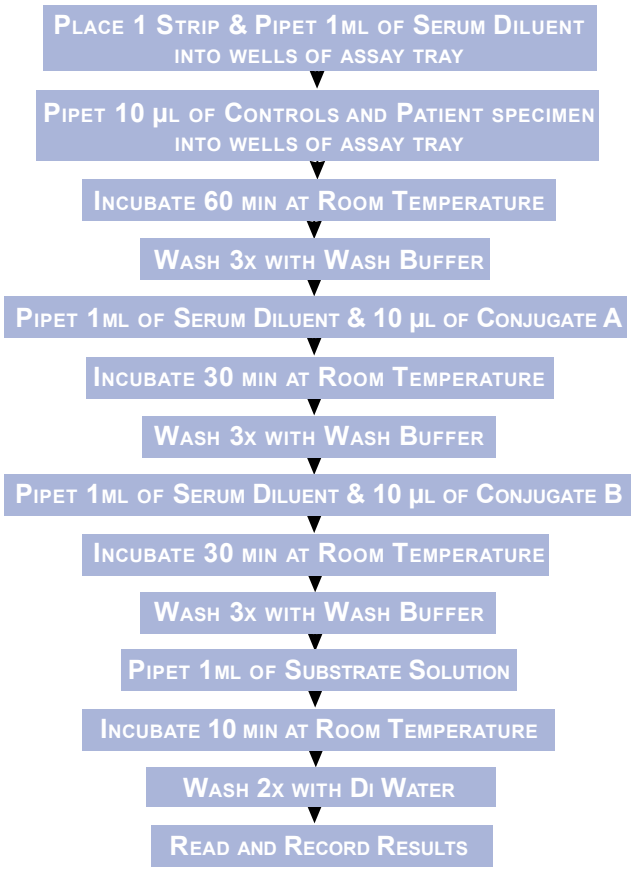
ImmuBlot™ Procedure at a Glance



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ImmuBlot™ Anti-Neuronal Antibody Western Blot Immunoassay

For Research Use Only

PRODUCT INSERT

Code: 1174 20 Determinations

INTENDED USE

A Western Blot Immunoassay for the detection of anti-neuronal autoantibodies against Hu, Yo, Ri and other neuronal antigens in human serum.

SUMMARY AND EXPLANATION

Autoimmune responses of the central nervous system (CNS), recognized as *paraneoplastic neurologic disorders* are manifestations of an immune response against tumors. These consist of a variety of neurological disorders like *paraneoplastic encephalomyelitis (PE)*, *sensory neuropathy (PSN)*, *cerebellar degeneration (PCD), paraneoplastic opsoclonus myoclonus ataxia (POMA) and stiff person syndrome*[1-3]. Clinical presentation includes sensory and memory loss, cerebellar, brainstem, motor or autonomic dysfunction (PE or PSN); involuntary saccadic eye movements, truncal and limbic myoclonus and ataxia (POMA). A reliable diagnosis of such conditions and the detection of the underlying tumor is difficult. In a significant number of cases in fact, the underlying tumor is not discovered until the patient presents with neurological symptoms[4,5]. *Paraneoplastic disorders* are characterized by the presence of neuronal autoantibodies in patient serum. The detection of these autoantibodies is useful for the clinician as it represents the presence of an underlying tumor. Tumors that have been known to initiate paraneoplastic disorders are *small-cell lung cancer, neuroblastoma, breast, ovarian and testicular cancers*. The following autoantibodies are found in *paraneoplastic syndromes*[6-8]:

a) anti-Hu, anti-neuronal nuclear antibody type I (ANNA-1) is associated with Small cell lung cancer resulting in PE
b) anti-Ri, anti-neuronal nuclear antibody type II (ANNA-2)is associated with Neuroblastoma (Children) and Fallopian or Breast cancer (Adults) resulting in POMA
c) anti-Yo , purkinje cell cytoplasmic antibody type I (PCA-1) is associated with certain gynecological cancers resulting in cerebellar degeneration.

Presence of one of these antibodies supports a clinical diagnosis of *paraneoplastic syndrome* and prompts a search for underlying neoplasm. These markers also help discriminate between true paraneoplastic disorders and other inflammatory disorders of the nervous system that mimic a paraneoplastic syndrome.

The Western Blot immunoassay provides a sensitive method for screening and confirmation of autoantibodies against various neuronal antigens present in the nucleus as well as the cytoplasm of granular and purkinje cells. Anti-Hu, anti-Yo and anti-Ri reactions can easily be observed at 35-40kD (Hu), 62kD (Yo) and 55kD (Ri). If the specimen yields no immunoreactivity on the blot strip, the result should be reported as negative.

PRINCIPLES OF PROCEDURE

To perform the test, strips are incubated with diluted patient serum. Antibodies specifically bind to the neuronal nuclear antigens on the strip. After washing the strips, and two incubation steps using Conjugate A and Conjugate B, strips are washed and incubated with enzyme substrate. Anti-Hu, anti-Yo and anti-Ri antibody positive reactions appear as blue-violet bands at 35-40 kD (Hu), 62kD (Yo), and 55 kD (Ri).

REAGENTS

Storage and Preparation

Store all reagents at 2-8°C. **Do not freeze.** Do not use, if liquid reagents are turbid or a precipitate is present. Prior to starting the assay, reagents must be equilibrated to room temperature (~22°). Antigen strips can only be used once. Do not interchange components of different lots. Do not use reagents beyond expiration date indicated on labels.

Precautions

All human derived components used have been tested for HBsAg, HCV, HIV-1 and 2 and HTLV-I and found negative by US FDA required tests. However, all human blood derivatives and patient specimens should be considered potentially infectious and good laboratory practices in storing, dispensing and disposing of these materials must be followed[9].

