# $^{35}S$ pulse Labelling of *Chlamydomonas* Chloroplast Proteins

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**[Abstract]** $^{35}S$ pulse labelling of proteins is used to attach a radioactive label to newly synthesized proteins, as sulfur is an element that is mainly present in proteins (Fleischmann and Rochaix 1999). Depending on your organism's uptake mechanisms you need cysteine, methionine or sulfuric acid as a source of radioactive sulfur. This example uses *Chlamydomonas* cells and $H_2{}^{35}SO_4$ (Schwarz *et al*., 2012).

## Materials and Reagents

1. Strain of interest
2. Control strains lacking the gene for the proteins of interest (as a negative control)
3. Tris (Applichem, catalog number: A1379)
4. Ammonium chloride (Carl Roth, catalog number: 5470)
5. Magnesium chloride (Carl Roth, catalog number: KK36)
6. Calcium chloride (Merck, catalog number: 1023780500)
7. $K_2HPO_4$ (Applichem, catalog number: A1363)
8. $KH_2PO_4$ (Applichem, catalog number: A1364)
9. $Na_2EDTA$ (Carl Roth, catalog number: 8043)
10. $ZnSO_4 \cdot 7H_2O$ (Carl Roth, catalog number: T884)
11. $H_3BO_3$ (Carl Roth, catalog number: P010)
12. $MnCl_2 \cdot 4H_2O$ (Carl Roth, catalog number: 0276)
13. $FeSO_4 \cdot 7H_2O$ (Carl Roth, catalog number: P015)
14. $CoCl_2 \cdot 6H_2O$ (Carl Roth, catalog number: T889)
15. $CuSO_4 \cdot 5H_2O$ (Carl Roth, catalog number: 8175)
16. $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (Carl Roth, catalog number: 3666)
17. HEPES (Carl Roth, catalog number: HN78)
18. Tricine (Carl Roth, catalog number: 6977)
19. Methanol (Applichem, catalog number: A3493)
20. Cycloheximide (Carl Roth, catalog number: 8682)
21. $^{35}S$ sulfuric acid (Hartmann Analytic, catalog number: S-RA-1)
22. Liquid nitrogen (Linde, inquire)

23. Protease inhibitor cocktail (F. Hoffmann-La Roche, catalog number: 04693159001)
24. TAP-B (see Recipes)
25. TAP-B/T(see Recipes)
26. Hutner trace elements (see Recipes)
27. Buffer A (see Recipes)
28. Buffer B (see Recipes)

## Equipment

1. Sterile Erlenmeyer flasks (Brand KG)
2. Photometer (GE Healthcare)
3. Hemocytometer (Brand KG)
4. Microscope (≥ 400x magnification, Leica)
5. Reaction tubes (Sarstedt)
6. Screw cap microreaction tubes (Sarstedt)
7. Microreaction tube centrifuge with cooling capacity (Eppendorf)
8. Gel dryer (Bio-Rad Laboratories)
9. Whatman filter paper (GE Healthcare)

## Procedure

A. Sulfur deprivation
1. Grow your strain of interest and the control strains at 23 °C to the early log phase (cell density < $2 \times 10^6$ cells/ml).
2. Spin down your cells (5 min, 1,000 *x g*, RT) in sterile 50 ml reaction tube and resuspend carefully in 10 ml TAP-B, centrifuge again (5 min, 1,000 *x g*, RT) and resuspend the cells in 10 ml TAP-B.
3. Transfer cells to a sterile 25 ml Erlenmeyer flask (it is best to set up the medium in this flasks and put the resuspended cells back in the same flask), shake for 16 h at 23 °C in medium light (30 - 50 $\mu mol/m^2/s$).
4. Spin down your cells (5 min, 1,000 *x g*, RT) and resuspend carefully in 10 ml TAP-B/T.
5. Centrifuge again (5 min, 1,000 *x g*, RT) and resuspend the cells in exactly 10 ml TAP-B/T, transfer cells back to 25 ml Erlenmeyer flask.
6. Agitate cells for 2 h in the dark (wrap flasks with aluminium foil or cover with cardboard box).

B. Adjustment of cells to the same amount of chlorophyll/cells

1. Transfer 0.5 ml of the cultures to a microreaction tube (keep the remaining culture shaking in the dark).
2. Centrifuge tube (2 min, 20,000 *x g*, 4 °C) and discard supernatant, resuspend pellet thoroughly in 1 ml methanol.
3. Centrifuge again (1 min, 20,000 *x g*, 4 °C) and use supernatant for chlorophyll measurement at 652 nm, dilute with methanol (prechilling not necessary] if optical density is higher than 1 (do not forget to adjust your calculation for that dilution factor).
4. Calculate chlorophyll content:

$$\frac{OD_{652} \times 2}{34{,}5} \times 1000 \times \text{dilution factor} = \mu g\ Chl/ml$$

5. Adjust chlorophyll content with TAP-B/T to 80 µg $ml^{-1}$:

$$\text{Total Chlorophyll } [\mu g] = \text{total Volume} \times \mu g\ Chl/ml$$

$$\frac{1\ ml \times \text{total chlorophyll } [\mu g]}{80\ \mu g} = \text{ml for resuspension of your cells}$$

6. Centrifuge cells (5 min, 1,000 *x g*, RT) and resuspend carefully in calculated volume of TAP-B/T to get 80 µg Chl/ml.
7. If your cells are lacking chlorophyll better adjust the cell number than the chlorophyll amount, prepare a 1:10 dilution of cultures and count the cells (hemocytometer, microscope), spin down cells and adjust cell number to $7.25 \times 10^7$ cells/ml (that corresponds to ~80 µg Chlorophyll/ml if using a green culture).

