Human Neuron-Specific Enolase (NSE) ELISA
Cat No: K12-0938

Principle:
The method employs sandwich ELISA technique. Monoclonal antibodies are pre-coated onto microwells. Samples and standards are pipetted into microwells and Human Neuron-Specific Enolase (NSE) present in the sample are bound by the antibodies. Biotin labeled antibody is added and followed by Streptavidin-HRP is pipetted and incubated to form a complex. After washing microwells in order to remove any non-specific binding, the substrate solution (TMB) is added to microwells and color develops proportionally to the amount of Human Neuron-Specific Enolase (NSE) in the sample. Color development is then stopped by addition of stop solution. Absorbance is measured at 450 nm.

Intended Use:
This Kit is used to assay the level of Human Neuron-specific Enolase (NSE) in Human serum, plasma and other biological samples. The Kit is For Laboratory / Research Use Only.

Materials provided in the Kit:
1. Anti-Human NSE Coated Microtiter Plate (96 wells) - 1 no
2. Human NSE Standard (lyophilized, concentrated, 150 ng/ml) - 2 vials
3. Biotinylated Anti-NSE (concentrated) - 120 ul
4. Streptavidin-HRP Conjugate (concentrated) - 120 ul
5. Sample Diluent - 20 ml
6. Biotin Antibody Dilution Buffer - 10 ml
7. HRP Conjugate Dilution Buffer - 10 ml
8. (25X) Wash Buffer - 30 ml
9. TMB Substrate - 10 ml
10. Stop Solution - 10 ml
11. Instruction Manual

Materials to be provided by the End-User:
1. Microplate Reader able to measure absorbance at 450 nm.
2. Adjustable pipettes to measure volumes ranging from 50 ul to 1000 ul.
3. Deionized (DI) water.
4. Wash bottle or automated microplate washer.
5. Clean tubes and Eppendorf tubes
6. Precision single and multi-channel pipette and disposable tips.
7. 37°C incubator
8. Timer.

Storage Information:
1. All reagents should be stored at 2ºC to 8ºC. For long term storage, store the biotin antibody and standards (preferably aliquoted) at -20ºC. Avoid multiple freeze-thaws as it leads to loss of activity of the components.
2. All the reagents and wash solutions are stable until the expiration date of the kit.
3. 20 minutes prior before use, bring all components to room temperature (18-25ºC). Store all the components of the kit at its appropriate storage condition after use.
4. The Substrate is light-sensitive and should be protected from direct sunlight or UV sources.

Health Hazard Warnings:
1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin. Refer to the MSDS online for details.
2. To reduce the likelihood of blood-borne transmission of infectious agents, handle all samples in accordance with NCCLS regulations.

Specimen Collection and Handling:
1. **Serum-** Place whole blood sample at room temperature for 2 hours or put it at 2-8°C overnight and centrifugation for 20 minutes at approximately 1000×g, Collect the supernatant and carry out the assay immediately. Blood collection tubes should be disposable, non-pyrogenic, and non-endotoxin.

2. **Plasma-** Collect plasma using EDTA-Na2 or heparin as an anticoagulant. Centrifuge samples for 15 minutes at 1000×g at 2-8°C within 30 minutes of collection. Collect the supernatant and carry out the assay immediately. Avoid hemolysis, high cholesterol samples.
3. **Tissue Homogenates-** As hemolysis blood has relation to the assay results, it is necessary to remove residual blood by washing tissue with pre-coating PBS buffer (0.01M, pH=7.4). Mince tissue after weighing it and get it homogenized in PBS (the volume depends on the weight of the tissue Normal 9 ml PBS would be appropriate to 1 gram tissue pieces. Some protease inhibitors are recommended to add into the PBS) with a glass homogenizer or ice. To further break the cells, you can sonicate the suspension with an ultrasonic cell disruptor or subject it to freeze-thaw cycles. The homogenates are then centrifuged for 5 minutes at 5000xg to get the supernatant. The total protein concentration was determined by BCA kit and the total protein concentration of each pore sample should not exceed 0.3 mg,
4. **Cell Culture Supernatant-** Centrifuge supernatant for 20 minutes at 1000xg at 2-8°C to remove insoluble impurity and cell debris. Collect the clear supernatant and carry out the assay immediately.
5. **Cell Culture Lysate-** Commercial RIPA kits are recommended to follow the instructions provided. Generally 0.5 ml RIPA lysis buffer would be appropriate to 2x10(6) cells, DNA must be removed. The total protein concentration was determined by BCA kit and the total protein concentration of each pore sample should not exceed 0.3 mg.
6. **Other Biological Fluids-** Centrifuge samples for 20 minutes are 1000xg at 2-8°C. Collect supernatant and carry out the assay immediately.

Note: Samples to be used within 5 days can be stored at 2-8°C, besides that, samples must be stored at -20°C (assay ≤1 month) or -80°C(assay≤2 months) to avoid loss of bioactivity and contamination. Avoid multiple freeze-thaw cycles. The hemolytic samples are not suitable for this assay.

