

Quantikine[®] ELISA

Human Enolase 2/Neuron-specific Enolase Immunoassay

Catalog Number DENL20

For the quantitative determination of human Enolase 2 concentrations in cell culture supernates, cell lysates, serum, and plasma.

Note: The standard reconstitution method has changed. Read this package insert in its entirety before using this product.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Enolase 2 is also known as gamma enolase or neuronal enolase. Enolase (2-phospho-D-glycerate hydrolase) is a cytoplasmic enzyme that is involved in the glycolytic pathway, in which it converts 2-phosphoglycerate to phosphoenolpyruvate. It has three members: Enolase 1, Enolase 2, and Enolase 3, which are also termed α , γ , and β enolase, respectively. They exist as several dimeric isoenzymes including $\alpha\alpha$, $\alpha\beta$, $\beta\beta$, $\alpha\gamma$, and $\gamma\gamma$. The $\alpha\gamma$ and $\gamma\gamma$ isoenzymes are abundant in neurons and neuroendocrine cells, and therefore, they are also termed neuron specific enolase (NSE) (1-2). Human Enolase 2 is 434 amino acids (aa) in length. It shares 83% aa identity with human enolases 1 and 3 and 99% with its mouse orthologue.

Serum Enolase 2 levels are low in normal subjects. However, when neuronal injury occurs, it is released from the injured cells into the cerebrospinal fluid and systemic circulation. Studies have shown that elevated serum levels of Enolase 2 are commonly found among a variety of conditions associated with central nervous system damage such as stroke, traumatic brain injury, multiple sclerosis, and Alzheimer's disease (3-6). In malignant tumors of neuroendocrine origin, Enolase 2 production is increased, which usually also results in elevated serum levels of Enolase 2 (7). Such examples include small cell lung cancer, APUDoma, and neuroblastoma (8-11).

The Quantikine® Human Enolase 2 Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human Enolase 2 in cell culture supernates, cell lysates, serum, and plasma. It contains *E. coli*-expressed recombinant human Enolase 2 and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human Enolase 2 showed linear curves that were parallel to the standard curves obtained using the Quantikine® kit standards. These results indicate that this kit can be used to determine relative mass values for naturally occurring human Enolase 2.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human Enolase 2 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Enolase 2 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human Enolase 2 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of Enolase 2 bound in the initial step. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, dilute the samples with calibrator diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine® Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human Enolase 2 Microplate	893813	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human Enolase 2.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of zip-seal. May be stored for up to 1 month at 2-8 °C.*
Human Enolase 2 Standard	893815	Recombinant human Enolase 2 in a buffer with preservatives; lyophilized. <i>Refer to the vial label for reconstitution volume.</i>	Aliquot and store for up to 1 month at ≤ -20 °C.*
Human Enolase 2 Conjugate	893814	21 mL of a polyclonal antibody specific for human Enolase 2 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-9	895167	11 mL of a buffered protein solution with preservatives. <i>May contain a precipitate. Warm to room temperature, and mix gently to dissolve. If the precipitate does not completely dissolve, mix well during use.</i>	
Calibrator Diluent RD5C Concentrate	895046	21 mL of a concentrated buffered protein base with preservatives. <i>Use diluted 1:5 in this assay.</i>	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservatives. <i>May turn yellow over time.</i>	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	6 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 100 mL and 500 mL graduated cylinders.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Test tubes for dilution of standards.
- Human Enolase 2 Controls (optional; R&D Systems®, Catalog # QC83).

If using cell lysate samples, the following is also required:

- Cell Lysis Buffer 1 (R&D Systems®, Catalog # 890713).

PRECAUTIONS

Calibrator Diluent RD5C contains sodium azide, which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the MSDS on our website prior to use.

SAMPLE COLLECTION & STORAGE

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Cell Lysates - Cells must be lysed prior to assay. See Sample Values section.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma on ice using heparin as an anticoagulant. Centrifuge for 15 minutes at 2-8 °C at 1000 x g within 30 minutes of collection. An additional centrifugation step of the plasma at 10,000 x g for 10 minutes at 2-8 °C is recommended for complete platelet removal. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Enolase 2 is present in platelet granules and is released upon platelet activation. Therefore, to measure circulating levels of Enolase 2, platelet-free plasma should be collected for measurement. It should be noted that many protocols for plasma preparation, including procedures recommended by the Clinical Laboratory Standards Institute (CLSI), result in incomplete removal of platelets from blood.

