store components for Modules I & II in the freezer.

Experiment Components

Each experiment module is designed for 6 groups

A *Pisum sativum* (Pea seeds)

Module I: Isolation of Mitochondria

B1 Homogenization Buffer I
C1 Potassium Phosphate pH 7.4
D1 Assay Mix
E1 Ascorbic Acid
F1 Sodium Hydrosulfite

Module II: Isolation of Chloroplasts

G2 Homogenization Buffer II
H2 60% Sucrose in Tricine buffer pH 7.5
I2 Tricine buffer, 10x concentrate, pH 7.5

Also Included:

- 1 ml pipets
- 10 ml pipets
- Transfer pipets
- Microcentrifuge tubes
- Filters

All components are intended for educational research only. They are not to be used for diagnostic or drug purposes, nor administered to or consumed by humans or animals.

Requirements

- Vermiculite (horticulture grade)
- Nursery flat, 21 x 10 x 2 inches
- Scissors or razor blades
- Pasteur pipets
- Blender
- Funnels
- Cheesecloth
- Microscope, slides, and coverslips
- Centrifuge (refrigerated Sorvall SS-34, HB4 or equivalent)
- Clinical centrifuge (optional)
- Microcentrifuge
- Centrifuge tubes
- Small paint brushes
- Spectrophotometer (optional, must be capable of measuring 1 ml sample)
- Ice bucket and ice
- Waterbath
- Distilled water
- Isopropanol (100%)
- Ethanol (70% and 95%)
- Acetone (optional)
- Beakers or flasks
- Pipet pumps
- Automatic micropipet and tips
- Test tubes (13 x 100 mm)

Isolation of Plant Mitochondria & Chloroplasts

Biologists recognize that cells are the basic units of structure for all living things. The cells of prokaryotes-like bacteria are relatively homogeneous. Their cytoplasm is differentiated into regions that differ in appearance, but these regions are not physically distinct and are not subdivided by physical barriers like membranes. In eukaryotic cells, smaller membrane-bound structures are regularly found. These have recognizable names: nucleus, mitochondrion, chloroplast, vacuole, etc. and are called organelles. Organelles are physically distinct structures, suspended within the cytoplasm of the cell but separated from it. Organelles are sites of particular chemical reactions and are defined by their functions as well as by their structure. It is useful to ask, "How do we know what functions each of the organelles perform?"

Much of what we know about organelle function comes from investigations in which organelles were removed from the rest of the cell and studied in isolation. Techniques for isolating organelles from their cells are based on physical differences between them. In this experiment, chloroplasts and mitochondria will be isolated from plant cells using density gradient centrifugation.

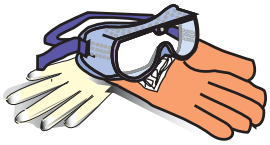
Because organelles differ from one another in mass and density, they can be separated from one another by centrifugation. If any two particles are suspended in water, the larger or more dense of the two will sediment more quickly through the solution in response to gravity. When the particles are small as organelles, sedimentation can take a long time. In a centrifuge, the rate of sedimentation is increased. As the rotor of a centrifuge spins, centrifugal force (a force outward - away from the center of the rotation) is applied to the particles in solution. This force is much larger than the force of gravity. Because centrifugal force replaces gravitational force as the principal force acting on the solution, its strength is normally reported as "times the force of gravity" or "X g". In density gradient centrifugation, the solution in which the organelles are suspended is prepared in a centrifuge tube such that the solution is least dense at the top of the tube and most dense at the bottom. This is what is meant by "density gradient". In the centrifuge, organelles sediment through the solution until they reach a place in the tube where their own density matches that of the solution around them. For example, chloroplasts in cells are of the same density, when the centrifuge is stopped the chloroplasts are all found in the same location in the tube. They form a distinct green band in the gradient.

Using this technique, students will isolate two different organelles: chloroplasts and mitochondria. Students will calculate yield of chloroplasts by measuring chlorophyll and assay an enzyme present in mitochondria to determine the efficiency of their isolation procedures.

EXPERIMENT OBJECTIVE:

In this experiment, students will learn to isolate and detect specific markers in mitochondria and chloroplasts from plant tissue.

LABORATORY SAFETY



Standard laboratory precautions are required. Gloves and goggles should be worn in the laboratory.

