Instructions for Use

Neopterin ELISA

Enzyme immunoassay for the in-vitro-diagnostic quantitative determination of neopterin in human serum, plasma and urine.

REF **40-371-25012**

Σ **96**

2-8 °C

EU: *For research use only.*

U.S.: *For research use only.*
Not for use in diagnostic procedures.

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1. INTENDED USE

Enzyme immunoassay for the *in-vitro-diagnostic* quantitative determination of neopterin in human serum, plasma and urine.

2. SUMMARY AND EXPLANATION

Neopterin is a low molecular weight molecule belonging to the chemical group known as pteridines. It is synthesised by cellular immune reaction of macrophages and dendritic cells upon stimulation with the cytokine interferon-g and as a consequence released. Neopterin has a higher stability in body fluids which makes the sample handling and measurement easier compared to other cytokines. The low molecular weight, let neopterin molecules rapidly pass the intravasal area, where it is releases in urine after glomerular filtration. The half life period in human bodies is only affected by renal excretion. So neopterin values reflect the totality of immunological processes for monocytes/macrophages and dendritic cells and can be seen as a general marker of immune activity. This characteristic feature of neopterin to reflect the different interactions of immunocompetent cells is the basis for the extraordinary status of measuring neopterin in immmunological diagnosis. As a non-invasive method, urinary neopterin to creatinine ratio determination is also helpful in monitoring disease progression and the effects of therapies, as well.
Neopterin biosynthesis is closely associated with activation of the cellular immune system. Increased concentrations of neopterin were reported in patients with viral infections, suggesting that increased values may originate from the immune response of patients directed against virally infected cells. It was shown that antigenic stimulation of human peripheral blood mononuclear cells leads to neopterin release into cell culture medium and that human macrophages produce neopterin in vitro when stimulated by interferon gamma.
The determination of neopterin levels in human body fluids offers a useful and innovative tool to monitor diseases associated with the activation of cell-mediated immunity.
Increasing neopterin levels in various infections precede the clinical manifestation and seroconversion.
Normally samples are not tested for all possible infections. Therefore, the measurement of neopterin in blood donor samples is a useful tool in order to reduce the risk of infections via blood transfusion.

Other diagnostic applications for the determination of neopterin are:

- follow-up of traumatized ICU patients
- use as prognostic indication in HIV infections and malignant diseases
- early indication of complications in allograft recipients
- indication of disease activity in autoimmune diseases
- diagnosis of viral infections
- differential diagnosis of acute viral and bacterial infections
- diagnosis of tumour diseases
- follow-up control of chronic infections and monitoring of immunostimulatory therapy

3. TEST PRINCIPLE

Solid phase enzyme-linked immunosorbent assay (ELISA) based on the basic principle of a competitive ELISA. An unknown amount of antigen in the sample and a fixed amount of enzyme labelled antigen compete for the antibody-binding sites (rabbit-anti-neopterin). Both antigen-antibody complexes bind to the wells of the microtiter strips coated with a goat-anti-rabbit antibody. Unbound antigen is removed by washing. The intensity of the color developed after the substrate incubation is inversely proportional to the amount of antigen in the sample. Results of samples can be determined directly using the standard curve.

4. WARNINGS AND PRECAUTIONS

1. For *in-vitro diagnostic* use only. For professional use only.
2. Before starting the assay, read the instructions completely and carefully. Use the valid version of the package insert provided with the kit. Be sure that everything is understood.
3. In case of severe damage of the kit package please contact us or your supplier in written form, latest one week after receiving the kit. Do not use damaged components in test runs, but keep safe for complaint related issues.
4. Obey lot number and expiry date. Do not mix reagents of different lots. Do not use expired reagents.
5. Follow good laboratory practice and safety guidelines. Wear lab coats, disposable latex gloves and protective glasses where necessary.
6. Reagents of this kit containing hazardous material may cause eye and skin irritations. See MATERIALS SUPPLIED and labels for details. Material Safety Data Sheets for this product are available upon request.
7. Chemicals and prepared or used reagents have to be treated as hazardous waste according to national biohazard and safety guidelines or regulations.
8. Avoid contact with Stop solution. It may cause skin irritations and burns.
9. All reagents of this kit containing human serum or plasma have been tested and were found negative for anti-HIV I/II, HBsAg and anti-HCV. However, a presence of these or other infectious agents cannot be excluded absolutely and therefore reagents should be treated as potential biohazards in use and for disposal.

