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NADH-linked microtiter plate-based assay for measuring Rubisco activity & activation state – PK-LDH

In 1 collection

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ABSTRACT

This protocol uses five reactions to couple RuBP carboxylation and 3-PGA formation to NADH oxidation to measure Rubisco activity, based on Scales et al. (2014).



During the NADH-linked assays, some Rubisco active sites might become carbamylated as the leaf extract is exposed to high CO₂ and Mg²⁺ in the assay buffer. Therefore, these assays are not suitable for measuring Rubisco initial activity and/or activation state at different levels in the canopy or in conditions in which low intercellular CO₂ is promoted (e.g., low light, drought stress, cold stress); the 30 second ¹⁴CO₂-based assay is recommended in such situations (protocol available in this collection).

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Sales CRG, Silva AB, Carmo-Silva E. 2020. Measuring Rubisco activity: challenges and opportunities of NADH-linked microtiter plate-based and ¹⁴C-based assays. *Journal of Experimental Botany*, <https://doi.org/10.1093/jxb/eraa289>

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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



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

KEYWORDS

Enzyme activity assay , Rubisco, Crop improvement , NADH-linked assay, PK-LDH, Plant phenotyping, Microtiter plate

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PARENT PROTOCOLS

Part of collection

[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 (MgCl ₂ ·6H ₂ O) | 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 (NaHCO ₃) | S6014 | Sigma Aldrich |
| Potassium chloride (KCl) | P9333 | Sigma Aldrich |

| NAME | CATALOG # | VENDOR |
|---|-----------|-------------------|
| 2,3-Diphospho-D-glyceric acid pentasodium salt (2,3-dPGA) | D5764 | Sigma Aldrich |
| Adenosine 5'-diphosphate sodium salt (ADP) | A2754 | Sigma Aldrich |
| β -Nicotinamide adenine dinucleotide reduced disodium salt hydrate (NADH) | N8129 | Sigma Aldrich |
| Enolase from bakers yeast (<i>S. cerevisiae</i>) | E6126 | Sigma Aldrich |
| Pyruvate kinase/Lactic dehydrogenase enzymes from rabbit muscle (PK/LDH) | P0294 | Sigma Aldrich |
| 2,3-dPGA-dependent phosphoglycerate mutase (dPGM) | | Home-made |
| Ethanol absolute 99.8 % | 10437341 | Fisher Scientific |

MATERIALS TEXT



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.
<https://doi.org/10.1104/pp.117.3.1059>



Lampinen J, Raitio M, Perälä A, Oranen H, Harinen R (2015). Microplate based pathlength correction method for photometric DNA quantification assay. Application Laboratory, Sample Preparation & Analysis, Thermo Fisher Scientific, Vantaa, Finland.
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https://doi.org/10.1007/978-1-4939-7786-4_14



Scales JC, Parry MA, Salvucci ME (2014). A non-radioactive method for measuring Rubisco activase activity in the presence of variable ATP: ADP ratios, including modifications for measuring the activity and activation state of Rubisco.. *Photosynthesis research* 119: 355-365.
<https://doi.org/10.1007/s11120-013-9964-5>



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

Before using the protocol always check the Safety Data Sheet (SDS) for each chemical.

BEFORE STARTING

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)
- Microtiter plate reader (BMG Labtec, SpectroStarNano)
- 96-well microtiter plate with clear flat bottom (Thermo Scientific, 442404)
- Pipette set
- Mortar and pestle
- 1.5 mL microtubes

REAGENTS & SOLUTIONS

1 REAGENTS & SOLUTIONS TO PREPARE BEFOREHAND



- Powder chemical stocks stored at -20°C: let warm up to room temperature on desiccant before opening container.
- Expensive chemicals purchased in very small amounts (mg), for which concentration in assay is not critical (e.g. in excess): trust quantity stated by the supplier and add ultrapure H₂O / solvent to container for final concentration (e.g. 2,3-dPGA).
- Protein and substrate solutions should typically be stored at -80°C.
- Freeze proteins in LN₂ before storing at -80°C. Store in small aliquots to prevent multiple freeze-thaw cycles. If using in consecutive days, protein solutions can be kept at 4°C.
- Buffers and solutions will last longer if filtered through 0.22 µm membrane.

