Version 2 Last updated 2 March 2017

ab211092 Gluconokinase Activity Assay Kit (Colorimetric)

For the rapid, sensitive and accurate measurement of Gluconokinase activity in a variety of samples.

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

Table of Contents

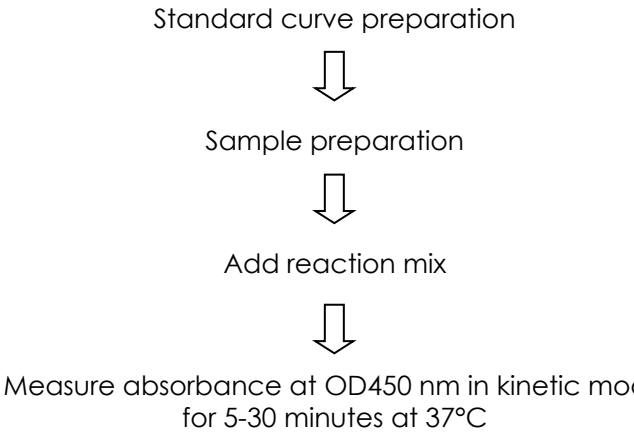
1. Overview

Gluconokinase Activity Assay Kit (Colorimetric) (ab211092) provides a simple, sensitive and convenient method for detecting gluconokinase activity in a variety of samples such as cell and tissue lysates, and even in prokaryotes such as *E. coli*. In this assay, Gluconokinase converts Gluconate into 6-Phosphate-D-Gluconate in an ATP dependent manner. 6-Phosphate-D-Gluconate and ADP in turn undergo a series of reactions to form an intermediate, which reacts with the probe to form a colored product with strong absorbance (OD 450 nm). The detection limit of this assay < 0.1 mU.

Gluconokinase (GntK, ATP:D-gluconate 6-phosphotransferase, Gluconate Kinase; EC:2.7.1.12) is a key enzyme for degradation of gluconate. In *E. coli* and yeast, Gluconokinase phosphorylates gluconate into 6-Phosphate-D-Gluconate in an ATP dependent manner. Via the Hexose Monophosphate Shunt (HMS) pathway, 6-Phosphate-D-Gluconate generates ribose-5-phosphate, which is critical for nucleotides and nucleic acid synthesis.

Despite the widespread use of D-gluconate as acidity regulator in food and medicine, little is known of the mechanism of gluconate metabolism in humans.

2. Protocol Summary



**For kinetic mode detection, incubation time given in this summary is for guidance only*

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 prep)	Storage temperature (after prep)
GntK Assay Buffer	25 mL	-20°C	-20°C
GntK Substrate (50 µmole)	1 vial	-20°C	-20°C
ATP (40 µmole)	1 vial	-20°C	-20°C
GntK Converting Enzyme (10 U)	1 vial	-20°C	-20°C
GntK Developer (10 U)	1 vial	-20°C	-20°C
GntK Probe (30 mg)	1 vial	-20°C	-20°C
NADH Standard (0.5 µmole)	1 vial	-20°C	-20°C
GntK Positive Control (200 mU)	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 clear plate with flat bottom
- Dounce homogenizer (if using tissue)
- (Optional) 10kD Spin Column (ab93349) – to remove small molecules from sample which may interfere with assay.

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 which generate 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 GntK Assay Buffer (25 mL):

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

9.2 GntK Substrate (lyophilized, 50 µmole):

Reconstitute in 220 µL ddH_2O and pipette up and down to dissolve completely. Aliquot substrate so that you have enough volume to perform the desired number of assays. Store at -20°C. Use within two months.

9.3 ATP (lyophilized, 40 µmole):

Reconstitute in 220 µL ddH_2O and pipette up and down to dissolve completely. Aliquot ATP so that you have enough volume to perform the desired number of assays. Store at -20°C. Use within two months.

9.4 GntK Converting Enzyme (lyophilized,10 U):

Reconstitute in 220 µL GntK Assay Buffer and pipette up and down to dissolve completely. Aliquot enzyme so that you have enough volume to perform the desired number of assays. Store at - 20°C. Use within two months.

