Technical Bulletin

# Pyruvate Dehydrogenase Activity Assay Kit

**Catalogue number MAK567**

## Product Description

Pyruvate Dehydrogenase (PDH) is a mitochondrial enzyme which has an important role in carbohydrate metabolism. Combining with dihydrolipoyl transacetylase (E2) and dihydrolipoyl dehydrogenase (E3), PDH (E1) forms a well-characterized enzyme complex (PDC).

In the presence of $NAD^+$ and CoA, PDH catalyzes the conversion of pyruvate into Acetyl-CoA and $CO_2$. Thus, PDH links the glycolysis metabolic pathway (cytoplasm) to the citric acid cycle (mitochondria).

The Pyruvate Dehydrogenase Activity Assay Kit provides a sensitive, simple, fast, direct, and convenient way for the detection of PDH activity in various samples, based on a colorimetric method. The amount of the colorimetric (450 nm) product is proportional to the enzymatic activity present in the sample.

One unit of pyruvate dehydrogenase is the amount of enzyme that will generate 1.0 µmole of NADH per minute at 37 °C at pH 7.5, in the presence of saturating levels of Coenzyme A. The kit can detect PDH activity that is lower than 0.1 mU, in a wide variety of samples, including tissues, cells as well as purified mitochondria.

## Components

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

- PDH Assay Buffer 25 mL
  Catalogue Number MAK567A
- PDH Substrate 1 Vial
  Catalogue Number MAK567B
- PDH Developer 220 µL
  Catalogue Number MAK567C.
- NADH Standard 400 µL
  Catalogue Number MAK567D.
- PDH Positive Control 20 µL
  Catalogue Number MAK567E.

## Reagents and Equipment Required but Not Provided

- 96-well plates, clear, flat bottom. It is recommended to use clear plates for colorimetric assays. (Catalogue Number M2936 or equivalent)
- Plate reader that is capable to read wavelength of 450 nm.
- Horizontal Shaker
- Vortex Mixer
- Saturated Ammonium Sulfate, ~4.1 M $(NH_4)_2SO_4$ (optional, for samples containing small interfering molecules)
- Mitochondria isolation kit (Catalogue Number MITOISO1 or MITOISO2) (optional, to assay purified mitochondria)

## Precautions and Disclaimer

For R&D use only. Not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

## Storage/Stability

The product is shipped at room temperature. Store at -20 °C upon receipt, protected from light.

## Preparation Instructions

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

PDH Assay Buffer - Allow buffer to come to room temperature before use. Store at 2 - 8°C or at –20 °C.

PDH Substrate - Reconstitute with 220 µL of ultrapure water. Aliquot and store at –20 °C. Keep on ice while in use. Use within two months. Avoid repeated freeze/thaw cycles.

PDH Developer - Aliquot and store protected from light at -20 °C. Keep on ice while in use. Avoid repeated freeze/thaw cycles.

NADH Standard - 1.25 mM NADH Standard Solution. Aliquot and store at –20 °C. Keep on ice while in use. Avoid repeated freeze/thaw cycles.

PDH Positive Control - Dilute with 100 µL of PDH Assay Buffer. Mix gently by pipetting, then aliquot and store protected from light at -20 °C. Use within 2 months of dilution. Keep on ice while in use. Avoid repeated freeze/thaw cycles.

## Procedure

All Samples and Standards should be run in technical triplicates. For your convenience, an online excel-based calculator is available on the product's webpage, at

https://www.sigmaaldrich.com/US/en/product/sigma/mak567

Sample Types:

Animal tissues: heart, liver, muscle, etc.

Purified mitochondria.

Cell culture: Adherent or suspension cells.

Before beginning, it is advisable to pre-heat the plate reader to 37 °C and setup the appropriate reading protocol. The protocol should be as follows:

- Preheat to 37 °C.
- Start a 60-minutes Kinetics program with 5-minutes intervals.

Each kinetic step includes a 10 second horizontal shake followed by absorbance reading at A450.

