Enzyme immunoassay (ELISA) for the quantitative determination of neopterin in serum, plasma and urine

Article number: 99R.096 (96 determinations)

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Contents of the Kit

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity for 96 det.</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1vial</td>
<td>Enzyme conjugate (neopterin/alkaline phosphatase conjugate), lyophilized, for reconstitution with 16 mL of buffer B</td>
</tr>
<tr>
<td>B</td>
<td>1 x 16 mL vial</td>
<td>Enzyme conjugate buffer, ready for use</td>
</tr>
<tr>
<td>C</td>
<td>1 plate</td>
<td>Microtitre plate, with 12 rows each of 8 wells coated with anti-neopterin antibodies (polyclonal, sheep), for 96 determinations, ready for use</td>
</tr>
<tr>
<td>C2</td>
<td>1 plate</td>
<td>Microtitre plate, uncoated for premixing of enzyme conjugate as well as standards, controls and samples for 96 determinations, ready for use</td>
</tr>
<tr>
<td>T</td>
<td>2 tablets of substrate</td>
<td>Substrate (4-nitrophenyl phosphate), for dissolution in 15 mL of buffer E</td>
</tr>
<tr>
<td>E</td>
<td>1 x 15 mL vial</td>
<td>Substrate buffer, for dissolution of tablets T</td>
</tr>
<tr>
<td>F</td>
<td>1 x 15 mL vial</td>
<td>Stop solution (2 N sodium hydroxide), ready for use</td>
</tr>
<tr>
<td>W</td>
<td>1 x 11 mL vial</td>
<td>B-R-A-H-M-S Washing solution universal, concentrate, 11 mL</td>
</tr>
<tr>
<td>S1 – S6</td>
<td>6 x 0.4 mL vials</td>
<td>Neopterin standards (human serum), ready for use, concentrations: def. 2; 6.4; 16; 40; 100; 250 nmol/L</td>
</tr>
<tr>
<td>K1, K2</td>
<td>2 x 0.4 mL vials</td>
<td>Control sera, ready for use. Concentrations see leaflet enclosed</td>
</tr>
<tr>
<td></td>
<td>1 black cover sheet</td>
<td></td>
</tr>
</tbody>
</table>


Date: 2015-05-24
(This version supersedes all earlier instruction manuals.)

Content changes versus previous version

- Labelling in accordance with Regulation (EC) No 1272/2008 on the classification, labelling and packaging of substances and mixtures, which implements the Global Harmonised System for Labelling and Classification of Hazardous Chemicals (GHS).
- B-R-A-H-M-S service USA deleted

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Introduction

The B·R·A·H·M·S Neopterin EIA is a competitive enzyme immunoassay for the quantitative determination of neopterin in serum, plasma and urine using coated microtitre plates. The wells of the microtitre plate are coated with anti-neopterin antibodies (polyclonal, sheep). After addition of the enzyme conjugate (neopterin/alkaline phosphatase conjugate) to standards and control sera and to patient samples (serum, plasma or urine) the neopterin of the patient samples competes with the neopterin/enzyme conjugate for the binding sites of these antibodies, thus forming an immune complex bound to the solid phase (anti-neopterin antibody/neopterin or neopterin/enzyme conjugate).

The subsequent intensive washing step ensures the complete removal of all unbound components. The addition of the 4-nitrophenyl phosphate substrate solution starts the enzyme reaction in which the alkaline phosphatase contained in the neopterin/enzyme conjugate catalyses the cleavage of the phosphate of 4-nitrophenyl phosphate, thus forming the yellow 4-nitrophenol. The enzymatic reaction is stopped by alkalisation with sodium hydroxide. The intensity of the colour (measured in optical density OD) depends on the quantity of enzyme bound for a constant reaction time and consequently is inversely proportional to the neopterin concentration in the patient sample. Thus, high neopterin values correspond to a low optical density.

The optical density is measured by means of a microtitre plate reader at an absorption maximum of 405 nm. The results are calculated by plotting a standard curve (optical density versus concentrations of neopterin standard), from which the neopterin concentrations in the patient samples can be read off directly.

