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$^{14}CO_2$-based assay for measuring Rubisco activity & activation state

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We use this protocol and it's working

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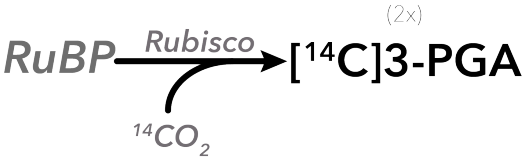
Abstract

The Rubisco activity $^{14}CO_2$-based assay measures the incorporation of $^{14}CO_2$ into the acid-stable product 3-phosphoglycerate (3-PGA). This protocol is based on Parry et al. (1997).



Guidelines

1. Check the "Materials" tab for a list of all the chemicals used in this protocol.
2. In the "Steps" tab, there is a brief description of the materials and equipment necessary for the protocol execution.
3. In the "Steps" tab, there is information on preparation of solutions, procedures for determining Rubisco initial and total activities, and notes to take into consideration to ensure reliable results.
4. The references cited are at the end of the "Materials" tab.

Materials

MATERIALS

- Bicine **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**B3876
- Magnesium chloride hexahydrate (MgCl2.6H2O) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**M2393
- Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**E1644
- Benzamidine **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**B6506
- ε-Aminocaproic acid **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**A2504
- Sodium hydroxide (NaOH) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S5881
- 2-Mercaptoethanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**M6250
- DL-Dithiothreitol (DTT) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**43819
- Phenylmethanesulfonyl fluoride (PMSF) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P7626
- Protease inhibitor cocktail **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P9599
- D-Ribulose 1.5-bisphosphate sodium salt hydrate (RuBP) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**83895
- Sodium bicarbonate [14C] (NaH14CO3) **Perkin Elmer Catalog #**NEC086H005MC
- Potassium hydroxide (KOH) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P5958
- Formic acid **Honeywell Fluka Catalog #**F0507
- Gold star quanta scintillation cocktail **Meridian Catalog #**QSQ1
- Ethanol absolute 99.8 % **Fisher Scientific Catalog #**10437341

Citation

Carmo-Silva E, Andralojc PJ, Scales JC, Driever SM, Mead A, Lawson T, Raines CA, Parry MAJ (2017)
. Phenotyping of field-grown wheat in the UK highlights contribution of light response of photosynthesis and flag leaf longevity to grain yield.
Journal of Experimental Botany 68: 3473-3486.

https://doi.org/10.1093/jxb/erx169 LINK

Citation

Kane HJ, Wilkin JM, Portis AR, Andrews TJ (1998)
. Potent inhibition of ribulose-bisphosphate carboxylase by an oxidized impurity in ribulose-1,5-bisphosphate.
Plant Physiology 117: 1059-1069.

10.1104/pp.117.3.1059 LINK

Citation

Parry MAJ, Andralojc PJ, Parmar S, Keys AJ, Habash D, Paul MJ, Alred R, Quick WP, Servaites JC (1997)
. Regulation of Rubisco by inhibitors in the light. Plant, Cell & Environment 20: 528-534.

https://doi.org/10.1046/j.1365-3040.1997.d01-85.x LINK

Citation

Sharwood RE, Sonawane BV, Ghannoum O, Whitney SM (2016)
. Improved analysis of C4 and C3 photosynthesis via refined in vitro assays of their carbon fixation biochemistry.
Journal of Experimental Botany 67: 3137-3148.

https://doi.org/10.1093/jxb/erw154 LINK

Citation

Wong C-H (1980). Practical enzymatic syntheses of ribulose 1,5-bisphosphate and ribose 5-phosphate.
Journal of the American Chemical Society 102: 7938-7939.

https://doi.org/10.1021/ja00547a023 LINK

Troubleshooting

Safety warnings

Work with radiation should follow local safety procedures.
Before using the protocol always check the Safety Data Sheet (SDS) for each chemical.