CTAB, cetyltrimethylammonium bromide (in the extraction buffer) is a strong detergent and can cause burns to the skin. Follow your instructor's direction for proper handling and disposal of these materials.

Experiment Procedure

MODULE 1A: Isolation of Mitochondria

All solutions should be kept ice-cold. Keep cell homogenate on ice while you are working.

1. Harvest 5 grams of 7-day-old pea roots, shake off vermiculite in which they are growing, and rinse in a beaker of distilled water.

2. Chop the roots into small pieces with a razor blade or scissors and put into a chilled blender with 20 ml ice-cold Homogenization Buffer I (diluted B1).

   Note: More than one 5 gram batch of roots can be homogenized at a time. If several lab groups are sharing the blender, they should use 20 ml of buffer for each 5 grams of roots homogenized, then divide the homogenate.

3. Homogenize the tissue with five 2-3 second bursts of the blender at high speed.

4. Wearing gloves, filter the homogenate through four layers of cheesecloth plus one layer of Filters. It may be necessary to squeeze the filtrate through the cloth.

5. Pour the filtrate into a centrifuge tube, balance against a tube of water or against a tube from another lab group, and centrifuge at 4˚C , 700 xg, 10 minutes (2500 rpm in a Sorvall SS34 rotor).

6. Decant the supernatant from step 5 into a clean centrifuge tube and centrifuge at 4˚C, 10,000 xg, 10 minutes (9500 rpm in a Sorvall SS34 rotor).

MODULE 1A: Isolation of Mitochondria

7. Decant the supernatant from step 6 into a beaker, label "10,000 xg supernatant" and store on ice. This will be used for the assay of the mitochondrial marker. The pellet at the bottom of the centrifuge tube should contain isolated mitochondria. Wash them by gently resuspending them in 20 ml of fresh homogenization buffer. This can be done by pipeting 1-2 ml of the buffer into the tube and using a small paint brush to break up the pellet. Once the pellet is resuspended in this small volume, it can be diluted with the remaining 18-19 ml of buffer.

8. Re-centrifuge the washed mitochondria as in step 6 above.

9. Discard the supernatant from this spin and resuspend the mitochondrial pellet in 5 ml of homogenization buffer. Label "mitochondria preparation" and store on ice.

MODULE 1B: Assay of Mitochondrial Marker Enzyme

Cytochrome c oxidase is a large complex of enzymes that catalyzes a whole series of reactions. The overall reaction catalyzed by the complex is:

$$2 \text{ cytochrome } c^{(2+)} + 2\ H^{+} + {}^{1}/_{2}\ O_2 \longrightarrow \text{cytochrome } c^{(3+)} + H_2O$$

2+ = reduced form of cytochrome c
3+ = oxidized form of cytochrome c

In this reaction, you are measuring how much color develops or changes over time. The more cytochrome c oxidase enzyme found in the mitochondria, the faster the color change. Cytochrome c is the substrate, ascorbic acid is a proton donor which allows the cytochrome c to be reduced so it can then be oxidized.

1. From the ice bucket, remove 0.2 ml of "mitochondria preparation" to each of two small test tubes and 0.2 ml of "10,000 xg supernatant" to one small test tube and allow them to come to room temperature on the lab bench. To one of the tubes containing mitochondria, add 0.8 ml of Potassium Phosphate buffer (C1) for a 1:5 dilution (prep 1). To the other tube of mitochondria, add 1.8 ml of Potassium Phosphate buffer (C1) for a 1:10 dilution (prep 2). Store at 4°C.

Tube	mitochondria Preparation	Potassium Phosphate Buffer
Mito prep 1	0.2 ml	0.8 ml
Mito prep 2	0.2 ml	1.8 ml

MODULE 1B: Assay of Mitochondrial Marker Enzyme

Experiment Procedure

2. Assemble five reaction mixtures in five small test tubes. Label the tubes as follows: (a) blank, (b) reference, (c) 10,000 xg super., (d) Mitochondria prep 1 and (e) prep 2. Add 1 ml of distilled water to the blank tube and 0.8 ml 1x Assay Mix (diluted D1) to tubes b, c, d and e.

3. Add 0.2 ml of Potassium Phosphate buffer (C1) to the reference tube (b) and 0.2 ml of each fraction to the appropriate tubes.

