DRG® NSE (EIA-2353)

Revised 29 July 2010 rm (Vers. 5.1) **RUO in the USA**

Please use only the valid version of the package insert provided with the kit.

Intended Use

The NSE ELISA kit is intended for the determination of NSE in human serum.

SUMMARY AND EXPLANATION OF THE ASSAY

The glycolytic enzyme enolase (2-phospho-D-glycerate hydrolase, EC42.1.11) exists as several dimeric isoenzymes (αα, αβ, αγ, ββ and γγ) composed of three distinct subunits α, β and γ. The γ unit is found either in a homologous γγ- or in a heterologous αγ-isoenzyme and is known as neuron-specific enolase (NSE). The monoclonal antibodies used in the NSE ELISA bind to the γ-subunit of the enzyme and thereby detects both the γγ and the αγ forms (1, 2). The NSE levels are low in healthy subjects and subjects with benign diseases. Elevated levels are commonly found in donors with malignant tumours with neuroendocrine differentiation, especially small cell lung cancer (SCLC) (3) and neuroblastoma (4). Quantitative determination of NSE in serum may be valuable in the management of donors with suspected or diagnosed SCLC or neuroblastoma, to aid in the differential diagnosis and to monitor the effect of treatment (5, 6).

PRINCIPLE OF THE TEST

The NSE ELISA is a solid phase, non-competitive immunoassay based on two monoclonal antibodies (derived from mice) directed against two separate antigenic determinants of the NSE molecule.
The monoclonal antibodies (MAb) used bind to the γ-subunit of the enzyme and thereby detects both the γγ and the αγ form. Calibrators and donor samples are incubated together with biotinylated Anti-NSE MAb E21 and horseradish peroxidase (HRP) labelled Anti-NSE MAb E17 in streptavidin coated micro strips. After washing, buffered Substrate/Chromogen reagent (hydrogen peroxide and 3, 3', 5, 5' tetramethylbenzidine) is added to each well and the enzyme reaction is allowed to proceed. During the enzyme reaction a blue colour will develop if antigen is present. The intensity of the colour development is proportional to the amount of NSE present in the samples.
The colour intensity is determined in a microplate spectrophotometer at 620 nm (or optionally at 405 nm after addition of Stop Solution).
Calibration curves are constructed for each assay by plotting absorbance value versus the concentration for each standard. The NSE concentrations of donor samples are then read from the calibration curve.

Revised 29 July 2010 rm (Vers. 5.1) **RUO in the USA**

REAGENTS

- Each NSE ELISA kit contains reagents for 96 tests.
- The expiry date of the kit is stated on the label on the outside of the kit box.
- Do not use the kit beyond the expiry date.
- Do not mix reagents from different kit lots.
- Store the kit at 2 °C - 8 °C. Do not freeze.
- Opened reagents are stable according to the table below provided they are not contaminated, stored in resealed original containers and handled as prescribed. Return to 2 °C - 8 °C immediately after use.

Component	Quantity	Storage and stability after first opening
Microtiterplate [MICROPLA]	1 plate	2 °C - 8 °C until expiry date stated on the plate
12 x 8 breakable wells coated with streptavidin. After opening, immediately return unused strips to the aluminium pouch containing desiccant and reseal carefully to keep dry.		
NSE Calibrators [Cal NSE A – E]	5 vials, lyophilised, 0.75 mL each	4 weeks at 2 °C - 8 °C 3 months at – 20°C
The lyophilised calibrators contain human NSE in a protein matrix with 0.01 % of a non-azide preservative. *To be reconstituted* with 0.75 mL distilled water before use. ***NOTE:*** The exact NSE concentration is lot specific and is indicated on the label of each vial.		
Biotin Anti-NSE [BIOTIN Anti-NSE]	1 x 15 mL	2 °C - 8 °C until expiry date stated on the vial
Biotin Anti-NSE monoclonal antibody from mouse, approximately 2 µg/mL. Contains phosphate buffer (pH 7.1), bovine serum albumin, blocking agents, an inert blue dye and 0.01 % methyl-isothiazolone (MIT) as preservative. *To be mixed* with Tracer, HRP Anti-NSE before use.		
Tracer, HRP Anti-NSE [CONJ Anti-NSE]	1 x 0.75 mL	2 °C - 8 °C until expiry date stated on the vial
Stock solution of HRP Anti-NSE monoclonal antibody from mouse, approximately 40 µg/mL. *To be mixed* with Biotin Anti-NSE prior to use. Contains 0.02 % methyl-isothiazolone (MIT), 0.02 % bromonitrodioxane and 20 ppm Proclin 300 as preservatives.		
TMB HRP-Substrate [SUBS TMB]	1 x 12 mL	2 °C - 8 °C until expiry date stated on the vial
Ready for use. Contains buffered hydrogen peroxide and 3,3´,5,5´ tetramethylbenzidine (TMB).		
Stop Solution [STOP]	1 x 15 mL	2 °C - 8 °C until expiry date stated on the vial
Ready for use. Contains 0.12 M hydrochloric acid.		
Wash Concentrate [WASHBUF 25X]	1 x 50 mL	2 °C - 8 °C until expiry date stated on the bottle
To be diluted with water 25 times before use. A Tris-HCl buffered salt solution with Tween 20. Contains Germall II as preservative.		