5. STORAGE AND STABILITY

The kit is shipped at ambient temperature and should be stored at 2-8 °C. Keep away from heat or direct sun light. The storage and stability of specimen and prepared reagents is stated in the corresponding chapters.

The microtiter strips are stable up to the expiry date of the kit in the broken, but tightly closed bag when stored at 2–8 °C.

6. SPECIMEN COLLECTION AND STORAGE

Serum, Plasma (EDTA)

The usual precautions for venipuncture should be observed. It is important to preserve the chemical integrity of a blood specimen from the moment it is collected until it is assayed. Do not use grossly hemolytic, icteric or grossly lipemic specimens. Do not use specimens containing NaN_3. Samples appearing turbid should be centrifuged before testing to remove any particulate material.			
Storage:	2-8°C	≤ -20°C (Aliquots)	Keep away from heat or direct sun light.
Stability:	72 h	6 months	Avoid repeated freeze-thaw cycles.

Urine

It is possible to use spontaneous as well as 24 h urine. The total volume of urine excreted during a 24 h period should be collected and mixed in a single bottle. Preservation is not necessary. Determine total volume for calculation of results. **Mix and centrifuge samples before use in the assay.**			
Storage:	2-8°C	≤ -20°C (Aliquots)	Keep away from heat or direct sun light.
Stability:	72 h	6 months	Avoid repeated freeze-thaw cycles.

7. MATERIALS SUPPLIED

Quantity	Symbol	Component
1 x 12 x 8	MTP	**Microtiter Plate** Break apart strips. Coated with anti-rabbit IgG (goat, polyclonal).
1 x 5 mL	ANTISERUM	**Neopterin Antiserum** Ready to use. Contains: Antiserum (rabbit), phosphate buffer, stabilizers.
1 x 13 mL	ENZCONJ	**Enzyme Conjugate** Ready to use. Contains: Neopterin, conjugated to peroxidase, phosphate buffer, stabilizers. **Store protected from light.**
1 x 1.5 mL	CAL A-F	**Standard A-F** 0; 1.35; 4.0; 12.0; 37.0; 111 nmol/L Ready to use. Contains: Neopterin, phosphate buffer, stabilizers.
1 x 2 x 1.5 mL	CONTROL 1+2	**Control 1+2** Ready to use. Concentrations / acceptable ranges see QC Certificate.
1 x 21 mL	ASSAYBUF	**Assay Buffer** Ready to use. Contains: phosphate buffer, BSA, stabilizers.
1 x 50 mL	WASHBUF CONC	**Wash Buffer** Concentrate (20x) Contains: Tween, stabilizers.
1 x 19 mL	TMB SUBS	**TMB Substrate Solution** Contains: TMB, Buffer, stabilizers.
1 x 19 mL	TMB STOP	**TMB Stop Solution** Ready to use. 1 M H_2SO_4.
1 x	FOIL	**Adhesive Foil** 5 x black

8. MATERIALS REQUIRED BUT NOT SUPPLIED

1. Micropipettes (Multipette Eppendorf or similar devices, < 3 % CV). Volume: 10; 50; 100; 1000 µL
2. Vortex mixer
3. Orbital shaker (500 rpm)
4. 8-Channel Micropipettor with reagent reservoirs
5. Wash bottle, automated or semi-automated microtiter plate washing system
6. Microtiter plate reader capable of reading absorbance at 450 nm (reference wavelength 600-650 nm)
7. Bidistilled or deionised water
8. Paper towels, pipette tips and timer

