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Product Information

PEP Colorimetric/Fluorometric Assay Kit

Catalog Number **MAK102**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

Phospho(enol)pyruvate (PEP) in an intermediate in carbohydrate metabolism involved in glycolysis and gluconeogeneis. In glycolysis, PEP is metabolized by pyruvate kinase to yield pyruvate. In plants, PEP is involved in the formation of aromatic amino acids as well as in the carbon fixation pathway.

PEP concentration is determined by a coupled enzyme assay, in which PEP is converted to ATP and pyruvate, resulting in a colorimetric (570 nm)/fluorometric (λ_{ex} = 535/λ_{em} = 587 nm) product, proportional to the PEP present.

Components

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

PEP Assay Buffer Catalog Number MAK102A	25 mL
PEP Probe, in DMSO Catalog Number MAK102B	0.2 mL
PEP Converter Catalog Number MAK102C	1 vl
PEP Developer Mix Catalog Number MAK102D	1 vl
PEP Standard, 1 μmole Catalog Number MAK102E	1 vl

Reagents and Equipment Required but Not Provided.

- 96 well flat-bottom plate – It is recommended to use black plates with clear bottoms for fluorescence assays and clear plates for colorimetric assays.
- Fluorescence or spectrophotometric multiwell plate reader
- Liquid N_2 or dry/ice methanol
- Perchloric acid (Catalog Number 311413, 1.00518, or equivalent)
- Potassium bicarbonate (Catalog Number 60339 or equivalent)

Precautions and Disclaimer

This product is 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.

Preparation Instructions

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

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

PEP Probe Solution – Warm to room temperature to melt frozen solution prior to use. Aliquot and store, protected from light and moisture, at –20 °C. Upon thawing, the PEP Probe is ready-to-use in the colorimetric assay.

For the fluorescence assay, dilute an aliquot of the colorimetric PEP Probe Solution 5 to 10-fold with PEP Assay Buffer, just prior to use. This will reduce the background of the fluorescence assay.

PEP Converter and PEP Developer Mix – Reconstitute each with 220 µL of PEP Assay Buffer. Mix well by pipetting (do not vortex), then aliquot each and store, protected from light, at –20 °C. Use within 2 months of reconstitution and keep cold while in use.

PEP Standard – Reconstitute with 100 µL of water to generate a 10 mM (10 nmole/µL) PEP stock solution. Mix well by pipetting, then aliquot and store 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.

PEP Standards for Colorimetric Detection
Dilute 10 µL of the 10 mM PEP Standard (10 nmole/µL) with 90 µl of the PEP Assay Buffer to prepare a 1 nmole/µL standard solution. Add 0, 2, 4, 6, 8, 10 µL of the 1 nmole/µL standard solution into a 96 well plate, generating 0 (blank), 2, 4, 6, 8, and 10 nmole/well standards. Add PEP Assay Buffer to each well to bring the volume to 50 µL.

PEP Standards for Fluorometric Detection
Prepare a 1 nmole/µL standard solution as for the colorimetric assay. Dilute 10 µL of the 1 nmole/µL standard solution with 90 µL of the PEP Assay Buffer to make a 0.1 nmole/µL standard solution. Add 0, 2, 4, 6, 8, 10 µL of the diluted 0.1 nmole/µL standard solution into a 96 well plate, generating 0 (blank), 0.2, 0.4, 0.6, 0.8, and 1.0 nmole/well standards. Add PEP Assay Buffer to each well to bring the volume to 50 µL.

Sample Preparation
Cells or tissue (20–50 mg) should be frozen in liquid nitrogen or a dry ice/methanol solution then powdered thoroughly with mortar and pestle at –80 °C. Transfer to a microcentrifuge tube. Add 100 µL of 3 M ice-cold perchloric acid and vortex until contents are thoroughly mixed. Neutralize by adding 10 µL of 3 M potassium bicarbonate until the pH reaches 6.5–7.5. Vortex between potassium bicarbonate additions. Centrifuge at 12,000 × *g* for 3 minutes to remove insoluble material. Some plant extracts may need to be decolorized with activated charcoal. To decolorize, add 5 mg of activated charcoal per tube prior to centrifugation step. Incubate for 5 minutes and then proceed to centrifugation. Use samples immediately or store at –80 °C.
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 PEP Assay Buffer.

Notes: High concentrations of pyruvate in the samples can generate a background signal. To remove the effect of pyruvate background, a sample blank may be set up for each sample by omitting the PEP Converter from the reaction mix.

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

Table 1.
Reaction Mixes

Reagent	Samples and Standards	Sample Blank
PEP Assay Buffer	44 µL	46 µL
PEP Probe	2 µL	2 µL
PEP Converter	2 µL	–
PEP Developer	2 µL	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, and incubate the reaction for 60 minutes at room temperature. Cover the plate and protect from light during the incubation.

3. For colorimetric assays, measure the absorbance at 570 nm (A_{570}). For fluorometric assays, measure fluorescence intensity (λ_{ex} = 535/λ_{em} = 587 nm).

Results

Calculations

The background for either assay is the value obtained for the 0 (blank) PEP standard. Correct for the background by subtracting the blank value from all readings. Use the values obtained from the appropriate PEP standards to plot a standard curve.

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

Sample background values can be significant and must be subtracted from all sample readings. Subtract the sample blank value from the sample reading to obtain the corrected measurement. Using the corrected measurement, the amount of PEP present in the samples may be determined from the standard curve.

Concentration of PEP

$$S_a/S_v = C$$

S_a = Amount of PEP in unknown sample (nmole) from standard curve

S_v = Sample volume (μL) added to reaction well

C = Concentration of PEP in sample

PEP molecular weight: 168.04 g/mole

Sample Calculation

Amount of PEP (S_a) = 5.84 nmole (from standard curve)

Sample volume (S_v) = 50 μL

Concentration of PEP in sample

$$5.84 \text{ nmole}/50\ \mu\text{L} = 0.1168 \text{ nmole}/\mu\text{L}$$

$$0.1168 \text{ nmole}/\mu\text{L} \times 168.04 \text{ ng/nmole} = 19.62 \text{ ng}/\mu\text{L}$$

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 fluorescence assays, use black plates with clear bottoms. 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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