Version 4a, Last updated 2 July 2025

ab211103 Hexokinase Activity Assay Kit (Fluorometric)

For the rapid, sensitive and accurate measurement of Hexokinase (HK) activity in a variety of samples.

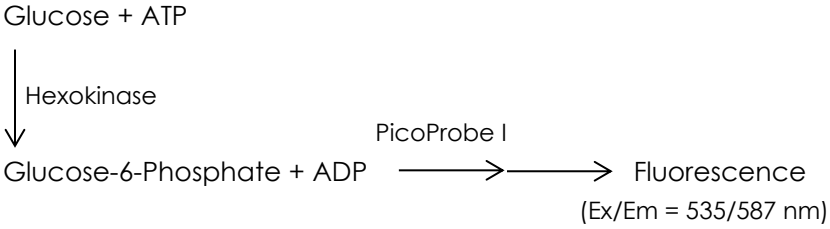
This product is for research use only and is not intended for diagnostic use.

Table of Contents

1. Overview

Hexokinase Activity Assay Kit (Fluorometric) (ab211103) provides a simple, sensitive and quick method for monitoring hexokinase (HK) activity in cells, serum, and animal or plant tissues. In this assay, HK converts glucose into glucose-6-phosphate, which in turn undergoes a series of reactions and reduces the sensitive probe to generate an intense fluorescent product that can be easily detected at Ex/Em = 535/587 nm.

This assay can detect as low as 2 µU of HK activity.



Hexokinase (HK, 6-Phosphate glucose kinase, ATP: D-Hexose 6- Phosphotransferase, ATP-dependent hexokinase, EC 1.1.1.49) is responsible for phosphorylating hexoses (six-carbon sugars) to form hexose phosphate. Hexokinases play an important role in glucose metabolism, as glucose is the most important substrate of hexokinases. Hexokinases are found in many organisms including bacteria, plants and mammals. In mammals, there are four isoforms (HK-I, II, III and IV). HK-I, HK-II, and HK-III are referred as "low Km" because of their high affinity for glucose (Km < 1mM), while HK-IV (also known as Glucokinase) has a Km for glucose 100-fold higher and can only phosphorylate glucose when the substrate concentration is high enough.

Hexokinase deficiency leads to diseases such as X-linked muscular dystrophy and rare autosomal recessive hemolytic anemia. On the other hand, increased hexokinase activity is detected in various human tumors and is associated with metastasis.

2. Protocol Summary

Standard curve preparation

Sample preparation

Add reaction mix

Measure fluorescence (Ex/Em = 535/587 nm) immediately in kinetic mode for 10-40 minutes at 25°C.

**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)
Assay Buffer 60	25 mL	-20°C	-20°C
PicoProbe I	0.4 mL	-20°C	-20°C
HK Substrate	1 mL	-20°C	-20°C
ATP II	1 vial	-20°C	-20°C
Developer Mix G	1 vial	-20°C	-80°C
Developer Mix P	1 vial	-20°C	-20°C
HK Positive Control	1 vial	-20°C	-20°C
NADPH Standard	1 vial	-20°C	-20°C

PLEASE NOTE: Assay Buffer 60 was previously labelled as Assay Buffer LX and HK Assay Buffer, and Developer Mix G as Development Enzyme Mix IX and HK Enzyme. Also, Developer Mix P as Developer IX and HK Developer. The composition has not changed.

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 fluorescence at Ex/Em = 535/587 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, preferably white
- Dounce homogenizer (if using tissue)

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 ATP II:

Reconstitute in 220 µL ddH_2O. Pipette up and down to dissolve completely. Aliquot ATP II/ATP so that you have enough volume to perform the desired number of assays. Store at -20°C. Use within 2 months.

9.2 Assay Buffer 60:

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

9.3 Developer Mix P:

Reconstitute in 220 µL Assay Buffer. 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 2 months.

9.4 Developer Mix G:

Reconstitute in 220 µL Assay Buffer. Pipette up and down to dissolve completely. Aliquot enzyme so that you have enough volume to perform the desired number of assays. Store at - 80°C after preparation. Use within 2 months.

9.5 HK Positive Control:

Reconstitute in 200 µL Assay Buffer and mix thoroughly by pipetting up and down. Aliquot positive control so that you have enough volume to perform the desired number of assays. Store at -20°C. Avoid freeze/thaw. Use within 2 months. Keep on ice while in use.

Immediately before performing the assay, dilute HK positive control 1:200 in Assay Buffer. Do not store diluted positive control.

