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Product Information

Hexokinase Colorimetric Assay Kit

Catalog Number **MAK091**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

Hexokinases, which catalyze the ATP-dependent phosphorylation of aldo- and keto-hexoses to hexose-6-phosphate, catalyze the first step in a number of important biochemical pathways. In mammalian tissues, glucose is the predominant substrate for the hexokinases. There are four isozymes of hexokinase in mammalian tissue, (HK-I, -II, -III, and –IV), which differ in their kinetic and regulatory properties as well as tissue distribution and cofactor use. The hexokinases catalyze the first step in most of the relevant glucose metabolism pathways. Alterations in hexokinase activity are associated with multiple disorders such as X-linked muscular dystrophy, hemolytic anermias, and cancer.

The Hexokinase Colorimetric Assay kit provides a simple and direct procedure for measuring hexokinase activity in a variety of samples. Hexokinase activity is determined by a coupled enzyme assay, in which glucose is converted to glucose-6-phosphate by hexokinase, which is oxidized by glucose-6-phosphate dehydrogenase to form NADH. The resulting NADH reduces a colorless probe resulting in a colorimetric (450 nm) product proportional to the hexokinase activity present. One unit of HK is the amount of enzyme that will generate 1.0 μmole of NADH per minute at pH 8.0 at room temperature.

Components

The kit is sufficient for 100 assays in 96 well plates.

Component	Quantity
HK Assay Buffer Catalog Number MAK091A	25 mL
HK Substrate Catalog Number MAK091B	1 mL
HK Coenzyme Catalog Number MAK091C	1 vl
HK Enzyme Mix Catalog Number MAK091D	1 vl
HK Developer Catalog Number MAK091E	1 vl
NADH Standard Catalog Number MAK091F	1 vl
HK Positive Control Catalog Number MAK091G	1 vl

Reagents and Equipment Required but Not Provided.

- 96 well flat-bottom plate – It is recommended to use clear plates for colorimetric assays.
- Spectrophotometric multiwell plate reader

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

Briefly centrifuge vials before opening. Use ultrapure water for the preparation of reagents. To maintain reagent integrity, avoid repeated freeze/thaw cycles.

HK Assay Buffer – Allow buffer to come to room temperature before use.

HK Coenzyme and HK Enzyme Mix – Reconstitute each with 220 μL of HK Assay Buffer. Mix well by pipetting (don't vortex), then aliquot each and store, protected from light, at –20 °C. Use within 2 months of reconstitution and keep cold while in use

HK Developer – Reconstitute with 220 μL of water. Mix well by pipetting, then aliquot and store, protected from light, at –20 °C. Use within 2 months of reconstitution.

NADH Standard – Reconstitute with 400 μL of water to generate a 1.25 mM (1.25 nmole/μL) NADH stock solution. Mix well by pipetting, then aliquot and store at –20 °C. Use within 2 months of reconstitution.

HK Positive Control – Reconstitute with 100 μL of HK Assay Buffer. Mix well by pipetting, then aliquot and store, protected from light, at –20 °C. Use within 2 months of reconstitution.

Storage/Stability

The kit is shipped on wet ice and storage at –20 °C, protected from light, is recommended.

Procedure

All samples and standards should be run in duplicate.

NADH Standards for Colorimetric Detection

Add 0, 2, 4, 6, 8, and 10 μL of the 1.25 mM standard solution into a 96 well plate, generating 0 (blank), 2.5, 5, 7.5, 10, and 12.5 nmole/well standards. Add HK Assay Buffer to each well to bring the volume to 50 μL.

Sample Preparation

Tissue (10 mg) or cells (1×10^6) can be homogenized in 200 μL of ice-cold HK Assay Buffer. Centrifuge the samples at 13,000 × *g* for 10 minutes to remove insoluble material.

Note: For unknown samples, it is suggested to test several sample dilutions to ensure the readings are within the linear range of the standard curve.

Bring samples to a final volume of 50 μL with HK Assay Buffer.

Notes: NADH in the samples can generate a background signal. To remove the effect of NADH background, a sample blank may be set up for each sample by omitting the HK Substrate.

For the positive control, dilute 1 μL of the HK positive control into 99 μL of HK Assay Buffer. Add 1–10 μL of the diluted positive control into wells and bring to a final volume of 50 μL with HK Assay Buffer.

Assay Reaction

1. Set up the Reaction Mixes according to the scheme in Table 1. 50 μL of the apppropriate Reaction Mix is required for each reaction (well).