Important Notes

Hazard classification:
Stop solution (2 N sodium hydroxide). Danger.
P280: Wear protective gloves/protective clothing/eye protection/face protection.
P313: Get medical advice/attention.
P305+351+338: IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P361: Remove/Take off immediately all contaminated clothing.
The stop solution contains dilute sodium hydroxide.
Substrate buffer: Attention, Danger
H315: Causes skin irritation.
H318: Causes serious eye damage.
H373: May cause damage to organs through prolonged or repeated exposure.
P280: Wear protective gloves/protective clothing/eye protection/face protection.
P313: Get medical advice/attention.
P305+351+338: IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P361: Remove/Take off immediately all contaminated clothing.
The substrate buffer contains the preservative sodium azide at concentrations of < 0.1% by weight.

Quality control: National quality assurance guidelines for quantitative tests in the medical laboratory (current version) must be complied with. For instance, test accuracy and precision can be monitored by means of laboratory in-house and/or commercially available control materials. If unacceptable control values are obtained, proceed as outlined in standard laboratory diagnostic procedures to determine the cause and implement corrective measures.

This kit contains materials of human origin (e.g. serum). Only materials are used in their preparation that materials have been screened for HBsAg, HIV I/II antibodies and HCV antibodies and found to be negative. However, the reagents and patient samples should be handled with the generally acknowledged precautions and laboratory techniques as potential contagion cannot be ruled out.

The following kit reagents contain the preservative sodium azide at concentrations of < 0.1% by weight: standards, controls, enzyme conjugate and substrate buffer. These reagents should not be swallowed or allowed to come in contact with the skin or mucous membranes. p-nitrophenol is generated on conversion of the substrate.

Our Customer Service Department, phone: +49(0)3302/883 300, will gladly send the reagent-specific EU Safety Data Sheets in accordance to regulation 1907/2006-EC upon request.

In the event that glass vials are included in the reagent kit, we explicitly point out that there will be a breakage hazard, and consequently a risk of injury.

The reagents as well as waste generated by the test must be disposed of in accordance with local regulations.

Bibliography

Test Procedure

Incubation Scheme

Manual as well as machine version (premixing in uncoated microtitre plate [C 2])

Blank S1 – S6K1, K2P 1 etc.

1. Pipette standards µL – 50 – –
controls µL – – 50 –
serum samples µL – – – 50
enzyme conjugate µL – 150 150 150
– Mix thoroughly by shaking briefly

2. Pipette
– Transfer a 150 µL aliquot of this mixture to the neopterin antibody-coated microtitre plate (C) [multichannel pipette].

3. Incubate
– 2 hours at room temperature away from light

4. Aspirate or decant
– Aspirate or decant completely.

5. Pipette washing solution µL – 350 350 350

6. Aspirate or decant
– Aspirate or decant completely and shake out on cellulose. Repeat step 5 and 6 three times to ensure a total of 4 washing steps.

7. Pipette substrate solution µL 100 100 100 100

8. Incubate
30 ± 5 minutes at room temperature (cf. test procedure).

9. Pipette stop solution µL 100 100 100 100
– Mix thoroughly by shaking briefly.

10. Measurement of optical density in a photometer (wavelength 405 nm)

Calculation of results

Specimen Handling

Both human serum and also plasma and urine can be used in the B-R-A-H-M-S Neopterin EIA. When performing the test, the samples should be free from microbial contamination.

Samples that cannot be studied on the day they are obtained may be stored for 3 days at 2…8°C or longer at −20°C away from light. The sample container should at all events be closed. Avoid repeated freezing and thawing.

Before storage at −20°C the serum or plasma should be separated from blood clots.

Neopterin and its hydrated derivatives are sensitive to light. Patient samples, standards and tracers should therefore be stored cool and protected from light. Exposure to direct sunlight should be avoided.

Insoluble material should be removed from the samples by centrifugation prior to the test.

Notes on Test Execution

Do not use any reagents that have exceeded the expiration date printed on the label.

The individual components of the kit are perfectly attuned to each other. If components from different batches are exchanged or mixed, B-R-A-H-M-S GmbH can assume no liability for the accuracy of results.

In large test series, reagents of the same batch designation are pooled. The indicated sequence of steps must be followed.

Patient samples with a concentration above the measuring range are to be rated as “> highest standard”. The result must not be extrapolated. The patient sample in question should be diluted and retested. Further information can be obtained from B-R-A-H-M-S GmbH customer service.

Test Procedure

Adjust the reagents to room temperature before undertaking the test and ensure the homogeneity of the solution.

