

***In organello* Protein Synthesis**

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[Abstract] *In organello* protein synthesis method allows the analysis of mitochondrial translation products. The principle of this method relies on incubation of isolated intact mitochondria with radiolabeled amino acids such as ³⁵S methionine. After protein synthesis, the radiolabeled translation products are subsequently separated by SDS polyacrylamide gel electrophoresis and analysed by autoradiography. For *in organello* analysis of protein synthesis, the isolated intact mitochondria must retain their bioenergetics capacity, and in consequence be fully functional and able to perform coupled respiration. This in turn requires a quick and gentle purification of mitochondria during their isolation.

Materials and Reagents

1. Youngest leaves harvested from 9- to 10-week-old *Arabidopsis thaliana*
2. Common chemicals
Klorin, NaCl, Tween-20, sucrose, tetrasodiumpyrophosphate, PVP-40, EDTA, KH₂PO₄, sodium ascorbate, L-cysteine, TES, BSA, GTP, mannitol, KCl, DTT, HEPES, MgCl₂, sodium acetate, ADP, malic acid, pyruvate, puromycin, L-methionine, isopropanol, acetic acid, Commassie Blue G-250, glycerol, SDS, Tris, β-mercaptoethanol, bromophenol blue
3. Sand (50-70 mesh particle size) (Sigma-Aldrich, catalog number: 274739)
4. Percoll (pH 8.5-9.5) (25 °C) (Sigma-Aldrich, catalog number: P1644)
5. L-³⁵S methionine (HARTMANN ANALYTIC, catalog number: SRM-01H)
6. Amino acid mixture without L-methionine (Promega corporation, catalog number: L9961)
7. DC Protein Assay Kit (Bio-Rad Laboratories, catalog number: 500-0112)
8. 12% SDS-polyacrylamide gel (Leammli electrophoresis system)
9. 10% chlorox solution (see Recipes)
10. Grinding medium (see Recipes)
11. 2x wash buffer (see Recipes)
12. Synthesis mix (see Recipes)
13. Stop solution (see Recipes)
14. Isopropanol fixing solution (see Recipes)
15. Rapid Coomassie Blue G-250 staining solution (see Recipes)

16. Destaining solution (see Recipes)
17. 1x solubilization buffer (see Recipes)

Equipment

1. Mortar
2. Miracloth (Calbiochem®, catalog number: 475855-1R)
3. Polycarbonate centrifuge tubes with round bottom (30 ml and 90 ml)
4. 1.5 ml microcentrifuge tubes (SARSTEDT AG, catalog number: 72.690.001)
5. Paint brushes
6. Tubes with round bottom (SARSTEDT AG, catalog number: 55.484.001)
7. Pasteur pipette
8. Gradient former model 485 (Bio-Rad Laboratories, catalog number: 165-4120)
9. Peristaltic pump –PumpP-1 (GE Healthcare, catalog number: 18-111--91)
10. Centrifuges 1K15 and 3K18 (Sigma-Aldrich)
11. Microcentrifuges (Eppendorf, catalog number: 5452 000.018)
12. Incubator shaker (IKA KS4000i control shaker, catalog number: 3510001)
13. Spectrophotometer UV-1800 (Schimadzu, catalog number: 206-25400-32)
14. SDS-PAGE system - Mini-Protean Tetra Cell (Bio-Rad Laboratories, catalog number: 165-8000)
15. Slab Gel dryer SGD5040 (Thermo Fisher Scientific, catalog number: SGD5040-230)
16. Radioactive room
17. Carestream Kodak BioMax MR film (Sigma-Aldrich, catalog number: Z353949-50EA)

Procedure

- A. Isolation of mitochondria from leaves of *Arabidopsis* by a modification of the method described by Lister *et al.* (2007)

Note: All subsequent steps perform at 4 °C in sterile conditions.

1. Prepare 10% chlorox solution and store in cold room.
2. Immediately prior to isolation, freshly add the following ingredients to previously prepared (see Recipe 2 below) Grinding medium and 2x wash buffer.