9. PROCEDURE NOTES

1. Any improper handling of samples or modification of the test procedure may influence the results. The indicated pipetting volumes, incubation times, temperatures and pretreatment steps have to be performed strictly according to the instructions. Use calibrated pipettes and devices only.
2. Once the test has been started, all steps should be completed without interruption. Make sure that required reagents, materials and devices are prepared ready at the appropriate time. Allow all reagents and specimens to reach room temperature (18-25 °C) and gently swirl each vial of liquid reagent and sample before use. Mix reagents without foaming.
3. Avoid contamination of reagents, pipettes and wells/tubes. Use new disposable plastic pipette tips for each component and specimen. Do not interchange caps. Always cap not used vials. Do not reuse wells/tubes or reagents.
4. It is advised to determine samples in duplicate to be able to identify potential pipetting errors.
5. Use a pipetting scheme to verify an appropriate plate layout.
6. Incubation time affects results. All wells should be handled in the same order and time sequences. It is recommended to use an 8-channel Micropipettor for pipetting of solutions in all wells.
7. Microplate washing is important. Improperly washed wells will give erroneous results. It is recommended to use a multichannel pipette or an automatic microplate washing system. Do not allow the wells to dry between incubations. Do not scratch coated wells during rinsing and aspiration. Rinse and fill all reagents with care. While rinsing, check that all wells are filled precisely with Wash Buffer, and that there are no residues in the wells.
8. Humidity affects the coated wells/tubes. Do not open the pouch until it reaches room temperature. Unused wells/tubes should be returned immediately to the resealed pouch including the desiccant.

10. PRE-TEST SETUP INSTRUCTIONS

10.1. Preparation of concentrated components

⚠	The contents of the kit for 96 determinations can be divided into 3 separate runs. The volumes stated below are for one run with 4 strips (32 determinations).

Dilute / dissolve	Component	Diluent		Relation	Storage	Stability
15 mL	WASHBUF CONC	285 mL	bidist. water	1:20	2-8°C	1 month

10.2. Dilution of Samples

Sample	to be diluted	with	Relation	Remarks
Serum, Plasma	no			Avoid direct sun light.
Urine	generally	Assay Buffer	1:101	e.g. 10 µL + 1000 µL Avoid direct sun light.

Samples containing concentrations higher than the highest standard have to be diluted further.

⚠	Samples from patients treated with ATG (anti-T lymphocyte globulin from rabbit) after transplantation will cause erroneous high results. This effect can be avoided by a pre-incubation of the samples: Pipette 100 µL of serum into a Sarstedt or glass tube and add 200 µL of Assay Buffer. Close tubes (use pierced stopper for glass tubes) and incubate for 10 min in a waterbath at 95 - 100 °C. Vortex and withdraw 10 µL of the gel for the assay. Results have to be multiplied 3-fold.

11. TEST PROCEDURE

This enzyme immunoassay is evaluated for the manual use and especially for the automated use with the DadeBehring BEP2000 ELISA processor for the determination of neopterin in serum and plasma. Therefore the manual contains two different working procedures. The usage of this assay with other automated systems is possible. However in this case please contact us for further advices.

11.1. MANUAL PROCEDURE

1.	Pipette **20 µL** of each **Standard, Control, serum, plasma and diluted urine sample** into the respective wells of the Microtiter Plate.
2.	Pipette **100 µL Enzyme Conjugate** into each well.
3.	Pipette **50 µL** of **Neopterin Antiserum** into each well.
4.	Cover plate with black adhesive foil. **Incubate 90 min** at **RT (18-25 °C)** on an orbital shaker (500 rpm) in the dark.
5.	Remove adhesive foil. Discard incubation solution. Wash plate **4 x 300 µL** with diluted **Wash Buffer**. Remove excess solution by tapping the inverted plate on a paper towel.
6.	For adding of Substrate and Stop Solution use, if available, an 8-channel Micropipettor. Pipetting should be carried out in the same time intervals for Substrate and Stop Solution. Use positive displacement and avoid formation of air bubbles.
7.	Pipette **150 µL** of **TMB Substrate Solution** into each well.
8.	**Incubate 10 min** at **RT (18-25 °C)**.
9.	Stop the substrate reaction by adding **150 µL** of **TMB Stop Solution** into each well. Briefly mix contents by gently shaking the plate.
10.	**Measure** optical density with a photometer at **450 nm** (Reference-wavelength: 600-650 nm) within **15 min**.

