

ab117994 – ENO1 Human Activity Assay Kit

Instructions for Use

For the measurement of Human Enolase 1
(ENO1) activity

This product is for research use only and is not
intended for diagnostic use.

Table of Contents

| | | |
|----|-------------------------------|----|
| 1. | Introduction | 3 |
| 2. | Assay Summary | 7 |
| 3. | Kit Contents | 8 |
| 4. | Storage and Handling | 8 |
| 5. | Additional Materials Required | 9 |
| 6. | Reagent Preparation | 9 |
| 7. | Test sample Preparation | 10 |
| 8. | Assay Procedure | 13 |
| 9. | Data Analysis | 15 |

1. Introduction

Principle: ab117994 is used to determine enolase 1 (ENO1) activity in a sample. The native enzyme is immunocaptured within the wells of the microplate; this removes all other enzymes. The enolase activity is determined by following the consumption of NADH in an assay buffer that includes pyruvate kinase, lactate dehydrogenase and required substrates (Figure 1). The consumption of NADH is monitored as decrease of absorbance at 340 nm.

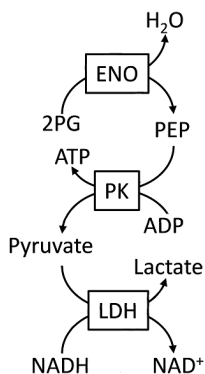


Figure 1. Scheme of reactions used in this kit. Abbreviations are as follows: enolase (ENO), 2-phospho-D-glycerate (2PG), phosphoenolpyruvate (PEP), pyruvate kinase (PK), lactate dehydrogenase (LDH).

Background: Enolase (EC 4.2.1.11) is a multifunctional enzyme catalyzing conversion of 2-phospho-D-glycerate to phosphoenolpyruvate. Besides its role in glycolysis, it plays a part in various processes such as growth control, hypoxia tolerance and allergic responses. It may also function in the intravascular and pericellular fibrinolytic system due to its ability to serve as a receptor and activator of plasminogen on the cell surface of several cell-types such as leukocytes and neurons. It stimulates immunoglobulin production. Mammalian enolase is composed of 3 isozyme subunits, alpha (ENO1, P06733), beta (ENO3) and gamma (ENO2), which can form homodimers or heterodimers which are cell-type and development-specific. The alpha/alpha homodimer is expressed in embryo and in most adult tissues. The alpha/beta heterodimer and the beta/beta homodimer are found in striated muscle, and the alpha/gamma heterodimer and the gamma/gamma homodimer in neurons. Alpha enolase is localized to cytoplasm. It can translocate to the plasma membrane in either the homodimeric (alpha/alpha) or heterodimeric (alpha/gamma) form. MBP1, a shorter isoform of the ENO1, binds to the myc promoter and acts as a transcriptional repressor. It may be a tumor suppressor. It locates mainly in nucleus. Used as a diagnostic marker for many tumors and, in the heterodimeric form, alpha/gamma, as a marker for hypoxic brain injury after cardiac arrest. Also it is a marker for endometriosis. Antibodies against alpha-enolase are present in sera from patients with cancer-associated retinopathy syndrome (CAR), a progressive blinding disease which occurs in the presence of systemic tumor

growth, primarily small-cell carcinoma of the lung and other malignancies. Is identified as an autoantigen in Hashimoto encephalopathy (HE) a rare autoimmune disease associated with Hashimoto thyroiditis (HT). HT is a disorder in which destructive processes overcome the potential capacity of thyroid replacement leading to hypothyroidism.

Limitations:

- FOR RESEARCH US ONLY. NOT FOR DIAGNOSTIC PROCEDURES.
- Use this kit before expiration date.
- Do not mix or substitute reagents from other lots or sources.
- If experimental samples generate values above the range of the control or normal sample standard curve, further dilute the samples with 1X Incubation buffer and repeat the assay.
- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.

Technical Hints:

- To avoid cross contamination, change pipette tips between additions of each standard, sample and between reagent additions. Also use separate clean, dry reservoirs for each reagent.
- Cover plate during incubation steps.
- Thorough and consistent wash technique is essential for proper assay performance. Wash buffer must be forcefully dispensed and completely removed from the wells by aspiration or decanting. Remove remaining wash buffer by inverting the plate and blotting on paper towels.

