

ab136957

Hexokinase Assay Kit (Colorimetric)

For the sensitive and accurate measurement of hexokinase in serum, animal tissue and cell culture samples (adherent and suspension).

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

PLEASE NOTE: With the acquisition of BioVision by Abcam, we have made some changes to component names and packaging to better align with our global standards as we work towards environmental-friendly and efficient growth. You are receiving the same high-quality products as always, with no changes to specifications or protocols.

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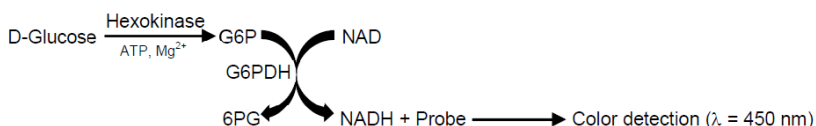
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1. BACKGROUND

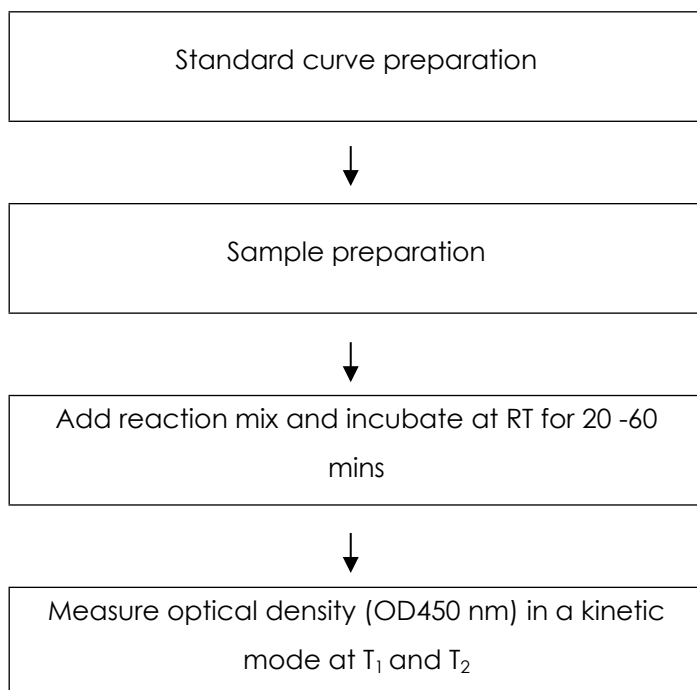
Hexokinase Assay kit (Colorimetric) (ab136957), glucose is converted to glucose-6-phosphate by hexokinase; the glucose-6-phosphate is oxidized by glucose-6-phosphate dehydrogenase to form NADH, which reduces a colorless probe to a colored product with strong absorbance at 450 nm. The assay is simple, sensitive and rapid and can detect hexokinase activity even less than 0.1 mU/well.

Hexokinases (HK) are found in many organisms including bacteria, plants and mammals and play an important role in glucose metabolism. Hexokinases phosphorylate glucose and generate glucose-6-phosphate for glycolysis. Hexokinases have four isoforms (HKI, II, III and IV). HK-I, HK-II and HK-III have low K_m , while HK-IV has 100 fold high K_m . Hexokinase deficiency leads to severe human diseases such as X-linked muscular dystrophy and a rare autosomal recessive hemolytic anemia.

However, increased hexokinase activity is detected in various human tumors and is associated with metastasis. Early detection of abnormal hexokinase activity is crucial for diagnosis, prediction and treatment of the disease.



2. ASSAY SUMMARY



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. Modifications to the kit components or procedures may result in loss of performance.

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 section 5.

