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ab211071 6-Phosphogluconate Assay Kit (Colorimetric)

For the sensitive and accurate measurement of 6-phosphogluconate (6-PGA) in various biological samples.

This product is for research use only and is not intended for diagnostic use.

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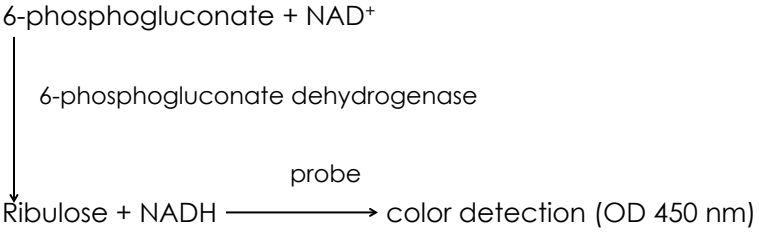
1. Overview

6-Phosphogluconate Assay Kit (Colorimetric) (ab211071) provides a convenient method to detect 6-phosphogluconate (6-PGA) in cell and tissue lysates. In this assay, 6-phosphogluconate is converted to ribulose-5-phosphate by 6-phosphogluconate dehydrogenase in the presence of NAD^+. NADH formed in the reaction reduces the probe present in the reaction mix, generating a strong absorbance at OD 450 nm. This absorbance is proportional to the amount of 6-PGA present in the samples.

This assay kit is fast, sensitive and high-through adaptable. It can measure less than 20 µM of 6-phosphogluconate present in the sample.



6-phosphogluconate (6-phosphogluconic acid, 6-PGA) is an intermediate of both Pentose Phosphate Pathway (PPP) and Entner-Doudoroff Pathway. In the Pentose Phosphate Pathway, 6-PGA is converted by 6-phosphogluconate dehydrogenase into ribulose-5-phosphate, a precursor for the synthesis of nucleic acids. In prokaryotes, 6-PGA is the main metabolite of the Entner-Doudoroff pathway, and is converted into pyruvate by both 6-phosphogluconate dehydratase and 2-keto-3-Deoxyphosphogluconate aldolase.

Recent studies show that long-term exposure to glucose perturbs the PPP, causing significant accumulation of 6-PGA and impairment of beta cell function in the pancreas. Measurement of 6-PGA levels is important when evaluating therapies for diabetes research.

In pathological bacteria, measurement of 6-PGA levels and functionality of Entner-Doudoroff pathway can be used for clinical and diagnostic purposes.

2. Protocol Summary

Standard curve preparation

Sample preparation

Add reaction mix and incubate for 60 minutes at 37°C

Measure optimal density (OD450 nm)

3. Precautions

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipette by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage and Stability

Store kit at -20°C in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature.

Δ Note: Reconstituted components are stable for 2 months.

5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. Materials Supplied

Item	Quantity	Storage temperature (before preparation)	Storage temperature (after preparation)
6-PGA Assay Buffer	25 mL	-20°C	-20°C
6-PGA Enzyme (15 mg)	1 vial	-20°C	-20°C
6-PGA Substrate Mix (25 mg)	1 vial	-20°C	-20°C
6-PGA Standard (10 mg)	1 vial	-20°C	-20°C

7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Microplate reader capable of measuring absorbance at OD 450 nm
- MilliQ water or other type of double distilled water (ddH_2O)
- Cold PBS
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions
- 96 well plate with clear flat bottom
- Dounce homogenizer (if using tissue)
- (Optional) 0.22 µm filter or 10kD Spin Column (ab93349) – to clarify samples if not clear after preparation

8. Technical Hints

- **This kit is sold based on number of tests. A "test" simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Ensure all reagents and solutions are at the appropriate temperature before starting the assay.
- Samples generating values that are greater than the most concentrated standard should be further diluted in the appropriate sample dilution buffer.
- Make sure all necessary equipment is switched on and set at the appropriate temperature.

9. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

9.1 6-PGA Assay Buffer (25 mL):

Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C.