Indications of instability
The TMB HRP-Substrate should be colourless or slightly bluish. A blue colour indicates that the reagent has been contaminated and should be discarded.

WARNINGS AND PRECAUTIONS

For research use only
- For Professional Use Only
- Please refer to the U.S. Department of Health and Human Services (Bethesda, Md., US) publication No. (CDC) 88-8395 on laboratory safety or any other local or national regulation.
- Handle all donor specimens as potentially infectious.
- Follow local guidelines for disposal of all waste material.

Caution
Each donor unit used in the preparation of human source reagent has been tested and found to be Non Reactive for HIV-1/2 Antibody, HCV Antibody and Hepatitis B Surface Antigen (HBsAg). Since no method can completely rule out the presence of blood borne diseases, the handling and disposal of human source reagents from this product should be made as if they were potentially infectious.

SPECIMEN COLLECTION AND HANDLING

The NSE ELISA is intended for use with serum.
Collect blood by venipuncture and separate the serum according to common procedures. Serum should be separated from the clot within 60 minutes of collection to avoid leaking of NSE from blood cells.
Do not use haemolysed samples.
Plasma is not recommended since significant amounts of NSE can be released from platelets.
Samples can be stored at 2 °C - 8 °C for 24 hours. For longer periods store samples at -70°C or below.
Samples should not be stored in a self-defrosting freezer and not be thawed and refrozen before analysis.
Bring frozen samples to room temperature and mix THOROUGHLY by gently inverting multiple times before analysis.
Samples that contain gross particulates should be centrifuged at 10.000 x g for 10 minutes, prior to use to eliminate any particulate matter that may have developed from the thawing process. Analyze thawed samples within one hour.

PROCEDURE

Materials required but not supplied with the kit

1. **Microplate shaker**
   Shaking should be medium to vigorous. Longitudinal shaking approximately 200 strokes/min, oscillations 700 - 900/min.
2. **Microplate wash device**
   Automatic platewash capable of performing 1 and 6 washing cycles, or semi manual microplate washing device connected to vacuum pump or water-jet vacuum and a liquid trap for retaining aspirated liquid.
   The Nunc Immuno-8 manual strip washer is recommended if an automatic microplate wash is not used.
3. **Microplate spectrophotometer**
   with a wavelength of 620 nm and/or 405 nm, and an absorbance range of 0 to 3.0.

4. **Precision pipettes**
   with disposable plastic tips for dispensing microlitre volumes. An 8-channel pipette or respenser pipette with disposable plastic tips for delivery of 100 µL is useful but not essential. Pipettes for dispensing millilitre volumes.
5. **Distilled or deionized water**
   For reconstitution of NSE Calibrators and for preparation of diluted wash solution.

