

[¹⁴C] Glucose Cell Wall Incorporation Assay for the Estimation of Cellulose Biosynthesis

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[Abstract] Cellulose is synthesized by Cellulose Synthase A proteins at the plasma membrane using the substrate UDP glucose. Herein, we provide a detailed method for measuring the incorporation of radiolabeled glucose into the cellulose fraction of the cell wall. In this method *Arabidopsis* seedlings are treated for 2 h with a cellulose biosynthesis inhibitor in the presence of radiolabeled glucose, and are subsequently boiled in acetic-nitric acid to solubilize non-cellulosic material. The radiolabeled glucose detected in the insoluble fraction indicates the amount of cellulose synthesized during the experimental timeframe. The short-term nature of this method is a useful tool in determining if inhibition of cellulose biosynthesis is the herbicides primary mode of action.

Materials and Reagents

1. *Arabidopsis* seed (*Arabidopsis thaliana* L.)
2. Dextrose (Sigma-Aldrich, catalog number: D9434)
3. Radiolabeled glucose [glucose D-¹⁴C(U)] (American Radiolabeled Chemicals, catalog number: ARC 0122D)
4. Acetic Acid, Glacial (Certified ACS) (Fisher Scientific, catalog number: A38-212)
5. Nitric Acid, Fuming (Certified ACS) (Fisher Scientific, catalog number: A202-212)
6. Counting cocktail (Biodegradable Scintillation Cocktails) (Biosafe II, catalog number: 111195)
7. Deionized sterile water (autoclaved at 120 °C for 30 min)
8. Murashige & Skoog (MS) Basal Salt Mixture (*PhytoTechnology Laboratories*[®], catalog number: M524)
9. MES monohydrate (Sigma-Aldrich, catalog number: 69889)
10. Model A307 Sample Oxidizer (PerkinElmer, catalog number: A030700)
11. Aluminum foil
12. Eppendorf tubes (1.5 ml) (autoclaved at 120 °C for 30 min)
13. MS media (see Recipes)
14. Acetic-nitric acid reagent (see Recipes)

Equipment

1. Eppendorf tube holders
2. Pasteur pipets and tips
3. 1 L Erlenmeyer flask (autoclaved at 120 °C for 30 min)
4. Microscale
5. Orbital shaker
6. Microcentrifuge
7. Glass scintillation vials (20 ml)
8. Scintillation Counter (Packard Tri-Carb Liquid Scintillation Counter)
9. Autoclave

Procedure

A. Setup (Notes 1 and 2)

1. In a 1.5 ml Eppendorf tube, add *Arabidopsis* seeds (~100 mg of seed = ~ 2 g of seedling weight wet) and surface sterilize. Briefly, in a laminar flow hood, add 1 ml of 30% household bleach solution supplemented with 20 µl of 5% v/v sodium dodecyl sulfate (SDS) and shake for 15 min. Remove the bleach supernatant with the seed settling at the bottom of the tube, then sequentially wash three times with 1 ml of autoclaved deionized water. Remove the final wash solution and aliquot 500 µl of sterilized water into the Eppendorf tube. Transfer the seed to a 4 °C refrigerator and store in darkness for 2 days to promote even germination.
2. Prepare two bottles of liquid MS media (a 250 ml) with and without glucose (2% w/v), respectively. Autoclave in a liquid cycle at 120 °C for 30 min.
3. Add the seed and the cooled and sterilized MS media supplemented with 2% glucose into a pre-sterilized 1 L Erlenmeyer flask.
4. Place and secure the flask on an orbital shaker and agitate (180 rpm) the mixture for 2 h in light to promote germination. Afterwards, wrap the flask with aluminum foil to prevent light penetration. Place the flask back on the shaker and agitate for 72 h. (Note 3).

B. Experimental Procedure

Note: Seedlings need to be kept in the dark from steps B5-8.

5. After 72 h, remove the glucose supplemented media and wash seedlings to remove all traces of glucose (Note 4). A depiction of how the seedlings should look is in Figure 1.



Figure 1. A representation of dark-grown etiolated *Arabidopsis* seedlings that were grown in liquid MS media for 72 h. A square is equal to 1 cm.

6. Add 20 mg (wet weight) worth of etiolated seedling to an Eppendorf tube. Wrap each tube in aluminum foil to prevent light penetration (Notes 5, 6, and 7).

Note: All the following steps should be conducted in an area designed for radioactive experiments. Wear and use the appropriate personal protective equipment. Waste material must be disposed in a proper manner.