Add buffer (B) to the enzyme conjugate (A) to prepare the ready to use form. To facilitate proper identification of reconstituted tracer (lot, expiry date) blank labels (100) can be ordered under article number 105359 free of charge.

Prepare washing solution: dilute 11 mL concentrate with distilled water to yield 550 mL. We strongly recommend to contact the manufacturer or distributor before using other washing solutions.

Ensure the substrate tablets (T) are completely dissolved in substrate buffer (E). The ready to use substrate solution (T + E) is stable for 3 days at 2…8°C.

Do not interrupt the individual stages in the procedure. Keep the order and the incubation times constant for all samples. The Wells in the plate should always be filled in the same order.

Incubation must be carried out in the dark because of the sensitivity of neopterin to light (black cover sheet); the substrate/enzyme reaction can also be carried out in normal daylight.

A standard series and negative and positive controls must be carried out for each microtitre plate.

It is recommended that a blank be determined, for which a corresponding well should be left free. Pipette 100 µL of the substrate 4-nitrophenyl phosphate into this well at a later point – as described under point 7 – and add 100 µL of the stop solution (F) after incubation for 30 minutes.

Duplicates are recommended.

1. Pipette 50 µL of the standards (in increasing concentrations), 50 µL of the controls and 50 µL of the serum samples each with 150 µL of the enzyme conjugate into the uncoated microtitre plate (C2).

Thorough mixing of the contents by shaking briefly or aspirating and ejecting is recommended.

When using an automatic pipette, ensure the contents are thoroughly mixed by aspirating and ejecting twice.

2. Transfer an aliquot of 150 µL of this mixture into the neopterin-antibody coated microtitre plate (C) using a multichannel pipette (recommended in the manual version). The transfer time should not exceed 5 minutes.

3. Incubate the microtitre plate for 2 hours at room temperature in the dark (using the black cover sheet).

The black cover sheet is not required with the machine version. The light protection within the apparatus e.g. FAME or Behring processor, is sufficient.

4. Aspirate or decant the incubation volume for both the manual and the machine version.

6. Aspirate or decant the wells again completely. **Repeat step 5 and 6 three times to ensure a total of 4 washing steps.**

After the last washing step in the manual version, remove any remaining droplets by tapping the plate sharply on blotting paper, taking care to ensure no droplets remain in the wells.

7. Pipette 100 µL of the dissolved substrate (4-nitrophenyl phosphate) into all wells – including that of the blank where appropriate.

8. Incubate the microtitre plate (C) again for 30 ± 5 minutes at room temperature. This reaction can also be performed in normal daylight.

Because of the temperature dependency of the enzyme activity, it is recommended the incubation time of the enzyme/substrate reaction should be as follows:

<table>
<thead>
<tr>
<th>Room temperature range (°C)</th>
<th>Duration of enzyme/substrate interaction (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17.0 – 20.4</td>
<td>30</td>
</tr>
<tr>
<td>20.5 – 24.9</td>
<td>30</td>
</tr>
<tr>
<td>≥ 25.0</td>
<td>25</td>
</tr>
</tbody>
</table>

**Calculation of Results**

For computer-aided evaluation, an evaluation programme (spline/unsmoothed) must be selected that suits the specific combination of processor and measuring equipment used.

Alternatively, the results can also be calculated using semilogarithmic graph paper. In this case the concentrations of the standards (x-axis, logarithmic) are plotted against the mean values of the optical density of the standards (y-axis, linear), thus creating a standard curve with the 6 points obtained. The optical density of the samples can now be used to read off the corresponding neopterin concentrations directly in nmol/L.

For technical support, please contact the customer service of B·R·A·H·M·S GmbH or the appropriate distribution partner / sales representative.

**Example (example “after deduction of the blank”)**

<table>
<thead>
<tr>
<th></th>
<th>OD₁</th>
<th>OD₂</th>
<th>Mean</th>
<th>Neopterin [nmol/L]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard 1</td>
<td>2.00</td>
<td>1.98</td>
<td>1.99</td>
<td>def. 2</td>
</tr>
<tr>
<td>Standard 2</td>
<td>0.99</td>
<td>1.05</td>
<td>1.02</td>
<td>6.4</td>
</tr>
<tr>
<td>Standard 3</td>
<td>0.58</td>
<td>0.56</td>
<td>0.57</td>
<td>16</td>
</tr>
<tr>
<td>Standard 4</td>
<td>0.27</td>
<td>0.29</td>
<td>0.28</td>
<td>40</td>
</tr>
<tr>
<td>Standard 5</td>
<td>0.13</td>
<td>0.15</td>
<td>0.14</td>
<td>100</td>
</tr>
<tr>
<td>Standard 6</td>
<td>0.08</td>
<td>0.06</td>
<td>0.07</td>
<td>250</td>
</tr>
<tr>
<td>Patient sample 7</td>
<td>1.13</td>
<td>1.09</td>
<td>1.11</td>
<td>6.0</td>
</tr>
</tbody>
</table>

