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Protocol status: Working
 We use this protocol and it's working

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$^{14}\text{CO}_2$ -based assay for measuring Rubisco activity & activation state

In 1 collection

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ABSTRACT

The Rubisco activity $^{14}\text{CO}_2$ -based assay measures the incorporation of $^{14}\text{CO}_2$ 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 **Sigma Aldrich Catalog #B3876**

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PROTOCOL integer ID:
36836

Keywords: Enzyme activity assay, Rubisco, Radiometric assay, Crop improvement

⊗	Magnesium chloride hexahydrate (MgCl ₂ ·6H ₂ O) Sigma Aldrich Catalog #M2393
⊗	Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) Sigma Aldrich Catalog #E1644
⊗	Benzamidine Sigma Aldrich Catalog #B6506
⊗	ε-Aminocaproic acid Sigma Aldrich Catalog #A2504
⊗	Sodium hydroxide (NaOH) Sigma Aldrich Catalog #S5881
⊗	2-Mercaptoethanol Sigma Aldrich Catalog #M6250
⊗	DL-Dithiothreitol (DTT) Sigma Aldrich Catalog #43819
⊗	Phenylmethanesulfonyl fluoride (PMSF) Sigma Aldrich Catalog #P7626
⊗	Protease inhibitor cocktail Sigma Aldrich Catalog #P9599
⊗	D-Ribulose 1,5-bisphosphate sodium salt hydrate (RuBP) Sigma Aldrich Catalog #83895
⊗	Sodium bicarbonate [14C] (NaH ¹⁴ CO ₃) Perkin Elmer Catalog #NEC086H005MC
⊗	Potassium hydroxide (KOH) Sigma Aldrich Catalog #P5958
⊗	Formic acid Honeywell 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.

LINK

<https://doi.org/10.1093/jxb/erx169>

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.

LINK

[10.1104/pp.117.3.1059](https://doi.org/10.1104/pp.117.3.1059)

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.

LINK

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

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.

LINK

<https://doi.org/10.1093/jxb/erw154>

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.

LINK

<https://doi.org/10.1021/ja00547a023>

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 INSTRUCTIONS

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 8.2

[M] 20 millimolar (mM) MgCl₂·6H₂O

[M] 1 millimolar (mM) EDTA

[M] 2 millimolar (mM) Benzamidine

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

- Dissolve in ultrapure H₂O; 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 H₂O. 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) $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$

- 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 ($\geq 99\%$) 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) $\text{NaH}^{14}\text{CO}_3$ (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 μ L 2x Basic assay buffer (from step 1.5) - final concentration in the solution = 1x

🧴 50 μ L 100 mM $\text{NaH}^{14}\text{CO}_3$ (from step 1.7) - final concentration in the solution = 10 mM

🧴 165 μ L 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 absence 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 $25\text{ }^{\circ}\text{C}$ (standard) and $30\text{ }^{\circ}\text{C}$, depending on the species. Assays can be performed at a range of temperatures, however special care should be taken at high temperatures (e.g. $50\text{ }^{\circ}\text{C}$), 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 $4\text{ }^{\circ}\text{C}$.
- Collect samples from $-80\text{ }^{\circ}\text{C}$ 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 CO₂ and Mg²⁺ 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 $^{\circ}\text{C}$ 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:

- $\text{NaH}^{14}\text{CO}_3$: 0.5 Ci/mol CO_2 = 0.5 $\mu\text{Ci}/\mu\text{mol CO}_2$
- 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: $0.010491 / 0.5 = 0.0210$ $\mu\text{mol CO}_2$

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 ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) or protein content ($\mu\text{mol CO}_2 \text{ min}^{-1} \text{ mg}^{-1}$).

From the Rubisco activity calculations above for initial (V_i) and total activity (V_t), the Rubisco

activation state (AS, %) can be calculated:

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