7. Make a master mix of glucose free-MS media that is supplement with radiolabeled glucose ($1 \mu\text{Ci ml}^{-1}$ per tube) and the respective drug concentration for each treatment (Notes 8 and 9). For example, say I want to know the inhibitory effects of isoxaben at 100 nM. For 3 replications, I would create a 4x master mix. The master mix would contain 2 ml of glucose free-MS media, 2 μl of isoxaben at 100 μM , and 2 μl of ^{14}C -glucose from the 1 mCi ml^{-1} stock.
8. Transfer 0.5 ml of the master-mix to their respective Eppendorf tube and place in an Eppendorf holder. Cover the holder with aluminum foil and allow seedlings to sit in tubes for 2 h (Note 10). Shake holder every 30 min or agitate if possible.
9. After treatment, remove unincorporated radiolabeled glucose by centrifuging for 10 min at max speed and decant supernatant. Then add 1 ml of water, centrifuged for 10 min at max speed, and decant supernatant (Note 11). Repeat twice.
10. Bring a water bath to boil.
11. Add 0.5 ml of acetic-nitric acid reagent in each tube.
12. Submerge closed tubes in boiling water bath for 30 min.
13. Let samples cool, then centrifuge for 15 min at max speed. Carefully remove 400 μl of supernatant and place into a scintillation vial. This is a representative sample of the soluble cell wall fraction. While removing supernatant make sure not to disturb the pelleted insoluble fraction (Note 12).

14. Next, wash the remaining sample to remove radioactivity that was not incorporated into the insoluble fraction. Wash each sample with 1 ml of water, centrifuged for 10 min at max speed, and carefully discard supernatant. Repeat 2 more times.
15. Re-suspend pelleted material with 1 ml of water and transfer it to a scintillation vial. The insoluble fraction is considered cellulose (Updegraff, 1960) (Note 13).
16. Add 10 and 5 ml of scintillation cocktail to the vials containing the insoluble and soluble material, respectively. Place vials in a scintillation counter and choose appropriate program for ^{14}C to determine disintegrations per minute (dpm). Include a blank containing only the scintillation cocktail to determine the background radioactivity count (Note 14).
17. Analyze the results.

Notes

1. It is important that steps 1 through 4 are done in a sterile environment and all growth media kept sterile.
2. It is extremely important to keep seedlings in the dark until step B11.
3. This timing is important because the hypocotyls are rapidly elongating and require large amounts of glucose.
4. Etiolated seedlings were placed in a seed-cleaning sieve and subsequently washed with deionized water.
5. The amount of seedling-wet weight can change if needed; however a significant quantity of seedlings is needed to take up a statistically relevant amount of radioactivity.
6. It is extremely important to keep your weight similar among all tubes (replication). If not, this will have a significant effect on the amount of radioactivity uptake. If you are so inclined you can present data as dpm gram^{-1} . Each tube is an independent replication within a trial. We suggest at least using 3 analytic replications per treatment and repeat the experiment in time.
7. The majority of excess water was drained and a subset of etiolated seedlings were placed in a petri dish. Three tubes were filled at a time then a new subset of seedlings was used. During this step we relied on the light source from the scale display as are only light source.
8. Greater than $1 \mu\text{Ci ml}^{-1}$ of radiolabeled glucose is required to detect radioactivity in the insoluble fraction over background noise in the scintillation counter ($> 400 \text{ dpm}$).
9. At minimum a 2X rate should be used, however we suggest a saturating 20X rate. X is equal to the rate that reduces hypocotyl or root growth by 50% (GR_{50}) and should be predetermined in a dose response experiment. For example the GR_{50} for isoxaben is $\sim 5 \text{ nM}$ and thus a 100 nM concentration would be used. The compound carrier and

control, DMSO, should not exceed more than 0.01% of the solution. DMSO at higher concentrations can be detrimental to seedling growth.

The 2-hour time point is important in determining if inhibition of cellulose biosynthesis is a direct effect of the drugs mode of action.

10. Important to use water to remove excess radioactivity, as MS media or sulfuric acid does not mix with the counting cocktail solution.
11. 400 μ l was chosen as a representative sample of the soluble fraction because the leftover solution is hard to remove without removing the potential insoluble fraction (cellulose).
12. Transfer can be aided by cutting the tip off of a 1 ml tip and pipette with it.
13. Important that the cocktail be >75% of the mixture otherwise the solution will become cloudy and counting will not be possible. If this happens dilute into another vial and add the count sums.
14. This method is only applicable to 72-hour-old *Arabidopsis* seedlings. To measure the insoluble fraction in thicker tissue, for example grass roots, tissue should be first burned in an oxidizer. The created $^{14}\text{CO}_2$ is captured and rinsed into a vial to be counted in a scintillation counter.

Recipes

1. MS media
 - a. Half MS (Murashige and Skoog) basal salt media (2.32 g/L)
 - b. MES monohydrate (0.6 g/L)
 - c. Adjust pH to 5.7 with 5M KOH
2. Acetic-nitric acid reagent [acetic acid:nitric acid:water (8:1:2)]

Add 15 ml of concentrated nitric acid to 150 ml of 80% acetic acid

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