Tube	Description	DH_20	1x Assay Mix	10,000 xg Supernatant	Mito prep 1	Mito prep 2	Potassium Phosphate Buffer
a	blank	1 ml					
b	reference		0.8 ml				0.2 ml
c	10,000 xg super		0.8 ml	0.2 ml			
d	Mito prep 1		0.8 ml		0.2 ml		
e	Mito prep 2		0.8 ml			0.2 ml	

Note:
If your spectrophotometer uses a reference cuvette, this step is not necessary.

4. Transfer the contents of the blank (tube A) to a cuvette and use it to blank the spectrophotometer. (Note: If your spectrophotometer uses a sample cuvette and a reference cuvette, both should be filled with distilled water to blank the instrument.) Place the reference sample into the spectrophotometer then measure and record the change in absorbance at 550 nm of this sample at 20 second intervals for 1 minute. This change represents non-enzymatic oxidation of the substrate. The rate of this change is used as a correction factor and should be subtracted from the rate of change when enzyme is present in step 5.

   Note: If your spectrophotometer uses both sample and reference cuvettes, after blanking the instrument with water, place the reference solution into the reference cuvette and the enzyme-containing sample into the sample cuvette. Calculation of a correction factor as described above is not necessary.

5. To perform the assay, transfer a reaction mix from each of the tubes containing sample (tubes c, d, and e) to a cuvette. Measure and record the absorbance for each sample at 550 nm at 20 second intervals for one minute to determine the rate of the reaction in the absence of substrate. This value (the slope of this line) will be used in step 7 below as a correction factor in your calculation of the rate of the enzyme-catalyzed reaction.

6. Start the reaction (zero time) by adding 10 µl of 0.8M ascorbic acid (dissolved E1) to the cuvette containing sample from tube c. Mix contents of the cuvette by inversion and quickly replace the cuvette into the spectrophotometer. Record the absorbance at 550 nm at 20 second intervals for 2 minutes.

MODULE 1B: Assay of Mitochondrial Marker Enzyme

7. Subsequently, repeat step 6 for samples d and e. You can scatter the readings as shown in the chart.

   Molar concentration of enzyme in the preparation being assayed is not known. How is specific activity related to the rate of reaction just calculated? Specific activity = rate/mg protein (total) in the assay mixture.

Tube	0.8 M Ascorbic acid	Time	Reading
C (10,000 xg)	10 µl	0 20 40 60 80 100 120	First reading 2 min. last reading
D (Mito prep 1)	10 µl	10 30 50 70 90 110 130	First reading 2 min. last reading
E (Mito prep 2)	10 µl	30 50 70 90 110 130 150	First reading 2 min. last reading

MODULE 1B: Assay of Mitochondrial Marker Enzyme

8. Calculate the rate of Cytochrome c oxidation for each of the samples:

$$\text{Rate} = \frac{\text{Difference in Absorbance between steps 6 and 5/minute}}{\text{Molar absorptivity of Cytochrome c multiplied by path length of light}}$$

Molar absorptivity of cytochrome c = $18.5 \times 10^6\ M^{-1}$
Path length through cuvette is usually 1 cm.

The units attached to rate are moles of Cytochrome c oxidized per minute. In general, this value would be reported as micromoles Cytochrome c oxidized per minute; thus, calculation is simplified to:

$$\text{Rate} = \frac{\text{Change in absorbance/minute (corrected as above)}}{18.5}$$

Note: Enzyme activity is usually reported in the literature as specific activity. This is a way of standardizing the reporting of enzyme activity since the exact

Experiment Procedure

Module IIA: Isolation of Chloroplast

All solutions should be ice-cold. Keep solutions, samples, gradients, etc. on ice while you are working.

1. Use 60% sucrose (H2) and 1x Tricine buffer (diluted I2) to prepare sucrose gradient as follows:

Solution Being Prepared	60% Sucrose	1x Tricine
50% sucrose	4.2 ml	0.8 ml
40% sucrose	6.7 ml	3.3 ml
30% sucrose	2.5 ml	2.5 ml

2. Prepare sucrose gradient in a 50 ml centrifuge tube.

   A. Pipette 5 ml 60% sucrose solution into bottom of the tube.

   B. Layer 5 ml 50% sucrose, then 10 ml 40% sucrose into the tube. Layers should be distinct from one another if you are careful.