Before start

MATERIAL & EQUIPMENTS (for list of chemicals check "Materials" tab)

- Leaf sample frozen in -80°C
- Centrifuge for microtubes (speed 14000 *g*, 4 °C; VWR, Mega Star 600R)
- Dry block heating system (Grant Instruments QBD4)
- Vortex
- Chronometer
- Fume hood
- Pipette set
- Mortar and pestle
- Glass vials for liquid scintillation counting (Perkin Elmer 6000167, 7 mL)
- 1.5 mL microtubes

REAGENTS & SOLUTIONS

1

REAGENTS & SOLUTIONS TO PREPARE BEFOREHAND

1.1 **Basic extraction buffer (1x)**

[M] 50 millimolar (mM) Bicine-NaOH pH 8.2

[M] 20 millimolar (mM) $MgCl_2.6H_2O$

[M] 1 millimolar (mM) EDTA

[M] 2 millimolar (mM) Benzamidine

[M] 5 millimolar (mM) ε-Aminocaproic acid

- Dissolve in ultrapure H_2O; adjust pH to 8.2 with NaOH; degas the solution bubbling with nitrogen (5 min/100 mL), then add:

[M] 50 millimolar (mM) 2-Mercaptoethanol

- Adjust for the final volume; it can be dispensed in aliquots (e.g. 50 mL Falcon tubes).

-20 °C (storage)

1.2 [M] 1 Molarity (M) DTT

- Dissolve in ultrapure H_2O. 4 °C (storage)

1.3 [M] 100 millimolar (mM) PMSF

- Dissolve in ethanol 99%. 4 °C (storage)

1.4 Plant protease inhibitor cocktail

-20 °C (storage)

1.5 **Basic assay buffer (2x)**

[M] 200 millimolar (mM) Bicine-NaOH

[M] 40 millimolar (mM) $MgCl_2.6H_2O$

- Dissolve in ultrapure H_2O; adjust pH to 8.2 with NaOH; adjust for the final volume; degas the solution bubbling with nitrogen (5 min/100 mL). It can be dispensed in aliquots (e.g. 50 mL Falcon tubes).

-20 °C (storage)

1.6 [M] 30 millimolar (mM) RuBP

-20 °C (storage)

Note

High purity RuBP (≥99%) is required to avoid interference in measurable activity due to the presence of RuBP-analogs that inhibit carboxylation (Kane et al., 1998; Sharwood et al., 2016). It is available commercially or it can be produced enzymatically from AMP-5' monohydrate and ATP disodium salt (Wong, 1980).

1.7 [M] 0.1 Molarity (M) $NaH^{14}CO_3$ (0.5 Ci/mol)

-20 °C (storage)

1.8 [M] 0.3 Molarity (M) KOH

Room temperature (storage)

1.9 [M] 10 Molarity (M) Formic acid

Room temperature (storage)

1.10 Gold Star Quanta scintillation cocktail

Room temperature (storage)

2 **SOLUTIONS TO PREPARE JUST BEFORE USE**

- Prepared with reagents/solutions described in step 1.

2.1 **Complete extraction buffer**

1x Basic extraction buffer (from step 1.1)

[M] 10 millimolar (mM) DTT (from step 1.2)

[M] 1 millimolar (mM) PMSF (from step 1.3)

[M] 1 % (v/v) Plant protease inhibitor cocktail (from step 1.4)

- Prepare the volume considering the number of extractions to be performed throughout the day plus two extras (to have a little excess). Mix all together.

On ice

Note

The volume of extraction buffer will depend on the size of the leaf sample and the protein content, therefore it is species dependent and should be tested beforehand. It is important to ensure that the Rubisco concentration in the assays does not compromise the sensitivity of the assays (e.g. too much would consume all the substrate too quickly).

2.2 **Complete assay buffer (volume per vial or reaction)**

250 µL 2x Basic assay buffer (from step 1.5) - final concentration in the solution = 1x

50 µL 100 mM $NaH^{14}CO_3$ (from step 1.7) - final concentration in the solution = 10 mM

165 µL ultrapure H_2O

- Prepare the volume considering that the activities are measured in duplicates (2 technical replicates).
- If the goal is to assay Rubisco initial and total activities, for each sample extract, it is necessary to have the volume for 4 vials (2 initials + 2 totals).
- In addition, account for additional volume for at least two more vials for Blanks (background counts, in the asbence of RuBP), plus one extra (to have a little excess).