9.2 6-PGA Enzyme (Lyophilized, 15 mg):

Reconstitute in 220 µL of 6-PGA Assay Buffer. Pipette up and down to dissolve completely. Keep on ice while in use. Aliquot enzyme so that you have enough to perform the desired number of assays. Store at -20°C. Use reconstituted enzyme within 2 months.

9.3 6-PGA Substrate Mix (Lyophilized, 25 mg):

Dissolve in 220 µL of ddH_2O. Pipette up and down to dissolve completely. Keep on ice while in use. Aliquot substrate mix so that you have enough to perform the desired number of assays. Store at -20°C. Use within to 2 months.

9.4 6-PGA Standard (Lyophilized, 10 mg):

Reconstitute in 100 µL of ddH_2O to generate a 100 mM (100 nmol/µL) standard stock solution. Keep on ice while in use. Aliquot standard so that you have enough to perform the desired number of assays. Store at -20°C. Use within 2 months.

10. Standard Preparation

- Always prepare a fresh set of standards for every use.
- Discard working standard dilutions after use as they do not store well.

10.1 Prepare a 1 mM standard (1 nmol/µL) by diluting 10 µL of the 100 mM 6-PGA standard stock solution (Step 9.4) with 990 µL ddH_2O. Mix well.

10.2 Use 1 mM 6-PGA Standard to prepare the standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

Standard #	6-PGA Standard (µL)	Assay Buffer (µL)	Final volume standard in well (µL)	End amount 6-PGA in well (nmol/well)
1	0	150	50	0
2	6	144	50	2
3	12	138	50	4
4	18	132	50	6
5	24	126	50	8
6	30	120	50	10

Each dilution has enough amount of standard to set up duplicate readings (2 x 50 µL).

11. Sample Preparation

General sample information:

- We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
- We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you snap freeze your samples in liquid nitrogen upon extraction and store them immediately at -80°C. When you are ready to test your samples, thaw them on ice and proceed with the Sample Preparation step. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.

11.1 Cell lysates:

11.1.1 Harvest the amount of cells (adherent/suspension) necessary for each assay (initial recommendation: 1 x 10^7 cells).
11.1.2 Wash cells with cold PBS.
11.1.3 Resuspend cells in 100 µL of 6-PGA Assay Buffer on ice.
11.1.4 Homogenize cells quickly by pipetting up and down a few times.
11.1.5 Incubate cell homogenate for 10 minutes on ice.
11.1.6 Centrifuge sample for 5 minutes at 4°C at 10,000 x*g* in a cold microcentrifuge to remove any insoluble material.
11.1.7 Collect supernatant and transfer to a new tube.
11.1.8 Keep on ice.

Δ Note: if samples are not clear, they need to be clarified through a 0.22 µm filter or spin filtered using a 10kD Spin column (ab93349) in order to remove insoluble components. Use flow through for the assay.

11.2 Tissue lysates:

11.2.1 Harvest the amount of tissue necessary for each assay (initial recommendation = 10 mg).
11.2.2 Wash tissue in cold PBS.
11.2.3 Resuspend tissue in 100 µL of 6-PGA Assay Buffer.
11.2.4 Homogenize tissue with a Dounce homogenizer or pestle sitting on ice, with 10 – 15 passes.
11.2.5 Incubate tissue homogenate for 10 minutes on ice.

11.2.6 Centrifuge 5 minutes at 4°C at 10,000 x*g* in a cold microcentrifuge to remove any insoluble material.
11.2.7 Collect supernatant and transfer to a new tube.
11.2.8 Keep on ice.

Δ Note: if samples are not clear, we need to be spin filtered using a 0.22 µm filter or a 10kD Spin column (ab93349) to remove insoluble components. Use flow through for the assay.

Δ Note: We suggest using different volumes of sample to ensure readings are within the standard curve range.

12. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all standards, controls and samples in duplicate.
- Prepare all reagents, working standards, and samples as directed in the previous sections.

Δ Note: NADH present in cell or tissue extracts will generate background in this assay. Set up Sample Background Controls to correct for background noise.