      Hint: Tip the tube as you add each layer of sucrose. Let tip of the pipette just touch the surface of liquid in tube.

   C. With the tip of a Pasteur pipette or stirring rod, gently mix at the interface of the 50% and 40% layers to diffuse slightly.

   D. Layer 5 ml 30% sucrose on top of the gradient.

   E. Keep the gradient on ice while you prepare tissue sample.

3. Harvest 5 grams of 7-day-old pea seedlings at the soil line with a razor blade.

4. Chop the tissue into small pieces with a razor blade or scissors and transfer them to a chilled blender containing 20 ml ice-cold homogenization buffer II (diluted G2).

   Note: More than one 5 gram batch of seedlings can be blended at a time. If several lab groups are sharing the blender, they should use 20 ml of buffer for each 5 grams of seedlings homogenized, then divide the homogenate.

5. Homogenize with five 2-3 second bursts of the blender at high speed.

6. Filter the homogenate into a beaker (on ice) through four layers of cheesecloth, squeezing the cloth gently to remove most of the liquid (wear gloves).

Handle gradients very carefully, so as not to disturb the layers of sucrose.

Module IIA: Isolation of Chloroplast

Experiment Procedure

Use extreme caution when working with acetone. It is very flammable and should be handled in a fume exhaust hood.

7. Re-filter the first filtrate through one layer of Filter, moistened in homogenization buffer (G2), by gravity. Do not squeeze. You may want to prepare a wet-mount slide of the residue left in the cheesecloth or Filter for the microscope. What have you removed from the homogenate by filtration?

8. Layer 10 ml of the filtrate onto the top of the gradients (prepared in step 2). Balance your tube against the tube of another group for centrifugation. If necessary, add 1x homogenization buffer II to balance the tubes. Centrifuge at 4°C in HB4 rotor, 4000 rpm for 5 minutes, then increase speed to 10,000 rpm for 10 minutes. Allow the centrifuge to coast to a stop. Carefully remove your gradient from the rotor.

9. You should see two green bands in the gradient. The green band toward the bottom of the tube is the fraction containing intact chloroplasts. Remove the top of the gradient carefully with a Pasteur pipet. Save the two chlorophyll-containing fractions in clean tubes on ice.

10. Prepare wet-mount slides of the chlorophyll-containing fractions and examine on the microscope. What differences do you notice between them?

Read cautionary statement at left!

Module IIB: Quantitation of Chlorophyll in Chloroplasts (Optional Step)

1. Assay chlorophyll content of the two green bands. For each sample:

   A. Into a clean centrifuge tube, pipette 50 µl of the gradient fraction to be assayed and 0.95 ml distilled water.

   B. Add 4 ml acetone.

   C. Centrifuge in a clinical centrifuge, 5 minutes.

   D. Measure the absorbance of the solution in a spectrophotometer at 652 nm. (The appropriate blank for this measurement is 80% acetone in water.)

   E. Calculate chlorophyll content:
   A_{652} x 29 = µg chlorophyll/10 µl chloroplast fraction.

Study Questions

1. What fractions of marker enzyme activity are found in the 10,000 X g supernatant?
2. Explain the utility of marker enzymes in a cell fractionation experiment.
3. What are some reasons that marker enzyme activity might be formed in the "wrong" fraction?
4. What is Cytochrome c (the substrate for this marker enzyme) and what does it do in the mitochondrion?
5. Make a diagram of your chloroplast/sucrose gradient. Have materials other than chloroplasts banded in the tube? Where are they? What do they look like? Why was the cell homogenate filtered before being loaded on the gradient? What was removed by filtration? What fraction of the chloroplasts in the homogenate have you isolated intact?

Notes:

Instructor's Guide

Notes to the Instructor

Class size, length of laboratory sessions, and availability of equipment are factors which must be considered in the planning and the implementation of this experiment with your students. These guidelines can be adapted to fit your specific set of circumstances.

If you do not find the answers to your questions in this section, a variety of resources are continuously being added to the EDVOTEK® web site. In addition, Technical Service is available from 9:00 am to 6:00 pm, Eastern time zone. Call for help from our knowledgeable technical staff at 1-800-EDVOTEK (1-800-338-6835).