Note

Example for 10 extractions: 10x (2xInitial + 2xTotal activity assays) + 2xBlanks + extra = volume for 43 vials.

Note

[M] 2 millimolar (mM) KH_2PO_4 can be added in the complete assay buffer for field samples to maximise the measurable Rubisco activity (Carmo-Silva et al., 2017).

PROCEDURE

3 **START**

- Thaw the frozen solutions that will be used in the day.

- Turn on the heat block and set to the temperature to be used for Rubisco activity measurements.

Note

The temperature to be used for the Rubisco activity measurement depends on the experiment goals. Typical measurement temperatures are 25 °C (standard) and 30 °C, depending on the species. Assays can be performed at a range of temperatures, however special care should be taken at high temperatures (e.g. 50 °C), as this can lead to evaporation of the assay mix; rates will be faster, i.e. the assay might become less sensitive; and may cause difficulty in handling hot vials.

- Turn on the centrifuge and set to 4 °C.
- Collect samples from -80 °C into liquid nitrogen.
- Prepare the complete extraction buffer (step 2.1) and the complete assay buffer (step 2.2) and keep it On ice

4 EXTRACTIONS & RUBISCO ASSAYS

4.1

- Immediately before starting the extraction, place 4 vials in the heat block (for 2 initial and 2 total activity assays).
- Add 465 µL of complete assay buffer (from step 2.2.) to each of the 4 vials.
- Add 10 µL of 30 mM RuBP (from step 1.6) to the 2 vials for initials.

4.2 Extraction

- Add the complete extraction buffer to an ice-cold mortar.
- Take a sample from the liquid nitrogen container and add to the mortar.
- Grind the sample thoroughly for 00:00:30 to maximum of 00:01:00.
- Collect the homogenate into an ice-cold 1.5 mL microtube and centrifuge 14000 x g, 4°C, 00:01:00.

Note

To prevent Rubisco deactivation (or even denaturation) the extraction should not take more than 1 min and it should be done in a ice-cold mortar, keeping the sample cold at all times. In our hands, with the extraction buffer described (containing protease inhibitors, mercaptoethanol and DTT, which keeps the enzyme reduced) 1 min centrifugation does not impact Rubisco activity. However, this should be tested for each species and extraction buffer used.

- When centrifugation stops, take the extract supernatant into another ice-cold 1.5 mL microtube.
- Proceed with the Rubisco assays straight away.
- Add 25 µL of sample extract consecutively to each of 4 vials, with 15 s intervals (Total1, Total2, Initial1, Initial2). All additions are completed within 1.5 min of finishing centrifugation.

Note

The Initial activity assays start with extract addition, while the Total activity assays start with addition of RuBP after 3 min of extract incubation with CO_2 and Mg^{2+} to allow for Rubisco carbamylation.

Note

This protocol can be adapted for measuring Rubisco activity with purified enzyme. In this case, Rubisco is frequently pre-activated and initial activity assays are performed.

- Quench the assays after 30s by adding 100 µL 10 M formic acid (from step 1.9).
- A possible time-line for the assays would be:

Step	Solution to be added	Initial activity		Total activity	
		Rep 1	Rep 2	Rep 1	Rep 2
1	Add 465 µL assay buffer	Before extraction	Before extraction	Before extraction	Before extraction
2	Add 10 µL of 30 mM RuBP	Before extraction	Before extraction	NA	NA
Proceed to the extraction. Follow the next steps when the extract supernatant is ready					
Start the chronometer					
3	Add 25 µL of extract	00:45	01:00	00:15	00:30
4	Quench with 100 µL 10M formic acid	01:15	01:30	NA	NA
5	Add 10 µL of 30 mM RuBP	NA	NA	03:15	03:30
6	Quench with 100 µL 10 M formic acid	NA	NA	03:45	04:00

