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# Product Information

**Enolase Activity Assay Kit**

Catalog Number **MAK178**
Storage Temperature –20 °C

## TECHNICAL BULLETIN

### Product Description

Enolase is a multifunctional glycolytic enzyme that catalyzes the conversion of D-2-phosphoglycerate to phospho(enol)pyruvate (PEP) and water. It can function as a plasminogen receptor in endothelial, epithelial, and hematopoietic cells and hence, may be involved in fibrinolytic and intravascular systems. It is also known to act as a heat shock protein, which may have implications in transcriptional and pathological functions. Enolase has been implicated in autoimmune and systemic diseases.[1] Furthermore; serum neuron-specific enolase is known to function as a predictor of patient outcome post cardiac arrest.[2] Thus enolase assays can be used for studying cellular functions like carbohydrate metabolism, transcription and other pathophysiological processes.

The Enolase Activity Assay Kit provides a simple and sensitive procedure for measuring enolase activity in a variety of samples. Enolase activity is determined by a coupled enzyme assay in which D-2-phosphoglycerate is converted to PEP, resulting in the formation of an intermediate that reacts with a peroxidase substrate, generating a colorimetric (570 nm) or fluorometric ($\lambda_{ex}$ = 535/$\lambda_{em}$ = 587 nm) product proportional to the enolase activity present. One milliunit of enolase is the amount of enzyme that will generate 1.0 nmole of $H_2O_2$ per minute at pH 7.2 at 25 °C.

### Components

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

| | |
|---|---|
| Enolase Assay Buffer<br>Catalog Number MAK178A | 25 mL |
| Peroxidase Substrate, in DMSO<br>Catalog Number MAK178B | 0.2 mL |
| Enolase Substrate Mix<br>Catalog Number MAK082C | 1 vl |
| Enolase Converter<br>Catalog Number MAK178D | 1 vl |
| Enolase Developer<br>Catalog Number MAK178E | 1 vl |
| Enolase Positive Control<br>Catalog Number MAK178F | 1 vl |
| Hydrogen Peroxide Standard, 0.88 M<br>Catalog Number MAK178G | 0.1 mL |

### 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 (ELISA reader)

### 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. To maintain reagent integrity, avoid repeated freeze/thaw cycles.

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

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

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

Peroxidase Substrate – Allow reagent to come to room temperature before use. Mix well by pipetting, then aliquot and store, protected from light and moisture, at –20 °C. Upon thawing, the Fluorescent Peroxidase Substrate is ready-to-use in the colorimetric assay.

For the fluorescence assay, dilute an aliquot of the Fluorescent Peroxidase Substrate 5 to 10-fold with Enolase Assay Buffer, just prior to use. This will reduce background in the fluorescence assay.

Enolase Converter and Developer – Reconstitute each with 220 µL of Enolase Assay Buffer. Mix well by pipetting, then aliquot each 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.

$H_2O_2$ Standards for Colorimetric Detection
Notes: Use ultrapure water for the preparation of $H_2O_2$ Standards. Prepare working dilutions of $H_2O_2$ standards just before use. Do not store the diluted standards.

Dilute 4 µL of 0.88 M $H_2O_2$ Standard with 348 µL of water to prepare a 10 mM $H_2O_2$ standard solution. Dilute the 10 mM $H_2O_2$ standard further to 1 mM by adding 100 µL of 10 mM $H_2O_2$ into 900 µL of water. 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 water to each well to bring the volume to 50 µL.

$H_2O_2$ Standards for Fluorometric Detection
Prepare a 1 mM $H_2O_2$ standard solution according to the instructions for the $H_2O_2$ standard solution for colorimetric detection. Add 100 µL of 1 mM $H_2O_2$ standard into 900 µL of water to prepare a 0.1 mM $H_2O_2$ standard solution. Add 0, 2, 4, 6, 8, and 10 µL of the 0.1 mM standard solution into a 96 well plate, generating 0 (blank), 200, 400, 600, 800, and 1,000 pmole/well standards. Add water to each well to bring the volume to 50 µL.

Sample Preparation
Tissue (10 mg) or cells ($1 \times 10^6$) can be homogenized in 100 µL of ice-cold Enolase Assay Buffer. Centrifuge the samples at 10,000 × *g* for 5 minutes to remove insoluble material.