Safety Data Sheets can be found on our website:
www.edvotek.com/safety-data-sheets

Pre-Lab Preparations

Tissue Requirement Summary

For Mitochondria:
5 g of freshly harvested roots

For Chloroplasts:
5 g of freshly harvested shoots

PLANT MATERIAL,
Component A *Pisum sativum* (pea seeds)
(for both modules)

All of the experiments on cell fractionation included in this kit were planned using pea seedlings as starting material. From a single nursery flat (21 x 10 x 2 inches) enough plant material can be harvested to perform all three experiments, for each of the six lab groups. Plan to plant seeds one to two weeks before they will be used in the lab. To plant:

1. Soak the pea seeds (A) in a large beaker overnight.
2. Sow them in a layer of about 1.5 inches of wet horticulture grade vermiculite in a standard nursery flat. The seeds can be very close together.
3. Cover the seeds with 0.5 - 1.0 inch of vermiculite and water well.
4. Cover the flat with plastic wrap to hold in moisture while the seeds germinate.

Once the seedlings have begun to emerge from the vermiculite, remove the plastic and keep the flat well watered. Seedlings can be grown on a windowsill, in a growth chamber, or in the greenhouse. For best results, however, do not grow the seedlings under intense light. Under very bright lights, chloroplasts accumulate large starch granules which can disrupt the chloroplasts during isolation.

Module I: Use freshly harvested roots, 5 grams of tissue per lab group, for isolation of mitochondria.

Module II: For chloroplast isolation, use 5 grams of freshly harvested shoots per lab group. The shoots should be cut just at the soil line.

NOTE:
If Homogenization Buffer, component B1, has come out of solution, heat it to 65°C until it has completely dissolved.

Pre-Lab Preparations

PRE-LAB FOR MODULE I

1. Dilute Homogenization Buffer I (B1) by mixing 128 ml of component B1 with 192 ml of distilled water. Label and aliquot 50 ml per lab group and place on ice.

2. Cut cheesecloth to fit funnels. Arrange the filters and the cut cheesecloth so that they fit the funnels for filtering plant homogenates.

3. Aliquot 3 ml of 0.1 M Potassium Phosphate buffer (C1) for each group. Label 0.1 M Potassium Phosphate buffer.

4. Prepare reaction mixture for assay of mitochondrial marker enzyme. Add 1 or 2 granules of Sodium Hydrosulfite (F1) to the 10x Assay Mix (D1). Shake vigorously to dissolve the granules and mix the entire contents with 27 ml of distilled water. Label "1x Assay Mix" and aliquot 4 ml per group.

5. Dissolve Ascorbic Acid (E1) in 0.75 ml distilled water to make 0.8 M Ascorbic Acid. Label and aliquot 100 µl per group.

PRE-LAB FOR MODULE II

1. Dilute Homogenization Buffer II (G2) by mixing 94 ml of component G2 with 47 ml distilled water. Label and aliquot 20 ml per lab group and place on ice.

2. Mix 5 ml of Tricine Buffer, 10x concentrate pH 7.5 (I2) with 45 ml of distilled water. Label and aliquot 8 ml per group.

3. Label and aliquot 20 ml of 60% Sucrose (H2) for each of the six groups.

For Module I, each student group requires:

- Ice and bucket
- 5 g 7-day-old pea roots
- Razor or scissors
- 50 ml ice cold diluted Homogenization Buffer I (diluted B1)
- Blender
- Cheesecloth
- Filters
- Funnel
- Centrifuge tubes (2 or 3)
- Small paint brush
- 3 ml 0.1 M Potassium Phosphate buffer (C1)
- 4 ml 1x Assay Mix (diluted D1)
- 100 µl 0.8M Ascorbic Acid (dissolved E1)

For Module II, each student group requires:

- Ice and bucket
- 5 g 7-day-old pea shoots
- Razor or scissors
- 20 ml ice cold Homogenization Buffer II (diluted G2)
- 20 ml 60% sucrose solution (H2)
- 8 ml Tricine buffer (diluted I2)
- Blender
- Cheesecloth
- Filters
- Centrifuge tubes (2 or 3)
- 10 ml pipets and pumps
- Microscope slides and coverslips
- Microscope
- Spectrophotometer (optional)
- Acetone (optional)

Please refer to the kit insert for the Answers to Study Questions