2. Assay Summary

Prepare samples as instructed.

Determine the protein concentration of extracts.



Equilibrate all reagents to room temperature.



Dilute sample to desired protein concentration in 1X Incubation Buffer.

Add 50 μ L sample to each well used. Incubate 2 hours at room temperature.



Aspirate and wash each well twice.

Add 200 μ L 1X Activity Solution to each well.

Pop bubbles and immediately begin recording the color development with elapsed time at 340 nm for 20-60 minutes.

3. Kit Contents

Sufficient materials are provided for 96 measurements in a microplate.

| Item | Quantity |
|---|------------------------------|
| Extraction Buffer | 15 mL |
| 20X Buffer | 20 mL |
| 10X Blocking Buffer | 6 mL |
| Base Buffer | 24 mL |
| Reagent Mix | Add 1.00 mL H ₂ O |
| PK (Pyruvate kinase) | 187.5 units |
| LDH (Lactate dehydrogenase) | 187.5 units |
| Microplate (8 x 12 well antibody coated strips) | 1 |

4. Storage and Handling

All components are shipped cold. Reagent Mix is shipped lyophilized. Store all components at 4°C. This kit is stable for 6 months from receipt.

5. Additional Materials Required

- Standard absorbance microplate reader capable of kinetic reading at 340nm
- Multichannel pipette (50 - 300 µL) and tips
- 1.5-mL microtubes
- Paper towels
- Deionized water

6. Reagent Preparation

1. Prepare 1X Wash Buffer by adding 20 mL 20X Buffer to 380 mL nanopure water.
2. Prepare 1X Incubation Buffer by adding 6 mL 10X Blocking Buffer to 54 mL 1X Wash Buffer.

3. Immediately prior to use prepare 1X Activity Solution.

Rehydrate the Reagent Mix by adding 1.00 mL pure H₂O and incubate 3 minutes at room temperature, then vortex the tube thoroughly to dissolve. Ensure that the Reagent Mix has been dissolved. To prepare 1X Activity Solution add 1.00 mL of rehydrated Reagent Mix and 187.5 units each of PK and LDH (the PK and LDH concentrations in units/ μ L are indicated on the PK and LDH stocks) to 24 mL Base Buffer. The unused amounts of Assay Solution should be aliquoted and stored at -80°C.

7. Test sample Preparation

Note: Extraction buffer can be supplemented with PMSF and protease inhibitor cocktail prior to use. Supplements should be used according to manufacturer's instructions.

1. Collect non-adherent cells by centrifugation or scrape to collect adherent cells from the culture flask. Typical centrifugation conditions for cells are 500 x g for 10 min at 4°C.
2. Rinse cells twice with PBS.
3. Solubilize cell pellet at 2×10^7 /mL in Extraction Buffer.

4. Incubate on ice for 20 minutes. Centrifuge at 16,000 x g, 4°C for 20 minutes. Transfer the supernatants into clean tubes and discard the pellets. Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.

Tissue lysates:

1. Tissue lysates are typically prepared by homogenization of tissue that is first minced and thoroughly rinsed in PBS to remove blood (dounce homogenizer recommended).
2. Suspend the homogenate to 25 mg/mL in PBS.
3. Solubilize the homogenate by adding 4 volumes of Extraction Buffer to a sample protein concentration of 25 mg/mL.
4. Incubate on ice for 20 minutes. Centrifuge at 16,000 x g, 4°C for 20 minutes. Transfer the supernatants into clean tubes and discard the pellets. Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.

Sub-cellular organelle lysates e.g. mitochondria:

1. Prepare the organelle sample by, for example, sub-cellular fractionation.
2. Pellet the sample.
3. Solubilize the pellet by adding 9 volumes Extraction Buffer.
4. Incubate on ice for 20 minutes. Centrifuge at 16,000 x g, 4°C for 20 minutes. Transfer the supernatants into clean tubes and discard the pellets. Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.

These test samples should be diluted to within the working range of the assay in 1X Incubation Buffer. As a guide, typical ranges of sample concentration for commonly used sample types are shown below in Data Analysis.