11.2. AUTOMATED PROCEDURE

In the following chapter is described the procedure for a typical ELISA processor using BEP 2000 from Dade Behring as an example. we provides also protocols for other commercially available devices e.g. Triturus from Grifols, DSX from Dynex, DS2 from Dynex, Tecan Genesis RSP, BEP3 from Dade Behring, Gemini from Stratec etc. Please contact us if you want to automatize your ELISA. Our application specialists are glad to assist you.

Procedure for BEP2000 (Dade Behring) for serum and plasma

For valid runs on the Behring ELISA Processor BEP2000 only use the program file and reagent data base that is recommended. These files can be ordered easily.

1.	Aspirate **110 µL Enzyme Conjugate** in one reagent tip (300 µL), and than aspirate additionally to that volume **20 µL** of each **Standard, Control or Sample in the same tip**.
2.	Pipette **120 µL** of that mixture (**of Standard, Control or Sample with Enzyme Conjugate**) in the respective wells of the microtiter plate.
3.	Pipette **50 µL** of **Antiserum** into each well.
4.	**Incubate 90 ± 5min** at **RT (18-25 °C)** on an orbital shaker (frequency of 10 Hz; with an amplitude of 4 mm) in the dark.
5.	Aspirate supernatant. Wash plate **6 x** with **300 µL** diluted **Wash Buffer**.
6.	Pipette **150 µL** of **TMB Substrate Solution** into each well.
7.	**Incubate 10 ± 1 min** at **RT (18-25 °C)**.
8.	Pipette **150 µL** of **TMB Stop Solution** into each well.
9.	**Measure** optical density with a photometer at **450 nm** (Reference-wavelength: 600-650 nm) within **15 min**.

12. QUALITY CONTROL

The test results are only valid if the test has been performed following the instructions. Moreover the user must strictly adhere to the rules of GLP (Good Laboratory Practice) or other applicable standards/laws. All kit controls must be found within the acceptable ranges as stated on the labels and the QC certificate. If the criteria are not met, the run is not valid and should be repeated. Each laboratory should use known samples as further controls. It is recommended to participate at appropriate quality assessment trials.

In case of any deviation the following technical issues should be proven: Expiration dates of (prepared) reagents, storage conditions, pipettes, devices, incubation conditions and washing methods.

13. CALCULATION OF RESULTS

The obtained OD of the standards (y-axis, linear) are plotted against their concentration (x-axis, logarithmic) either on semi-logarithmic graph paper or using an automated method. A good fit is provided with cubic spline, 4 Parameter Logistics or Logit-Log.

For the calculation of the standard curve, apply each signal of the standards (one obvious outlier of duplicates might be omitted and the more plausible single value might be used).

The concentration of the samples can be read directly from the standard curve.

Due to the dilution of urine samples the urine values obtained have to be multiplied by the factor 101.

Samples showing concentrations above the highest standard have to be diluted as described in PRE-TEST SETUP INSTRUCTIONS and reassayed.

Conversion:
Based on the molecular weight of Neopterin (MW: 253.2 g/mol) and Creatinine (MW: 113.1 g/mol) a calculation in different units can be made as follows:

Serum/Plasma:

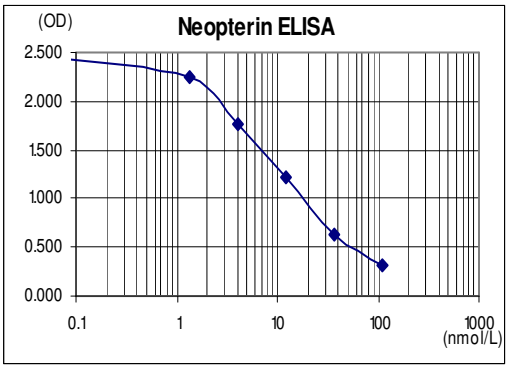
Neopterin	(nmol/L) x 0.253 = (ng/mL)
	(ng/mL) / 0.253 = (nmol/L)

Urine:
Usually neopterin in urine is correlated to creatinine (which hast o be analyzed by separate method) and expressed in neopterin to creatinine -ratio (UNCR) in µmol neopterin/mol creatinine:

Creatinine	(mg/dL) x 88.4 = (µmol/L)
	(µmol/L) / 1000 = (mmol/L)
	(mmol/L) / 1000 = (mol/L)
Neopterin	(nmol/L) / 1000 = (µmol/L)

Typical Calibration Curve
(Example. Do not use for calculation!)

