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

In 1 collection

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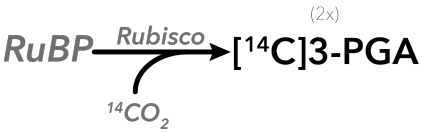
ABSTRACT

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).



THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

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COLLECTIONS

Protocols from Sales et al. (2020) Rubisco activity: challenges and opportunities of NADH-linked microtiter plate-based and 14C-based assays

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KEYWORDS

Enzyme activity assay, Rubisco, Radiometric assay, Crop improvement

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PARENT PROTOCOLS

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Protocols from Sales et al. (2020) Rubisco activity: challenges and opportunities of NADH-linked microtiter plate-based and 14C-based assays

Protocols from Sales et al. (2020) Rubisco activity: challenges and opportunities of NADH-linked microtiter plate-based and 14C-based assays

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

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

MATERIALS TEXT

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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

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. http://10.1104/pp.117.3.1059

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

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

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

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 STARTING

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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 Milimolar (mM)** Bicine-NaOH pH**8.2**

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

[M]**1 Milimolar (mM)** EDTA

[M]**2 Milimolar (mM)** Benzamidine

[M]**5 Milimolar (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 Milimolar (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 Milimolar (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 Milimolar (mM)** Bicine-NaOH

[M]**40 Milimolar (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)

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1.6 [M]**30 Milimolar (mM)** RuBP

-20 °C (storage)

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 Milimolar (mM)** DTT (from step 1.2)

[M]**1 Milimolar (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**

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).

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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).

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

[M] **2 Milimolar (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.

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.

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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** .

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.

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.

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:

		Initial activity		Total activity	
Step	**Solution to be added**	**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

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- 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.

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:

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

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