1.1 Basic extraction buffer (1x)

[M]50 Milimolar (mM) Bicine-NaOH pH8.2

[M]20 Milimolar (mM) MgCl₂·6H₂O

[M]1 Milimolar (mM) EDTA

[M]2 Milimolar (mM) Benzamidine

[M]5 Milimolar (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 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 H₂O. ⚡ **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 [M]**20 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).

2 STOCK COMPONENTS FOR THE ASSAY BUFFER

[M]**1 Molarity (M)** Bicine-NaOH pH8

- Dissolve in ultrapure H₂O; adjust pH to 8.2 with NaOH; filter through 0.22 µm membrane for long shelf life.
⚡ **4 °C (storage)**

[M]**1 Molarity (M)** MgCl₂·6H₂O

- Dissolve in ultrapure H₂O; filter through 0.22 µm membrane for long shelf life. ⚡ **4 °C (storage)**

[M]**0.5 Molarity (M)** NaHCO₃

- Dissolve in ultrapure H₂O; filter through 0.22 µm membrane for long shelf life. ⚡ **4 °C (storage)**

[M]**3 Molarity (M)** KCl

- Dissolve in ultrapure H₂O; filter through 0.22 µm membrane for long life shelf. ⚡ **4 °C (storage)**

[M]**0.1 Molarity (M)** 2,3-dPGA

- Dissolve all solid by adding ultrapure H₂O to the container; aliquot. ⚡ **-80 °C (storage)**

[M]**0.5 Molarity (M)** ADP

- Add powder to a tube, add some ultrapure H₂O to dissolve, add **[M]4 Molarity (M)** NaOH (~10 µL/mL) to approximately **pH7**; add ultrapure H₂O to the final volume; aliquot. **⚡ -80 °C (storage)**



To test the pH use universal pH paper, add **1 µl** of the solution and check colour.

[M]14 Milimolar (mM) NADH

- Dissolve in **[M]100 Molarity (m)** Bicine-NaOH **pH8**; aliquot. **⚡ -80 °C (storage)**



Protect from light as it is light sensitive.

Please, check notes in section 2.1 to a more efficient way of aliquoting NADH.

[M]36 U/ml Enolase

- Dissolve all solid by adding **[M]100 Molarity (m)** Bicine-NaOH **pH8** to the container; aliquot. **⚡ -80 °C (storage)**

[M]1 KU/ml PK + **[M]1.4 KU/ml** LDH (PK/LDH)

⚡ -20 °C (storage)

[M]3 KU/ml d-PGM

- Home-made (for more detail, please see the protocol "Purification of 2,3-bisphosphate-dependent phosphoglycerate mutase (d-PGM)"). **⚡ -80 °C (storage)**

2.1 Complete assay buffer for Rubisco activity

- The basic assay buffer (table below) can be prepared the day before the assays and kept at **⚡ 4 °C**, or prepared in advance (e.g. at the start of an experiment), snap-frozen in aliquots and kept at **⚡ -80 °C**.
- Each assay buffer aliquot should only be thawed once, as repeated freeze thawing can result in degradation of the coupling enzymes; thus, it is important to aliquot adequate volumes for use in a day.
- NADH is prepared separately, snap-frozen in aliquots and kept at **⚡ -80 °C**, and added to the assay buffer just before the assays.

| Final concentration component | Stock | Volume in 200 μ L assay (μ L) |
|---|-----------------------|--|
| 100 mM Bicine-NaOH pH 8.2 | 1 M | 20 |
| 20 mM $MgCl_2$ | 1 M | 4 |
| 10 mM $NaHCO_3$ | 0.5 M | 4 |
| 20 mM KCl | 3 M | 1.3 |
| 5 mM DTT | 1 M | 1 |
| 0.2 mM 2,3-dPGA | 0.1 M | 0.4 |
| 2 mM ADP | 0.5 M | 0.8 |
| 5 U mL^{-1} Enolase | 36 U mL^{-1} | 28 |
| ~12.5 / 17.5 U mL^{-1} PK-LDH | ~1 / 1.4 KU mL^{-1} | 2.5 |
| 3.75 U mL^{-1} d-PGM | 3 KU mL^{-1} | 0.25 |
| Total | | 62.3 |
| <i>To be prepared separately and added just before the assays</i> | | |
| 0.4 mM NADH | 14 mM | 5.71 |
| Total for each well | | 68.0 |



We recommend using the same assay buffer for all the samples of the same experiment.