12.1 Set up reaction wells:

- Standard wells = 50 µL standard dilutions.
- Sample wells = 1 – 50 µL samples (adjust volume to 50 µL/well with 6-PGA Assay Buffer).
- Background Control Sample wells =1 – 50 µL samples (adjust volume to 50 µL/well with 6-PGA Assay Buffer).

12.2 6-PGA Reaction preparation:

12.2.1 Prepare 50 µL of Reaction Mix and Background Mix for each reaction. Mix enough reagents for the number of assays to be performed. Prepare a master mix to ensure consistency.

Component	Reaction Mix (µL)	Background Reaction Mix (µL)
6-PGA Assay Buffer	46	48
6-PGA Enzyme	2	0
6-PGA Substrate Mix	2	2

12.2.2 Add 50 µL of Reaction Mix into each standard and sample wells.

12.2.3 Add 50 µL of Background Reaction Mix into the background control sample wells.

12.3 Plate measurement:

12.3.1 Mix and incubate at 37°C for 60 min protected from light.

12.3.2 Measure output on a microplate reader at OD 450 nm.

13. Calculations

– Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiply the concentration found by the appropriate dilution factor.

13.1 Subtract the sample background control from sample reading.

13.2 Subtract the mean absorbance value of the blank (Standard #1) from all standard and sample readings. This is the corrected absorbance.

13.3 Average the duplicate reading for each standard and sample.

13.4 Plot the corrected absorbance values for each standard as a function of the final concentration of 6-PGA.

13.5 Draw the best smooth curve through these points to construct the standard curve. Most plate reader software or Excel can plot these values and curve fit. Calculate the trendline equation based on your standard curve data (use the equation that provides the most accurate fit).

13.6 Apply the corrected OD reading to the standard curve to get 6-PGA (B) amount in the sample wells.

13.7 Concentration of 6-PGA (nmol/µL, mmol/L or mM) in the test samples is calculated as:

$$6\text{ - PGA concentration} = \frac{B}{V} * D$$

Where:

B = amount of 6-PGA in the sample well calculated from standard curve (nmol).

V = sample volume added in the sample wells (µL).

D = sample dilution factor.

6-phosphogluconate acid – MW = 276.135 g/mol.

Sample 6-phosphogluconate acid concentration can also be expressed in nmol/mg or µmol/g of sample.

14. Typical Data

Typical standard curve – data provided **for demonstration purposes only**. A new standard curve must be generated for each assay performed.

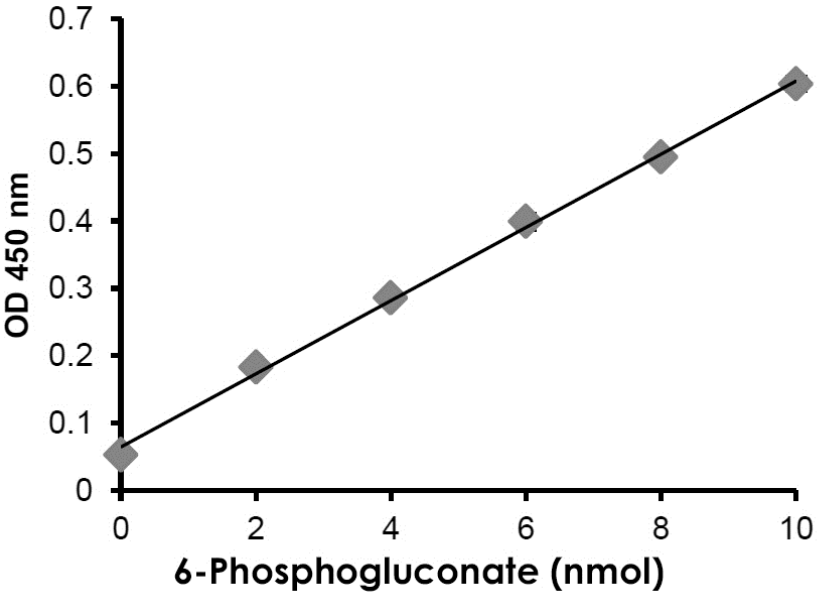


Figure 1. Typical 6-PGA standard calibration curve.