Sample Dilution

The user should estimate the concentration of target protein in the test sample, and select a proper dilution factor to make the diluted target protein concentration fall in the optimal detection range of the kit. Dilute the sample with the provided diluent, and several trials may be necessary. The test sample must be well mixed with the diluent. If samples are expected to have very high concentrations of the analyte, dilute the samples with PBS (pH 7.4) first and then further dilute with the Sample Diluent.

Reagent Preparation (all reagents should be diluted immediately prior to use):

1. Label any aliquots made with the kit Lot No and Expiration date and store it at appropriate conditions mentioned.
2. Bring all reagents to Room temperature before use.
3. To make **Wash Buffer (1X); dilute 30 ml of (25X) Wash Buffer in 720 ml of DI water.**
4. **Biotinylated Anti-NSE Working Solution**: Prepare it within 1 hour before experiment. Calculate required total volume of the working solution: 0.1 ml / well x quantity of wells. (Allow 0.1-0.2 ml more than the total volume. Dilute the Biotinylated Anti-NSE Antibody (concentrated) with Biotin Antibody Dilution Buffer at 1:100 and mix them thoroughly. (i.e. Add 1 ul Biotinylated Anti-NSE into 99 ul Biotin Antibody Dilution Buffer.)
5. **Streptavidin:HRP Conjugate Working Solution**: Prepare it within 30 minutes before experiment. Calculate required total volume of the working solution: 0.1ml / well x quantity of wells. (Allow 0.1-0.2 ml more than the total volume. Dilute the Streptavidin:HRP Conjugate with Streptavidin:HRP Conjugate Dilution Buffer at 1:100 and mix them thoroughly. (i.e. Add 1 ul of Streptavidin:HRP Conjugate into 99 ul of Streptavidin:HRP Conjugate Dilution Buffer.)
6. **Standards Preparation**: Reconstitute original NSE Standard with 1 ml of Sample Diluent. Keep the standard for 15 mins with gentle agitation before making further dilutions. Prepare the additional Standards by serially diluting the standard stock solution as per the below table.

Standard Concentration	Standard Vial	Dilution Particulars
150 ng/ml	Standard No.8	Reconstitute with 1ml Sample Diluent
75 ng/ml	Standard No.7	300 ul Standard No.8 + 300 ul Sample Diluent
37.5 ng/ml	Standard No.6	300 ul Standard No.7 + 300 ul Sample Diluent
18.75 ng/ml	Standard No.5	300 ul Standard No.6 + 300 ul Sample Diluent
9.375 ng/ml	Standard No.4	300 ul Standard No.5 + 300 ul Sample Diluent
4.688 ng/ml	Standard No.3	300 ul Standard No.4 + 300 ul Sample Diluent
2.344 ng/ml	Standard No.2	300 ul Standard No.3 + 300 ul Sample Diluent
0 ng/ml	Standard No.1	300 ul Sample Diluent only

Procedural Notes:

1. In order to achieve good assay reproducibility and sensitivity, proper washing of the plates to remove excess un-reacted reagents is essential.
2. High Dose Hook Effect may be observed in samples with very high concentrations of Human Neuron-specific Enolase (NSE). High Dose Hook Effect is due to excess of antibody for very high concentrations of Human Neuron-specific Enolase (NSE) present in the sample. High Dose Hook effect is most likely encountered from samples early in the purification process. If Hook Effect is possible, the samples to be assayed should be diluted with a compatible diluent. Thus if the Human Neuron-specific Enolase (NSE) concentration of the undiluted sample is less than the diluted sample, this may be indicative of the Hook Effect.
3. Avoid assay of Samples containing Sodium Azide (NaN_3), as it could destroy the HRP activity resulting in under-estimation of the amount of Human Neuron-specific Enolase (NSE).
4. It is recommended that all Standards and Samples be assayed in duplicates.
5. Maintain a repetitive timing sequence from well to well for all the steps to ensure that the incubation timings are same for each well.
6. If the Substrate has a distinct blue color prior to use it may have been contaminated and use of such substrate can lead to poor sensitivity of the assay.
7. The plates should be read within 30 minutes after adding the Stop Solution.
8. Make a work list in order to identify the location of Standards and Samples.

Assay Procedure:

1. It is strongly recommended that all Standards and Samples be run in duplicates or triplicates. A standard curve is required for each assay.

2. Add **100 ul prepared Standards and diluted Samples** to respective wells.

3. Cover the plate with a sealer and incubate for 90 minutes at 37°C.

4. Aspirate and wash plate 4 times with diluted Wash Buffer (1X) and blot residual buffer by firmly tapping plate upside down on absorbent paper. Wipe of any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step.

5. Pipette **100 ul Biotinylated Anti-NSE Working Solution** to all wells.

6. Cover the plate with a sealer and incubate for 60 minutes at 37°C.

7. Aspirate and wash as per Step (4) above.

8. Pipette **100 ul Streptavidin:HRP Conjugate Working Solution** to all wells. Mix well.

9. Cover the plate with a sealer and incubate for 30 minutes at 37°C.

10. Aspirate and wash as per Step (4) above.

11. Pipette **90 ul TMB Substrate** in all the wells.

12. Incubate the plate at **37°C** for **10 minutes**. DO NOT SHAKE or else it may result in higher backgrounds and worse precision. Positive wells should turn bluish in color.