9.5 GntK Developer (lyophilized,10 U):

Reconstitute in 220 µL GntK Assay Buffer and pipette up and down to dissolve completely. Aliquot developer so that you have enough volume to perform the desired number of assays. Store at - 20°C. Use within two months.

9.6 GntK Probe (lyophilized, 30 mg):

Reconstitute in 220 µL ddH_2O and pipette up and down to dissolve completely. Aliquot probe so that you have enough volume to perform the desired number of assays. Store at -20°C. Use within two months.

9.7 NADH Standard (lyophilized, 0.5 µmole):

Reconstitute in 400 µL ddH_2O to generate a 1.25 mM NADH standard stock solution. Pipette up and down to dissolve completely. Aliquot standard so that you have enough volume to perform the desired number of assays. Store at -20°C.Use within two months.

9.8 GntK Positive Control (lyophilized,200 mU):

Reconstitute in 200 µL GntK Assay Buffer and mix thoroughly. Keep on ice while in use. Aliquot positive control so that you have enough volume to perform the desired number of assays. Use within two months. Store at - 20°C.

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 Using 1.25 mM NADH standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

Standard #	NADH 1.25 mM standard (µL)	Assay Buffer (µL)	Final volume standard in well (µL)	End amount NADH in well (nmol/well)
1	0	150	50	0
2	6	144	50	2.5
3	12	138	50	5
4	18	132	50	7.5
5	24	126	50	10
6	30	120	50	12.5

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 complete the Sample Preparation step before storing the samples. Alternatively, if that is not possible, 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. 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 necessary for each assay (initial recommendation: 1 x 10^6 cells).

11.1.2 Wash cells with cold PBS.

11.1.3 Homogenize cells with 100 µL ice cold GntK Assay Buffer quickly by pipetting up and down a few times.

11.1.4 Keep on ice for 10 minutes.

11.1.5 Centrifuge sample for 5 minutes at 4°C at 10,000 x*g* using a cold microcentrifuge to remove any insoluble material.

11.1.6 Collect supernatant and transfer to a new tube.

11.1.7 Keep on ice.

11.1.8 Cell samples may contain small molecules that can interfere with the assay. Remove these from the sample by using a 10 kD Spin Column (ab93349). Add sample to the spin column, centrifuge at 10,000 x*g* for 10 min at 4°C. Collect the filtrate.

11.2 Tissue lysates:

11.2.1 Harvest the amount of tissue necessary for each assay (initial recommendation: 5 mg).
11.2.2 Wash tissue in cold PBS.
11.2.3 Homogenize tissue in 100 µL ice cold GntK Assay Buffer with a Dounce homogenizer sitting on ice, with 10 – 15 passes.
11.2.4 Keep on ice for 10 minutes.
11.2.5 Centrifuge sample for 5 minutes at 4°C at 10,000 x*g* using a cold microcentrifuge to remove any insoluble material.
11.2.6 Collect supernatant and transfer to a new tube.
11.2.7 Keep on ice.
11.2.8 Lysate samples may contain small molecules that can interfere with the assay. Remove these from the sample by using a 10 kD Spin Column (ab93349). Add sample to the spin column, centrifuge at 10,000 x*g* for 10 min at 4°C. Collect the filtrate.

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 If you suspect your samples contain substances that can generate background, set up Sample Background Controls to correct for background noise.

12.1 Plate Loading:

- Standard wells = 50 µL standard dilutions.
- Sample wells = 10 -50 µL samples (adjust volume to 50 µL/well with GntK Assay Buffer).
- Sample Background Control wells = 10- 50 µL samples (adjust volume to 50 µL/well with GntK Assay Buffer).
- Positive control = 1 -10 µL Positive Control (adjust volume to 50 µL/well with GntK Assay Buffer).