Grinding medium	300 ml
1% (w/v) BSA	3.00 g
18 mM sodium ascorbate	1.06 g
20 mM L-cysteine	0.74 g
2 x Wash buffer	150 ml

0.2% (w/v) BSA 0.30 g

The remaining 50 ml of prepared 2x wash buffer (see Recipe 3 below) leave without BSA.

Put these buffers in the cold room.

3. Prepare heavy and light gradient solutions (35 ml). These recipes are sufficient for 4 gradient tubes.

HEAVY (4.4% PVP-40)

2x wash buffer 17.5 ml

Percoll 9.8 ml

20% (w/v) PVP-40 7.7 ml

LIGHT (0% PVP-40)

2x wash buffer 17.5 ml

Percoll 9.8 ml

MiliQ water 7.7 ml

Put these solutions in the cold room.

4. Immediately after making a heavy and light gradient solutions prepare 1x wash buffer with BSA. For this purpose mix the residual volume of 2x wash buffer with BSA with the same volume of MiliQ water to obtain 1x wash buffer with BSA. Store this buffer in cold room.
5. Weigh 25 g youngest leaves harvested from 9- to 10-week-old *Arabidopsis thaliana* growing under short-day conditions (SD, 22 °C) per sample.
6. Sterilize leaves for 5 min in 10% cold chlorox solution. Then rinse the leaves 2-3 times for 1 min in cold sterile MiliQ water. Store leaves on ice, in dark.
Note: Store leaves in dark avoid activation of photosynthesis inside chloroplasts (which can hamper the isolation of purified mitochondria).
7. Put the leaves in 100 ml Grinding medium and cut the leaves with scissors. Then add 2.5 g of sterile sand (50-70 mesh particle size) and grind leaves energetically in a mortar for 30 sec.
8. Filter homogenate through four layers of Miracloth into a conical flask.
9. Transfer filtered homogenate (volume of Grinding medium + volume of grinding tissue) to 2 pre-chilled plastic centrifuge tubes (volume of tubes 90 ml).
10. Centrifuge for 5 min at 2,450 x g at 4 °C → pellets cell debris and nuclei (general scheme of isolation mitochondria is presented in Figure 1).

