

## Radiolabeling of Chlorophyll by [<sup>14</sup>C]Glutamic Acid *in vivo* and Relative Quantification of Labeled Chlorophyll by Using Thin Layer Chromatography (TLC)

Roman Sobotka<sup>1\*</sup>, Luděk Kořený<sup>2</sup>, Jana Kopečná<sup>2</sup> and Miroslav Oborník<sup>2</sup>

<sup>1</sup>Department of Phototrophic Microorganisms, Institute of Microbiology ASCR, Trebon, Czech Republic; <sup>2</sup>Department of Molecular Parasitology, Institute of Parasitology, Biology Centre ASCR, Ceske Budejovice, Czech Republic

\*For correspondence: [sobotka@alga.cz](mailto:sobotka@alga.cz)

**[Abstract]** This is an accurate method to assess the rate of chlorophyll biosynthesis *in vivo* in cyanobacteria. Given that labeled glutamate is used as the very early precursor of chlorophyll together with a short pulse of labeling (30 min), this method provides information about the metabolic flow through the whole chlorophyll biosynthetic pathway on a short timescale.

### **Materials and Reagents**

1. *Synechocystis* PCC 6803
2. Glutamic acid [U-C<sup>14</sup>] (ARC 0165A, American Radiolabeled Chemicals) ([<sup>14</sup>C]Glu)
3. Methanol
4. 25% ammonia solution
5. 1 M NaCl
6. Hexane
7. 10% KOH
8. Petroleum ether
9. Chloroform
10. 1 M Na<sub>2</sub>HPO<sub>4</sub>

11. 1 M NaH<sub>2</sub>PO<sub>4</sub>
12. X-ray film (Eastman Kodak Company)
13. NH<sub>4</sub>OH
14. 1 M TES (pH 8.2)  
[2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethanesulfonic acid]/NaOH
15. Growth medium BG11

### **Equipment**

1. 10 ml Headspace vials (Sigma-Aldrich)
2. Water bath shaker
3. 2 ml o-ring cap tubes
4. Glass beads (100-200 μm)
5. Vortex
6. Tabletop centrifuge (MiniSpin plus, Eppendorf)
7. Speedvac Concentrator plus (Eppendorf)
8. Silica gel TLC plate (SIL G-25, MACHEREY-NAGEL)
9. Rectangular TLC developing tank (Sigma-Aldrich)
10. Mikro 22R centrifuge (Hettich)
11. MiniSpin centrifuge (Eppendorf)

### **Procedure**

1. Labeling requires a 25-ml culture of cyanobacterial cells at the exponential phase of growth. Protocol works very well for the cyanobacterium *Synechocystis* PCC 6803 grown at 30 °C in growth medium BG11 (Rippka *et al.*, 1979) to optical density at 750 nm~0.4.

- Harvest cells by centrifuging at 3,500 x g for 5 min at room temperature using brake 5 (Mikro 22R centrifuge).
2. Discard supernatant and resuspend cell pellet in 2 ml of growth medium BG11, supplemented by 20 mM TES (pH 8.2). Transfer cells into a 2 ml tube and centrifuge at 3,500 x g for 2 min at room temperature (MiniSpin centrifuge).
  3. Discard supernatant and resuspend cell pellet in 450 µl of growth medium/20 mM TES. Transfer cells into a glass vial.
  4. Incubate cells in a water bath shaker for 30 min at 100 rpm under light and temperature conditions you prefer for your experiment.
  5. Add 180 µM of [<sup>14</sup>C]Glu dissolved in growth medium. Incubate under the same conditions for another 30 min.
  6. Transfer the labeled culture into a 2 ml o-ring cap tube. Spin down the cells at 7,000 x g for 2 min at room temperature and discard supernatant. At this point, the cell pellet can be frozen in liquid nitrogen and stored at -70 °C or used immediately for following pigment extraction.
  7. Resuspend cells in 1 ml of H<sub>2</sub>O and pellet cells at 7,000 x g for 2 min at room temperature. Discard water and repeat wash twice by 1 ml of H<sub>2</sub>O to remove all traces of labeled [<sup>14</sup>C]Glu. Resuspend cells in 1 ml of methanol/0.2% NH<sub>4</sub>OH. Add 50 µl of glass beads and vortex for 1 min to facilitate pigment extraction. Spin down 4 min at max rpm. Work under a dim light for all following steps
  8. Transfer supernatant (~1 ml) into 2 ml tube and add another 300 µl of methanol/0.2% NH<sub>4</sub>OH to cells, vortex and spin down again. Combine supernatants and add 140 µl of 1 M NaCl.
  9. Add 400 µl of hexane, vortex and spin down 30 sec at max rpm to accelerate phase separation. Collect upper phase containing chlorophyll. Repeat step 9 three times and combine all hexane into a 2 ml tube.
  10. Evaporate hexane using SpeedVac concentrator set to V-AL and 30 °C for 5 min.

11. Resuspend the pellet in 190  $\mu$ l of methanol and add 10  $\mu$ l of 10% KOH. Incubate at room temperature for 15 min to convert chlorophyll into phytol-less Mg-chlorin.
12. Extract this solution by 200  $\mu$ l of hexane and discard upper phase, repeat 4-times.
13. Transfer remaining ~150  $\mu$ l of the methanol phase into a new 0.5 ml tube. Evaporate this solution using a SpeedVac concentrator to final volume of 30-50  $\mu$ l.
14. Extract this solution 5 times by 150  $\mu$ l of petroleum ether and discard upper phase containing carotenoids.
15. Evaporate completely at 30 °C for 30 min and resuspend pellet in 30  $\mu$ l of methanol: chloroform (1:1). Load 10  $\mu$ l on the TLC plate. 5  $\mu$ l can be used for measurement using scintillation counter (see next step).
16. Load 10  $\mu$ l of pigment solution on a silica gel TLC plate. Place the plate in a TLC developing tank with 300 ml of mobile phase – methanol: 10 mM  $\text{Na}_x\text{H}_y\text{PO}_4$ , pH 6.8 (3: 1, v/v). Remove the plate from the tank after 30 min of incubation and dry it at 37 °C for 10 min. Expose the plate to X-ray film for 3-5 days and develop. After development only a one signal should be detected corresponding to Mg-chlorin 'green' band on TLC.

*Note: An alternative to TLC followed by detection on an X-ray film is to use a scintillation counter and measure directly radioactivity in the final pigment fraction (5  $\mu$ l). The later method is faster; however it is less sensitive and also accurate due to presence of  $^{14}\text{C}$  labeled impurities.*

### **Acknowledgments**

We acknowledge the financial support from the project Algatech (CZ.1.05/2.1.00/03.0110) and Czech Science Foundation, project no. P506/12/1522.

---

**References**

1. Koreny, L., Sobotka, R., Janouskovec, J., Keeling, P. J. and Obornik, M. (2011). [Tetrapyrrole synthesis of photosynthetic chromerids is likely homologous to the unusual pathway of apicomplexan parasites](#). *Plant Cell* 23(9): 3454-3462.
2. Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M. and Stanier, R. Y. (1979). [Generic assignments, strain histories and properties of pure cultures of cyanobacteria](#). *J Gen Microbiol* 111(1): 51-61.