ab211114
Hexokinase II Inhibitor Screening Kit (Colorimetric)

For the rapid, sensitive and accurate screening of potential Hexokinase II inhibitors.

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

Hexokinase II Inhibitor Screening Kit (Colorimetric) (ab211114) provides a sensitive, quick, and easy method for screening potential inhibitors of Hexokinase II (HK-II) in less than 30 minutes. The assay is based in the ability of HK-II to convert glucose into glucose-6-phosphate. Glucose-6-phosphate is oxidized by glucose-6-phosphate dehydrogenase to form NADH, which reduces a probe that shows strong absorbance at OD 450 nm. In the presence of a HK-II inhibitor such as Bromopyruvic Acid, the reaction is impeded, decreasing the rate or extent of generation of HK-II–dependent absorbance at OD 450 nm.

This simple and high-throughput adaptable assay kit can be used to screen, study or characterize potential inhibitors of Hexokinase II.

Hexokinases (HK, EC: 2.7.1.1) are found in many organisms and play an important role in glucose metabolism. The Hexokinase family phosphorylates glucose and generates glucose-6-phosphate for glycolysis. Four Hexokinase isoforms (HK-I, II, III and IV) are found in numerous species. Hexokinase II (HK-II) is the main isoform of the Hexokinases and is responsible for malignant phenotypes. HKII binds to the outer mitochondrial membrane via the Voltage-Dependent Anion Channel (VDAC), a Porin-like protein. HKII has become an attractive therapeutic target for its role in cancer metastasis.
2. Protocol Summary

Screening compound & controls preparation

↓

Enzyme and substrate solution preparation

↓

Add enzyme solution to wells. Incubate for 5 minutes at 25°C

↓

Add substrate solution to wells

↓

Measure absorbance at OD450 nm in kinetic mode for 5 -30 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.

△ 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

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Storage temperature (before prep)</th>
<th>Storage temperature (after prep)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HK Assay Buffer</td>
<td>25 mL</td>
<td>-20°C</td>
<td>4°C/ -20°C</td>
</tr>
<tr>
<td>HK Substrate</td>
<td>1 mL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>HK Coenzyme</td>
<td>1 vial</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>HK Converter</td>
<td>1 vial</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>HK Developer</td>
<td>1 vial</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Hexokinase II (human)</td>
<td>10 µL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>HK Inhibitor Control (Bromopyruvic Acid)</td>
<td>1 vial</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
</tbody>
</table>
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 (ddH₂O)
- 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, preferably white
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 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.

- 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 HK Assay Buffer (25 mL):
Ready to use as supplied. Equilibrate to room temperature before use. Store at 4°C or -20°C.

9.2 HK Substrate (1 mL):
Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C.

9.3 HK Coenzyme (lyophilized, 25 mg):
Reconstitute HK Coenzyme with 220 µL of ddH₂O. Pipette up and down to dissolve completely. Aliquot coenzyme 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.

9.4 HK Converter (lyophilized, 10 U):
Reconstitute HK Converter with 220 µL of HK Assay Buffer by pipetting up and down. Aliquot converter 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.

9.5 HK Developer (lyophilized, 30 mg):
Reconstitute HK Developer with 220 µL of ddH₂O. 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. Keep on ice while in use.

9.6 Hexokinase II (human) (10 µL):
Ready to use as supplied. Equilibrate to room temperature before use. Aliquot enzyme so that you have enough volume to perform the desired number of assays. Store at -20°C.

9.7 HK Inhibitor Control (Bromopyruvic Acid) (lyophilized, 50 µmol):
Reconstitute HK Inhibitor Control with 100 µL of ddH₂O. Aliquot inhibitor 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.

Immediately prior to use, dilute 1:50 in Assay Buffer:
5 µL
Inhibitor Control + 245 µL Assay Buffer
10. Sample Preparation

General sample information:
- Always prepare a fresh set of samples and controls for every use.

10.1 Screening Compounds:
10.1.1 Dissolve test compounds into proper solvent.
10.1.2 Dilute to 2X the desired test concentration with HK Assay Buffer before use.

△ Note: We suggest using different concentrations of test compounds if effective concentration is unknown.
11. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all controls and samples in duplicate.

⚠️ Note: preferred final solvent concentration should not be more than 5% by volume. If solvent exceeds 5%, include solvent control to test the effect of the solvent on enzyme activity.

11.1 Set up Reaction wells:
- Sample compound wells (S) = 50 µL test compounds.
- Inhibitor Control wells (IC) = 50 µL diluted Inhibitor Control (1:50).
- Enzyme Control wells (EC) = 50 µL HK Assay Buffer.
- OPTIONAL: Solvent control (SC) = 10 µL solvent.

11.2 Prepare Hexokinase II Enzyme Solution:
11.2.1 Dilute HK-II 1:100 with HK Assay Buffer. Mix well. Prepare enough mix for all tests (eg. 2 µL HK-II +198 µL Assay buffer).
11.2.2 Add 5 µL diluted HK-II Enzyme Solution to each well.
11.2.3 Incubate for 5 minutes at 25°C.
⚠️ Note: Do not store unused diluted HK-II. Always prepare a fresh stock when needed.

11.3 Hexokinase Substrate Mix:
11.3.1 Prepare 45 µL of Substrate Mix for each reaction. Mix enough reagents for the number of assays to be performed. Prepare a master mix to ensure consistency.