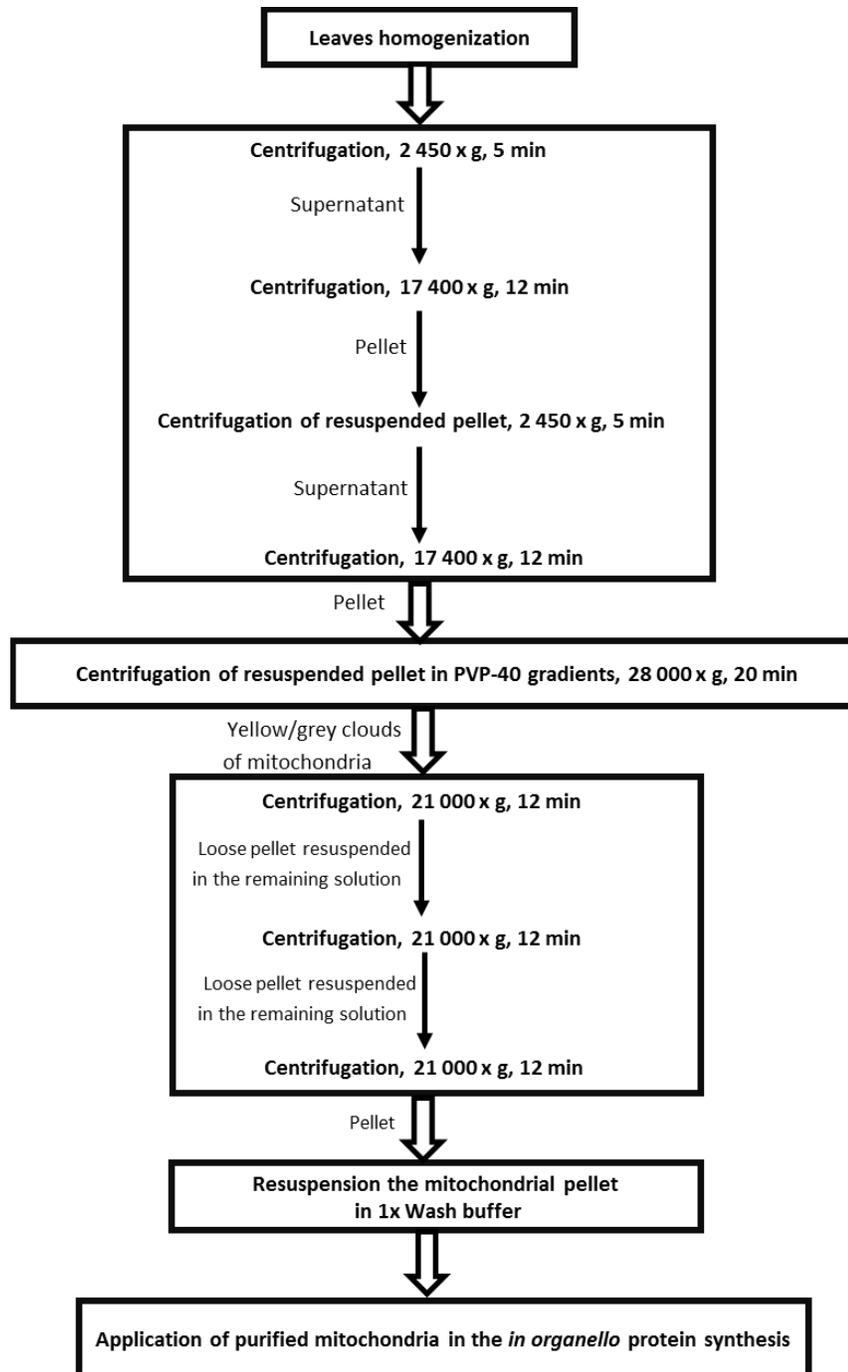


Figure 1. Scheme for the isolation of mitochondria from leaves of *Arabidopsis thaliana*

11. Transfer supernatant to new tubes (volume of tubes 90 ml) and centrifuge for 12 min at 17,400 x g at 4 °C → pellets mitochondria, peroxisomes, etc.
12. Discard supernatant and resuspend pellets in residual supernatant using a pre-wet with Grinding medium small paintbrush. Gently mix the residual supernatant touching with use

- of paintbrush the bottom of the tubes (volume of tubes 90 ml) to completely dissolve the pellet of mitochondria.
13. Fill the tubes with 1x wash buffer with BSA and repeat centrifuge from steps 10-11 in order to pellet purified mitochondria.
 14. In the meantime make PVP-40 gradients in pre-chilled centrifuge tubes (volume of tubes 30 ml) using a gradient former model 485 (see Figure 2).