8. Assay Procedure

Bring all reagents and samples to room temperature before use. It is recommended all samples and standards be assayed in duplicate.

1. Prepare all reagents, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 50 μL of each diluted test sample per well. It is recommended to include a dilution series of a control (normal) sample as a reference. Also include a 1X Incubation Buffer as a zero standard.
4. Cover/seal the plate and incubate for 2 hours at room temperature. If available use a plate shaker for all incubation steps at 300 rpm.
5. Aspirate each well and wash, repeat this once more for a total of **two** washes. Wash by aspirating or decanting from wells then dispensing 300 μL 1X Wash Buffer into each well as described above. Complete removal of liquid at each step is essential to good performance. After the last wash,

remove the remaining buffer by aspiration or decanting. Invert the plate and blot it against clean paper towels to remove excess liquid.

6. Gently add 200 μ L 1X Activity Solution (step 6.3 to each well minimizing the production of bubbles.
7. Pop any bubbles immediately and record absorbance in the microplate reader prepared as follows:

| | |
|--------------|---|
| Mode: | Kinetic |
| Wavelength: | 340 nM |
| Time: | 20 min - 60 min (as desired) |
| Interval: | 20 sec - 1 min |
| Shaking: | Shake between readings (if possible) |

Alternative– In place of a kinetic reading, at a **user defined** time/s record the OD 340 nm in all wells.

Record the data for analysis and proceed to Data Analysis.

9. Data Analysis

Example data sets are shown below illustrating data analysis of ENO1 activity measurements in HepG2 cells (an example of a human cell line derived from liver), and homogenate samples from whole human liver tissue.

The starting concentration of 2-phospho-D-glycerate in the assay is 0.5 mM and NADH is 0.25 mM. 2-phospho-D-glycerate consumption is in 1:1 proportion with consumption of NADH measured as decrease in absorbance at 340 nm ($\text{NADH } \epsilon_{340\text{nm}} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$). For simplicity the activity can be expressed as the change in absorbance per minute per amount of sample loaded into the well. Activity was collected as described in this protocol using a Molecular Dynamics microplate reader. Standard curves of reference sample data were exported to graphing software capable of a 4-parameter data analysis (Fig. 2). Activity is clearly measurable in the 6-400 $\mu\text{g/mL}$ range when such a fit is applied for HepG2, or human liver tissue extracts.

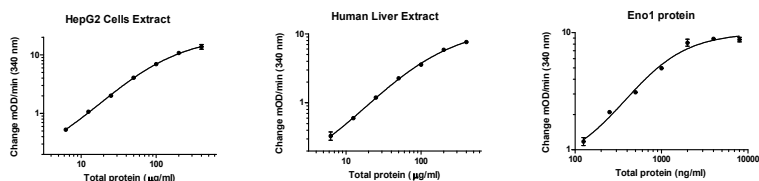


Figure 2. Examples of dilution series prepared from HepG2 cells extract, human liver tissue extract or recombinant Eno1 protein (ab89248)

Unknown samples should be interpolated from these reference sample graphs. This determined *relative activity* is the amount of reference sample required to generate the same amount of activity as the unknown sample and usual expressed as a per cent value.

WORKING RANGE

This assay has been demonstrated with extracts prepared from cultured cells, such as HepG2, HeLa and SHSY5Y cells, as well as with human liver and heart tissue extracts or recombinant Eno1. Typical ranges for several sample types are described below. It is highly recommended to prepare multiple dilutions for each sample to ensure that each is in the working range of the assay.

| Sample Type | Range |
|--|---------------------|
| Cultured whole cell extracts (type dependant) e.g. HepG2 | 0.006 – 0.200 mg/mL |
| Tissue extract | 0.006 – 0.200 mg/mL |
| ENO1 protein (ab89248) | 125 -2000 ng/mL |

SENSITIVITY

Calculated minimum detectable dose of ENO1 activity (zero dose $n=39 + 2$ standard deviations) is present in 2.9 $\mu\text{g/mL}$ HepG2 extract.