<table>
<thead>
<tr>
<th>Component</th>
<th>Hexokinase II Substrate Mix (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HK Assay Buffer</td>
<td>29</td>
</tr>
<tr>
<td>HK Substrate</td>
<td>10</td>
</tr>
<tr>
<td>HK Coenzyme</td>
<td>2</td>
</tr>
<tr>
<td>HK Converter</td>
<td>2</td>
</tr>
<tr>
<td>HK Developer</td>
<td>2</td>
</tr>
</tbody>
</table>
11.3.2 Add 45 µL of Hexokinase II Substrate Mix into each well.
11.3.3 Mix well with gentle shaking.

11.4 Measurement:
11.4.1 Measure immediately absorbance (OD = 450 nm) on a microplate reader in kinetic mode for 5 - 30 minutes at 25°C.
12. Calculations

- Use only the linear rate for calculation.

12.1 Plot readings for each sample test compound (S), inhibitor control (IC) and enzyme control (EC).

12.2 Draw the line of the best fit to construct the curve (most plate reader software or Excel can do this step). Calculate the trend line equation (use the equation that provides the most accurate fit).

12.3 Choose two points (T1 and T2) in the linear range of the plot and obtain the corresponding values for the absorbance (OD1 and OD2).

12.4 Calculate Slope ($\Delta OD/\Delta T$) for all samples (S), Enzyme Control (EC) and Inhibitor control (IC), if desired, as follows:

$$\Delta OD/\Delta T = (OD2 - ODU1)/ (T2 - T1)$$

12.5 Average the slope for each duplicate reading.

12.6 Calculate the % Relative Inhibitions as follows:

$$\% \text{ Relative Inhibition} = \frac{Slope \ of \ EC - Slope \ of \ S}{Slope \ of \ EC} \times 100$$

△ Note: The Relative Activity of the Enzyme Control should be set as 100%.

△ Note: Irreversible inhibitors that inhibit the Hexokinase II activity completely at the tested concentration will have $\Delta OD = 0$ and thus % Relative Inhibition will be 100%.

△ Note: If OD of SC < OD of EC = make a higher stock of test inhibitor, or dissolve the inhibitor in lower concentration of the solvent; or use a different solvent if possible.

If OD of S < OD of EC = treat as 100% inhibition and further dilute the test inhibitor and repeat the assay.
13. Typical Data

Data provided for demonstration purposes only.

**Figure 1.** Typical inhibition curve of human Hexokinase II activity by the hexokinase inhibitor Bromopyruvic Acid. IC$_{50}$ was determined to be 3 µM. Assay was performed following the kit protocol.
14. 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 reagents and aliquot; get equipment ready.
- Prepare test compounds in suitable solvents; dilute if appropriate.
- Set up Sample compound wells (S) (50 µL test compounds), Inhibitor Control wells (IC) (50 µL diluted 1:50 Inhibitor Control), Enzyme Control wells (EC) (50 µL HK Assay Buffer), Solvent control (SC) (50 µL solvent).
- Prepare HK-II Enzyme Solution (5 µL/well) by diluting HK-II 1:100 with HK Assay Buffer. Prepare a mix for all wells.
- Add 5 µL HK-II Enzyme Solution to each well. Incubate for 5 minutes at 25°C.
- Prepare Hexokinase Substrate Mix (45 µL/well) master mix as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Hexokinase Substrate Mix (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HK Assay Buffer</td>
<td>29</td>
</tr>
<tr>
<td>HK Substrate</td>
<td>10</td>
</tr>
<tr>
<td>HK Coenzyme</td>
<td>2</td>
</tr>
<tr>
<td>HK Converter</td>
<td>2</td>
</tr>
<tr>
<td>HK Developer</td>
<td>2</td>
</tr>
</tbody>
</table>

- Add 45 µL/substrate mix to each well.
- Measure absorbance (OD = 450 nm) on a microplate reader in kinetic mode for 5 - 30 minutes at 25°C.
## 15. Troubleshooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Assay not working</strong></td>
<td>Use of ice-cold buffer</td>
<td>Buffers must be at assay temperature</td>
</tr>
<tr>
<td></td>
<td>Plate read at incorrect wavelength</td>
<td>Check the wavelength and filter settings of instrument</td>
</tr>
<tr>
<td></td>
<td>Use of a different microplate</td>
<td>Colorimetric: clear plates</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fluorometric: black wells/clear bottom plates</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Luminometric: white wells/clear bottom plates</td>
</tr>
<tr>
<td><strong>Assay with erratic readings</strong></td>
<td>Pipetting errors</td>
<td>Avoid pipetting small volumes (&lt; 5 µL) and prepare a master mix whenever possible</td>
</tr>
<tr>
<td></td>
<td>Air bubbles formed in well</td>
<td>Pipette gently against the wall of the tubes</td>
</tr>
<tr>
<td><strong>No fluorescence above background in inhibitor wells</strong></td>
<td>Inhibitor concentration is too high</td>
<td>Reduce concentration of inhibitor and re-do assay</td>
</tr>
<tr>
<td><strong>No inhibition seen in test compound wells</strong></td>
<td>Inhibitor concentration is not high enough</td>
<td>Increase concentration of inhibitor and re-do assay</td>
</tr>
<tr>
<td></td>
<td>Compound is not an inhibitor</td>
<td>Use another compound for your test</td>
</tr>
</tbody>
</table>
16. Notes
Technical Support

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