- In the interval between quenching initial activities and initiating the reaction for total activities, it is possible to prepare the vials for the next assays (steps 1 and 2 in this table).
- Repeat for all the extractions of the day.
- Blanks can be prepared at the start or end of the day, by adding to each vial 465 µL of complete assay buffer (from step 2.2.) and 100 µL 10M formic acid (from step 1.9).
- In addition, it is useful to prepare at least two background checks per experiment by adding to each vial 465 µL of complete assay buffer (from step 2.2.) , 25 µL sample extract, and 3 minutes later 100 µL 10M formic acid (from step 1.9) to test for background levels of carboxylation due to RuBP that may be present in the leaf extracts. In our hands, this tends to be negligible.

Note

We typically do a simple test to verify the total amount of radioactivity present in the complete assay buffer, prepared just before starting the extractions. This serves to verify that the amount of radioactivity in the solution is reliable and comparable accross days of assays. For this,
Add 490 µL 0.3 M KOH (from step 1.8) plus 10 µL complete assay buffer (from step 2.2). Add 3.6 mL Gold Star Quanta scintillation cocktail (from step 1.10). Close the vial as soon as possible and mix well.

- Dry all the vials at 100 °C in the heat block (it takes approximately 01:00:00).

- Let vials cool, then add 400 µL ultrapure H_2O to each vial to re-hydrate acid stable compounds. Wait 00:05:00
- Add 3.6 mL of Gold Star Quanta scintillation cocktail (from step 1.10). Close the vials and vortex/mix well.
- Determine ^{14}C radioactivity using a scintillation counter.

CALCULATIONS

5 **Assumptions:**

- $NaH^{14}CO_3$: 0.5 Ci/mol CO_2 = 0.5 µCi/µmol CO_2
- 1 µCi = 2220000 disintegration per minute (dpm);

Example:
Blank: 89.03 dpm
Vial 1: 23378.90 dpm

1. correct vial DPM value by subtracting background counts (Blank): $23378.9—89.03 = 23289.87$ dpm
2. convert dpm to µCi: $23289.87/2220000 = 0.010491$ µCi
3. convert to CO_2 concentration: $0.010491/0.5 = 0.0210$ µmol CO_2

Rubisco activity is then converted to the unit of interest by accounting for the reaction time, the volume of sample extract used and the corresponding sample leaf area (µmol CO_2 m^{-2} s^{-1}) or protein content (µmol CO_2 min^{-1} mg^{-1}).

From the Rubisco activity calculations above for initial (*Vi)* and total activity (*Vt*), the Rubisco activation state (AS, %) can be calculated:

$$AS = 100 \times V_i/V_t$$

Citations

Kane HJ, Wilkin JM, Portis AR, Andrews TJ. Potent inhibition of ribulose-bisphosphate carboxylase by an oxidized impurity in ribulose-1,5-bisphosphate
10.1104/pp.117.3.1059

Sharwood RE, Sonawane BV, Ghannoum O, Whitney SM. Improved analysis of C4 and C3 photosynthesis via refined in vitro assays of their carbon fixation biochemistry
https://doi.org/10.1093/jxb/erw154

Wong C-H. Practical enzymatic syntheses of ribulose 1,5-bisphosphate and ribose 5-phosphate
https://doi.org/10.1021/ja00547a023

Carmo-Silva E, Andralojc PJ, Scales JC, Driever SM, Mead A, Lawson T, Raines CA, Parry MAJ. Phenotyping of field-grown wheat in the UK highlights contribution of light response of photosynthesis and flag leaf longevity to grain yield
https://doi.org/10.1093/jxb/erx169

Parry MAJ, Andralojc PJ, Parmar S, Keys AJ, Habash D, Paul MJ, Alred R, Quick WP, Servaites JC. Regulation of Rubisco by inhibitors in the light
https://doi.org/10.1046/j.1365-3040.1997.d01-85.x