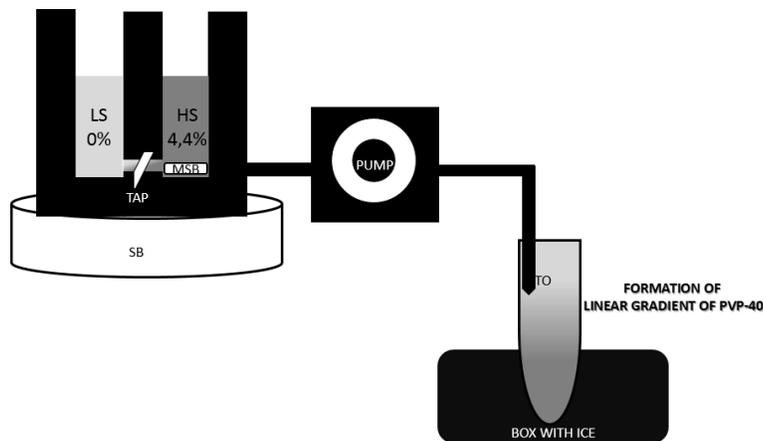


Figure 2. Preparation of linear PVP-40 gradient with a gradient former. Set up tubes on ice and tape outflow tubes (TO) to the inside of the tubes. Close off the connection (TAP) between chambers. Pour 8,75 ml light gradient solution into chamber without tubing outlet. Pour 8,75 ml heavy gradient solution into chamber with tubing outlet and place a small magnetic stir bar (MSB) in this chamber, place gradient former on stirring block (SB). A magnetic stir bar should rotate at a speed sufficient to ensure complete mixing. Set peristaltic pump and allow for a moment only heavy solution to run. Open connection (TAP) between the chambers and allow solution to mix. It is important that the end of the outflow tube (TO) is held below the level of the gradient former so that solution will flow by gravity to down the side of a tubes held below the apparatus.

15. After centrifugation from step 13 discard supernatant and resuspend pellets in 1.75 ml of 1x wash buffer with BSA using a small paintbrush. Combine the two resuspended pellets of the same type to one tube.
16. Load sample carefully on PVP-40 gradient.
17. Balance tubes and centrifuge for 20 min at 28,000 $\times g$ at 4 °C with the brakes off → separation of mitochondria from chloroplasts and thylakoids.
18. After centrifugation mitochondria should form a light yellow/grey clouds at the bottom of the tubes. Carefully remove and discard the layer above the mitochondrial fraction by aspiration with a pasteur pipette.

19. Collect mitochondrial fraction with use of truncated tip to a six 1.5 ml Eppendorf tubes filling with 1 ml of 1x wash buffer with BSA. To each tube collect 2 x 200 μ l of mitochondrial fractions.

Note: Use of truncated tips allow to collect dense and cloudy mitochondrial fractions.

20. Centrifuge for 12 min at 21,000 x g at 4 °C.

21. Remove supernatant carefully by pipette. Pellet might sit very loose.

22. Resuspend gently pellets in all Eppendorf tubes in the remaining medium by snapping the tube. Combine the resuspended pellets of the same type to two arbitrarily chosen tubes.

23. Fill these two tubes with 1x wash buffer without BSA and centrifuge for 12 min at 21,000 x g at 4 °C.

24. Remove supernatant and resuspend pellets in the remaining medium by gently snapping the tube. Combine the two resuspended pellets of the same type to one tube.

Note: The purpose of this step was purification of isolated mitochondria from Percoll. First the washes in more Eppendorf tubes allowed to discard the remaining contamination from gradients. In the next centrifugation steps the number of Eppendorf tubes were reduced to the one, containing purified mitochondria.

25. Fill final tube with 1x wash buffer without BSA-centrifuge for 12 min at 21,000 x g at 4 °C.

26. Remove supernatant and resuspended mitochondria in 30 μ l of 1x wash buffer without BSA.

27. Keep mitochondrial fraction on ice to use immediately in the *in organello* protein synthesis.

B. Protein assay

Determine the mitochondrial protein levels by DC Protein Assay Kit II, follow the instructions provided by the manufacturer. Estimate the protein concentrations for two independent amount of sample by comparison to a standard curve generated by the measurement of BSA of known concentrations. Using standard procedure, the assay is used with samples having protein concentrations between 0.2 and 1.5 mg/ml. The concentrations of mitochondrial proteins which we obtained are between 20-25 μ g/ μ l (mitochondrial fraction dissolved in 30 μ l of 1x wash buffer).

C. *In organello* protein synthesis

For each mitochondrial preparation, set up two labeling reactions: one for the synthesis mix and one for control synthesis mix, to estimate the bacterial contamination. Use sodium acetate in control reactions instead of malic acid and pyruvate. Sodium acetate is a

nonoxidizable substrate that can be utilized by bacteria but not by mitochondria, hence amino acid incorporation in its presence gives an indication of bacterial contamination.

