ab136957– Hexokinase Assay Kit (Colorimetric)

For rapid, sensitive and accurate measurement of hexokinase levels in various samples.
For research use only - not intended for diagnostic use.

For overview, typical data and additional information please visit:
http://www.abcam.com/ab136957 (use http://www.abcam.cn/ab136957 for China, or http://www.abcam.co.jp/ab136957 for Japan)

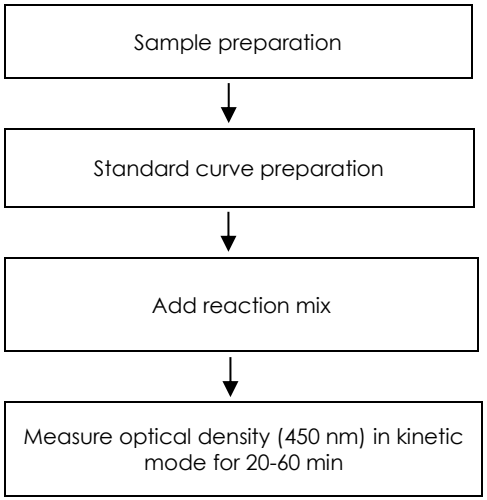
Background:

Hexokinase Assay kit (ab136957, Colorimetric) provides a simple, rapid and reliable procedure for measuring hexokinase activity in tissue, tissue extracts, serum, suspension and adherent cells.

Hexokinase catalyses the reaction where glucose is converted to glucose-6-phosphate, which 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 can detect hexokinase activity less than 0.1 mU/well.

Assay Summary:

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



QUICK ASSAY PROCEDURE

- Prepare standard, Developer Solution III and Developer Mix G (aliquot if necessary); get equipment ready.
- Prepare samples in duplicate
- Prepare appropriate standard curve.
- Set up plate for standard (50 µL) and samples (50 µL)
- Prepare and add 50 µL Reaction Mix to each well
- Measure plate at OD 450 nm in kinetic mode for 20 to 60 min.

Precautions & Limitations:

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.
- 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.

This kit is incompatible with EDTA and other chelators. Please ensure any serum/plasma samples used do not use EDTA or citrate as an anticoagulant (heparin plasma is suggested).

Storage and Stability:

Store kit at -20ºC in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted. Reconstituted components are stable for 2 months. Do not use kit or components if they have exceeded the expiry date.

Materials Supplied:

Item	Quantity	Storage Temperature (on receipt)	Storage temperature (reconstituted)
Assay Buffer 60	25 mL	-20°C	-20°C
HK Substrate	1 mL	-20°C	-20°C
HK Coenzyme	1 vial	-20°C	-20°C
Developer Mix G	1 vial	-20°C	-20°C
Developer Solution III	1 vial	-20°C	-20°C
NADH Standard I	1 vial	-20°C	-20°C
Hexokinase Positive Control	1 vial	-20°C	-20°C

PLEASE NOTE: Assay Buffer 60 was previously labeled as Assay Buffer LX and Assay Buffer, and Developer Mix G as Development Enzyme Mix IX and Enzyme Mix (Lyophilized). The composition has not changed.

Materials Required, Not Supplied:

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

- Microplate reader capable of measuring absorbance (OD) at 450 nm
- 96 well clear plate with clear flat bottom
- Orbital shaker
- Vortex
- Heat block or water bath
- Microcentrifuge
- Dounce homogenizer (if using cells or tissue)
- MilliQ water or other type of double distilled/deionized water (ddH_2O)
- 1x PBS, pH 7.4 (ab285410 or similar)

Reagent Preparation:

- Briefly centrifuge small vials at low speed prior to opening.
- Equilibrate reagents to room temperature before use.
- Aliquot reagents so that you have enough volume to perform the desired number of assays.

Assay Buffer 60: Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C.

HK 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.

HK Coenzyme: Reconstitute with 220 µL Assay Buffer 60 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.

Developer Mix G: Reconstitute with 220 µL Assay Buffer 60. Pipette up and down to dissolve completely. Aliquot Developer Mix G 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.

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

NADH Standard I: Reconstitute with 400 µL dH_2O to generate 1.25 mM (1.25 nmol/µL) NADH Standard I 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.

Hexokinase Positive Control: Reconstitute with 100 µL Assay Buffer 60 and mix thoroughly. Aliquot Hexokinase 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 solution 1:99 in Assay Buffer 60.

Sample Preparation:

1. We recommend performing several dilutions or testing multiple doses of your sample to ensure the readings are within the standard value range.
2. 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, 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.
3. Interferences: For optimal enzymatic activity samples should not contain EDTA or other chelating agents.
   -NADH, other enzymes and intermediates in the samples can contribute to background signals. These can be accounted for by performing a paired background control.
   -Care must be taken in finding the linear range when high glucose is present in the sample, as glucose can over-inflate background signals. Further dilution or a buffer exchange may be required.

Cells (adherent or suspension) samples:
1. Harvest the number of cells necessary for each assay (initial recommendation = 2 x 10^6 cells – equivalent of 1-5 x 10^4 cells/well required).
2. Wash cells in cold PBS.
3. Resuspend cells in 200 µL of Assay Buffer 60.
4. Homogenize cells with a Dounce homogenizer sitting on ice.
5. Centrifuge sample for 2-5 minutes at 4°C at top speed using a cold microcentrifuge to remove any insoluble material.
6. Collect supernatant and transfer to a clean tube.
7. Keep on ice.