### Preparation of NADH Standards

1. Add 0, 2, 4, 6, 8, and 10 µL of the 1.25 mM NADH Standard solution into a 96-well plate to generate 0 (blank), 2.5, 5, 7.5, 10, and 12.5 nmole/well standards.
2. Add PDH Assay Buffer to each Standard well to bring the volume to 50 µL. See "Table A" if using the online Kit Calculator.

### Sample Preparation

Liquid Samples may be assayed directly.

Tissue Samples (10 mg) or cells ($10^6$) can be homogenized in 100 µL of ice-cold PDH Assay Buffer. Keep on ice for 10 minutes. Centrifuge the samples at 10,000 x g for 5 minutes to remove insoluble material. Transfer supernatant to a fresh tube.

For unknown Samples, it is suggested to test several different Sample dilutions to ensure that the readings are within the linear range of the Standard curve.

Add 5–50 µL of Sample to triplicate wells. Bring Samples to a final volume of 50 µL with PDH Assay Buffer.

**Note**: Although fresh Samples are always preferred, cell\tissue lysates can be stored at -80 °C before the assay. The lysates should be aliquoted if the assay will be repeated with the same samples to minimize freezing and thawing. Alternately, all cells may be stored at -80 °C and the lysates can be prepared on the day of the experiment.

**Note**: For samples exhibiting significant background, include a Sample Blank for each sample by omitting the PDH Substrate. The Sample Blank readings can then be subtracted from the sample readings. See Table 1.

**Note**: Small molecules in some tissues, such as liver, may interfere with the assay. To remove small molecules, it is suggested to use an ammonium sulfate precipitation method. Pipette 50–100 µL of lysate into a fresh tube, add 2X volume of saturated ammonium sulfate (~4.1 M, at room temperature) and keep on ice for 20 minutes. Centrifuge at 10,000 x g for 5 minutes, remove and discard the supernatant, and resuspend the pellet to the original volume with the PDH Assay Buffer.

**Note**: It is extremely important that no air bubbles are formed in any well. Pipette gently to avoid bubbling and blow air lightly to burst any bubbles. Air bubbles will yield significantly erratic readings and adverse results.

### Positive Control – Optional

Add 1–10 µL of the PDH Positive Control solution to triplicate wells and adjust to a final volume of 50 µL with PDH Assay Buffer.

### Assay Reaction

1. Set up the Reaction Mixes according to the scheme in "Table B" in the Customer Calculator or see Table 1.
2. 50 µL of the Reaction Mix is required for each reaction well. Briefly, multiply the number of wells by 46 to obtain the PDH Assay Buffer volume (µL). Multiply number of wells by 2 to obtain the PDH Substrate volume (µL) and PDH Developer volume (µL).

   All samples and standards should be run in technical triplicates. It is highly advisable to use a multi-channel pipettor and troughs, when possible, in order to minimize the technical error.

   The volume in each well is 50 µL, for both standard wells and sample wells.

**Table 1.**

Preparation of Reaction Mixes

| Component | Samples and Standards | Blank |
|---|---|---|
| PDH Assay Buffer | 46 µL | 48 µL |
| PDH Developer | 2 µL | 2 µL |
| PDH Substrate | 2 µL | - |

3. Add 50 µL of the appropriate reaction mix to each well. Mix by using a horizontal shaker or pipetting. The volume in each well is now 100 µL, for both standard wells and sample/positive control wells.
4. Insert the plate to the pre-heated (37 °C) plate reader. Immediately measure the absorbance at 450 nm (Initial A450).
5. Continue to incubate the plate at 37 °C, taking measurements (A450) every 5 minutes, for 60 min. Protect the plate from light during the incubation.
6. The final absorbance measurement (Final A450) for calculating the enzyme activity would be the penultimate reading or the value before the most active sample is near or exceeds the end of the linear range of the standard curve. The time of the penultimate reading is T final.

**Note**: It is essential that Final A450 falls within the linear range of the standard curve.

## Results

### Calculations

For your convenience, an online excel-based calculator is available on the product's webpage, at https://www.sigmaaldrich.com/US/en/product/sigma/mak567.
Simply copy and paste the raw data obtained from the plate reader according to the instructions.

Alternatively, follow the following steps.