12.2 Gluconokinase Reaction Mix:

12.2.1 Prepare 50 µL of Reaction 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 Control Mix (µL)
GntK Assay Buffer	40	44
GntK Substrate	2	0
ATP	2	0
GntK Converting Enzyme	2	2
GntK Developer	2	2
GntK Probe	2	2

12.2.2 Add 50 µL of Reaction Mix into each well containing standard, positive control and samples. Mix well.

12.2.3 *Add 50 µL of Background Control Mix to background control samples. Mix well.

12.3 Measurement:

12.3.1 Measure immediately absorbance at OD = 450 nm in a colorimetric microplate reader in kinetic mode for 5 – 30 minutes at 37°C.

Δ Note: Incubation time depends on the Gluconokinase activity in the samples. Longer incubation time may be required if activity in the sample is low. We recommend measuring absorbance in kinetic mode, and choosing two time points (T1 and T2) to calculate the gluconokinase activity of the samples. The NADH Standard curve can be read in endpoint mode (ie. at the end of incubation time).

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.
- Use only the linear rate for calculation.

13.1 Standard curve calculation:

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

13.1.2 Average the duplicate reading for each standard.

13.1.3 Plot standard curve readings and draw the line of the best fit to construct the standard curve (most plate reader software or Excel can do this step). Calculate the trend line equation based on your standard curve data (use the equation that provides the most accurate fit).

13.2 Measurement of Gluconokinase activity in the sample:

13.2.1 For all reaction wells, choose two time points (T1 and T2) in the linear phase of the reaction progress curves and obtain the corresponding absorbance values at those points (OD1 and OD2).

13.2.2 Calculate ΔOD450 as follows:

$$\Delta OD450 = OD2 - OD1$$

13.2.3 If sample background control reading is significant, subtract sample background control reading from sample reading.

13.2.4 Apply the ΔOD to the NADPH Standard Curve to get B nmol of NADPH generated during the reaction time.

13.2.5 Gluconokinase activity (nmol/min/µL or mU/µL or U/mL) in the test samples is calculated as:

$$Gluconokinase\ Activity = \left(\frac{B}{\Delta T x\ V}\right) x\ D$$

Where:

B = amount of NADH in sample well calculated from standard curve (nmol).

ΔT = linear phase reaction time T2 – T1 (minutes).

V = original sample volume added into the reaction well (µL)

D = sample dilution factor.

Unit definition:

1 Unit Gluconokinase activity = amount of enzyme that generates 1.0 µmol of NADH per minute at pH7.2 at 37°C.

14. Typical Data

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

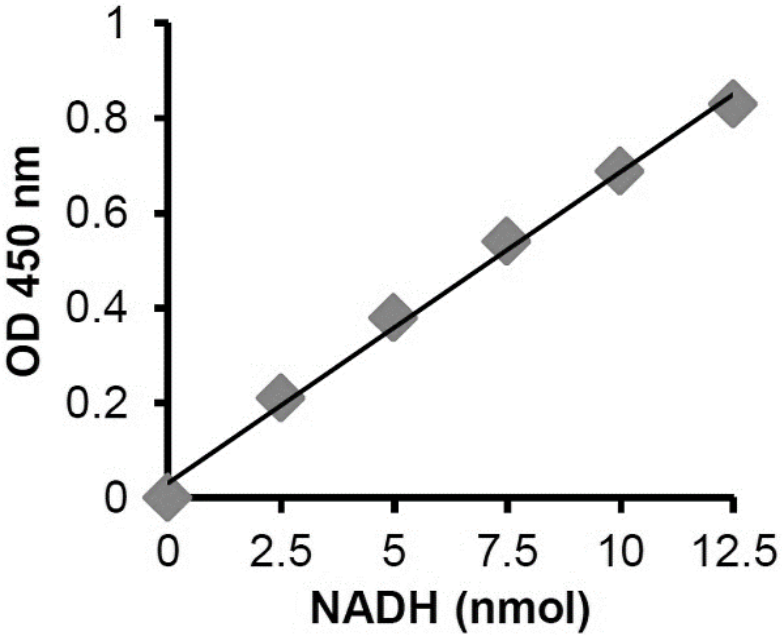


Figure 1. Typical NADH standard calibration curve.