13. Pipette **50 ul** of **Stop Solution** to all wells. The wells should turn from blue to yellow in color.

14. Read the absorbance at 450 nm with a microplate within 10-15 minutes after addition of Stop solution.

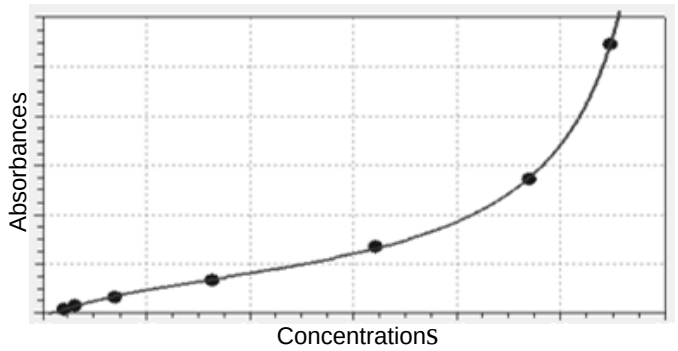
Calculation of Results:

Determine the Mean Absorbance for each set of duplicate or triplicate Standards and Samples. Use the Net Absorbance (Absorbance of Standard/Sample - Absorbance of Blank) to calculate the Mean Absorbances. Using standard graph paper, plot the average value (absorbance 450nm) of each standard on the Y-axis versus the corresponding concentration of the standards on the X-axis.

Draw the best fit curve through the standard points. To determine the unknown concentrations, find the unknown's Mean Absorbance value on the Y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the concentration. If samples were diluted, multiply by the appropriate dilution factor.

Software which is able to generate a cubic spline curve-fit, 4PL or a polynomial regression to the 2nd order is best recommended for automated results.

Typical Graph



Precautions:
Do not mix reagents from different kits or lots. Reagents and/or antibodies from different manufacturers should not be used with this set.

Performance Characteristics:
Please note that this validation is performed in our laboratory and will not necessarily be duplicated in your laboratory. This data has been generated to enable the user to get a preview of the assay and the characteristics of the kit and is generic in nature. We recommend that the user performs at the minimum; the spike and recovery assay and the dilutional linearity assay to assure quality results. For a more comprehensive validation, the user may run the protocols as suggested by us herein below to develop the parameters for quality control to be used with the kit.

Sensitivity:
Limit Of Detection: It is defined as the lowest detectable concentration corresponding to a signal of Mean of '0' standard plus 2* SD. 10 replicates of '0' standards were evaluated and the LOD was found to **1.406ng/ml**.

Specificity:
This assay has high sensitivity and excellent specificity for detection of NSE. No significant cross-reactivity or interference between NSE and analogues was observed.

Recovery
Matrices listed below were spiked with certain level of NSE and the recovery rates were calculated by comparing the measured value to the expected amount of NSE in samples.

Matrix	Recovery Range (%)	Average (%)
Serum(n=5)	85-105	97
EDTA Plasma(n=5)	86-105	96
Heparin Plasma(n=5)	85-103	96

Assay Range:
2.344 ng/ml – 150 ng/ml

Precision:
Intra-Assay: CV<8%
Inter-Assay: CV<10%

Linearity:
The linearity of the kit was assayed by testing samples spiked with appropriate concentration of NSE and their serial dilutions. The results were demonstrated by percentage of calculated concentration to the expectation.

Sample	1:2	1:4	1:8
serum (n=5)	88-105%	90-103%	86-103%
EDTA plasma (n=5)	88-101%	83-99%	82-95%
heparin plasma (n=5)	82-98%	80-97%	80-98%

LIMITED WARRANTY

KinesisDx does not warrant against damages or defects arising in shipping or handling, or out of accident or improper or abnormal use of the product; against defects in products or components not manufactured by KinesisDx, or against damages resulting from such non-KinesisDx made products or components. KinesisDx passes on to customer the warranty it received (if any) from the maker thereof of such non- KinesisDx made products or components. This warranty also does not apply to product to which changes or modifications have been made or attempted by persons other than pursuant to written authorization by KinesisDx.

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ASSAY PROCEDURE

1	Bring all reagents to room temperature before use.		
2	Pipette Standards 1 - 8 Samples	100 ul	100 ul
3	Incubate	90 minutes (37ºC)	
4	Wash 1X Wash Buffer	Decant, 4 x 300 ul	
5	Pipette Biotinylated Anti- NSE	100ul	100ul
6	Incubate	60 minutes (37ºC)	
7	Wash 1X Wash Buffer	Decant, 4 x 300 ul	
8	Pipette Streptavidin: HRP Conjugate	100 ul	100 ul
9	Incubate	30 minutes (37ºC)	
10	Wash 1X Wash Buffer	Decant, 4 x 300 ul	
11	Pipette TMB Substrate	90 ul	90 ul
12	Incubate in the dark	10 minutes (37ºC)	
13	Pipette Stop Solution	50 ul	50 ul
14	Measure 450 within 15 mins		