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

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[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-C14] (ARC 0165A, American Radiolabeled Chemicals) ([^{14}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_2HPO_4

11. 1 M NaH_2PO_4
12. X-ray film (Eastman Kodak Company)
13. NH_4OH
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 [^{14}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_2O 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_2O to remove all traced of labeled [^{14}C]Glu. Resuspend cells in 1 ml of methanol/0.2% NH_4OH. 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_4OH 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 µl of methanol and add 10 µl of 10% KOH. Incubate at room temperature for 15 min to convert chlorophyll into phytol-less Mg-chlorin.
12. Extract this solution by 200 µl of hexane and discard upper phase, repeat 4-times.
13. Transfer remaining ~150 µl of the methanol phase into a new 0.5 ml tube. Evaporate this solution using a SpeedVac concentrator to final volume of 30-50 µl.
14. Extract this solution 5 times by 150 µl of petroleum ether and discard upper phase containing carotenoids.
15. Evaporate completely at 30 °C for 30 min and resuspend pellet in 30 µl of methanol: chloroform (1:1). Load 10 µl on the TLC plate. 5 µl can be used for measurement using scintillation counter (see next step).
16. Load 10 µl 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 $Na_XH_YPO_4$, 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 µl). The later method is faster; however it is less sensitive and also accurate due to presence of ^{14}C labeled impurities.

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References

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