**ravo PNS-Blot**  
Recombinant Immunoblot  
for the Detection of the Paraneoplastic Autoantibodies  
anti-HuD, Anti-Yo, anti-Ri, anti-CV2 (anti-CRMP5),  
anti-Amphiphysin, anti-Ma1 and anti-Ma2

**8, 16 or 24 Determinations**

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**Version 07/2015**  
Please pay attention to the differences in comparison to the previous version 06/2013

**Complete revision:** details on specimen added (page 3), information on performance data and interfering substances added (page 7). Improvement of test procedure (page 5)  
**Page 5: Procedure:**  
Dilution of serum samples 1:200 in one step  
Sample incubation: only 30 minutes instead of 60 minutes  
Substrate incubation: reduced to 20 minutes
Summary:

Paraneoplastic neurological syndromes (PNS) are a group of neurological disorders associated with a tumor and its metastasis that are not the cause of the syndromes. An autoimmune process is considered as the underlying pathophysiological mechanism. Specific antineuronal autoantibodies can be detected in a majority of patients with PNS. Demonstration of the well characterized autoantibodies anti-HuD, anti-Yo, anti-Ri, anti-CV2/CRMP5, anti-Amphiphysin, anti-Ma1 and anti-Ma2 in the presence of paraneoplastic neurological symptoms provides strong diagnostic evidence of a – possibly occult – neoplasm. In two-thirds of the cases, a paraneoplastic neurological syndrome precedes the discovery of an underlying neoplasm by up to five years. Thus, demonstration of paraneoplastic antineuronal autoantibodies may lead to an early discovery of cancer (1-10, table).

<table>
<thead>
<tr>
<th>Table:</th>
<th>Paraneoplastic neurological syndromes</th>
<th>Most frequently associated tumors</th>
</tr>
</thead>
</table>
| Anti-Hu-Antibodies (ANNA-1) | • Sensory and autonomic neuropathy  
• Cerebellar ataxia  
• Encephalomyelitis  
• Limbic Encephalitis | Small-cell-lung cancer  
Non-small-cell lung cancer  
Extrapulmonary small cell cancer |
| Anti-Yo-Antibodies (Purkinje-cell-antigen) | • Cerebellar ataxia | Breast cancer  
Ovarian cancer  
Uterus cancer |
| Anti-Ri-Antibodies (ANNA-2, anti-Nova-1) | • Brainstem encephalitis  
(incl. Opsoclonus-Myoclonus-Syndrome)  
• Cerebellar ataxia | Breast cancer  
Small-cell-lung cancer  
Medullary carcinoma of the thyroid gland |
| Anti-CV2-(CRMP5-) Antibodies | • Sensory and sensorimotor neuropathy  
• Encephalomyelitis  
• Cerebellar ataxia  
• Limbic Encephalitis  
• Autonomic neuropathy  
• Chorea | Small-cell-lung cancer  
Thymom |
| Anti-Amphiphysin-Antibodies | • Stiff-person-syndrom  
• Various symptoms | Breast cancer  
Small-cell-lung cancer |
| Anti-Ma1 and Anti-Ma2- (Ta-) Antibodies | • Limbic Encephalitis  
• Brainstem encephalitis*  
• Cerebellar ataxia* | Testicular cancer  
Lung-cancers |

* Brainstem encephalitis and cerebellar ataxia usually associated with tumors different from testicular and immunoreactivity against Ma2 and Ma1 proteins.
Generally, these autoantibodies have been identified by immunohistochemical techniques. Due to problems with specificity a positive result in immunohistochemistry needs to be confirmed by an immunoblot, employing crude extracts from neuronal tissue as antigen. Immunohistochemistry is also laborious and requires a high degree of experience for reliable interpretation.

**Principle:**

Nitrocellulose strips which are coated with the recombinant antigens HuD, Yo, Ri, CV2 (CRMP5), Amphiphysin, Ma1 and Ma2 are incubated with a specimen of patient serum. Specific antibodies in the specimen will bind to the antigens. Non specific molecules in serum specimens will be removed by washing the strips. Bound antibodies are detected by alkaline phosphate conjugated anti-human IgG using BCIP/NBT as substrate.