C. Pulse

1. Add 25 µl of cycloheximide stock solution (100 µg/ml, final: 10 µg/ml) per culture to a screw cap tube (cycloheximide is for inhibition of cytosolic protein synthesis).
2. Take your cultures and prepared tubes and go to fume hood in the radioactive lab (don't inhale $^{35}S$! Release of radioactive gaseous $SO_2$).
3. Add 225 µl of each culture to the cycloheximide.
4. Incubate for 10' in rotary shaker in the dark (covered with cardboard box).
5. Add 12.5 µl of $^{35}S$ ($H_2{}^{35}SO_4$, 10 mCi/ml).
6. Light pulse: incubate for 5-20 min in front of a appropriate light source and agitate cells occasionally.
7. Spin down cells, discard supernatant (radioactive waste) and freeze the tube with the pellet in liquid nitrogen for at least 5 min to stop cellular activity.

D. Cell lysis

1. Thaw your cells on ice and keep them on ice from now on.
2. Add 200 µl of buffer A and break the cells by pipetting up and down for ~ 1 min or sonication (three times 5 pulses at 50% output with 30'' pauses in between).
3. Remove soluble material: spin down membranes at 20,000 *x g* for 25 min at 4 °C, discard supernatant into radioactive waste.
4. Resuspend membrane pellet with 100 µl of buffer B.

E. Protein electrophoresis

Use a protocol for Laemmli-SDS-PAGE and adjust conditions for your protein depending on its molecular weight, *e.g.* a 6 M urea-16% polyacrylamide-SDS-PAGE gel for separation of the photosystem II reaction center proteins D1 (encoded by the *psbA* gene) and D2 (encoded by the *psbD* gene).

F. Coomassie staining and drying of gel

1. Use a protocol for Coomassie Blue staining (to visualize size marker and lanes).
2. Put gel (upside down) on plastic tray, put Whatman paper (moistened with water) on top, flip over (Figure 1).
3. Place Whatman paper and gel on top of two more layers of Whatman paper, cover with plastic foil and dry in gel dryer.

G. Autoradiography

1. Place phosphor imaging screen on top of your gel and expose for 1-3 days (or use X-ray film for longer time).
2. Scan screen / develop X-ray film.
3. Compare bands in your strains of interest with negative controls to identify affected protein, e.g. the band for the photosystem II reaction center protein D1 (encoded by the *psbA* gene) is missing in the investigated mutants as in the *psbA* mutant *FuD7* in Figure 5 of (Morais *et al.,* 1998).

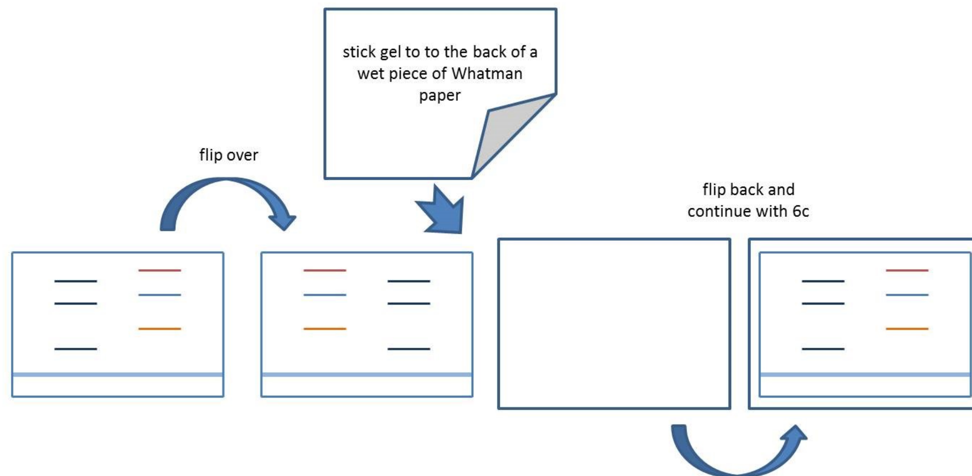


**Figure 1. Scheme of step (VI-2)**

## Recipes

1. TAP-B
   20 mM Tris
   7.5 mM ammonium chloride
   0.805 mM magnesium chloride
   0.34 mM calcium chloride
   0.537 mM $K_2HPO_4$
   0.463 mM $KH_2PO_4$
   0.1% Hutner trace elements
   Adjust to pH 7 with acetic acid
2. TAP-B/T
   20 mM tris
   7.5 mM ammonium chloride
   0.805 mM magnesium chloride
   0.34 mM calcium chloride
   0.537 mM $K_2HPO_4$
   0.463 mM $KH_2PO_4$
   Adjust to pH 7 with acetic acid
3. Hutner trace elements (Hill and Kafer, 2001)
   50 g $Na_2EDTA \cdot 2H_2O$
   22 g $ZnSO_4 \cdot 7H_2O$
   11.4 g $H_3BO_3$
   5 g $MnCl_2 \cdot 4H_2O$

5 g $FeSO_4 \cdot 7H_2O$
1.6 g $CoCl_2 \cdot 6H_2O$
1.6 g $CuSO_4 \cdot 5H_2O$
1.1 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$
Fill up with $dH_2O$ to 1,000 ml

4. Buffer A
   10 mM EDTA
   10 mM HEPES (pH 7.8)
   Protease inhibitor cocktail (according to the manufacturer's instructions)
5. Buffer B
   10 mM EDTA
   10 mM tricine (pH 7.8)
   Protease inhibitor cocktail (according to the manufacturer's instructions)

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## References

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