Note: Citrate plasma has not been validated for use in this assay. EDTA plasma is not suitable for use in this assay.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

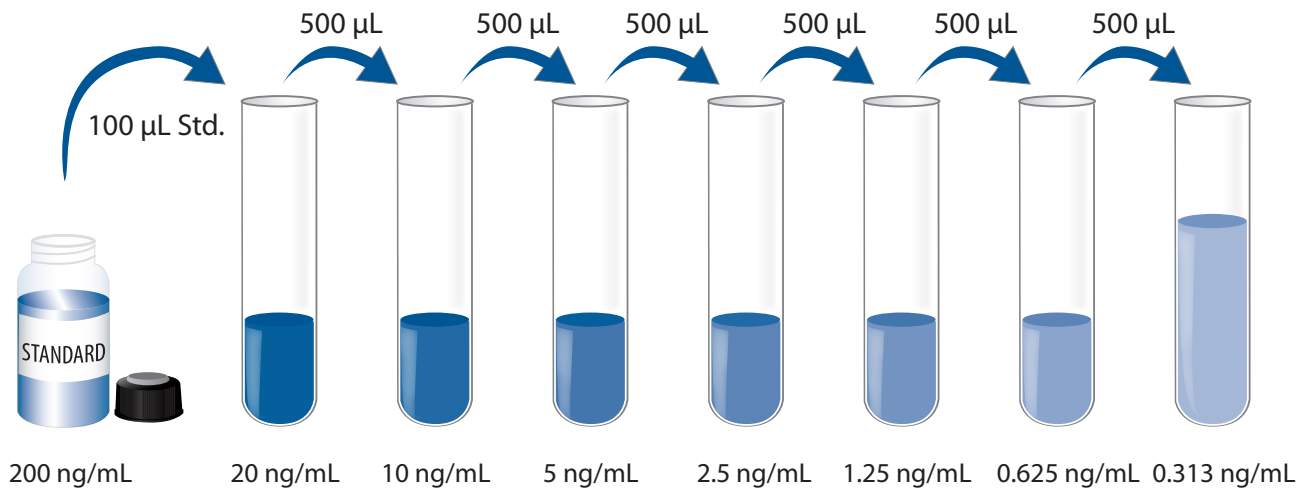
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 μ L of the resultant mixture is required per well.

Calibrator Diluent RD5C (diluted 1:5) - Add 10 mL of Calibrator Diluent RD5C Concentrate to 40 mL of deionized or distilled water to prepare 50 mL of Calibrator Diluent RD5C (diluted 1:5).

Human Enolase 2 Standard - Refer to the vial label for reconstitution volume. Reconstitute the Human Enolase 2 Standard with deionized or distilled water. This reconstitution produces a stock solution of 200 ng/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes. Mix well prior to making dilutions.

Pipette 900 μ L of Calibrator Diluent RD5C (diluted 1:5) into the 20 ng/mL tube. Pipette 500 μ L into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 20 ng/mL standard serves as the high standard. Calibrator Diluent RD5C (diluted 1:5) serves as the zero standard (0 ng/mL).



ASSAY PROCEDURE

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

1. Prepare all reagents, working standards, 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 100 μL of Assay Diluent RD1-9 to each well. *May contain a precipitate. Warm to room temperature, and mix gently to dissolve. If the precipitate does not completely dissolve, mix well during use.*
4. Add 50 μL of standard, control, or sample per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 μL) using a squirt bottle, manifold dispenser, or auto washer. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200 μL of Human Enolase 2 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on the shaker.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 μL of Substrate Solution to each well. **Protect from light.** Incubate for 30 minutes at room temperature **on the benchtop.**
9. Add 50 μL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

CALCULATION OF RESULTS

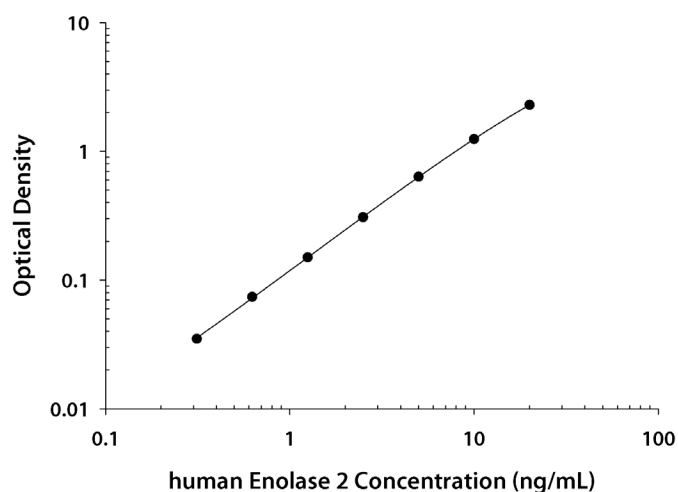
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the human Enolase 2 concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(ng/mL)	O.D.	Average	Corrected
0	0.014 0.018	0.016	—
0.313	0.051 0.051	0.051	0.035
0.625	0.088 0.092	0.090	0.074
1.25	0.165 0.166	0.166	0.150
2.5	0.322 0.325	0.324	0.308
5	0.650 0.652	0.651	0.635
10	1.259 1.262	1.261	1.245
20	2.280 2.339	2.310	2.294