Table 1.
Reaction Mixes

Reagent	Samples and Standards	Sample Blank
HK Assay Buffer	34 μL	44 μL
HK Enzyme Mix	2 μL	2 μL
HK Developer	2 μL	2 μL
HK Coenzyme	2 μL	2 μL
HK Substrate	10 μL	–

2. Add 50 μL of the appropriate Reaction Mix to each of the wells. Mix well using a horizontal shaker or by pipetting. Protect the plate from light during the incubation

3. Incubate the plate at room temperature. After 5 minutes, take the initial measurement ($T_{initial}$). Measure the absorbance at 450 nm at the initial time $(A_{450})_{initial}$.
   Note: It is essential $(A_{450})_{initial}$ is in the linear range of the standard curve.

4. Continue to incubate the plate at room temperature taking measurements (A_{450}) every 5 minutes. Protect the plate from light during the incubation.

5. Continue taking measurements until the value of the most active sample is greater than the value of the highest standard (10 nmole/well). At this time the most active sample is near or exceeds the end of the linear range of the standard curve.

6. The final measurement $[(A_{450})_{final}]$ for calculating the enzyme activity would be penultimate reading or the value before the most active sample is near or exceeds the end of the linear range of the standard curve, see step 5. The time of the penultimate reading is T_{final}.
   Note: It is essential the final measurement falls within the linear range of the standard curve.

Results

Calculations

Correct for the background by subtracting the final measurement [$(A_{450})_{final}$] obtained for the 0 (blank) NADH standard from the final measurement [$(A_{450})_{final}$] of the standards and samples. Background values can be significant and must be subtracted from all readings. Plot the NADH standard curve.

Note: A new standard curve must be set up each time the assay is run.

Subtract the final blank sample value from the final sample reading to obtain the corrected measurement. Using the corrected measurement, the amount of NADH present in the samples may be determined from the standard curve.

Using the corrected measurements, calculate the change in measurement from $T_{initial}$ to T_{final} for the samples.

$$\Delta A_{450} = (A_{450})_{final} - (A_{450})_{initial}$$

Compare the ΔA_{450} of each sample to the standard curve to determine the amount of NADH generated between $T_{initial}$ and T_{final} (B).

The HK activity of a sample may be determined by the following equation:

$$\text{HK Activity} = \frac{B \times \text{Sample Dilution Factor}}{(\text{Reaction Time}) \times V}$$

B = Amount (nmole) of NADH generated between $T_{initial}$ and T_{final}.

Reaction Time = $T_{final} - T_{initial}$ (minutes)

V = sample volume (mL) added to well

HK activity is reported as nmole/min/mL = milliunit/mL

One unit of HK is the amount of enzyme that will generate 1.0 μmole of NADH per minute at pH 8.0 at room temperature.

Example:

NADH amount (B) = 5.84 nmole

First reading ($T_{initial}$) = 5 minute

Second reading (T_{final}) = 35 minutes

Sample volume (V) = 0.01 mL

Sample dilution is 1

HK activity is:

$$\frac{5.84 \times 1}{(35 - 5) \times 0.01} = 19.47 \text{ milliunits/mL}$$

Troubleshooting Guide

Problem	Possible Cause	Suggested Solution
Assay not working	Cold assay buffer	Assay Buffer must be at room temperature
	Omission of step in procedure	Refer and follow Technical Bulletin precisely
	Plate reader at incorrect wavelength	Check filter settings of instrument
	Type of 96 well plate used	For colorimetric assays, use clear plates
Samples with erratic readings	Samples prepared in different buffer	Use the Assay Buffer provided or refer to Technical Bulletin for instructions
	Cell/Tissue culture samples were incompletely homogenized	Repeat the sample homogenization, increasing the length and extent of homogenization step.
	Samples used after multiple freeze-thaw cycles	Aliquot and freeze samples if needed to use multiple times
	Presence of interfering substance in the sample	If possible, dilute sample further
	Use of old or inappropriately stored samples	Use fresh samples and store correctly until use
Lower/higher readings in samples and standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Use of expired kit or improperly stored reagents	Check the expiration date and store the components appropriately
	Allowing the reagents to sit for extended times on ice	Prepare fresh Master Reaction Mix before each use
	Incorrect incubation times or temperatures	Refer to Technical Bulletin and verify correct incubation times and temperatures
	Incorrect volumes used	Use calibrated pipettes and aliquot correctly
Non-linear standard curve	Use of partially thawed components	Thaw and resuspend all components before preparing the reaction mix
	Pipetting errors in preparation of standards	Avoid pipetting small volumes
	Pipetting errors in the Reaction Mix	Prepare a Master Reaction Mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the plate well
	Standard stock is at incorrect concentration	Refer to the standard dilution instructions in the Technical Bulletin
	Calculation errors	Recheck calculations after referring to Technical Bulletin
	Substituting reagents from older kits/lots	Use fresh components from the same kit
Unanticipated results	Samples measured at incorrect wavelength	Check the equipment and filter settings
	Samples contain interfering substances	If possible, dilute sample further
	Sample readings above/below the linear range	Concentrate or dilute samples so readings are in the linear range

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