Add 1–50 µL of sample per well and bring samples to a final volume of 50 µL with Enolase Assay Buffer.

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

Some samples may have a high background. To remove the effect of high background, a sample blank may be set up for each sample by omitting the Enolase Substrate mix. The blank readings can then be subtracted from the sample readings.

For the positive control (optional), add 10 µL of the Positive Control to 990 µL of Enolase Assay Buffer. For colorimetric assays, add 1–10 µL of diluted Positive Control into the desired wells and bring the final volume to 50 µL with Enolase Assay Buffer. For fluorometric assays, dilute the positive control as indicated for the colorimetric assay. Further dilute the Positive Control by adding 50 µL of the diluted Positive Control to 450 µL of Enolase Assay Buffer. Add 1–10 µL of the 1,000-fold diluted Positive Control into the desired wells and bring the final volume to 50 µL with Enolase Assay Buffer.
Note: Prepare the dilutions of the Enolase Positive Control just prior to use. Do not store the diluted solutions.

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 |
|---|---|---|
| Enolase Assay Buffer | 42 µL | 44 µL |
| Enolase Substrate Mix | 2 µL | – |
| Enolase Converter | 2 µL | 2 µL |
| Enolase Developer | 2 µL | 2 µL |
| Peroxidase Substrate | 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. Protect the plate from light during the incubation

3. Incubate the plate at 25 °C. After 5–10 minutes, take the initial measurement ($T_{initial}$). Measure the absorbance at 570 nm at the initial time $(A_{570})_{initial}$ or the fluorescence intensity ($FLU_{initial}$, $\lambda_{ex}$ = 535/ $\lambda_{em}$ = 587 nm).
   Note: There is typically a lag phase, which lasts 5–10 minutes. It is essential $(A_{570})_{initial}$ or $FLU_{initial}$ are in the linear range of the standard curve.

4. Continue to incubate the plate at 25 °C taking measurements ($A_{570}$) every 2–3 minutes or FLU 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 for colorimetric standards or 1,000 pmole/well for fluorometric standards). 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 [$(A_{570})_{final}$ or $FLU_{final}$] 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 [$(A_{570})_{final}$ or $FLU_{final}$] obtained for the 0 (blank) $H_2O_2$ standard from the final measurement [$(A_{570})_{final}$ or $FLU_{final}$] of the standards and samples. Background values can be significant and must be subtracted from all readings. Plot the $H_2O_2$ standard curve.
Note: A new standard curve must be set up each time the assay is run.

Subtract the sample blank values from the sample readings to obtain the corrected measurements. Using the corrected measurements, the amount of $H_2O_2$ 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.

$$\Delta A_{570} = (A_{570})_{final} - (A_{570})_{initial}$$

or

$$\Delta FLU = FLU_{final} - FLU_{initial}$$

Compare the $\Delta A_{570}$ or $\Delta FLU$ of each sample to the standard curve to determine the amount of $H_2O_2$ generated (B) between $T_{initial}$ and $T_{final}$.

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

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

where:
B = Amount (nmole) of $H_2O_2$ generated between $T_{initial}$ and $T_{final}$
Reaction Time = $T_{final} - T_{initial}$ (minutes)
V = sample volume (mL) added to well

Enolase activity is reported as:

nmole/min/µL = milliU/µL = U/mL
or
pmole/min/µL = µU/µL = milliU/mL

One milliunit of Enolase is the amount of enzyme that will generate 1.0 nmole of $H_2O_2$ per minute at pH 7.2 at 25 °C.

Example:
$H_2O_2$ amount (B) = 5.84 nmole
First reading ($T_{initial}$) = 18 minutes
Second reading ($T_{final}$) = 47 minutes
Sample volume (V) = 0.01 mL
Sample dilution is 1

Enolase activity is:

$$\frac{5.84 \times 1}{(47-18) \times 0.01} = 20.14 \text{ milliunits/mL}$$

**References**


1. Pancholi, V., Multifunctional alpha-enolase: its role in diseases. Cell Mol. Life Sci., **58(7)**, 902-920, (2001).
2. Fogel, W. et al., Serum neuron-specific enolase as early predictor of outcome after cardiac arrest. Crit. Care Med., **25(7)**, 1133-1138 (1997).

**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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