Standard	Neopterin (nmol/L)	OD_{Mean}	OD/OD_{max}
A	0.00	2.449	100
B	1.35	2.238	91
C	4.00	1.772	72
D	12.0	1.209	49
E	37.0	0.634	26
F	111	0.325	13



14. INTERPRETATION OF RESULTS

Neopterin (Serum)	Interpretation
< 10 nmol/L	normal
> 10 nmol/L	elevated

The results themselves should not be the only reason for any therapeutical consequences. They have to be correlated to other clinical observations and diagnostic tests.

15. EXPECTED VALUES

Apparently healthy subjects show the following values:

Serum	
nmol/L	ng/mL
< 10	< 2.5

Serum				
Age	Sex	Neopterin nmol/L		(Neopterin Biochemistry – Methods - Clinical Application; H. Wachter et al. (1992), Walter de Gruyter, Berlin - New York)
		Mean	upper limit	
0-18	♂, ♀	6.78	13.5	
19-75	♂, ♀	5.34	8.7	
> 75	♂, ♀	9.67	19.0	

Urine				
Age	Sex	µmol Neopterin/mol Creatinine		(Neopterin Biochemistry – Methods - Clinical Application; H. Wachter et al. (1992), Walter de Gruyter, Berlin - New York)
		Mean	upper limit	
1-4	♂, ♀	267	432	
4-7	♂, ♀	226	405	
7-12	♂, ♀	181	374	
12-15	♂, ♀	171	343	
15-18	♂, ♀	144	320	
18-25	♂	123	195	
	♀	128	208	
26-35	♂	101	182	
	♀	124	209	
36-45	♂	109	176	
	♀	140	239	
46-55	♂	105	197	
	♀	147	229	
56-65	♂	119	218	
	♀	156	249	
>65	♂	133	229	
	♀	151	251	

It is recommended that each laboratory establishes its own range of normal values.

16. LIMITATIONS OF THE PROCEDURE

Specimen collection has a significant effect on the test results. See SPECIMEN COLLECTION AND STORAGE for details.

For cross-reactivities, see PERFORMANCE.

The following blood components do not have a significant effect (+/- 20 % of expected) on the test results up to the below stated concentrations:	Hemoglobin	5.0 mg/mL
	Bilirubin	2.5 mg/mL
	Triglyceride	45.5 mg/mL

Do not use samples containing sodium azide since these samples lead to erroneous high results.

Samples from patients who were treated with ATG (anti-T lymphocyte globulin from rabbit) after transplantation will cause erroneous high results. This effect can be avoided by a pre-incubation of the samples as described in PRE-TEST SETUP INSTRUCTIONS.

17. PERFORMANCE

Analytical Specificity (Cross-reactivity)	Substance		Cross Reactivity (%)	Cross-reactivity of other substances tested < 0.05 %	
	7,8-Dihydro-Neopterin		< 2.1		
	5,6,7,8-Tetrahydro-Neopterin		< 0.44		
	D-Monapterin		< 0.17		
	L-Monapterin		< 0.03		
	L-Biopterin		< 0.02		
	7,8-Dihydro-L-Biopterin		< 0.03		
Analytical Sensitivity (Limit of Detection)	0.7 nmol/L	Mean signal (Zero-Standard) - 2SD			
Precision		Range (nmol/L)	CV (%)		
Intra-Assay	Serum	3.1 - 43	4.3 – 11.7		
	Urine	932 - 5112	5.3 – 11.2		
Inter-Assay	Serum	4.67 – 29.98	8.8 – 13.8		
	Urine	2616 - 4419	9.3 – 14.4		
Linearity		Range (nmol/L)	Range (%)	Serial dilution up to	
	Serum	1.8 – 51.5	91 – 114	1:16	
	Urine	234 - 3622	87 – 120	1:8	
Recovery		Mean (%)	Range (%)	% Recovery after spiking	
	Serum	99	81 – 116		
	Urine	94	84 – 117		
Method Comparison versus HPLC	Serum	Assay = 1.18 x HPLC + 0.44		r = 0.92; n = 111	
	Urine	Assay = 1.17 x HPLC – 13.52		r = 0.99; n = 27	