Stock solutions and the assay buffer should thaw **On ice**. The assay buffer should be kept in a tube wrapped in aluminium foil **On ice** during the assays, as NADH is light sensitive.

3 SOLUTIONS TO PREPARE JUST BEFORE USE

- Prepared with reagents/solutions described in step 1.

3.1 Complete extraction buffer

1x Basic extraction buffer (from step 1.1)

10 Milimolar (mM) DTT (from step 1.2)

1 Milimolar (mM) PMSF (from step 1.3)

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. Rubisco concentration in the assays should be approx. 15 $\mu g mL^{-1}$ for purified enzyme and between 10-40 $\mu g mL^{-1}$ for non-purified enzyme. Rubisco amounts above these values may limit the sensitivity of the NADH-linked assays.

To test if the assay is giving reliable results (i.e, none of the chemicals are limiting the reactions) it is important to always perform a test when the plant species and/or growth conditions change. Perform the assay with different extract concentration (e.g. 1/2 the amount, 1/5 of the amount, etc) and check if the activity expressed by protein content (TSP or Rubisco) is maintained.

3.2 Complete assay buffer

- Thaw **On ice** the assay buffer and NADH aliquots (prepared according to step 2.1) to be used in the day.
- Mix the correspondent volume of both solutions together. Keep **On ice**, wrapped in aluminium foil.

Example of how to prepare the complete assay buffer: e.g. 20 samples in a day

- 20 samples x (1 Blank + 3xTotal + 3xInitial activity assays) = 140 assays/wells
- Assay buffer without NADH per well = 62.3, for 140 wells = 62.3 x 140 = 8722 µL
- NADH per well = 5.71, for 140 wells = 5.71 x 140 = 799.4 µL
- Mix both together (8722 + 799.4 µL)

PROCEDURE

4 START

- Thaw the frozen solutions that will be used in the day.
- Prepare CO₂-free ultrapure H₂O by bubbling with nitrogen (5 min/100 mL).
- Turn on the microplate reader and set up for the temperature that Rubisco activity will be performed, select kinetic protocol at 340 nm.

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 high temperatures might lead to evaporation of the assay mix and, since rates will be faster, the assay might become less sensitive.

- Turn on the centrifuge and set to **4 °C**.
- Collect samples from **-80 °C** into liquid nitrogen container.
- Prepare the complete extraction buffer (from step 3.1) and the complete assay buffer (from step 3.2) and keep it **On ice**.

5 EXTRACTIONS & RUBISCO ASSAYS

- 5.1
- Before starting the extraction, pipette to the 96-well microtiter plate **127 µL** CO₂-free ultrapure H₂O for the blank (singlet) and **121 µL** for the samples into each well (6 wells, i.e., triplicates for

initial activities and triplicates for total activities), followed by **68 µl** of complete assay buffer (from step 3.2).

- Gently mix components by pipetting up and down 5 times whilst stirring. Add **6 µl** of 20 mM RuBP (from step 1.5) to the wells for measuring initial Rubisco activity (see table below). Cover to protect from light. Proceed to extraction.

5.2 Extraction

- Add the complete extraction buffer (from step 3.1) 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 to the Rubisco assays straight away.
- Add **5 µl** of sample supernatant to the wells for total activity first, followed by those for initial activity, mixing well by pipetting up and down 10 times whilst stirring. Place microplate in the reader and start monitoring the change in absorbance at 340 nm immediately. The addition of the extract initiates the reaction for the initial activity assays, which is measured while incubating Rubisco with CO₂ and MgCl₂ in the absence of RuBP (total activity) for **00:03:00** at **30 °C** to enable carbamylation of the enzyme. The absorbance value should start decreasing in the wells for the initial activity assay (containing RuBP).
- Pause the reading in order to add **6 µl** of 20 mM RuBP (from step 1.5) to the wells for measurement of total Rubisco activity **00:03:00** after addition of sample supernatant.
- Place the microplate in the reader and continue monitoring the change in absorbance.
- The reading can be stopped once the reaction reaches a plateau.



Considering that the protein concentration in the extract is as suggested in step 3.1, 5 µL of sample supernatant will give a good NADH consumption rate. The rate can be adjusted by adding more or less supernatant, but note that the amount of CO₂-free ultrapure H₂O added in the wells will change (as the final volume needs to be 200 µL).



