# Amplite® Colorimetric Hexokinase Assay Kit

Catalog number: 11319
Unit size: 100 Tests

| Component | Storage | Amount (Cat No. 11319) |
|---|---|---|
| Component A: HK Assay Buffer | Freeze (< -15 °C), Minimize light exposure | 5 mL |
| Component B: HK Probe | Freeze (< -15 °C), Minimize light exposure | 1 mL |
| Component C: HK Enzyme Mix | Freeze (< -15 °C) | 1 Bottle |
| Component D: HK Substrate | Freeze (< -15 °C) | 1 Vial |
| Component E: HK Coenzyme | Freeze (< -15 °C) | 1 Vial |
| Component F: Hexokinase Positive Control (20 µg) | Freeze (< -15 °C) | 1 Vial |
| Component G: NADPH Standard (335 µg) | Freeze (< -15 °C), Minimize light exposure | 1 Vial |

## OVERVIEW

The Amplite® Colorimetric Hexokinase Assay Kit is designed to accurately measure the activity of hexokinases from various sources using a simple and quick protocol. This kit utilizes a coupled enzyme assay, where glucose is phosphorylated to glucose-6-phosphate and subsequently oxidized to produce NADH. The resulting NADH reduces a colorless substrate to a colored product, with absorbance measured at 450 nm, which is proportional to the hexokinase present in the samples. One unit (U) is the amount of enzyme that catalyzes the reaction of 1 µmol of substrate per minute. Hexokinases are intracellular enzymes that mediate ATP-dependent phosphorylation of hexoses (e.g., glucose, mannose, and fructose) to their respective hexose 6-phosphates. These phosphate esters can either be broken down to pyruvate by glycolysis or used for different biochemical/biosynthesis pathways. There are four isozymes of hexokinase known in vertebrates, Hexokinase I (A), II (B), III (C), and IV (D), which have different intracellular and tissue distribution, and kinetic characteristics. Changes in hexokinase level and activity are associated with diseases like X-linked muscular dystrophy, chronic non-spherocytic hemolytic anemia, insulin resistance, and cancer.

## AT A GLANCE

### Protocol Summary

1. Prepare the test samples and the serially diluted NADPH standards (50 µL).
2. Add the HK working solution (50 µL).
3. Incubate at room temperature for 10-30 minutes.
4. Measure the absorbance at 450 nm.

## KEY PARAMETERS

### Absorbance microplate reader

| | |
|---|---|
| Absorbance | 450 nm |
| Recommended plate | Clear bottom |

## PREPARATION OF STOCK SOLUTIONS

*Unless otherwise noted, all unused stock solutions should be divided into single-use aliquots and stored at -20 °C after preparation. Avoid repeated freeze-thaw cycles*

### NADPH Standard

1. Add 200 µL of PBS buffer to the vial of NADPH standard (Component G) to make a 2 mM (2 nmol/µL) NADPH stock solution. Store the solution at -80°C. Avoid repeated freeze/thaw cycles.

### HK Positive Control

1. To reconstitute the Hexokinase Positive Control (Component F), add 50 µL of ddH2O to prepare a 100 µg/mL stock solution. Mix well by pipetting and store at –20 °C.

   **Note:** Must be used within 2 months of reconstitution.

### 100X HK Substrate Stock Solution

1. To prepare a 100X HK Substrate stock solution, add 100 µL of ddH2O to the vial containing the HK Substrate (Component D). After mixing, store the solution at -20°C. Avoid repeated freezing and thawing.

### HK Coenzyme Stock Solution

1. Add 100 µL of ddH2O to the vial containing HK Coenzyme (Component E) to create a 100X stock solution. Store this solution at -20°C, and avoid repeated freeze/thaw cycles.

## PREPARATION OF STANDARD SOLUTIONS

For convenience, use the Serial Dilution Planner:
https://www.aatbio.com/tools/serial-dilution/11319

### NADPH Standard

Prepare a 200 µM NADPH solution (STD7) by add 3 µL of a 2 mM standard solution to 297 µL of PBS Buffer. Then, take 150 µL of the STD7 solution and perform 1:2 serial dilutions with PBS Buffer to create a series of diluted NADPH standards (STD7 to STD1).

## PREPARATION OF WORKING SOLUTION

### HK Working Solution

1. Add 1 mL of HK Probe (Component B) to 4 mL HK Assay Buffer (Component A), and mix well.
2. Transfer 5 mL of the above-prepared buffer mixture into the HK Enzyme Mix bottle (Component C) and mix thoroughly.
3. Add 50 µL of the HK Substrate stock solution to the same bottle and mix well.

4. Add 50 µL of the HK Coenzyme stock solution to the same bottle and mix well.

   **Note:** This HK working solution should be freshly prepared before each experiment and protected from light. A 5.0 mL solution is enough for 100 tests. Please prepare the necessary amount of HK working solution based on this proportion.

   **Note:** Alternatively, one can make a 50X HK Enzyme Mix stock solution by adding 100 µL of ddH2O into the bottle of HK Enzyme Mix (Component C) and then prepare the HK working solution by mixing the HK Enzyme Mix stock solution with other components listed above in the 'HK Working Solution' proportionally.

## SAMPLE EXPERIMENTAL PROTOCOL

**HK Positive Control**

1. Prepare one or more HK positive control samples along with the test sample. The recommended concentration for the HK positive control is between 0.5 and 0.1 µg/ml in PBS.