RECOVERY

| 200 $\mu\text{g/mL}$ HepG2 in: | Average % Recovery |
|--|---------------------------|
| Cell culture media | 63 |
| 10% Fetal Bovine serum | 140 |
| 10% Goat serum | 140 |
| 100% Extraction Buffer | 73 |

For more accurate comparison dilute all samples in equivalent buffers/media/supplements.

LINEARITY OF DILUTION

Linearity of dilution was determined by comparing dilution series of human heart muscle homogenate (HHH) extract to dilution series of HepG2 cells.

| 200 µg/mL HHH (fold of dilution) | % Expected |
|---|-------------------|
| undiluted | 114 |
| 2 | 100 |
| 4 | 97 |

REPRODUCIBILITY

| HepG2 (6 – 200 ng/mL) | % CV |
|------------------------------|-------------|
| Intra (n=3, 3 assays) | 9 |
| Inter (n=4 days) | 11 |

SPECIFICITY

Species– human reactive. Rat and mouse not reactive. Others untested.

Isoforms– ENO1 reactive. ENO2 and ENO3 not reactive.

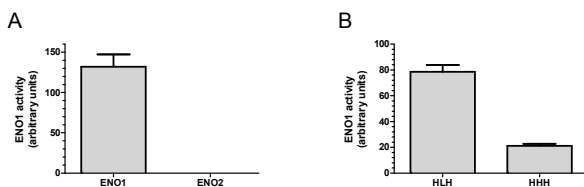


Figure 3. An experiment to determine the assay specificity.

Analysis of recombinant ENO1 (ab89248) and the neuron-specific recombinant ENO2 (ab78797), each at 2000 ng/mL (shown in A), and of extracts prepared from human liver homogenate (HLH) and human heart homogenate (HHH), each at 200 μ g/mL (shown in B) with the use of this kit. The relative ENO activity was determined from HepG2 standard curve as described in the protocol. The data show that the activity of the neuron-specific ENO2 can't be detected. The data also show that enolase activity is decreased in human heart homogenate by 73% compared to HLH. Since striated muscles of myocardium contain ENO3 homodimers and ENO1/ENO3 heterodimers, the data strongly suggest that the kit can't capture muscle-specific ENO3 homodimers.

The antibody (ab112994) used to capture ENO1 in this kit was generated by immunization of human cytosolic proteins. The resulting monoclonal mouse antibody isolates, by immunoprecipitation, a single ENO1 band to purity from human samples. The immunoprecipitate was confirmed to be ENO1 by mass spectrometry. This antibody labels a cytosolic intracellular pattern (Fig. 4). Finally the activity measured by this kit is 2PG dependent (Fig. 5).

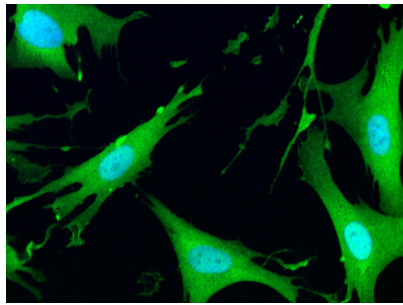


Figure 4. Immunofluorescent detection of ENO1 in cytosol in HeLa cells using ab112994.

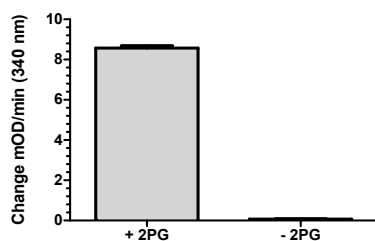


Figure 5. The ENO1 activity measured with this kit is 2-phospho-D-glycerate (2PG)-dependent in HepG2 cell extract

Complementary kit utilizing the same capture antibody to measure ENO1 protein quantity is also available (ab116694).

UK, EU and ROW

Email: technical@abcam.com

Tel: +44 (0)1223 696000

www.abcam.com

US, Canada and Latin America

Email: us.technical@abcam.com

Tel: 888-77-ABCAM (22226)

www.abcam.com

China and Asia Pacific

Email: hk.technical@abcam.com

Tel: 400 921 0189 / +86 21 2070 0500

www.abcam.cn

Japan

Email: technical@abcam.co.jp

Tel: +81-(0)3-6231-0940

www.abcam.co.jp