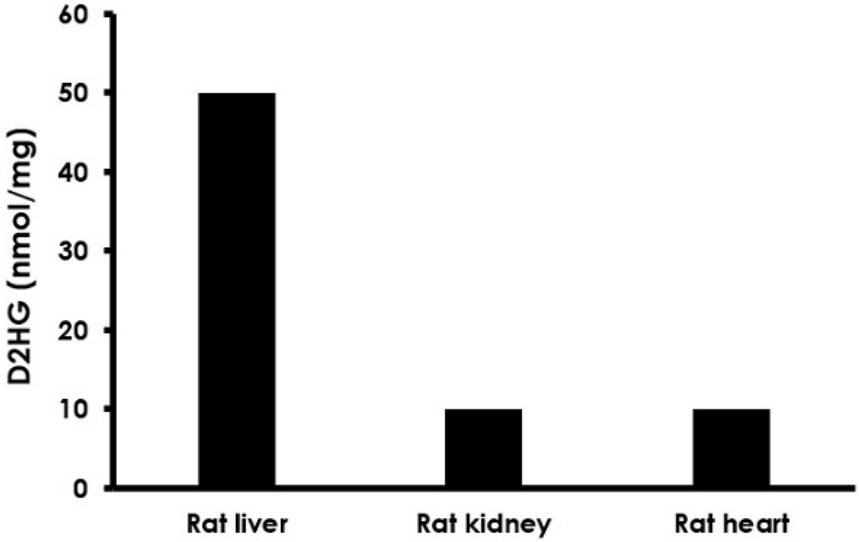


Figure 2. Measurement of 6-PGA in lysates from rat liver (160 µg), rat kidney (120 µg) and rat heart (60 µg).

15. Quick Assay Procedure

Δ Note: this procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.

- Prepare reagents, aliquot if necessary; get equipment ready.
- Prepare 6-PGA standard dilution [2 – 10 nmol/well].
- Prepare samples in optimal dilutions to fit standard curve readings.
- Set up plate in duplicate for standard (50 µL), samples (50 µL) and background sample control wells (50 µL).
- Prepare a master mix for Reaction Mix and (if appropriate) a master mix for Background Reaction Mix:

Component	Reaction Mix (µL)	Background Reaction Mix (µL)
6-PGA Assay Buffer	46	48
6-PGA Enzyme	2	0
6-PGA Substrate Mix	2	2

- Add 50 µL Reaction to standard and sample wells.
- Add 50 µL Background Reaction Mix to Sample Background control wells.
- Incubate plate at 37°C for 60 minutes protected from light.
- Measure plate at OD 450 nm.

16. Troubleshooting

Problem	Reason	Solution
Assay not working	Use of ice-cold buffer	Buffers must be at assay temperature
	Plate read at incorrect wavelength	Check the wavelength and filter settings of instrument
	Use of a different microplate	Colorimetric: clear plates Fluorometric: black wells/clear bottom plates Luminometric: white wells/clear bottom plates
Sample with erratic readings	Cells/tissue samples not homogenized completely	Use Dounce homogenizer, increase number of strokes
	Samples used after multiple free/ thaw cycles	Aliquot and freeze samples if needed to use multiple times
	Use of old or inappropriately stored samples	Use fresh samples or store at - 80°C (after snap freeze in liquid nitrogen) till use
	Presence of interfering substance in the sample	Check protocol for interfering substances; deproteinize samples
Lower/higher readings in samples and standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Allowing reagents to sit for extended times on ice	Always thaw and prepare fresh reaction mix before use
	Incorrect incubation times or temperatures	Verify correct incubation times and temperatures in protocol

Problem	Reason	Solution
Standard readings do not follow a linear pattern	Pipetting errors in standard or reaction mix	Avoid pipetting small volumes (< 5 µL) and prepare a master mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the tubes
	Standard stock is at incorrect concentration	Always refer to dilutions described in the protocol
Unanticipated results	Measured at incorrect wavelength	Check equipment and filter setting
	Samples contain interfering substances	Troubleshoot if it interferes with the kit
	Sample readings above/ below the linear range	Concentrate/ Dilute sample so it is within the linear range

17. Notes

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