1. Prepare pre-chilled disposable, sterile 3.5 ml tubes with round bottom .
2. Add 25 μ l amino acids (without L-methionine) and 1 mg BSA to 1 ml of Synthesis Mix with malic acid and pyruvate or 1 ml of Control Synthesis Mix with sodium acetate. BSA protects the freshly isolated mitochondria. Keep on ice.
3. Pipette to the pre-chilled tubes 100 μ l of Synthesis Mix or 100 μ l Control Synthesis Mix.
4. Add to each tubes 150 μ g of freshly isolated mitochondrial fractions.
5. Add 30 μ Ci 35 S L-methionine -1.5 μ l (20 mCi/ml) per sample and incubate at 25 $^{\circ}$ C for 30 or 60 min in incubator shaker (speed 180 rpm).

Note: During the assay keep the mitochondria well oxygenated using a shaker, to avoid the risk of anoxia due to their sedimentation to the bottom of the tube. In our experience, increasing the incubation times beyond 60 min does not increase the labeling of mitochondrial products.

6. Following incubation reactions are stopped by addition 350 μ l of ice cold 1x Wash buffer without BSA containing 10 mM unlabelled L-methionine and puromycin (50 μ g/ml).
7. Mix using pipette and transfer sample to new 1.5 ml Eppendorf tubes. Keep on ice.
8. Pellet the mitochondrial fractions at 21,000 x g for 5 min in centrifuge at 4 $^{\circ}$ C.
9. Remove the supernatant (containing the free 35 S-methionine) and resuspend the mitochondrial pellet in 30 μ l of 1x wash buffer without BSA.
10. Mitochondria may be frozen on dry ice and stored at -80 $^{\circ}$ C or resuspended in 20-30 μ l of electrophoresis sample buffer for immediate SDS-PAGE analysis (see D).

D. SDS-Polyacrylamide gel electrophoresis and autoradiography

1. Solubilize entire mitochondrial sample in a 1x solubilization buffer by heating for 5 min at 95 $^{\circ}$ C.
2. Analyse the samples on 12% SDS-polyacrylamide gel (Leammli electrophoresis system).
3. After electrophoresis gently agitate gel on a platform shaker in isopropanol fixing solution for 15 min.
4. Discard fixing solution and gently agitate gel in Rapid Commasie Blue G-250 staining solution for 30 min.
5. Remove staining solution and gently agitate gel in destaining solution for 40-60 min until a clear background with blue protein bands appears.
6. Agitate gel in water 3 times for 5 min.
7. After rinsing agitate gel in water containing 5% (v/v) of glycerol for 7 min. Agitation the gel in water containing glycerol may protect gel before cracking during drying.

8. Dry gel onto a Whatman 3 MM paper using gel dryer, and expose for minimum 3 days to a Kodak BioMax MR film.

Representative data

1. The example of autoradiogram of proteins synthesized *in organello* in *Arabidopsis* mitochondria is presented in below figure (Figure 3).

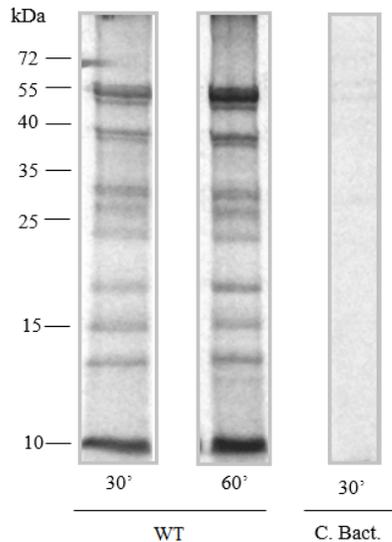


Figure 3. *In organello* protein synthesis. Fragments of autoradiogram of proteins synthesized *in organello* for 30 and 60 min by wild-type mitochondria isolated from *Arabidopsis* (Kwasniak *et al.*, 2013). After protein synthesis, 25 μ g of mitochondria were fractionated on 12% (w/v) SDS-PAGE. The lane marked C. Bact. is a specific control performed in the presence of a sodium acetate- substrate for bacterial translation.