Conversion factor: nmol/L x 0.253 = ng/mL

9. Pipette 100 µL of stop solution F into all wells to stop the enzyme reaction and the formation of colour. Sodium hydroxide must be added in the same order as for the substrate. Ensure the contents of the wells are thoroughly mixed by shaking the microtitre plate (C) briefly before measuring the optical density.

10. The optical density (OD) is measured in a photometer at a wavelength of 405 nm (reference wavelength 630 nm or 690 nm). Optionally, the measured blank is subtracted from the optical density results obtained.

**Follow the manufacturer’s instructions exactly!** Incorrect handling of the reagents may falsify the test results. B-R-A-H-M-S GmbH is not liable for faulty test results arising from improper storage, use or handling.

**Additionally required**

- Micropipettes (50 µL)
- Multichannel pipette (50, 100 µL)
- Distilled water
- Automatic washing machine or manual washing comb
- Microtitre plate reader (wavelength 405 nm, reference wavelength 630 nm or 690 nm)

**Interferences**

<table>
<thead>
<tr>
<th>Factor</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin</td>
<td>no significant effect up to 100 mg/dL</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>no significant effect up to 4 mg/dL</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>no significant effect up to 700 mg/dL</td>
</tr>
</tbody>
</table>
Stability and Storage Conditions

Store all reagents and the coated microtitre plate (C) at 2 to 8°C in their original shipping containers until immediately prior to use. Uncoated microtitre plates (C2) can be stored at room temperature. Observe carefully the expiry dates on the outer packaging and the kit labels.

Adjust the kit components to room temperature before use (about 30 minutes beforehand) and store cool again after use. If a fraction of the standard the controls, the enzyme conjugate buffer, the substrate buffer, the concentrated washing solution and the stop solution is removed aseptically, the unused quantity of the reagents is stable at 2…8°C in the original containers until the expiry date indicated.

Residual quantities of reconstituted enzyme conjugate must be frozen immediately. The reconstituted conjugate is usable for four weeks when stored at ≤ -15°C; one to three freezing and thawing cycles are possible.

Storage at 2…8°C is possible for four days as an option.

Microtitre plates (C) that are not fully utilised may be used until the expiry date if stored at 2…8°C.

The ready to use substrate solution is stable for 3 days at 2…8°C.

Diluted washing solution may be used for up to 4 weeks if stored at 2…8°C. Contaminated washing solution must not be used. This is the case either if the liquid is clouded or the pH value is < 6.

Symbols

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Usage</th>
<th>Symbol</th>
<th>Usage</th>
<th>Symbol</th>
<th>Usage</th>
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<td>Manufacturer</td>
<td>CE</td>
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<td>Use by</td>
<td>Temperature Limitation</td>
<td>REF</td>
<td>Article Number/ Catalogue Number</td>
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<td>Green Dot according to German Law</td>
<td>Buffer</td>
<td>CAL</td>
<td>Standard</td>
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<tr>
<td>CAL SET</td>
<td>Zero Standard</td>
<td>CONTROL</td>
<td>Control Serum</td>
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<tr>
<td>TRACER</td>
<td>Consult Instruction for use</td>
<td>LOT</td>
<td>Batch code</td>
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</tr>
<tr>
<td>BUF SUBS</td>
<td>Substrate buffer</td>
<td>SOLN STOP</td>
<td>Stop solution</td>
<td></td>
<td></td>
</tr>
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<td>LYOPH</td>
<td>Lyophilized, freeze dried</td>
<td>RCNS 16 mL</td>
<td>Reconstitute with 16 mL xxx</td>
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</tr>
<tr>
<td>GHS08 Health hazard</td>
<td></td>
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</tr>
</tbody>
</table>