Procedural notes

1. A thorough understanding of this package insert is necessary to ensure proper use of the NSE ELISA kit. The reagents supplied with the kit are intended for use as an integral unit. Do not mix identical reagents from kits having different lot numbers. Do not use the kit reagents after the expiry date printed on the outside of the kit box.
2. Reagents should be allowed to reach room temperature (20 °C – 25 °C) prior to use. The assay should only be performed at temperatures between 20 °C – 25 °C to obtain accurate results. Frozen sera must be gently but thoroughly mixed after thawing.
3. Before starting to pipette calibrators and donor specimens it is advisable to mark the strips to be able to clearly identify the samples during and after the assay.
4. The requirement for efficient and thorough washing for separation of bound and unbound antigen and reagents from the solid-phase bound antibody-antigen complexes is one of the most important steps in an EIA. In order to ensure efficient washing make sure that all wells are completely filled to the top edge with wash solution during each wash cycle, that wash solution is dispensed at a good flow rate, that the aspiration of the wells between and after the wash cycles is complete and that the wells are empty. If there is liquid left, invert the plate and tap it carefully against absorbent paper.

   Automatic strip washer:
   Follow the manufacturer's instructions for cleaning and maintenance diligently and wash the required number of wash cycles prior to and after each incubation step. It's highly recommended to use strip process mode and overflow wash mode with a dispensing volume of 800 µL. The aspiration/wash device should not be left standing with the Wash Solution for long periods, as the needles may get clogged resulting in poor liquid delivery and aspiration.

5. The TMB HRP-Substrate is very sensitive for contamination. For optimal stability of the TMB HRP-Substrate, pour the required amount from the vial to a carefully cleaned reservoir or preferably a disposable plastic tray to avoid contamination of the reagent. Be sure to use clean disposable plastic pipette tips (or respenser pipette tip).
6. Be sure to use clean disposable plastic pipette tips and a proper pipetting technique when handling samples and reagents. Avoid carry-over by holding the pipette tip slightly above the top of the well and avoid touching the plastic strip or surface of the liquid. A proper pipetting technique is of particular importance when handling the TMB HRP-Substrate solution.

Preparation of reagents

Preparation of reagents	Stability of prepared reagent
NSE Calibrators	4 weeks at 2 °C - 8 °C 3 months at – 20 °C

Add exactly 0.75 mL of distilled water to each vial and mix gently.
Allow standing for at least 15 minutes to reconstitute.
NOTE: The concentration of the calibrators is stated on the labels and should be used for calculation of the results.

Wash Solution 2 weeks at 2 °C – 25 °C in a sealed container
Pour the 50 mL Wash Concentrate into a clean container and dilute 25-fold by adding 1200 mL of distilled or deionised water to give a buffered Wash Solution.

Antibody Solution 3 weeks at 2 °C - 8 °C
Prepare the required quantity of Antibody Solution by mixing 50 µL of Tracer HRP Anti-NSE- with 1 mL of Biotin Anti-NSE per strip (see table below):

No. of Strips	Tracer, HRP Anti-NSE (µL)	Biotin Anti-NSE (mL)
1	50	1
2	100	2
3	150	3
4	200	4
5	250	5
6	300	6
7	350	7
8	400	8
9	450	9
10	500	10
11	550	11
12	600	12

Be sure to use a clean plastic or glass bottle for preparation of Antibody Solution.
Alternative: Pour the content of the HRP Anti-NSE-Tracer into the vial of Biotin Anti-NSE and mix gently. Be sure that all content of the Tracer is transferred to the vial of Biotin Anti-NSE.
NOTE: The Antibody Solution is stable for 3 weeks at 2 °C - 8 °C. Do not prepare more Antibody Solution than will be used within this period and make sure that it is stored properly.

Assay procedure

Perform each determination in duplicate for both calibrators and donor samples.
A calibration curve should be run with each assay. All reagents and samples must be brought to room temperature (20 °C - 25 °C) before use.

1. Start to prepare NSE Calibrators, Wash Solution and Antibody Solution. It is important to use clean containers. Follow the instructions carefully.
2. Transfer the required number of microplate strips to a strip frame. (Immediately return the remaining strips to the aluminium pouch containing a desiccant and reseal carefully). Wash each strip once with the Wash Solution. Do not wash more strips than can be handled within 30 min.
3. Pipette 25 µL of the NSE Calibrators (Cal A, B, C, D, E) and donor specimens (unknowns) into the strip wells according to the following scheme:

	1	2	3	4	5	6	7	8
A	Cal A	Cal E	Unkown 4					
B	Cal A	Cal E	etc.					
C	Cal B	Unkown 1						
D	Cal B	Unkown 1						
E	Cal C	Unkown 2						
F	Cal C	Unkown 2						
G	Cal D	Unkown 3						
H	Cal D	Unkown 3						