9.6 HK Substrate:

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

9.7 NADPH Standard:

Reconstitute in 200 µL ddH_2O to generate a 1 mM (1 nmol/µL) NADPH Standard solution. Aliquot satandard so that you have enough volume to perform the desired number of assays. Store at -20°C. Use within 2 months. Keep on ice while in use.

9.8 PicoProbe I:

Ready to use as supplied. Warm by placing in a 37°C bath for 1 – 5 minutes to thaw the DMSO solution before use.

Δ Note: DMSO tends to be solid when stored at -20°C, even when left at room temperature, so it needs to melt for a few minutes at 37°C. Repeat this step every time probe is needed.

Aliquot probe so that you have enough volume to perform the desired number of assays. Store at -20°C protected from light. Once the probe is thawed, use within two 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 40 µM (40 pmol/µL) NADPH working standard solution by diluting 40 µL of 1mM NADPH Standard in 960 µL of ddH_2O.

10.2 Using 40 µM NADPH working standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

Standard#	NADPH 40 µM standard (µL)	Assay Buffer (µL)	Final volume standard in well (µL)	End amount NADPH in well (pmol/well)
1	0	150	50	0
2	6	144	50	80
3	12	138	50	160
4	18	132	50	240
5	24	126	50	320
6	30	120	50	400

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 Assay Buffer 60 quickly by pipetting up and down a few times.
11.1.4 Keep on ice for 10 minutes.
11.1.5 Centrifuge sample for 10 minutes at 4°C at 10,000 xg using a cold microcentrifuge to remove any insoluble material.
11.1.6 Collect supernatant and transfer to a new tube.
11.1.7 Dilute supernatant 1:10 in Assay Buffer.
11.1.8 Keep on ice.

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 Homogenize tissue with 100 µL ice cold Assay Buffer 60 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 10 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 Dilute supernatant 1:10 in Assay Buffer
11.2.8 Keep on ice.

11.3 Serum:

Can be used directly; no sample preparation is required.

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 = 2 – 50 µL samples (adjust volume to 50 µL/well with Assay Buffer).
- Sample Background Control wells = 2 – 50 µL samples (adjust volume to 50 µL/well with Assay Buffer).
- Positive control = 2 – 10 µL diluted Positive Control (adjust volume to 50 µL/well with Assay Buffer).

12.2 Reaction Mix and Background Control 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)
Assay Buffer 60	32	44
PicoProbe I	2	2
Developer Mix G	2	2
Developer Mix P	2	2
ATP II	2	0
HK Substrate	10	0

12.2.2 Add 50 µL of Reaction Mix into each standard, sample and positive control well. Mix well. Do NOT add reaction mix to background control wells.

12.2.3 Add 50 µL of Background Control Mix into sample background control wells. Mix well.

12.3 Measurement:

12.3.1 Measure immediately fluorescence at Ex/Em = 535/587 nm in a microplate reader in kinetic mode for 10 – 40 minutes at 25°C.

Δ Note: Incubation time depends on the Hexokinase activity in the samples. Longer incubation time may be required if activity in the sample is low. We recommend measuring fluorescence in kinetic mode, and choosing two time points (T1 and T2) in the linear range to calculate the hexokinase activity of the samples. The NADPH Standard Curve can be read in endpoint mode (i.e. 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 fluorescence value of the blank (Standard #1) from all the standard readings. This is the corrected fluorescence.

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 de most accurate fit).

13.2 Measurement of Hexokinase 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 fluorescence values at those points (RFU1 and RFU2).

13.2.2 Calculate ΔRFU for sample as follows:

ΔRFU = RFU2 – RFU1.

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

13.2.4 Apply the ΔRFU to NADPH Standard Curve to get B pmol of NADPH generated during the reaction time.

13.2.5 HK activity (pmol/min/µL or mU/mL or µU/µL) in the test samples is calculated as:

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

Where:

B = amount of NADPH in sample well calculated from Standard Curve (pmol)

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 Hexokinase activity = amount of enzyme that will generate 1.0 µmol of NADPH per minute at pH8.0 at 25°C.

