# ISOLATION OF CHLOROPLASTS BY DIFFERENTIAL CENTRIFUGATION

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18Nov92, 23Nov93, 22Nov94, 27Nov95, 26Nov96, 21Nov00, 20Nov01, 22Sept03, 2Dec08, 1Dec09, 30Nov10
From DBF Notebook, C.C. III, p. 84
http://biology.clc.uc.edu/fankhauser/Labs/Cell_Biology/Chloroplasts/Chloroplasts.htm

**Equipment and Supplies per team of four students:**

- *Fresh* spinach (dark green, good turgor)
- mortar and pestle (or blender)
- clean sharp sand
- 50 mL 0.5 M sucrose (17% w/v)
- Styrofoam ice bath
- 25 mL graduated cylinder
- cheese cloth, 12 x 12 inches
- glass filter funnel
- two 16x150 mm test tubes in rack
- three 13x100 mm test tubes in rack: F1, S1, SO
- plastic capped 15 mL centrifuge tube
- double pan balance
- Pasteur pipet to adjust volumes, take samples
- table top clinical centrifuge
- glass stirring rods

**KEEP ALL EQUIPMENT AND MATERIALS ICE COLD:**

*Per team of two students:*

1. **Homogenize:** Grind 8 g deveined spinach with ½ tsp clean sharp sand in mortar and pestle to a paste. Measure out 16 mL ice-cold 0.5 M sucrose solution in a 25 mL graduated cylinder. Add in 3-4 mL increments, grind to smooth pulp with each addition. (A blender may be used for >100 mL volumes)
2. **Filter** homogenate through about eight layers of clean cheese cloth in a glass funnel into an iced 16x150 mm test tube. Do not squeeze. Pour filtrate back into 25 mL cylinder and **record volume**. Save ~0.5 mL of the filtrate (F1) in a labeled 13x100 mm test tube on ice to examine at 400x under microscope to determine composition and illustrate in notebook. Note appearance of components and degree of heterogeneity. (Label cells, ghosts, chloroplasts, mitochondria, debris.)
3. **Centrifuge** the filtrate against a balanced 16x150 tube at 50x g for 10 minutes (speed 2 on the clinical centrifuge)
4. **Decant** the top 10 mL in one steady motion, into a clean cold centrifuge tube. Avoid the sediment. Save, discard sediment. Record volume. Save ~0.5 mL supernatant (S1) on ice to examine under microscope to determine composition, illustrate and label as in step 2.
5. **Centrifuge** the supernatant from step 3 opposite a *carefully* balance tube at 1000x g for 10 minutes (speed 7) to precipitate chloroplasts. How does the supernatant appear? Precipitate? Do you see a well formed pellet? Discard the supernatant down the drain by turning the tube upside down. (You may lose some of the soft pellet, but not to worry.)
6. **Resuspend** pellet from step 4 by q.s.ing up to 0.5 mL with a few drops of ice-cold 0.5 M sucrose and suspending with a clean, ice cold stirring rod. Keep on ice at all times. Examine suspended organelles (SO) under microscope to determine composition, illustrate as is step 2. [You should haveF1, S1 and SO on ice. Make comparative illustrations of each in your book.]

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## Table for recording DCIP reduction $A_{600}$ versus time of light exposure.

18 Sept 00, 20 Nov 01, 20 Sept 04, 29 Nov 04, 27 Nov 07, 2Dec08, 1Dec09

Copy this table into your notebook prior to starting the chloroplast reduction experiment (protocol on next page). **Every blank square gets a reading**.

| **DCIP reduction: $A_{600}$ versus time of light exposure** | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| tube | vol. chloro plasts | light cond | Time of exposure in minutes | | | | | | | | | | | |
| | | | **0** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10** | **15** |
| 1 | 0 | dark | | x | x | x | x | x | x | x | x | x | x | |
| 2 | 0 | light | | x | x | x | x | | x | x | x | x | | |
| 3 | 5 uL | dark | | x | x | x | x | x | x | x | x | x | x | |
| 4 | 5 uL | light | | | | | | | | | | | | |
| 5 | 10 uL | dark | | x | x | x | x | x | x | x | x | x | x | |
| 6 | 10 uL | light | | | | | | | | | | | | |

# CHLOROPLAST REDUCTION OF INDOPHENOL 

David B. Fankhauser, Ph.D.

18Nov92, 23Nov93, 20Sept94, 21Nov95, 26Nov96, 20Sept99, 18Sept00, 20Nov01, 20Sept04, 29Nov05, 27Nov07, 1Dec09
From DBF Notebook, C.C. **III**, p. 84 & CC **IX**, p. 107

See previous protocol for the preparation of the purified chloroplasts. Continue to keep them ice cold until the moment you add them to the prepared tubes. Work in teams of two.

**Supplies:**

- purified spinach chloroplasts, ice cold
- 0.1 M $PO_4$ buffer, pH 6.5 (pH 7.0 OK?)
- 2.5 x $10^{-4}$ M 2,6 dichloroindophenol
  - (36.3 mg DCIP/500 mL, $A_{600}$ should be 3.0)
- displacement pipets, 10 uL and 50 uL
- seven unblemished 13 X 100 mm test tubes in rack
- 100 watt light — 1/ two teams
- 37°C incubator (can exclude light)
- Set up hot block next to spectrophotometer:
- hot block, 37°C — 1/ 2 teams
- spectrophotometer — 1/team
- vortex

**"Quick and dirty" version of expt:** 4 mL of reaction mix, 37 C. Add 5 or 10 uL chloroplasts, vortex, read $A_{600}$, start watch, place in front of light. Take readings every 30 seconds until three successive readings are the same. Repeat with 5 uL chloroplasts. Graph the decline in $A_{600}$.