Aliquot components in working volumes before storing at the recommended temperature. **Reconstituted components are stable for 2 months.**

5. MATERIALS SUPPLIED

Item	Amount †	Storage Condition (Before Preparation)	Storage Condition (After Preparation)
Assay Buffer LX/Assay Buffer	25 mL	-20°C	-20°C
HK Substrate/Hexokinase Substrate	1 mL	-20°C	-20°C
HK Coenzyme/Coenzyme (Lyophilized)	1 vial	-20°C	-20°C
Development Enzyme Mix IX/Enzyme Mix (Lyophilized)	1 vial	-20°C	-20°C
Developer Solution III/Developer (Lyophilized)	1 vial	-20°C	-20°C
NADH Standard I/NADH Standard (Lyophilized)	1 vial	-20°C	-20°C
Hexokinase Positive Control/Positive Control (Lyophilized)	1 vial	-20°C	-20°C

6. MATERIALS REQUIRED, NOT SUPPLIED

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

- MilliQ water or other type of double distilled water (ddH₂O)
- PBS
- Colorimetric microplate reader - equipped with filter for OD450 nm
- 96 well plate: clear plates for colorimetric assay
- Microcentrifuge
- Pipettes and pipette tips
- Heat block or water bath
- Vortex

- Dounce homogenizer or pestle (if using tissue)

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not use kit or components if it has exceeded the expiration date on the kit labels.
- 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.

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.
- Keep enzymes, heat labile components and samples on ice during the assay.
- Make sure all buffers and solutions are at room temperature before starting the experiment.
- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- 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 complete removal of all solutions and buffers from tubes or plates during wash steps.
- Make sure you have the right type of plate for your detection method of choice.
- Make sure the heat block/water bath and microplate reader are switched on.

9. REAGENT PREPARATION

- Briefly centrifuge small vials at low speed prior to opening.

9.1 **Assay Buffer LX/Assay Buffer:**

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

9.2 **HK Substrate/Hexokinase Substrate:**

Ready to use as supplied. Aliquot substrate so that you have enough volume to perform the desired number of assays. Store at -20°C.

9.3 **Coenzyme:**

Reconstitute with 220 μL Assay Buffer LX/Assay Buffer to generate 0.2 M solution. Aliquot co-enzyme so that you have enough volume to perform the desired number of assays. Store at -20°C. Keep on ice while in use.

9.4 **Development Enzyme Mix IX/Enzyme Mix:**

Reconstitute with 220 μL Assay Buffer LX/Assay Buffer. Pipette up and down to dissolve completely. Aliquot enzyme mix so that you have enough volume to perform the desired number of assays. Store at -20°C. Avoid repeated freeze/thaw cycles. Keep on ice while in use. Use within 2 months.

9.5 **Developer Solution III/Developer:**

Reconstitute with 220 μL dH_2O . Pipette up and down to dissolve completely. Aliquot Developer Solution III/developer so that you have enough volume to perform the desired number of assays. Store at -20°C.

9.6 **NADH Standard I/NADH Standard:**

Reconstitute with 400 μL dH_2O to generate 1.25 mM (1.25 nmol/ μL) NADH Standard I/NADH Standard solution. Aliquot standard so that you have enough

volume to perform the desired number of assays. Store at -20°C . Keep on ice while in use.

9.7 **Hexokinase Positive Control/Positive Control:**

Reconstitute with 100 μL Assay Buffer LX/Assay Buffer and mix thoroughly. Aliquot Hexokinase Positive Control/positive control so that you have enough volume to perform the desired number of assays. Store at -20°C .

Prior to use in the assay, dilute Hexokinase Positive Control/Positive control solution 1:99 in Assay Buffer LX/Assay Buffer.

10. STANDARD PREPARATION

- Always prepare a fresh set of standards for every use.
- Diluted standard solution is unstable and must be used within 4 hours.

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

Standard #	Volume of Standard (μL)	Assay Buffer LX/Assay Buffer (μL)	Final volume standard in well (μL)	End [NADH] in well
1	0	150	50	0 nmol/well
2	6	144	50	2.5 nmol/well
3	12	138	50	5.0 nmol/well
4	18	132	50	7.5 nmol/well
5	24	126	50	10.0 nmol/well
6	30	120	50	12.5 nmol/well

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 cells or tissue in liquid nitrogen upon extraction and store the samples 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 (adherent or suspension) samples:

- 11.1.1 Harvest the amount of cells necessary for each assay (initial recommendation = 1×10^6 cells).
- 11.1.2 Wash cells with cold PBS.
- 11.1.3 Resuspend cells in 200 μ L of ice-cold Assay Buffer LX/Assay Buffer.
- 11.1.4 Homogenize cells quickly by pipetting up and down a few times.
- 11.1.5 Centrifuge 5 minutes at 4°C at 12,000 rpm using a cold microcentrifuge to remove any insoluble material.
- 11.1.6 Collect supernatant and transfer to a clean tube.
- 11.1.7 Keep on ice.