Tissue Samples:
1. Harvest the amount of tissue necessary for each assay (initial recommendation = 10 mg).
2. Wash tissue in cold PBS.
3. Resuspend tissue in 200 µL of Assay Buffer 60.
4. Homogenize tissue with a Dounce homogenizer sitting on ice, with 10 – 15 passes.
5. Centrifuge samples for 5 minutes at 4°C at top speed using a cold microcentrifuge to remove any insoluble material.
6. Collect supernatant and transfer to a clean tube.
7. Keep on ice.

Serum Samples:
Serum samples can be tested directly by adding sample to the microplate wells. Bring volumes up to 50 µL with Assay Buffer 60.

However, to find the optimal values and ensure your readings will fall within the standard values, we recommend performing several dilutions of the sample (D=2, 5, 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.
- If your sample readings fall out the range of your fluorometric standard curve, you might need to adjust the dilutions and create a new standard curve.

1. Add 0, 2, 4, 6, 8 and 10 µl of reconstituted 1.25 mM NADH Standard I into a series of wells in duplicate in 96 well plate to generate 0, 2.5, 5.0, 7.5, 10 and 12.5 nmol/well of NADH. Adjust volume to 50 µl/well with Assay Buffer 60. Or prepare standard curve dilution as described in the table below in a microplate or microcentrifuge tubes (sufficient for 2 replicate curves):

Standard #	Volume of 1.25 mM Standard (µL)	Assay Buffer 60 (µL)	Final volume standard in well (µL)	End NADH Amount (nmol/well)
1	0	125	50	0
2	5	120	50	2.5
3	10	115	50	5.0
4	15	110	50	7.5
5	20	105	50	10
6	25	100	50	12.5

Assay Procedure:

- Keep enzymes and heat labile components and samples on ice during the assay.
- Equilibrate all other materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all standards, controls, and samples in duplicate.

1. Set up Reaction wells:
   - Standard wells = 50 µL standard dilutions.
   - Sample wells = 1 – 50 µL (40 µg) samples (adjust volume to 50 µL/well with Assay Buffer 60).
   - Sample Background wells = 1 – 50 µL (40 µg) samples (adjust volume to 50 µL/well with Assay Buffer 60).
   - Positive Control wells = 1 – 10 µL of **diluted** Hexokinase Positive Control (adjust volume to 50 µL/well with Assay Buffer 60).

2. Each well (standards, samples, and positive controls) requires 50 µL of Reaction Mix as shown in the table below. Each Sample Background well requires 50 µL of Background Reaction Mix.
3. To ensure consistency, use the table below to prepare a Master Mix of the appropriate Reaction Mix for your assay using the following calculation:
   X µL component x (Number reactions +1)

Component	Reaction Mix (µL)	Background Reaction Mix (µL)
Assay Buffer 60	34	44
Developer Mix G	2	2
Developer Solution III	2	2
HK Coenzyme	2	2
HK Substrate	10	0

4. Mix Master Reaction Mix by inversion. Add 50 µL of the Master Reaction Mix to each standard, sample and positive control well. Use a clean tip for each well
5. Mix Master Background Reaction Mix by inversion. Add 50 µL of the Master Reaction Mix to each background control well. Use a clean tip for each well.
6. Mix and immediately begin taking OD 450 kinetic readings every 1-2 minutes at room temperature for 20-60 minutes.

***NOTE: Required time depends on the Hexokinase activity in the samples. We recommend measuring the OD in a kinetic mode, and choosing 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.**

Calculations:

1. Average the duplicate reading for each standard and sample.
2. Subtract the mean absorbance value of the blank (Standard #1) from all standard readings. This is the corrected absorbance.
3. Plot the corrected absorbance values for each standard as a function of the final concentration of NADH.
4. Find the linear equation of the standard curve using a linear regression and determine the slope.
5. Subtract background control absorbance from the absorbance of the corresponding sample at each time point. This is the corrected sample absorbance.
6. Find the corrected sample absorbances (A_1, A_2) at two time points in the linear range (T_1, T_2). Calculate the change in absorbance due to hexokinase activity in the test sample:

$$\Delta A = A_2 - A_1$$

7. Use the ΔA to obtain B nmol of NADH generated by hexokinase during the reaction time ($\Delta T = T_2 - T_1$) from the standard curve.

$$B = \left(\frac{\Delta A - (y - intercept)}{Slope}\right)$$

8. Activity of hexokinase (nmol/min/mL or mU/mL) in the test samples is calculated as:

$$Hexokinase\ Activity = \frac{B}{(\Delta T \times V)} \times D = nmol/(min \times mL) = mU/mL$$

Where:

B = NADH amount from standard curve (in nmoles).
ΔT = reaction time (in minutes) ($\Delta T = T_2 - T_1$).
V = sample volume added into the reaction well (in mL).
D = sample dilution factor (before adding to the well).

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.

FAQ

1. **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 Km, while HKIV has 100 fold high Km. 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.

2. **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.

For additional helpful hints and tips on using our assay kits please visit:
https://www.abcam.com/en-us/support/product-support

Technical Support

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