4. Add 100 µL of Antibody Solution to each well using a 100 µL precision pipette (or an 8-channel 100 µL precision pipette). Avoid carry-over by holding the pipette tip slightly above the top of the well and avoid touching the plastic strip or surface of the liquid.
5. Incubate the plate for **1 hour (± 10 min) at room temperature (20 °C – 25 °C)** with constant shaking of the plate using a microplate shaker.
6. After the incubation aspirate and wash each strip 6 times.
7. Add 100 µL of TMB HRP-Substrate Solution to each well using the same procedure as in item 4. The TMB HRP-Substrate should be added to the wells as quickly as possible and the time between addition to the first and last well should not exceed 5 min.
8. Incubate for **30 min (± 5 min) at room temperature** with constant shaking.
   Avoid exposure to direct sunlight.
9. Immediately read the absorbance at 620 nm in a microplate spectrophotometer.

Option

If the laboratory does not have access to a microplate spectrophotometer capable of reading at 620 nm the absorbance can be determined as in item 10.

10. Add 100 µL of Stop Solution, mix and read the absorbance at 405 nm in a microplate spectrophotometer within 15 min after addition of Stop Solution.

Measurement range

The NSE ELISA measures concentrations between 1 µg/L and approximately 150 µg/L. If NSE concentrations above the measuring range are to be expected, it is recommended to dilute samples with normal human serum prior to analysis. **NOTE:** The serum used for dilution should also be measured in order to determine the endogenous NSE concentration (see "Calculation of results").

Quality control

Tumor Marker Control Sera Levels 1 and 2 (available separately, REF EIA-4458) are recommended for validation of the assay series. If values outside of the specified range are obtained, a complete check of reagents and reader performance should be made and the analysis repeated.

Reference materials

Since no common reference material is available for NSE antigen, NSE ELISA Calibrator values are assigned against a set of in-house reference calibrators.

CALCULATION OF RESULTS

If a microplate spectrophotometer with built-in data calculation program is used refer to the manual for the spectrophotometer and create a program using the concentration stated on the label of each of the NSE calibrators.
For automatic calculation of NSE results it is recommended to use either of the following methods:

- Cubic spline curve fit method. Calibrator A should be included in the curve with the value 0 µg/L.
- Spline smoothed curve fit method. Calibrator A should be used as plate blank.
- Interpolation with point-to-point evaluation. Calibrator A should be included in the curve with the value 0 µg/L.
- Quadratic curve fit method. Calibrator A should be included in the curve with the value 0 µg/L.

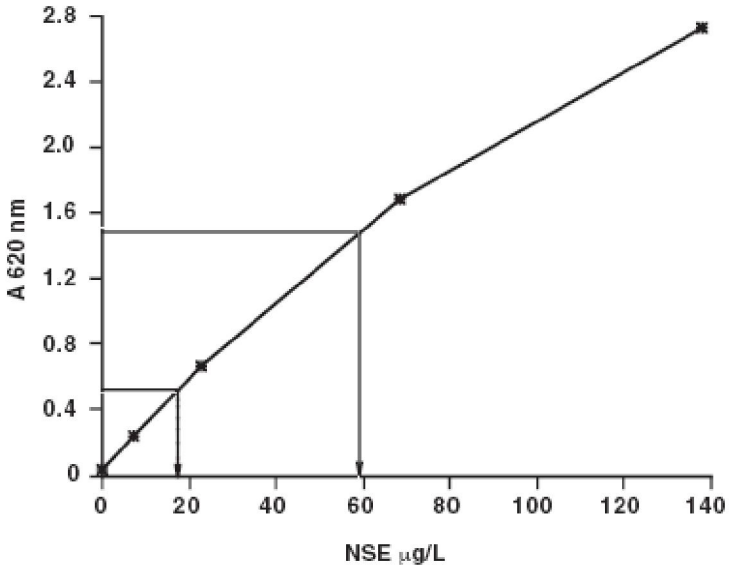
NOTE: 4-Parametric or Linear regression evaluation methods should not be used.