11.2 Tissue samples:

- 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 200 μ L ice cold Assay Buffer LX/Assay Buffer.
- 11.2.4 Homogenize tissue with a Dounce homogenizer sitting on ice, with 10 – 15 passes.
- 11.2.5 Centrifuge 5 minutes at 4°C at 12,000 rpm using a cold microcentrifuge to remove any insoluble material.
- 11.2.6 Collect supernatant and transfer to a clean tube.
- 11.2.7 Keep on ice.

11.3 Serum samples:

Serum samples can be tested directly by adding sample to the microplate wells. Bring volumes up to 50 μ L with Assay Buffer LX/Assay Buffer.

However, to find the optimal values and ensure your readings will fall within the standard values, we recommend performing several dilutions of the sample (1/2 – 1/5 – 1/10).

NOTE: *We suggest using different volumes of sample to ensure readings are within the Standard Curve range.*

12. ASSAY PROCEDURE and DETECTION

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- It is recommended to assay all standards, controls and samples in duplicate.

12.1 Set up Reaction wells:

- Standard wells = 50 μ L standard dilutions.
- Sample wells = 1 – 50 μ L (40 μ g) samples (adjust volume to 50 μ L/well with Assay Buffer LX/Assay Buffer).
- Sample Background wells = 1 – 50 μ L (40 μ g) samples (adjust volume to 50 μ L/well with Assay Buffer LX/Assay Buffer).
- Positive Control wells = 1 – 50 μ L (adjust volume to 50 μ L/well with Assay Buffer LX/Assay Buffer).

12.2 Reaction Mix:

Prepare Reaction Mix for each reaction

Component	Colorimetric Reaction Mix (μ L)	Background Reaction Mix (μ L)
Assay Buffer LX/Assay Buffer	34	44
Development Enzyme Mix IX/Enzyme Mix	2	2
Developer Solution III/Developer	2	2
Coenzyme	2	2
HK Substrate/Hexokinase Substrate	10	0

Mix enough reagents for the number of assays (samples, standards and background control) to be performed. Prepare a master mix of the Reaction Mix to ensure consistency. We recommend the following calculation:

$X \mu\text{L component} \times (\text{Number samples} + \text{standards} + 1).$

- 12.3 Add 50 μL of Reaction Mix to each standard, Hexokinase Positive Control/positive control, and sample wells.
- 12.4 Add 50 μL of Background Reaction Mix to each sample background control sample wells.
- 12.5 Incubate at room temperature for 20 - 60 minutes protected from light.
- 12.6 Measure output on a microplate reader.
 - Colorimetric assay: measure OD 450 nm.

NOTE: Incubation time depends on the Hexokinase activity in the samples. We recommend measuring the OD in a kinetic mode, and choose two time points (T_1 and T_2) in the linear range to calculate the hexokinase activity of the samples. The NADH standard curve can 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 multiplying the concentration found by the appropriate dilution factor.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).

- 13.1 Average the duplicate reading for each standard and sample.
- 13.2 Subtract the 0 standard reading from all standard readings.
- 13.3 Correct sample background by subtracting the value derived from the background control from all sample readings. This is the corrected absorbance.
- 13.4 Plot the corrected absorbance values for each standard as a function of the final concentration of NADH.
- 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 Calculate the hexokinase activity of the test sample:

$$\Delta A = A_2 - A_1$$
- 13.7 Use the ΔA to obtain B nmol of NADH generated by hexokinase during the reaction time ($\Delta T = T_2 - T_1$).
- 13.8 Activity of hexokinase (nmol/min/mL or mU/mL) in the test samples is calculated as:

$$\text{Sample Hexokinase Activity} = \frac{B}{(\Delta T \times V)} \times \text{Dilution Factor}$$

Where:

$$\Delta A = A_2 - A_1.$$

B = NADH amount from standard curve (nmol).