14. Typical Data

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

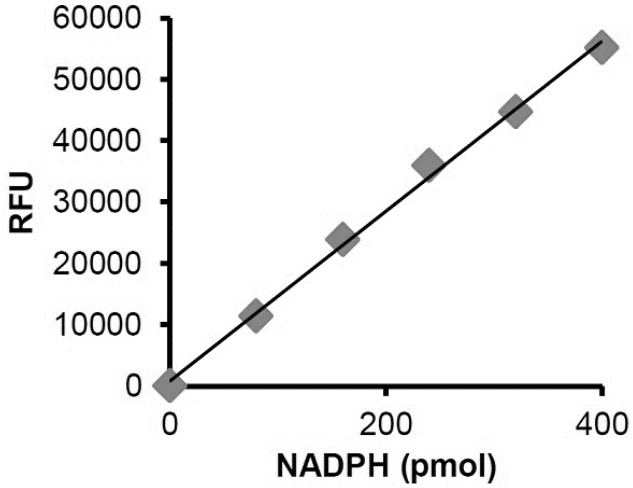


Figure 1. Typical NADPH standard calibration curve.

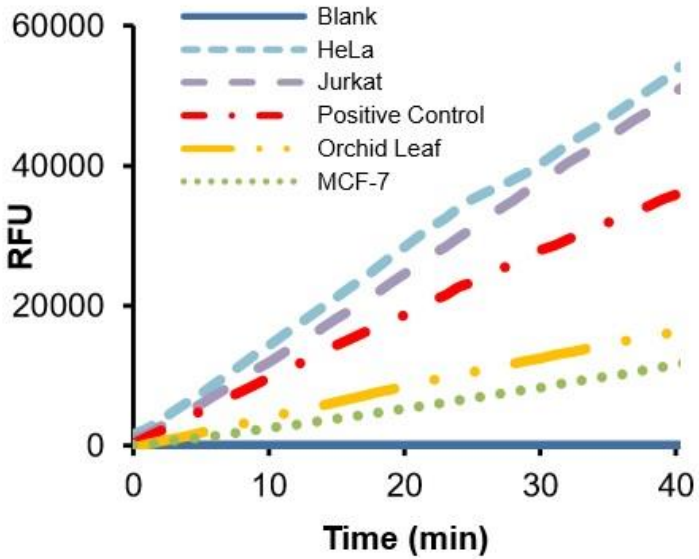


Figure 2. Kinetic curves showing Hexokinase activity in positive control (included in kit), lysates from HeLa (0.62 µg), Jurkat (0.65 µg) and MCF-7 cells (0.56 µg), and lysates from orchid leaf (4 µg).

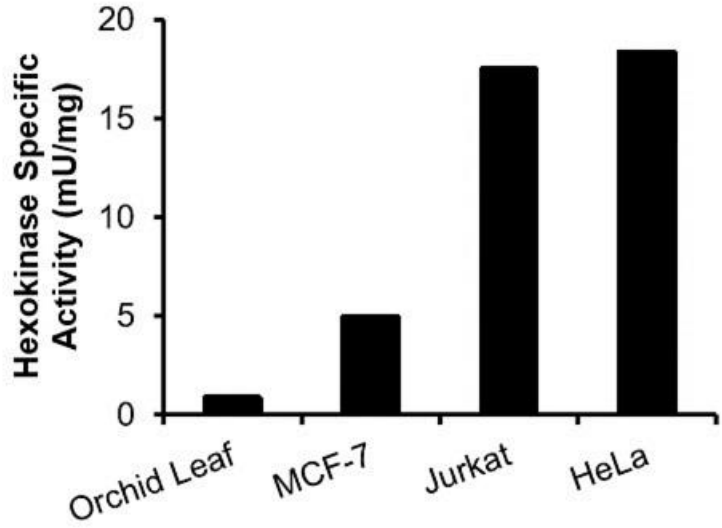


Figure 3. Hexokinase specific activity in lysates from orchid leaf (4 µg), MCF-7 (0.56 µg), Jurkat (0.65 µg) and HeLa cells (0.62 µg).

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; dilute positive control 1:200; get equipment ready.
- Prepare NADPH standard dilution [80 – 400 pmol/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 – diluted – wells (50 µL).
- Prepare a master mix for Reaction Mix and Background Control Mix:

Component	Reaction Mix (µL)	Background Control Mix (µL)
Assay Buffer 60	32	44
PicoProbe I	2	2
Developer Mix G	2	2
Developer Mix P	2	2
ATP II	2	0
HK Substrate	10	0

- Add 50 µL Reaction mix to each well containing standard, sample and positive control wells.
- Add 50 µL Background Control mix to each well containing background control wells.
- Measure fluorescence immediately at Ex/Em = 535/587 nm in kinetic mode for 10-40 minutes at 25°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

Technical Support

For all technical or commercial enquiries please go to:
www.abcam.com/contactus

www.abcam.cn/contactus (China)

www.abcam.co.jp/contactus (Japan)