**Storage:**

All kit components opened and unopened are stable until date of expiry stored at +2...8°C. Diluted wash buffer is stable for 4 weeks stored at +2 to +8°C.

**Specimen:**

Preferably freshly collected specimen (serum, plasma or cerebrospinal fluid) should be used. Specimen may be stored at 2 – 8°C for up to 5 days. For longer storage at 2 – 8°C antimicrobial agents (e.g. Thimerosal at a final dilution of 0.01%, ProClin300 at a final dilution of 0.03% or NaN₃ at a final dilution of 0.09%) should be added. For long term storage (several months) specimen should be stored in aliquots at – 20°C. Avoid repeated freeze thawing. Contaminated specimen may lead to false positive or negative results and should not be used.
## Reagents:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount/Volume</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrocellulose-Strips</td>
<td>8 (PNS001)</td>
<td>Nitrocellulose-Strips for the detection of IgG antibodies</td>
</tr>
<tr>
<td></td>
<td>16 (PNS002)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24 (PNS003)</td>
<td></td>
</tr>
<tr>
<td>Wash buffer</td>
<td>1 x 50 ml (PNS001)</td>
<td>Wash buffer, 20 x concentrated, blue screw cap</td>
</tr>
<tr>
<td></td>
<td>2 x 50 ml (PNS002)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 x 50 ml (PNS003)</td>
<td></td>
</tr>
<tr>
<td>Dilution buffer for samples</td>
<td>1 x 25 ml (PNS001)</td>
<td>ready to use, contains 0.03% ProClin300, green screw cap</td>
</tr>
<tr>
<td></td>
<td>2 x 25 ml (PNS002)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 x 25 ml (PNS003)</td>
<td></td>
</tr>
<tr>
<td>Positive control</td>
<td>1 x 2 ml (PNS001)</td>
<td>ready to use, contains ProClin300, purple screw cap</td>
</tr>
<tr>
<td></td>
<td>2 x 2 ml (PNS002)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 x 2 ml (PNS003)</td>
<td></td>
</tr>
<tr>
<td>Conjugate for samples and positive control</td>
<td>1 x 20 ml (PNS001)</td>
<td>ready to use, Alkaline Phosphatase Conjugate contains 0.03% ProClin300, red screw cap</td>
</tr>
<tr>
<td></td>
<td>2 x 20 ml (PNS002)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 x 20 ml (PNS003)</td>
<td></td>
</tr>
<tr>
<td>Substrate</td>
<td>1 x 20 ml (PNS001)</td>
<td>BCIP/NBT ready to use, black screw cap</td>
</tr>
<tr>
<td></td>
<td>2 x 20 ml (PNS002)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 x 20 ml (PNS003)</td>
<td></td>
</tr>
<tr>
<td>Incubation trays</td>
<td>1 x (PNS001)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 x (PNS002)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 x (PNS003)</td>
<td></td>
</tr>
<tr>
<td>Instruction for use</td>
<td>1 x</td>
<td></td>
</tr>
</tbody>
</table>

### Additional reagents available on request:

| Negative Control                    | 2 ml           | ready to use, contains 0.03% ProClin300                                   |
| Product No.:                         | PNSNKO         | Colourless screw cap                                                        |
Reconstitution:

- Make sure all kit components are at room temperature before use.
- Dilute the wash buffer concentrate 1:20 with distilled water. During storage at low temperatures, crystals may form in concentrated wash buffers, which can be dissolved by incubating the concentrate at 37°C for 30 minutes. Let the solution cool down again to room temperature before use. Diluted wash buffer is stable for 4 weeks stored at +4 to +8°C.
- The testing of cerebrospinal fluid (CSF) and the detection of intrathecal specific autoantibody synthesis is described on page 6

Procedure:

The nitrocellulose strips are labeled at the bottom (if not indicated otherwise). They must be incubated with the labels facing upwards and should be completely covered with fluid during all incubation steps.