The Initial activity assays start with extract addition, while total activity assays start with addition of RuBP after 3 min of extract incubation with CO₂ and Mg²⁺ 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.

- Below is a pipetting scheme for the microplate assay

| Solution (in pipetting order) | Volume to add (μL) | | |
|--|--------------------|------------------|----------------|
| | Blank | Initial activity | Total activity |
| <i>To be added before the extraction</i> | | | |
| CO ₂ -free H ₂ O | 127.0 | 121.0 | 121.0 |
| Complete assay buffer | 68.0 | 68.0 | 68.0 |
| 20 mM RuBP | 0 | 6 | 0 |
| <i>To be proceeded after the extraction</i> | | | |
| Leaf extract | 5 | 5 | 5 |
| <i>Start measuring absorbance at 340 nm while incubating the plate at the desired temperature (e.g. 30°C). Pause reading after 3 min to start reaction for total activity.</i> | | | |
| 20 mM RuBP | 0 | 0 | 6 |
| <i>Continue measuring absorbance at 340 nm until a plateau is reached.</i> | | | |



Conducting measurements at 30°C provides fast rates and reliable slopes, but the temperature can be adjusted according to the experimental aims and plant species used.



It is important to ensure that air bubbles are not introduced in the wells during the pipetting steps, as these will interfere with the absorbance measurements.

CALCULATIONS

- The activity of Rubisco is inferred from the consumption of RuBP ($\mu\text{mol s}^{-1}$) measured by absorbance change per second at 340 nm due to NADH oxidation, using an extinction coefficient of $6220 \text{ M}^{-1} \text{ cm}^{-1}$ or $6.22 \mu\text{mol}^{-1} \text{ mL cm}^{-1}$.
 - The carboxylation of one molecule of RuBP results in two molecules of 3-PGA, thus requiring two NADH in the final step. The rate of RuBP consumption ($\mu\text{mol s}^{-1}$) in the assay volume, is therefore calculated by:

$$RuBP_{consumption} = (Slope \times Volume) / (6.22 \times 2 \times Pathlength)$$

where the *Slope* represents the change in absorbance per second in the linear part of the absorbance trace change, *Volume* is the final volume per well in mL (0.2), 6.22 is the extinction coefficient of NADH in $\mu\text{mol}^{-1} \text{ mL cm}^{-1}$ and the factor 2 is used to account for the two molecules of NADH which are oxidized per molecule of RuBP. The *Pathlength* of the assay mix contained in each well is measured in cm.

- Rubisco initial (V_i) and total (V_t) activity expressed on a leaf area basis ($\mu\text{mol m}^{-2} \text{ s}^{-1}$) is then calculated by:

$$V_i \text{ or } V_t = (RuBP_{consumption} \times Extraction) / (Leafarea \times Aliquot)$$

where the *Extraction* is the volume of buffer in mL used for leaf extraction, leaf area is in m^2 , and *Aliquot* is the volume

of leaf extract supernatant used in the assay in mL.

- Rubisco activity can also be expressed on a Rubisco or total soluble protein (TSP) content basis ($\mu\text{mol min}^{-1}\text{mg}^{-1}$):

$$V_i \text{ or } V_t = (RuBP_{consumption} \times 60) / (Protein \times Aliquot)$$

where 60 is to convert seconds to minutes, *Protein* is the Rubisco or TSP content in mg mL^{-1} , and *Aliquot* is the volume of leaf extract supernatant used in the assay in mL.

- From the Rubisco activity calculations above for initial (V_i) and total activity (V_t), the Rubisco activation state (AS, %) can be calculated:

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



Measured absorbance values in a microtiter plate need to be normalized to a 1 cm pathlength, which would be found in a typical cuvette used in spectrophotometers. Measurements are corrected using Lambert-Beer's Law and considering both the volume in each well and the specific well dimensions for each type of microtiter plate. Modern microtiter plate readers frequently include a pathlength correction option, but this feature normally does not consider the properties of the solution. It is important to use the respective assay mix in determining the pathlength correction factor as the meniscus will affect the pathlength and absorbance reading in the microtiter plate. The pathlength can be determined according to Lampinen et al., (2012). Please, check the SI information in the publication linked to this protocol for more details.