For manual evaluation, a calibration curve is constructed by plotting the absorbance (A) values obtained for each NSE Calibrator against the corresponding NSE concentration (in µg/L), see figure. The unknown NSE concentrations can then be read from the calibration curve using the mean absorbance value of each donor specimen. If samples in an initial analysis give NSE levels above the concentration of calibrator E, it is necessary to dilute the sample 1/10 with normal human serum in order to obtain accurate results. The result should then be calculated according to the following procedure:

Dilution 1/10: $10 \times ([NSE]_{\text{Diluted sample}} - (0.9 \times [NSE]_{\text{Normal human serum}}))$

Example of results

Specimen	Calibrator values	Mean abs value (A)	NSE µg/L
Calibrator A	0 µg/L	0.037	
Calibrator B	7.5 µg/L	0.238	
Calibrator C	22.9 µg/L	0.663	
Calibrator D	68.4 µg/L	1.688	
Calibrator E	138.0 µg/L	2.720	
Specimen 1		0.518	17.5
Specimen 2		1.474	57.8



Example, do not use this curve to determine assay results.
The exact NSE concentration is indicated on the label of each standard vial.

LIMITATIONS OF THE PROCEDURE

The level of NSE cannot be used as absolute evidence for the presence or absence of malignant disease and the NSE test should not be used in cancer screening. The results of the test should be interpreted only in conjunction with other investigations and procedures in the diagnosis of disease and the NSE test should not replace any established clinical examination.

- Elevated NSE values not due to tumours may occur in dialysis donors and donors with leukaemic diseases.
- Serum should not contain visible haemolysis (the absorbance at 500 nm for non-turbid sample should not exceed 0.3) since erythrocytes contain significant amounts of NSE (7). Prolonged storage of whole blood can cause release of NSE from the blood cells.
- Anti-reagent antibodies (human anti-mouse antibody (HAMA) or heterophilic antibodies) in the donor sample may occasionally interfere with the assay, even though specific blocking agents are included in the buffer.

Revised 29 July 2010 rm (Vers. 5.1) **RUO in the USA**

WARRANTY

The performance data presented here were obtained using the assay procedure indicated. Any change or modification of the procedure not recommended by DRG may affect the results, in which event DRG disclaims all warranties expressed, implied or statutory including the implied warranty of merchantability and fitness for use.

LITERATURE REFERENCES / Literatur / BIBLIOGRAFIA

1. Paus E. and Nustad K., (989) Immunoradiometric Assay for αγ- and γγ-Enolase (Neuron-Specific Enolase), with Use of Monoclonal Antibodies and Magnetizable Polymer Particles. Clin. Chem. 35: 2034 - 2038.
2. Dahlén U., Karlsson B., Nilsson O. and Uhl W., (1995) Development of an Enzyme Immunoassay, NSE-Enzymun Test For Determination of Neuron-Specific Enolase. XXIII International Society for Oncodevelopmental Biology and Medicine, Montréal, Ouébec .
3. Cooper E.H., (1999) Neuron-specific enolase. The International Journal of Biological Markers 9(4):205-10.
4. Cooper E.H., Pritchard J., Bailey C.C. and Ninane J., (1987) Serum neuronspecific enolase in children's cancer. Br. J. Cancer 56: 65 - 67.
5. Schneider, P. M. et al., (2002) Lung Cancer. In "Tumor markers, Physiology, Pathobiology, Technology and Clinical Applications" Eds. Diamandis E. P. et al., AACC Press, Washington pp 287-303.
6. Bonner J. A., Sloan JA., Rowland KM., Klee GG., Kugler JW., Mailliard JA., Wiesenfeld M., Krook JE.,Maksymiuk AW., Shaw EG., Marks RS and Perez EA., (2000) Significance of Neuron-specific Enolase Levels before and during Therapy for Small Cell Lung Cancer. Clinical Cancer Research 6: 597-601.
7. Påhlman S., Esscher T., Bergvall P. and Odelstad L., (1984) Purification and characterization of human neuron-specific enolase: Radioimmunoassay development. Tumor Biol. 5: 127 - 139.
8. National Committee for Clinical Laboratory Calibrators, Evaluation of Precision Performance of Clinical Chemistry Devices. Approved Guideline EP5-A (1999).
9. National Committee for Clinical Laboratory Calibrators, National Evaluation Protocols for Interference Testing, Evaluation protocol Number 7, Vol. 6, No 13, August (1986).