DRG® Neopterin (EIA-2949)

Revised 20 Aug. 2013 rm (Vers. 14.1) **RUO in the USA**

This kit is intended for Research Use Only.

Not intended for use in diagnostic procedures.

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

INTENDED USE

Enzyme immunoassay for measurement of neopterin in human serum, plasma and urine.

SUMMARY

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.

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.

WARNINGS AND PRECAUTIONS

1. 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 DRG 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 HIV I/II, HBsAg and 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.

STORAGE AND STABILITY

The kit is shipped at ambient temperature and should be stored at 2 °C - 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 °C - 8 °C.

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 °C - 8 °C	≤ -20 °C (Aliquots)	Keep away from heat or direct sun light. Avoid repeated freeze-thaw cycles.
Stability:	72 h	6 months	

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 °C - 8 °C	≤ -20 °C (Aliquots)	Keep away from heat or direct sun light. Avoid repeated freeze-thaw cycles.
Stability:	72 h	6 months	

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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 8 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 6 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**, Ready to use 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**

MATERIALS REQUIRED BUT NOT SUPPLIED

1. Micropipettes (Multipette Eppendorf or similar devices, < 3% CV). Volumes: 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

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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 °C - 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.

PRE-TEST SETUP INSTRUCTIONS

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 °C - 8 °C	1 month

Dilution of Samples

Sample	to be diluted	with	Relation	Remarks
Serum	no			Avoid direct sun light.
Urine	generally	ASSAYBUF	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 of individuals 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:

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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 water bath at 95 °C - 100 °C. Vortex and withdraw 10 µL of the gel for the assay. Results have to be multiplied 3-fold.

TEST PROCEDURE

This enzyme immunoassay is evaluated for the manual use and especially for the automated use with the Siemens 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 DRG for further advices.

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** of **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 °C - 25 °C)** on an orbital shaker (500 rpm) in the dark.
5. Remove adhesive foil. Discard incubation solution. Wash plate **4 x** with **300 µL** 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 °C - 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.**

Automated Procedure

In the following chapter is described the procedure for a typical ELISA processor using BEP 2000 from Siemens as an example. DRG provides also protocols for other commercially available devices e.g. Triturus from Grifols, DSX from Dynex, DS2 from Dynex, Tecan Genesis RSP, BEP3 from Siemens, 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 (Siemens) for serum and plasma

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

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1. Aspirate **110 µL Enzyme Conjugate** in one reagent tip (300 µL), and then 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 ± 5 min** at **RT (18 °C - 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 °C - 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**.

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.

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)

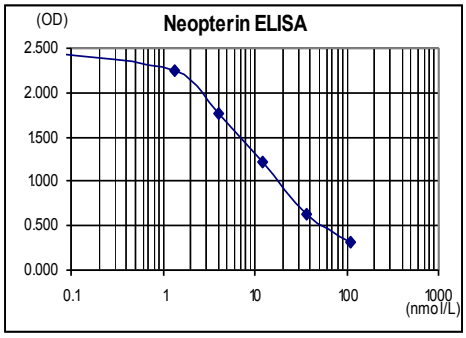
Urine:

Usually neopterin in urine is correlated to creatinine (which hast to 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

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.

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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 individuals 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.

REFERENCES/LITERATURE

1. Wachter H, Fuchs D, Hausen A, Reibnegger G, Weiss G, Werner ER, Werner-Felmayer G. Neopterin: Biochemistry - Methods - Clinical Application. Walter de Gruyter Berlin, New York, (1992)
2. Westermann J, Thiemann F, Gerstner L, Tatzber F, Kozák I, Bertsch T, Krüger C. Evaluation of a New Simple and Rapid Enzyme-Linked Immunosorbent Assay Kit for Neopterin Determination. Clin Chem Lab Med, 38 (4): 345-353 (2000)
3. X Garcia-Moll, D Cole, E Zouridakis, JC Kaski. Increased serum Neopterin: a marker of coronary artery disease activity in woman. Heart 83:346-350 (2000)
4. Smith D, Zouridakis, E, Mariani M, Fredericks S, Cole D, Kaski J. Neopterin levels in patients with coronary artery disease are independent of Chlamydia pneumoniae seropositivity. Am Heart J, 146 (1): 69-74 (2003)
5. B. Inci Fisenk, Durdal US, Osman I. Ozcebe & Gulsen Hascelik. The value of increased Neopterin levels in reducing transfusion-transmitted virus infections: Detection of a donation from a HbsAg positive chronic carrier by screening of neopterin in Turkish blood donors. Scandinavian Journal of Infectious disease, 37:599-604 (2005)
6. Michaela Bayer, Sven Schmitz, Jürgen Westermann, Frank Thiemann, Ralf Edelmann, Claudia Szakacs, Gerhardt Lanzer, Jens Blecken. Evaluation of a New-Linked Immunosorbent Assay for the Determination of Neopterin. Clin Lab. 51 (2005)
7. R. Weimer, C. Süsal, S. Yildiz, A. Staak, S. Pelzl, F. Renner, H. Dietrich, V. Daniel, S. Kamali-Ernst, W. Padberg, G. Opelz. Post-Transplant sCD30 and Neopterin as Predictors of Chronic Allograft Nephropathy: Impact of Different Immunosuppressive Regimes. Amercan Journal of Transplantation (2006)
8. Cangel P.Y. Chan, Junet W.Y. Choi , Kai-Yuan Cao, Ming Wang, Yang Gao, Duan-Hua Zhou, Biao Di, Hui-Fang Xu, Man-Fai Leung, Andreas Bergmann, Matthias Lehmann, Yong-Mei Nie, George W.H. Cautherley, Dietmar Fuchs, Reinhard Renneberg, Bo-Jian Zheng. Detection of serum neopterin for early assessment of dengue virus infection. Journal of Infection (2006)
9. Douglas T. Johnston, Marios Gagos, Nicolas Raio, Louis Ragolia, David Shenouda, Mark A. Davis-Lorton, Joshua R. De Leon. Alterations in serum neopterin correlate with thrombolysis in myocardial infarction risk scores in acute coronary syndromes. Coronary artery disease 2006, 17:511-516
10. SP Gieseg, EM Crone, EA Flavall, Z Amit. Potential to inhibit growth of atherosclerotic plaque development through modulation of macrophages neopterin/ 7,8-dihydroneopterin synthesis. British Journal of Pharmacology (2007)
11. Kausik K. Ray, David A. Morrow, Marc S. Sabatine, Amy Shui, Nader Rifai, Christopher P. Cannon, Eugene Braunwald. Circulation 2007; 115; 3071:3078