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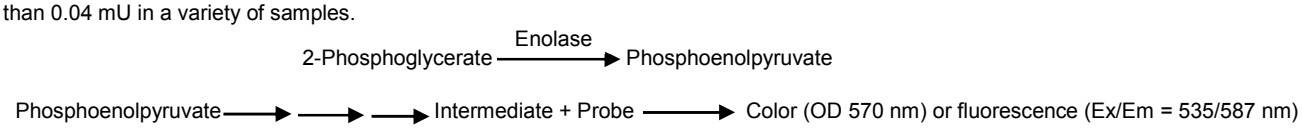
Enolase Activity Colorimetric/Fluorometric Assay Kit *rev. 8/13*

(Catalog # K691-100; 100 assays; Store at -20°C)

I. Introduction:

Enolase (EC 4.2.1.11), also called 2-phospho-D-glycerate hydrolase or 2-phosphoglycerate dehydratase, is a key enzyme in glycolysis. It converts 2-phosphoglycerate to phosphoenolpyruvate (PEP) & also catalyzes the reverse reaction, PEP to 2-phosphoglycerate under anabolic conditions during gluconeogenesis. In mammals, Enolase consists of three subunits: α, β and γ that combine into 5 isozymes, 3 of which are commonly found in human tissues. This enzyme exists in all organisms, which can undergo glycolysis. Besides its role in glycolysis, enolase has other functions too. Recent studies have shown that increased activity of enolase is associated with tumor invasion and metastasis; therefore, precise measurement of enolase activity may be of great interest for tumor diagnosis and prognosis. In BioVision's Enolase Activity Assay Kit, enolase catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate, which is subsequently used to generate an intermediate product. The intermediate product stoichiometrically reacts with OxiRed™ probe to generate color (OD 570 nm) or fluorescence (Ex/Em = 535/587 nm). This simple & sensitive assay Kit can detect enolase activity less than 0.04 mU in a variety of samples.



II. Applications:

- Measurement of enolase activity in various tissues/cells
- Analysis of glycolytic metabolism in different tissues/cells
- Mechanistic studies of various cancers
- Screening anti-cancer drugs

III. Sample Type:

- Animal tissues: Liver, muscle, heart, kidney etc.
- Cell culture: adherent or suspension cells

IV. Kit Contents:

Components	K691-100	Cap Code	Part Number
Enolase Assay Buffer	25 ml	WM	K691-100-1
OxiRed™ Probe (in DMSO)	0.2 ml	Red	K691-100-2A
Enolase Substrate Mix (Lyophilized)	1 vial	Orange	K691-100-3
Enolase Converter (Lyophilized)	1 vial	Purple	K691-100-4
Enolase Developer (Lyophilized)	1 vial	Green	K691-100-5
Enolase Positive Control (Lyophilized)	1 vial	Blue	K691-100-6
H_2O_2 Standard (0.88 M)	0.1 ml	Yellow	K691-100-7

V. User Supplied Reagents and Equipment:

- 96-well clear plate with flat bottom
- Multi-well spectrophotometer (ELISA reader)

VI. Storage and Handling:

Store kit at –20°C, protected from light. Warm Assay Buffer to room temperature before use. Briefly centrifuge small vials prior to opening.

VII. Reagent Preparation and Storage Conditions:

- **OxiRed™ Probe:** Ready to use as supplied. Warm at 37ºC for 1-2 min. to melt frozen DMSO. Store at -20ºC. Use within two months.
- **Enolase Substrate Mix:** Reconstitute with 220 µl Enolase Assay Buffer. Store at -20°C. Use within two months. Keep on ice while in use.
- **Enolase Converter and Developer:** Reconstitute each tube with 220 µl Enolase Assay Buffer. Pipette up and down to dissolve completely. Aliquot and store at -20°C. Use within two months.
- **Enolase Positive Control:** Reconstitute with 100 µl Enolase Assay Buffer and mix thoroughly. Aliquot and store at –20°C. Use within two months.

VIII. Enolase Activity Assay Protocol:

1. **Sample Preparation:** Homogenize tissue (10 mg) or cells (1 x 10^6) with 100 µl ice cold Enolase Assay Buffer on ice. Centrifuge at 10000 x g for 5 min. Collect the supernatant. Add 1-50 µl sample per well and adjust final volume to 50 µl with Enolase Assay Buffer.

   Note:

   a. For unknown samples, we suggest testing several doses to ensure the readings are within the Standard Curve range.

   b. For samples having high background, prepare a parallel sample well as the background control to correct the interference from the samples.

2. **H_2O_2 Standard Curve:** Dilute H_2O_2 Standard to 10 mM by adding 4 µl of 0.88 M H_2O_2 Standard into 348 µl dH_2O. Dilute 10 mM H_2O_2 Standard further to 1 mM by adding 100 µl of 10 mM H_2O_2 into 900 µl dH_2O.

Colorimetric Assay: Add 0, 2, 4, 6, 8 and 10 µl of diluted 1 mM H_2O_2 Standard into a series of wells in a 96-well plate to generate 0, 2, 4, 6, 8 and 10 nmol/well of H_2O_2 Standard. Adjust the volume to 50 µl/well with dH_2O.

Fluorometric Assay: Dilute 1 mM H_2O_2 Standard to 0.1 mM by adding 100 µl of 1 mM H_2O_2 Standard into 900 µl dH_2O. Add 0, 2, 4, 6, 8 and 10 µl of diluted 0.1 mM H_2O_2 Standard in a series of wells in 96-well plate to generate 0, 200, 400, 600, 800 and 1000 pmol/well of H_2O_2 Standard. Adjust the volume to 50 µl/well with dH_2O.