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## REDUCTION EXPERIMENT WITH DARK CONTROLS:

1. Set up apparatus at one location (need 3-way plug):
   - a: 37°C hot block (for 13x100 mm tubes) nearby and warmed up.
   - b: spectrophotometer on the same desk as the light exposure apparatus.
   - c: 100 watt bulb with reflector 25 cm from open test tube rack.
   - d: Have available a 37 Cincubator warmed up for dark incubation.
2. Construct a data table in your notebook to accept data (previous page in handouts).
3. **Select, label and polish seven *very clean, unblemished* test tubes** (B for blank + six tubes).
4. **Prepare DCIP reaction mix**. (Read $A_{600}$ before dispensing. $A_{600}$ should be At least 0.600).
   Per team:
   - 12 mL 0.1 M $PO_4$, pH 6.5 buffer
   - 12 mL 0.5 M sucrose
   - 12 mL 2.5 x $10^{-4}$ M DCIP (2,6 dichloroindophenol)
5. **Dispense 4 mL of the reaction mix** to each of your tubes. (Use 50 mL repeat pipet set on 4 mL.)

| tube: | Reaction Mix mL | chloroplast suspension µL | incubation conditions: | | $A_{600}$ |
|---|---|---|---|---|---|
| 1 | 4.00 | 0 | dark | 1 | |
| 2 | " | 0 | light | 2 | |
| 3 | " | 5 | dark | 3 | |
| 4 | " | 5 | light | 4 | |
| 5 | " | 10 | dark | 5 | |
| 6 | " | 10 | light | 6 | |

6. **Prewarm all prepared tubes** (lacking chloroplasts) in 37°C hot block for 2 min.
7. **DARK TUBES (control tubes): Add chloroplasts to tubes 3 and 5.** Mix and read the $A_{600}$ of 1, 3, and 5 against a water blank. *Immediately* place in 37°C incubator, *keep light excluded.*
8. **LIGHT TUBES:**
   **tube 2:** Place 25 cm from a 100 watt bulb.
   **tube 4**: Add 5 uL chloroplasts, mix, read $A_{600}$ and place in light as for tube 2. **Start stopwatch**.
   **tube 6:** Add 10 uL chloroplasts, mix, **read at 30 seconds** and place in front of light. Note time. Keep this 30 second time separation between reading of 2 and 4 for the next ten minutes.
9. **Read $A_{600}$ of 4 and 6 *every minute for 10 minutes***, read tube 2 only at 0, 5, and 10 min. (Keep tubes polished, read in consistent configuration.)
10. **Read $A_{600}$ of tubes which were kept in the dark** again at 15 minutes.
11. **Plot the absorbency of each tube versus time**.
12. Discuss the significance of the differences between the various six curves.

**Problems 2010:**

DCIP reaction mix read 1.500!
Altered protocol:
four tubes:two tubes got no chloroplasts, two tubes got chloroplasts.
Read non-chloroplast tubes, put one in dark incubator, one in front of light.
Added 10 uL chloroplasts to one tube, read, and placed immediately in dark incubator.
Added 10 uL chloroplasts to second tube, read, started stopwatch, and placed in front of light.
Read this tube every 30 seconds.
When two successive readings were the same, read the other three tubes (which should not have changed).

**Problems 2000:**

1. Emphasize the different decanting techniques for S1 (saving supernatant, do not want any pellet) and SO (saving pellet, do not want any supernatant, so pour off all supernatant in one motion, let pellet drain).
2. The chloroplasts seemed weak. (Degraded in RT conditions? Too few?) Have changed protocol to increase the concentration of chloroplasts (used to be 1:5, now have doubled SO, have no dilution, so should be 2.5x as much chloroplasts.
3. T-0 only read about 0.335. So have increased DCIP in Rxn Mx by 33%.
4. Be sure to illustrate the reduction of DCIP by NADPH.
5. Only had 3 teams, so had 50 mL Rxn Mx left over.

**Problems, suggestions from 1996:**

Took too long with chloroplast isolation, rushed through reduction phase
Students did not understand goal of reduction phase.
Need to set up station **for each pair of students:**

- spectrophotometer
- light
- meter stick
- vortex
- hot block

Problems, suggestions from 1995:

Ran out of 17% sucrose, needed about 500 mL for 10 students

Reaction Mix had an undiluted $A_{600}$ of about 1.9... Should either make less concentrated DCIP solution, or use less in rxn mix.

1:20 chloroplasts were not nearly strong enough. I tested 10 lambdas undiluted, got good reduction (maybe too fast)

Reaction mix made up for the entire class of 10 students:

60 mL   0.1 M $PO_4$ , pH 7.0
60 mL   0.5 M sucrose
14 mL   DCIP 36.3 mg/500 mL

gave OD at 600 nm of around 400.