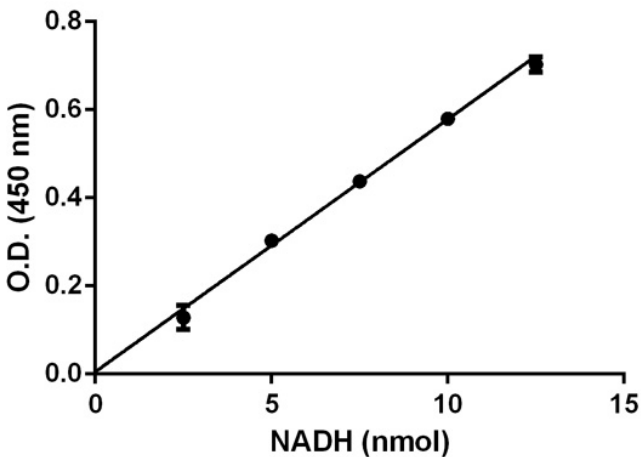
ΔT = reaction time (min) ($\Delta T = T_2 - T_1$).

V = sample volume added into the reaction well (mL).

Unit Definition: One unit of hexokinase is the amount of enzyme that will generate 1.0 μmol of NADH per min at pH 8 at room temperature.

14. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.



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Figure 1: Typical NADH standard calibration curve using endpoint colorimetric reading.

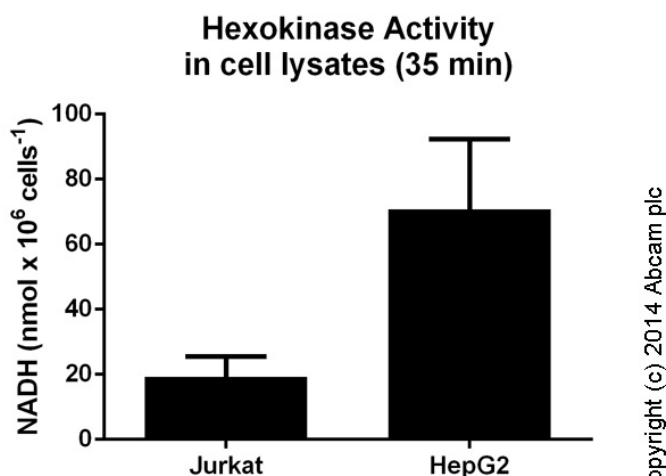


Figure 2: Hexokinase Activity measured in cell lysates showing quantity (nmol) per 1 mln cells after 35 minutes of incubation

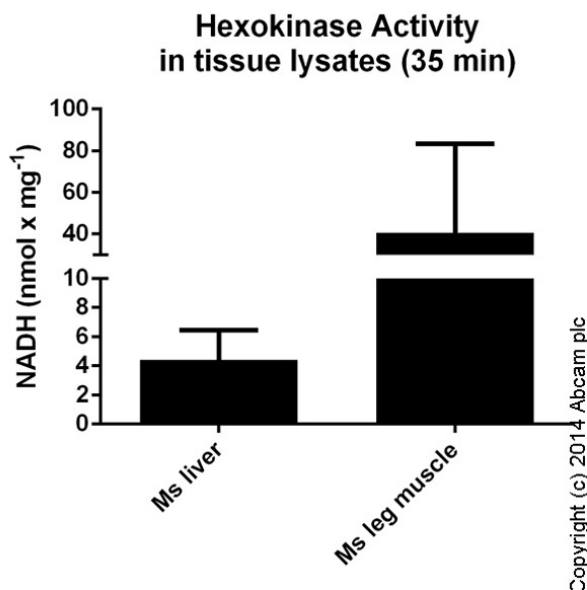


Figure 3: Hexokinase Activity measured in tissue lysates showing quantity (nmol) per mg of extracted protein after 35 minutes of incubation.

15. QUICK ASSAY PROCEDURE

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 standard, Developer Solution III/developer, coenzyme, and Development Enzyme Mix IX/enzyme mix (aliquot if necessary); get equipment ready.
- Prepare appropriate standard curve.
- Prepare samples in duplicate (find optimal dilutions to fit standard curve readings).
- Set up plate for standard (50 μ L), samples (50 μ L), Hexokinase Positive Control/positive control (50 μ L) and background sample controls (50 μ L).
- Prepare Reaction Mix (Number samples + standards + 1).

