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Product Information

Phosphofructokinase (PFK) Activity Colorimetric Assay Kit

Catalog Number **MAK093**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

Phosphofructokinase (PFK) catalyzes the third step of glycolysis, the conversion of fructose-6-phosphate to fructose-1,6-diphosphate, the rate limiting step of glycolysis. PFK activity is highly regulated by multiple cofactors in addition to post-translational modifications and can be used to measure glycolytic flux in tissues. Deficiencies in PFK activity can result in the glycogen storage disease, glycogenosis type VII (Tarui's disease).

The Phosphofructokinase Colorimetric Assay kit provides a simple and direct procedure for measuring PFK activity in a variety of samples. PFK activity is determined by a coupled enzyme assay, in which fructose-6-phosphate and ATP is converted to fructose-1,6-diphosphate and ADP by PFK. The ADP is converted by the enzyme mix to AMP and NADH. The resulting NADH reduces a colorless probe resulting in a colorimetric (450 nm) product proportional to the PFK activity present. One unit of PFK is the amount of enzyme that will generate 1.0 μmole of NADH per minute at pH 7.4 at 37 °C.

Components

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

PFK Assay Buffer 27 mL
Catalog Number MAK093A

PFK Substrate 1 vl
Catalog Number MAK093B

ATP 1 vl
Catalog Number MAK093C

PFK Enzyme Mix 1 vl
Catalog Number MAK093D

PFK Developer 1 vl
Catalog Number MAK093E

NADH Standard 1 vl
Catalog Number MAK093F

Positive Control 1 vl
Catalog Number MAK093G

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.

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

PFK Substrate and PFK Enzyme Mix – Reconstitute each with 220 μL of PFK 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.

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

NADH Standard – Reconstitute with 40 μL of PFK Assay Buffer to generate a 10 mM (10 nmole/μL) NADH stock solution. Mix well by pipetting, then aliquot and store at –20 °C. Use within 2 months of reconstitution.

Positive Control – Reconstitute with 100 µL of PFK 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
Dilute 10 µL of the 10 mM NADH Standard solution with 90 µl of PFK Assay Buffer to prepare a 1 mM (1 nmole/µL) standard solution. Add 0, 2, 4, 6, 8, and 10 µL of the 1 mM standard solution into a 96 well plate, generating 0 (blank), 2, 4, 6, 8, and 10 nmole/well standards. Add PFK Assay Buffer to each well to bring the volume to 50 µL.

Sample Preparation
Tissue (20 mg) or cells (2×10^6) can be homogenized in 200 µL of ice-cold PFK 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 PFK Assay Buffer.

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

Add 10–20 µL of the positive control into wells and bring to a final volume of 50 µL with PFK 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
PFK Assay Buffer	42 µL	44 µL
PFK Enzyme Mix	2 µL	2 µL
PFK Developer	2 µL	2 µL
ATP	2 µL	2 µL
PFK Substrate	2 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 37 °C. 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 37 °C 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 PFK activity of a sample may be determined by the following equation:

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

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

PFK activity is reported as nmole/min/mL = milliunit/mL
One unit of PFK is the amount of enzyme that will generate 1.0 μmole of NADH per minute at pH 7.4 at 37 °C.

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

PFK 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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