Recipes

Note: All the buffers and solutions are prepared with double-distilled water and sterilized by autoclave.

1. 10% chlorox solution (1 L)

Klorin	100 ml
NaCl	10 g
Tween-20	15 drops
2. Grinding medium (300 ml)

0.3 M sucrose	30.80 g
25 mM tetrasodiumpyrophosphate (anhydrous)	1.99 g

- | | |
|---------------------------------------|--------|
| 2 mM EDTA (disodium salt) | 0.22 g |
| 10 mM KH ₂ PO ₄ | 0.40 g |
| 1% (w/v) PVP-40 | 3.00 g |
- Dissolve well in ~250 ml of MiliQ water, adjust pH to 7.5 with HCl and then make up to 300 ml with MiliQ water.
3. 2x wash buffer (200 ml)
- | | |
|---------------|--------|
| 0.6 M sucrose | 41.0 g |
| 20 mM TES | 0.9 g |
- Dissolve well in ~150 ml of MiliQ water, adjust pH to 7.5 with NaOH and then make up to 200 ml with MiliQ water.
4. Synthesis mix (20 ml)
- | | |
|--------------------------------------|------------|
| 5 mM KH ₂ PO ₄ | 13.61 mg |
| 2 mM GTPNa | 20.93 mg |
| 0.4 M mannitol | 1457.00 mg |
| 60 mM KCl | 89.46 mg |
| 2 mM DTT | 6.17 mg |
| 50 mM HEPES | 238.31 mg |
| 10 mM MgCl ₂ | 40.66 mg |
| 4 mM ADP (K) | 40.10 mg |
- pH 7.0
- Mix solution and divide to
- a. 15 ml synthesis mix → add
- | | |
|--------------------------------------|-----------|
| 10 mM malic acid | 26.71 mg |
| 1 mM pyruvate (Na) | 1.65 mg |
| Filter sterilize (0.22 μm pore size) | 15 x 1 ml |
| Stored at -80 °C | |
- b. 5 ml control synthesis mix → add
- | | |
|--------------------------------------|----------|
| 20 mM Na-sodium acetate | 8.20 mg |
| Filter sterilize (0.22 μm pore size) | 5 x 1 ml |
| Stored at -80 °C | |
5. Stop solution (50 ml)
- 10 mM L-methionine (0.0746 g) dissolved in 50 ml of 1x wash buffer without BSA
6. Isopropanol fixing solution (1 L)
- | | |
|-------------|--------|
| Isopropanol | 250 ml |
| Acetic acid | 100 ml |
| Water | 650 ml |
7. Rapid Coomassie Blue G-250 staining solution (1 L)

Acetic acid	100 ml
Water	900 ml
Commassie brilliant blue G-250	60 mg
8. Destaining solution (1 L)	
Acetic acid	100 ml
Water	900 ml
9. 1x solubilization buffer	
2% (v/v) sodium dodecyl sulfate (SDS)	
10% glycerol	
62.5 mM Tris-HCl (pH 6.8)	
0.002 % (w/v) bromophenol blue	
5% β -mercaptoethanol	

Note: Indicated concentrations of components of solubilization buffer are final concentrations.

Acknowledgments

Functional mitochondria for *in organello* protein synthesis were isolated from leaves of Arabidopsis by a modification of the methods described by Lister *et al.*, 2007 and Millar *et al.*, 2007. *In organello* protein synthesis was performed for the first time on mitochondria isolated from leaves of Arabidopsis thaliana as described in the paper Kwasniak *et al.*, 2013, and was based on previously published papers including Boutry *et al.*, 1984. We are grateful to Christopher J. Leaver for several highly relevant papers that allow us to know details about the background of the *in organello* protein synthesis procedure.

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