Component	Colorimetric Reaction Mix (μ L)	Background Reaction Mix (μ L)
Assay Buffer LX/Assay Buffer	34	44
Development Enzyme Mix IX/Enzyme Mix	2	2
Developer Solution III/Developer	2	2
Coenzyme	2	2
HK Substrate/Hexokinase Substrate	10	0

- Add 50 μ L Reaction Mix or Background Reaction mix to appropriate wells.
- Incubate plate at RT for 20- 60 mins.
- Measure plate at OD 450nm.

16. TROUBLESHOOTING

Problem	Cause	Solution
Assay not working	Use of ice-cold buffer	Buffers must be at room temperature
	Plate read at incorrect wavelength	Check the wavelength and filter settings of instrument
	Use of a different 96-well plate	Colorimetric: Clear plates Fluorometric: black wells/clear bottom plate
Sample with erratic readings	Samples not deproteinized (if indicated on protocol)	Use PCA precipitation protocol for deproteinization
	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

RESOURCES

Problem	Cause	Solution
Standard readings do not follow a linear pattern	Pipetting errors in standard or reaction mix	Avoid pipetting small volumes (< 5 μ L) 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 on 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. FAQ

In measuring D-Glucose-rich sample (such as muscle), is there a possibility that the background control shows slightly higher values than the sample itself?

If there is higher NADH in the muscle sample than D-glucose, then background can be equivalent or higher. Since OD is measured in a kinetic mode, time points can be chosen within the linear regime such that background values are lower than D-glucose reactions. Sample volume in the well can also be optimized such that very high (~50 μ L) volumes are not used. Low volumes will prevent saturation and will allow better distinction between sample and background.

What time should the standard curve be read at?

Standards should be read at end-point (at the highest timepoint for the sample readings).

Do hemolysed samples affect the assay?

Any particulate matter in suspension/turbidity affects absorbance measurements. Hemoglobin color or hemolysis in the sample can influence the OD measurements but typically OD 450nm is the brown/yellow range and hence red color does not influence much. We would suggest diluting the sample, removing the hemolyzed chunks and then using the sample

Does this kit measure activity of HK-1 or HK-2 or other isoforms also? What about muscle tissue?

This kit is based on function of the Hexokinase enzyme (conversion of glucose to glucose-6-phosphate is measured). This kit does not distinguish between the isoforms. HKI, HKII and HKIII have low K_m , while HKIV has 100 fold high K_m . Hence it is very

likely that you will measure hexokinase activity from I, II and III and small contribution if any from HK IV depending on the relative amounts of these isoforms in your sample. HK-II is the predominant form in adipose and muscle cells. HKII is insulin-sensitive. So if the samples are from adipose tissue or muscle you will measure HK-II activity.

What is the source of the positive control enzyme?

Our positive control is HK-II from *Bacillus* sp.

Can I use other hexose-sugars as substrates, e.g. fructose?

This kit assays hexokinase activity in a variety of samples. Theoretically hexokinases in general can phosphorylate hexose sugars including fructose which is then phosphorylated to fructose-6-P. Nevertheless, the primary substrate is glucose for these enzymes in mammalian cells. Hexokinase IV (fastest hexokinase) is actually a glucokinase meaning it acts on glucose to form G6P. It might be possible to use fructose as a substrate to test since NADH formed after phosphorylation reacts with the probe to generate color. But this depends on whether the sample has fructokinase activity or is mainly a glucokinase. The substrate provided in the kit is D-glucose.

What is the activity in the positive control?

The positive control is a purified enzyme and the specific activity changes from lot to lot. The positive control is provided to be used as a benchmark sample to make sure all components of the kit are working. The positive control is not to be used to compare activity in the sample.

18. INTERFERENCES

These chemicals or biological materials will cause interferences in this assay causing compromised results or complete failure:

- NADH.

Technical Support

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For all technical or commercial enquiries please go to:

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