**Table 1.** Layout of NADPH standards and test samples in a 96-well solid black microplate. (STD = NADPH Standards (STD1-STD7, 3.125~200 µM), BL= Blank Control, TS = Test Samples.)

| BL | BL | HK Positive Control | TS |
|---|---|---|---|
| STD 1 | STD 1 | ... | ... |
| STD 2 | STD 2 | ... | ... |
| STD 3 | STD 3 | | |
| STD 4 | STD 4 | | |
| STD 5 | STD 5 | | |
| STD 6 | STD 6 | | |
| STD 7 | STD 7 | | |

**Table 2.** Reagent composition for each well.

| Well | Volume | Reagent |
|---|---|---|
| STD 1-STD 7 | 50 µL | Serial Dilutions (3.125 to 200 µM) |
| BL | 50 µL | PBS |
| HK Positive Control | 50 µL | HK Positive Control |
| TS | 50 µL | Test Sample |

1. Prepare NADPH standards (STD1-7), blank controls (BL), HK Positive Control, and test samples (TS) as outlined in Tables 1 and 2. For a 384-well plate, use 25 µL of reagent per well instead of 50 µL.

2. Add 50 µL of HK Working Solution to each well containing the NADPH standard, blank control, HK Positive Control, and test samples. For a 384-well plate, add 25 µL of HKNE Working Solution to each well instead.

3. Incubate at room temperature for 10-30 minutes, protected from light.

4. Monitor the absorbance intensity with an absorbance microplate reader at 450 nm.

## EXAMPLE DATA ANALYSIS AND FIGURES

The absorbance reading in the blank wells (containing only PBS) is used as a control. This reading is subtracted from the absorbance values of the wells with NADPH standards, HK positive, and test samples. Figure 1 shows the standard curve for NADPH. To calculate the NADPH concentrations in your samples using this standard curve, we recommend using the Online Linear Regression Calculator available at:

https://www.aatbio.com/tools/linear-logarithmic-semi-log-regression-online-calculator

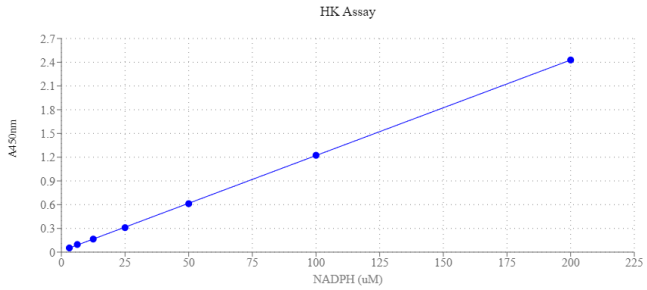



**Figure 1.** The NADPH dose response was measured using the Amplite® Colorimetric Hexokinase Assay Kit on a 96-well clear-bottom microplate. After a 10-minute incubation, readings were taken at 450 nm with a ClarioStar microplate reader (BMG).

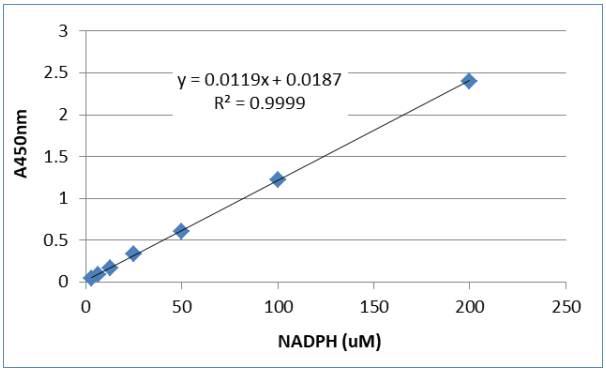
**Data Analysis Example**

Calculate HK Activity Example

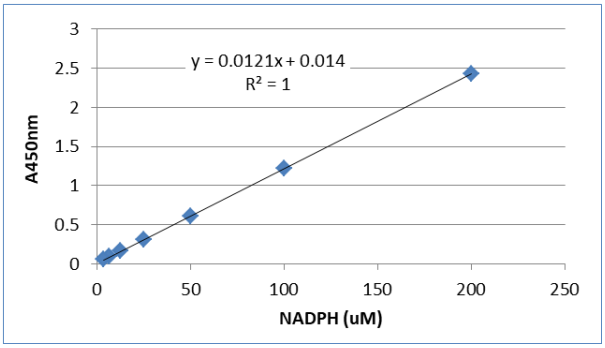
- HK positive control: 0.1ug/ml in PBS
- 0min to 10min kinetic reading,
- Use 0min and 10min absorbance readings to do the calculation

1. Plot NADPH Calibration curves at 0 min and 10min:

**0min Curve**



**10min Curve**




2. Calculate NADPH generated during 10min.

| HK-Positive control_0.1 µg/ml | HK-BG | NADPH (µM) @0min |
|---|---|---|
| 0.247 | 0.065 | 3.89 |

| HK-Positive Control_0.1 µg/ml | HK-BG | NADPH (µM) @ 10min |
|---|---|---|
| 0.9435 | 0.7615 | 61.78 |

| HK (µg/mL) | NADPH generated in 10 min | µM/min=mU/mL | HK Activity (U/mg) |
|---|---|---|---|
| 0.1 | 57.89 µM | 5.789 | 57.89 |

**Note:**

- 1 unit (U) is the amount of enzyme that catalyzes the reaction of 1 µmol of substrate per minute!
- nmole/min/mL= uM/min = mU/mL

**DISCLAIMER**

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