Note:

Prepare working solution of H_2O_2 Standard just before use. Don't store the diluted Standard.

3. **Enolase Positive Control:** Dilute Enolase Positive Control 100 times by adding 10 µl of Positive Control into 990 µl Enolase Assay Buffer. For colorimetric assay, add 1-10 µl of diluted Enolase Positive Control into desired well(s) and adjust the final volume to 50 µl with Enolase Assay Buffer. For fluorometric assay, dilute the 100 times diluted positive control further 10 times by adding 50 µl of 100 times diluted positive control into 450 µl Enolase Assay Buffer. Add 1-10 µl of 1000 times diluted Enolase Positive Control into desired well(s) and adjust the final volume to 50 µl with Enolase Assay Buffer.

   Note:

   Prepare diluted solution of Enolase Positive Control just before use. Don't store the diluted solution.

4. **Reaction Mix:** Mix enough reagents for the number of assays to be performed. For each well, prepare 50 µl Mix containing:

	Reaction Mix	* Background Control Mix
Enolase Assay Buffer	42 µl	44 µl
Enolase Substrate Mix	2 µl	----
Enolase Converter	2 µl	2 µl
Enolase Developer	2 µl	2 µl
OxiRed™ Probe	2 µl**	2 µl

   Add 50 µl of the Reaction Mix into each well containing the Standard, Positive Control and test samples. Mix well.

   * For samples having high background, add 50 µl of Background Control Mix to sample background control well(s). Mix well.

   ** For fluorometric Assay, dilute OxiRed™ Probe 10X with Enolase Assay Buffer.

5. **Measurement:** Measure the absorbance (OD 570 nm) or fluorescence (Ex/Em = 535/587 nm) in kinetic mode for 20-60 min. at 25ºC.

   Note: Incubation time depends on the Enolase Activity in the samples. We recommend measuring the absorbance or fluorescence in kinetic mode, and choosing two time points (T_1 & T_2) in the linear range to calculate the Enolase Activity of the samples. There is typically a lag phase, which lasts ~5-10 min. as seen in the figure below (c). The H_2O_2 Standard Curve can be read in Endpoint mode (i.e., at the end of incubation time).

6. **Calculation:** Subtract 0 Standard reading from all readings. Plot the H_2O_2 Standard Curve. If the sample background control reading is significant, subtract the background control reading from the sample. Apply the corrected sample reading to the H_2O_2 Standard Curve to get to get B nmol or B pmol of H_2O_2 generated by Enolase during the reaction time ($\Delta T = T_2 - T_1$).

Sample Enolase Activity = $B/(\Delta T \times V) \times D$ = nmol/min/µl or pmol/min/µl = mU/µl or µU/µl = U/ml or mU/ml

Where: **B** = H_2O_2 amount from Standard Curve (nmol or pmol)
Δ**T** = Reaction time (min.)
V = Sample volume added into the reaction well (µl)
D = Dilution Factor

Unit Definition: One milliunit of Enolase is the amount of enzyme that will generate 1.0 nmol of H_2O_2 per min. at pH 7.2 at 25ºC.

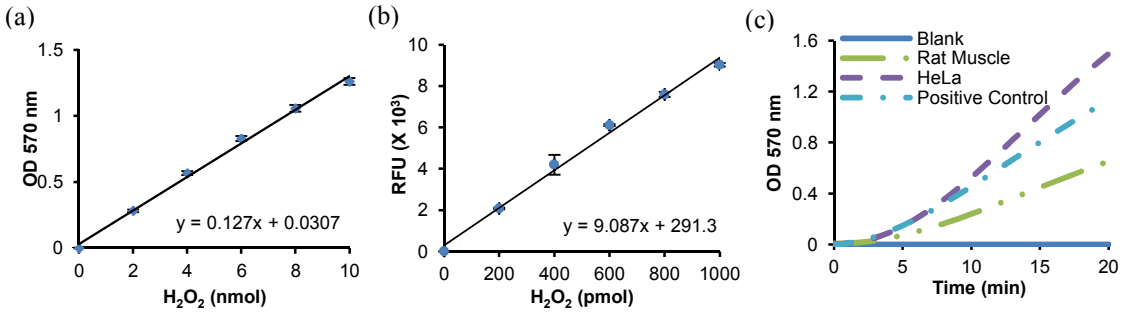


Figure: (a) H_2O_2 Standard Curve for Colorimetric Assay (b) H_2O_2 Standard Curve for Fluorometric Assay & (c) Enolase Activity in rat muscle lysate (1 µg), HeLa lysate (5 µg) and positive control. Assays were performed following the kit protocol.

IX. RELATED PRODUCTS:

Glucose Colorimetric Assay kit II (K686)
PicoProbe™ Glucose Fluorometric Assay Kit (K688)
Glucose-3-Phosphate Colorimetric Assay Kit (K641)
PEP Colorimetric/Fluorometric Assay Kit (K365)
Phosphoglucomutase Colorimetric Assay Kit (K774)
Pyruvate Colorimetric/Fluorometric Assay Kit (K609)
Glucose Colorimetric/Fluorometric Assay kit (K606)
2-Phosphoglycerate Colorimetric/Fluorometric Assay Kit (K778)
Hexokinase Colorimetric Assay Kit (K789)
Phosphofructokinase (PFK) Activity Colorimetric Assay Kit (K776)
Phosphoglucose Isomerase Colorimetric Assay Kit (K775)
Pyruvate Kinase Activity Colorimetric/Fluorometric Assay Kit (K709)

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