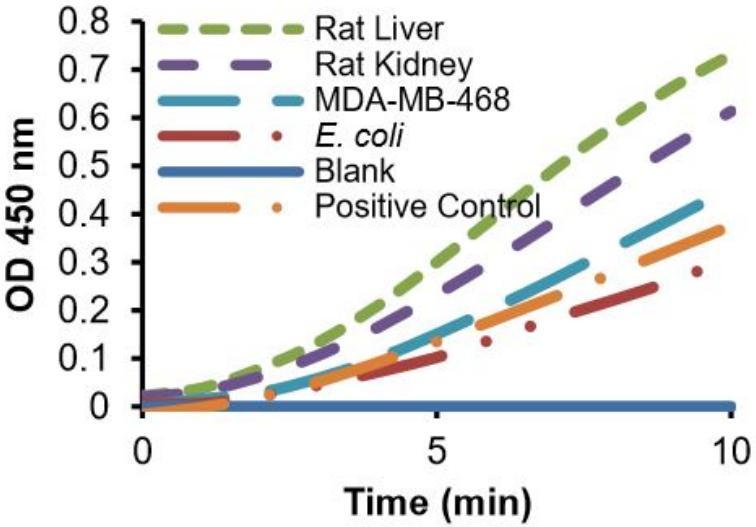


Figure 2. Kinetic curves showing Gluconokinase activity detection in positive control (1 µL; included in kit), lysates from rat liver and kidkey (1 µg, respectively), lysates from MDA-MB-468 cells (1 µg) and *E. coli* (0.1 µg).

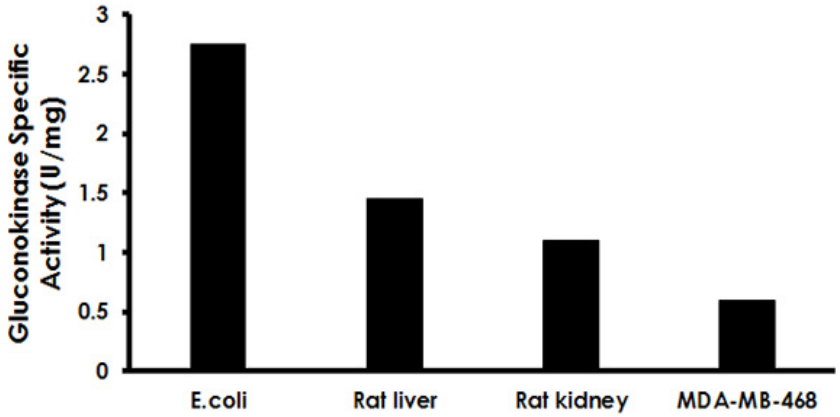


Figure 3. Gluconokinase Activity Assay Kit (ab211092) Gluconokinase specific activity in lysates from rat liver and kidkey (1 µg, respectively), lysates from MDA-MB-468 cells (1 µg) and *E. coli* (0.1 µg).

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 and aliquot if appropriate; get equipment ready.
- Prepare NADH standard dilution [2.5 – 12.5 nmol/well].
- Prepare samples in optimal dilutions to fit standard curve readings.
- Set up plate in duplicate for standard (50 µL), sample background control samples (50 µL), sample (50 µL) and positive control wells (50 µL).
- Prepare a master mix for Gntk Reaction Mix and Background Control Mix:

Component	Reaction Mix (µL)	Background Control Mix (µL)
GntK Assay Buffer	40	48
GntK Substrate	2	0
ATP	2	0
GntK Converting Enzyme	2	2
GntK Developer	2	2
GntK Probe	2	2

- Add 50 µL Reaction mix to each well containing standard, positive control and samples.
- Add 50 µL of Background Control Mix to background control samples.
- Measure absorbance immediately at OD = 450 nm in kinetic mode for 5-30 minutes at 37°C.

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
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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