18. PRODUCT LITERATURE REFERENCES

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Symbols / Symbole / Symbôles / Símbolos / Símbolos / Σύμβολα

REF	Cat.-No.: / Kat.-Nr.: / No.- Cat.: / Cat.-No.: / N.º Cat.: / N.–Cat.: / Αριθμός-Κατ.:
LOT	Lot-No.: / Chargen-Bez.: / No. Lot: / Lot-No.: / Lote N.º: / Lotto n.: / Αριθμός -Παραγωγή:
	Use by: / Verwendbar bis: / Utiliser à: / Usado por: / Usar até: / Da utilizzare entro: / Χρησιμοποιείται από:
	No. of Tests: / Kitgröße: / Nb. de Tests: / No. de Determ.: / N.º de Testes: / Quantità dei tests: / Αριθμός εξετάσεων:
CONC	Concentrate / Konzentrat / Concentré / Concentrar / Concentrado / Concentrato / Συμπύκνωμα
LYO	Lyophilized / Lyophilisat / Lyophilisé / Liofilizado / Liofilizado / Liofilizzato / Λυοφιλιασμένο
IVD	In Vitro Diagnostic Medical Device. / In-vitro-Diagnostikum. / Appareil Médical pour Diagnostics In Vitro. / Dispositivo Médico para Diagnóstico In Vitro. / Equipamento Médico de Diagnóstico In Vitro. / Dispositivo Medico Diagnostico In vitro. / Ιατρική συσκευή για In-Vitro Διάγνωση.
	Evaluation kit. / Nur für Leistungsbewertungszwecke. / Kit pour évaluation. / Juego de Reactivos para Evaluació. / Kit de avaliação. / Kit di evaluazione. / Κιτ Αξιολόγησης.
	Read instructions before use. / Arbeitsanleitung lesen. / Lire la fiche technique avant emploi. / Lea las instrucciones antes de usar. / Ler as instruções antes de usar. / Leggere le istruzioni prima dell'uso. / Διαβάστε τις οδηγίες πριν την χρήση.
	Keep away from heat or direct sun light. / Vor Hitze und direkter Sonneneinstrahlung schützen. / Garder à l'abri de la chaleur et de toute exposition lumineuse. / Manténgase alejado del calor o la luz solar directa. / Manter longe do calor ou luz solar directa. / Non esporre ai raggi solari. / Να φυλάσσεται μακριά από θερμότητα και άμεση επαφή με το φως του ηλίου.
	Store at: / Lagern bei: / Stocker à: / Almacene a: / Armazenar a: / Conservare a: / Αποθήκευση στους:
	Manufacturer: / Hersteller: / Fabricant: / Productor: / Fabricante: / Fabbricante: / Παραγωγός:
	Caution! / Vorsicht! / Attention! / ¡Precaución! / Cuidado! / Attenzione! / Προσοχή!

Symbols of the kit components see MATERIALS SUPPLIED.
Die Symbole der Komponenten sind im Kapitel KOMPONENTEN DES KITS beschrieben.
Voir MATERIEL FOURNI pour les symbôles des composants du kit.
Símbolos de los componentes del juego de reactivos, vea MATERIALES SUMINISTRADOS.
Para símbolos dos componentes do kit ver MATERIAIS FORNECIDOS.
Per i simboli dei componenti del kit si veda COMPONENTI DEL KIT.
Για τα σύμβολα των συστατικών του κιτ συμβουλευτείτε το ΠΑΡΕΧΟΜΕΝΑ ΥΛΙΚΑ.