PRECISION

Intra-assay Precision (Precision within an assay)

Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)

Three samples of known concentration were tested in twenty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

Sample	Intra-Assay Precision			Inter-Assay Precision		
	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (ng/mL)	2.15	8.41	13.0	1.96	8.50	12.6
Standard deviation	0.03	0.17	0.36	0.13	0.33	0.55
CV (%)	1.4	2.0	2.8	6.7	3.9	4.3

RECOVERY

The recovery of human Enolase 2 spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=8)	99	85-114%
Cell lysates (n=8)	105	85-115%
Serum (n=4)	95	85-110%
Platelet-poor heparin plasma (n=4)	95	85-111%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human Enolase 2 were serially diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=8)	Cell lysates* (n=4)	Serum (n=4)	Platelet-poor Heparin plasma (n=4)
1:2	Average % of Expected	101	100	100	98
	Range (%)	96-107	98-103	95-102	92-101
1:4	Average % of Expected	106	102	99	101
	Range (%)	103-110	100-103	94-101	96-106
1:8	Average % of Expected	110	103	101	98
	Range (%)	105-115	99-106	96-106	93-107
1:16	Average % of Expected	111	101	101	98
	Range (%)	99-115	98-102	95-107	91-106

*Samples were diluted prior to assay.

SENSITIVITY

Twenty assays were evaluated and the minimum detectable dose (MDD) ranged from 0.013-0.038 ng/mL. The mean MDD was 0.020 ng/mL.

The MDD was determined by adding two standard deviations to the mean O.D. value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against a highly purified *E. coli*-expressed recombinant human Enolase 2 produced at R&D Systems®.

SAMPLE VALUES

Serum/Plasma - Samples from apparently healthy volunteers were evaluated for the presence of human Enolase 2 in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Serum (n=15)	3.02	1.85-4.14	0.613
Heparin plasma (n=15)	6.74	3.39-13.7	3.37
Platelet-poor heparin plasma (n=20)	2.82	1.97-4.98	0.686

Cell Culture Supernates/Cell Lysates:

U-87 MG human glioblastoma/astrocytoma cells were cultured in MEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, 1 mM sodium pyruvate, and 100 µg/mL streptomycin sulfate until confluent.

SK-Mel-28 human malignant melanoma cells were cultured in MEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, 1 mM sodium pyruvate, and 100 µg/mL streptomycin sulfate until confluent.

MDA-MD-453 human breast cancer cells were cultured in RPMI supplemented with 10% fetal bovine serum, and 2 mM L-glutamine until confluent.

IMR-32 human neuroblastoma cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin sulfate until confluent.

After the supernates were harvested, cells were gently washed with cold PBS. Any excess PBS was poured off and discarded. For a T75 flask, 10 mL of Cell Lysis Buffer 1 was added and incubated at room temperature for one hour with gentle agitation. The lysates were then collected and centrifuged at 12,000 x g for 10 minutes to remove insoluble cell debris. The total protein concentration of the lysate supernates was determined by the Bradford method [Bradford, M.M. (1976) Anal. Biochem. **72**:248]. Lysates were aliquoted and stored at -70 °C.

Cell Line	Cell Culture Supernates (ng/mL)	Cell Lysates (ng/mg)
U-87 MG	1.44	221
SK-Mel-28	1.13	245
MDA-MB-453	1.16	121
IMR-32	1.15	44.4

SPECIFICITY

This assay recognizes natural and recombinant human Enolase 2.

The factors listed below were prepared at 200 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 200 ng/mL in a mid-range recombinant human Enolase 2 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

Enolase 1

Enolase 3

REFERENCES

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PLATE LAYOUT

Use this plate layout to record standards and samples assayed.

The diagram shows a 12x8 microplate layout. The rows are numbered 1 through 12 on the left side, and the columns are labeled A through H at the bottom. Each well is represented by a circle. The layout is as follows:

	A	B	C	D	E	F	G	H
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								

NOTES

NOTES

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