<table>
<thead>
<tr>
<th>Add the ready to use positive control (2ml) to one strip</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cover the other strips with 2 ml dilution buffer. Add 10 µl of specimen and mix carefully (end-dilution: 1 : 200)</td>
</tr>
<tr>
<td>Incubate for 30 minutes at room temperature on a rocking table.</td>
</tr>
</tbody>
</table>

- wash with diluted wash buffer: Carefully remove the fluid using a pipet or pour away the fluid from each strip. Add ca. 2 ml of diluted wash buffer to each strip and shake for ca. 30 seconds. Repeat five times.
- Add 2 ml alkaline phosphatase IgG conjugate, ready to use, per strip.

| Incubate for 30 minutes at room temperature on a rocking table. |

- wash with diluted wash buffer: Carefully remove the fluid using a pipet or pour away the fluid from each strip. Add ca. 2 ml of diluted wash buffer to each strip and shake for ca. 30 seconds. Repeat five times.
- Incubate each strip in 2 ml ready to use substrate-solution.

| Incubate for 20 minutes at room temperature until the bands become clearly visible. |
| See control scan for comparison. |

- Transfer the strips to distilled water to stop the reaction. Put the strips onto filter paper and let them dry. Interpret the results. Store the strips in the dark.
Testing of cerebrospinal fluid (CSF), detection of intrathecal specific autoantibody synthesis:

- Determination of the total IgG concentration of a serum-CSF-sample pair
- Dilute both, serum and CSF, in ready to use sample buffer to a concentration of 1 mg/liter as working solution (see example)
- Perform test in parallel as described above. Use 2 ml each of the respective calculated dilution.

Example:

<table>
<thead>
<tr>
<th>1 mg/l</th>
<th>Determined Concentration of IgG</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF</td>
<td>97,3 mg/l</td>
<td>1: 97</td>
</tr>
<tr>
<td>Serum</td>
<td>10,8 g/l</td>
<td>1 : 10.000</td>
</tr>
</tbody>
</table>

Compare the intensity of the bands of the serum-CSF-pair.
A more intensive band for the CSF-sample hints at an intrathecal specific antibody synthesis. If the intensity of the serum-CSF-pair is almost equal, run of the test at an IgG- concentration of 0.1 mg/liter may be helpful for easier interpretation.

**Interpretation:**

Put the strips side by side to assign the proteins. Only strips from the same batch should be compared.
The positive control and control scan helps to assign the proteins, the documentation sheet to stick the strips on the latter for documentation.

Usually strong bands are observed in serum samples from patients with clinically defined paraneoplastic syndroms.
The meaning of weak reactions is still unknown with the exception of autoantibodies to HuD. For HuD- auto-antibodies weak reactions are described in 18% of patients suffering from small cell lung cancer without paraneoplastic neurological disease. Thus, in case of low concentrations of anti-Hu antibodies a thorough search for a possible underlying tumor is recommended. Concerning weak reactions with the other antigens there is nothing known at present about the incidence of underlying tumours so that no clear recommendation can be given.
At least a control of the antibody status is recommended in the course of the disease.

**Ma1/Ma2 Reactivity**

**Ma1 - / Ma2 +:**
If patient sera are reactive with the Ma2 antigen exclusively this hints at a testicular cancer as underlying malignancy.

**Ma1 + /Ma2 + :**
The presence of both antibodies specific for Ma1 and Ma2 antigens proofs a paraneoplastic etiology of a neurological disease but it does not hint at a special type of cancer.
Sensitivity: 98.9%

Specificity: 200 samples of blood donors have been tested to determine the specificity of the assay. 3/200 samples showed very faint bands resulting in a specificity of 98.5%.

Limitations of the method
Interpretation of laboratory test results must always be made within the context of the clinical symptoms.

Some serum samples show a more or less strong violet background due to serum components of unknown origin. Such samples should be retested using another method e.g. IFA.

Interfering substances: Although no interference was observed for hemoglobin up to a concentration of 2 mg/ml, bilirubin up to a concentration of 0.2 mg/ml and triglycerides up to a concentration of 32 mg/ml hemolytic, icteric and lipemic samples should not be used if possible.

Remarks:
- Avoid contact of the skin with substrate solution.

References:
Product-No:
PNS001 (8 Determinations)
PNS002 (16 Determinations)
PNS003 (24 Determinations)

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