Correct for the background by subtracting the final measurement (Final A450) obtained for the 0 (blank) NADH Standard from the final measurement (Final A450) of the standards and samples. Background values can be significant and must be subtracted from all readings. Use the values obtained from the appropriate NADH Standards to plot a standard curve.

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

Calculate the change in absorbance measurement from T initial to T final for the samples.

$\Delta A_{450}$ = (Final A450) – (Initial $A_{450}$)

Determine the amount of NADH (nmole/well) generated by the PDH assay between T initial and T final (Sa), by dividing the sample's value of DA450 by the slope of the NADH Standard Curve's linear trendline.

PDH Activity:

$$\text{PDH Activity} = \frac{\text{Sa}}{(\text{Reaction time}) \times \text{Sv}}$$

Where:

Sa = Amount of NADH (nanomole) generated in unknown sample well, between T initial and T final, using the NADH standard curve

Reaction Time = T final – T initial (minutes)

Sv = sample volume (mL) added to well

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

**Note**: One unit of pyruvate dehydrogenase is the amount of enzyme that will generate 1.0 1.0 µmole of β-NAD$^+$ to β-NADH per minute at pH 7.5 at 37 °C, in the presence of saturating levels of Coenzyme A.

Sample Calculation:

Amount of NADH (Sa) = 5.84 nmole

(Calculated from the standard curve)

(T initial) = 3 minutes

(T final) = 32 minutes

Sample volume (Sv) = 0.05 mL

PDH activity in sample well:

$$(\text{milliunits/mL}) = \frac{5.84 \text{ nmole/well}}{(32\text{min} - 3\text{min}) \times 0.05 \text{ mL/well}} = 4.03$$

**Figure 1.**

An exemplary standard curve.

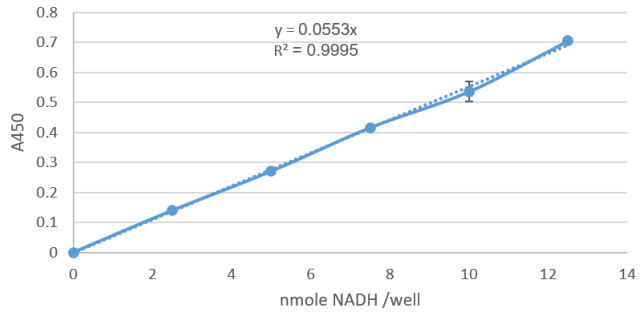



**Figure 2.**

An exemplary result of the assay performed on HeLa cells.

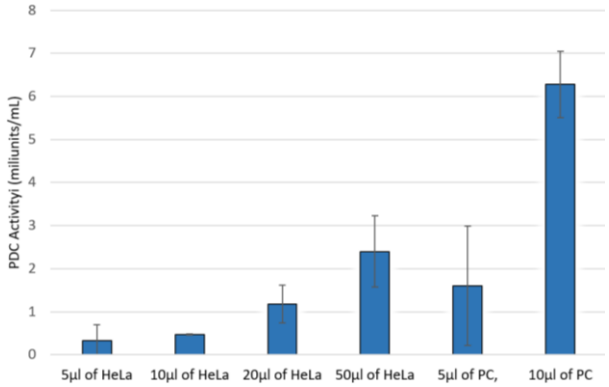
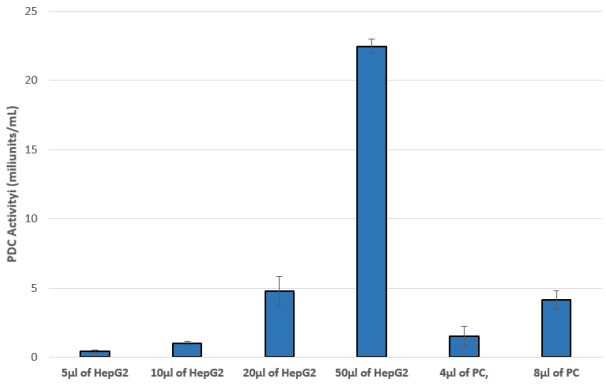



**Figure 3.**

An exemplary result of the assay performed on HepG2 cells.

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## 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 flat bottom, 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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## Notice

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