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NADH-linked microtiter plate-based assay for measuring Rubisco activity & activation state – PEPC-MDH

 In 1 collection

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Cristina Rodrigues Gabriel Sales¹, Anabela Silva², Elizabete Carmo-Silva¹

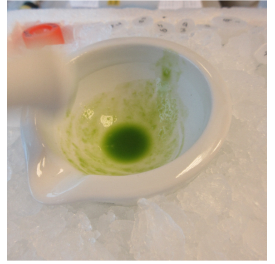
¹Lancaster Environment Centre, Lancaster University, Library Avenue, Lancaster, LA1 4YQ, UK;

²Biosystems & Integrative Sciences Institute (BioISI), Science Faculty of Lisbon University, Lisbon, 1749-016, Portugal



Cristina Rodrigues Gabriel Sales

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We use this protocol and it's working

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Keywords: Enzyme activity assay, Rubisco, Crop improvement , NADH-linked assay, PEPC-MDH, Plant phenotyping, Microtiter plate, measuring rubisco activity, rubisco activity, reactions to couple rubp carboxylation, nadh oxidation, pga formation to nadh oxidation, couple rubp carboxylation, based assay, reaction, pepc, mdh this protocol,

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).



Note

During the NADH-linked assays, some Rubisco active sites might become carbamylated as the leaf extract is exposed to high CO_2 and Mg^{2+} 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 CO_2 is promoted (e.g., low light, drought stress, cold stress); the 30 second $^{14}\text{CO}_2$ -based assay is recommended in such situations (protocol available in this collection).

Note

Rubisco activase (Rca) requires ATP and is inhibited by ADP, so the PEPC-MDH route can be used for measuring Rubisco activation by Rca (Scales et al., 2014).

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 ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$) **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 (NaHCO_3) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #S6014**
- ✕ Potassium chloride (KCl) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P9333**
- ✕ 2,3-Diphospho-D-glyceric acid pentasodium salt (2,3-dPGA) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D5764**
- ✕ β -Nicotinamide adenine dinucleotide reduced disodium salt hydrate (NADH) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #N8129**
- ✕ Enolase from bakers yeast (*S. cerevisiae*) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #E6126**
- ✕ 2,3-dPGA-dependent phosphoglycerate mutase (dPGM) **Home-made**
- ✕ Phosphoenolpyruvate carboxylase microbial (PEPC) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #C1744**
- ✕ Malic dehydrogenase from porcine heart (MDH) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M1567**
- ✕ Ethanol absolute 99.8 % **Fisher Scientific Catalog #10437341**



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.

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

LINK

Citation

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.

<https://assets.thermofisher.com/TFS-Assets/LCD/Application-Notes/AN-SkanIT-Microplate-Based-Pathlength-Correction-Technical-Note>

LINK

Citation

Sales CRG, Degen GE, Silva AB, Carmo-Silva E (2018)

. Spectrophotometric determination of Rubisco activity and activation state in leaf extracts. In: Covshoff S, ed. Methods in Molecular Biology. New York: Humana Press 1770: 239-250.

https://doi.org/10.1007/978-1-4939-7786-4_14

LINK



Citation

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>

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

Safety warnings

! 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)
- 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

Note

- 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 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/10 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 Mass Percent 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 [M] 20 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).

2 STOCK COMPONENTS FOR THE ASSAY BUFFER

[M] 1 Mass Percent Bicine-NaOH  8

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

[M] 1 Mass Percent MgCl₂·6H₂O

- Dissolve in ultrapure H₂O; filter through 0.22 μ m membrane for long shelf life. 🌡️ 4 °C (storage)

[M] 0.5 Mass Percent NaHCO₃

- Dissolve in ultrapure H₂O; filter through 0.22 μ m membrane for long shelf life. 🌡️ 4 °C (storage)

[M] 3 Mass Percent KCl

- Dissolve in ultrapure H₂O; filter through 0.22 μ m membrane for long shelf life. 🌡️ 4 °C (storage)

[M] 0.1 Mass Percent 2,3-dPGA

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

[M] 14 millimolar (mM) NADH

- Dissolve in [M] 100 Molarity (M) Bicine-NaOH  8 ; aliquot. 🌡️ -80 °C (storage)

Note

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  8 to the container; aliquot.  -80 °C (storage)

[M] 5 KU/ml PEPC

- Dissolve all solid by adding [M] 100 Molarity (M) Bicine-NaOH  8 to the container; aliquot.  -80 °C (storage)

[M] 8.5 KU/ml MDH

- Store at  4 °C when in ammonium sulfate suspension.
- Before use, it is necessary to buffer exchange as ammonium sulfate can affect the assay.
- Pipette a portion, e.g., 100 µL.
- Proceed to a buffer exchange using Amicon molecular weight cut-off of 10000 (Sigma-Aldrich); substitute ammonium sulfate for [M] 100 Molarity (M) Bicine-NaOH  8 in the same volume centrifuged (in this example 100 µL)
- Assay for TSP to know the concentration; aliquot.  -80 °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 $-80\text{ }^{\circ}\text{C}$, and added to the assay buffer just before the assays.

Final concentration components	Stock	Volume in 200 μL assay (μL)
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
5 U mL^{-1} Enolase	36 U mL^{-1}	28
3.75 U mL^{-1} PEPC	5 KU mL^{-1}	0.15
5 U mL^{-1} MDH*	2.2 KU mL^{-1}	0.46
3.75 U mL^{-1} d-PGM	3 KU mL^{-1}	0.25
Total		59.6
<i>To be prepared separately and added just before the assays</i>		
0.4 mM NADH	14 mM	5.71
Total for each well		65.3

Note

*As MDH needs to be buffer exchanged, the stock concentration will change in each preparation, thus the volume added in 200 μL assay will change.

Note

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

Note

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)

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

Note

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.

Note

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 wells
- Assay buffer without NADH per well = 59.6, for 140 wells = $59.6 \times 140 = 8344 \mu\text{L}$
- NADH per well = 5.71, for 140 wells = $5.71 \times 140 = 799.4 \mu\text{L}$
- Mix both together ($8344 + 799.4 \mu\text{L}$)

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.

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 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  129.7 µL CO₂-free ultrapure H₂O for the blank (singlet) and  123.7 µL for the samples into each well (6 wells, i.e., triplicates for initial activities and triplicates for total activities), followed by  65.3 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 .

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 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 CO_2 and MgCl_2 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.

Note

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 CO_2 -free ultrapure H_2O added in the wells will change (as the final volume needs to be 200 μL).

Note

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.

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.

- 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	129.7	123.7	123.7
Complete assay buffer	65.3	65.3	65.3
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>			

Note

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.

Note

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

- 6
- 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:

$$RuBPconsumption = (Slope \times Volume) / (6.22 \times 2 \times Pathlength)$$

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:

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

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 ($\mu\text{mol min}^{-1}\text{mg}^{-1}$):

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

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:

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

Note

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.

Citations

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

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

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