WARNING - Sodium azide (NaN_3) may react with lead and copper plumbing to form highly explosive metal azides. Upon disposal of liquids, flush with large volumes of water to prevent azide buildup. NaN_3 is toxic if ingested. Report incidents immediately to laboratory director or poison control center.

Follow good laboratory practices to minimize microbial and cross contamination of reagents.

Materials provided

ImmuBlot™ anti-neuronal antibody Western Blot *Product Code: 1174*

Kit contains sufficient reagents to perform 20 determinations.

1 x 20	**Western Blot Strips**
1 x 120 µl	***anti-Hu/Yo Positive Control** *(purple vial cap)*
1 x 120 µl	***Negative Control** *(yellow vial cap).*
1	**Control Card**
1 x 250 µl	***Conjugate A** (*blue vial cap)*
1 x 250 µl	***Conjugate B** *(white vial cap)*
1 x 60 ml	***Serum Diluent**
1 x 25 ml	**Enzyme Substrate** *(amber bottle)*
1 vial	***Powdered Wash Buffer**; reconstitute to one liter with deionized or distilled water.
3	**Assay trays**
2	**Report Forms**

**Contains <0.1% NaN_3*

LIMITATIONS OF THE PROCEDURE

The ImmuBlot™ Anti-Neuronal Antibody Western Blot should be used as an aid in diagnosis. Positive results may be found in other autoimmune conditions and/or certain infectious diseases. Results should be evaluated and interpreted by the clinician or neurologist in light of patient clinical history and other laboratory findings. Some sera may react to the MW marker, the significance of which is not known. It is recommended that positive reactions at 35-40 kD (anti-Hu), 62kD (anti-Yo), and 55 kD (anti-Ri) be supported by specific fluorescence pattern on the ImmuGlo™ Ant-Neuronal Antibody IFA (*Product Code: 1111).*

TROUBLESHOOTING GUIDE

- **Strong band/s on Negative Control strip.** Likely cause: contaminated Negative Control vial, or cross contamination from well containing a positive serum.
- **Positive Control appears like Negative Control strip.** Likely cause: Negative Control vial was confused as Positive Control vial.
- **Strips are completely blank.** Likely cause: addition of Conjugate (A or B) or Substrate was omitted.
- **High background and poor contrast between bands and background.** Likely cause: wash step(s) may have been omitted or incorrectly performed, or incubations were overextended.

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EXPECTED VALUES

There is a strong association among *paraneoplastic syndromes*, anti-neuronal antibody specificities and the associated tumor type. Paraneoplastic syndrome may occur months or years before a tumor is detected. The presence of neuronal autoantibodies helps in the detection of the underlying tumor. Antibodies against Hu are detected in about 20% of patients with lung cancer, while anti-Yo and anti-Ri ar present in about 2%-4% of cases. However, in a minority of patients with paraneoplastic syndrome with anti-neuronal antibodies, no tumor may be found. Certain patients with *paraneoplastic syndromes* may not have detectable antibody levels of anti-neuronal antibodies. 5-6% patients with Ovarian cancer have circulating anti-Ri antibodies in the absence of any paraneoplastic neurological syndrome[10]. The following table signifies the utility of antibody tests for anti-neuronal antibodies.

***Clinical Significance of Paraneoplastic anti-Neuronal Antibodies*[11-14]**

Antibody Specificity	Paraneoplastic Neurological Syndrome	Most frequently associated tumors	Western Immunoblot criteria for detection
Anti-Hu (ANNA-1)	Encephalomyelitis, sensory neuropathology autonomic neuropathy.	Small cell lung cancer, neuro-Blastoma; rarely non small cell lung cancer, prostate cancer, seminoma	35-40 kD reactive bands on extracts of isolated CNS neurons
Anti-Ri (ANNA-2)	Opsoclonus, ataxia, nystagmus dizziness dysarthria.	Breast cancer small cell lung Cancer	55 and 80 kD bands on neuronal proteins or with recombinant protein
Anti-Yo (PCA-1)	Subacute cerebellar syndrome, dysarthria and nystagmus	Ovarian cancer, breast cancer, other gyneco-logical cancer	62 kD band and 34 kD band on purified Purkinje cell extracts

***Frequency and Types of Cancer in Seropositive Patients with Paraneoplastic Antibodies*[17]**

	% Carcinomas Detected							
	Lung				Fallopian			Patients w/
Antibody	SCLC	NSCLC	Breast	Ovary	Tube/ Uterus	Thymoma	Other	Histologically Proven Cancer
Hu (ANNA-1)	66	4	0	0	0	2	8	80%
Yo (PCA-1)	0	0	13	63	13	0	1	91%
Ri (ANNA-2)	21	14	21	0	0	0	0	57%

SCLC = small cell lung carcinoma NSCLC = non-SCLC

Materials Required But Not Provided

- Clean 1000 ml graduated cylinder
- Non-serrated forceps (Filter forceps)
- Rocker or rotating platform shaker
- Absorbent paper or paper towels
- Deionized or distilled water
- Squeeze bottle to hold diluted wash buffer
- Pipettes capable of delivering 10 to 1000 µl
- Disposable pipet tips
- Timer

SPECIMEN COLLECTION AND HANDLING

Only serum specimens should be used in this procedure. Grossly hemolyzed, lipemic or microbially contaminated specimens may interfere with the performance of the test and should not be used. Store specimens at 2-8°C for no longer than one week. For longer storage, serum specimens should be frozen. Avoid repeated freezing and thawing of samples.

PROCEDURE

Procedural Notes

- Read Product Insert carefully before starting with the assay.
- Let serum specimens and test reagents equilibrate to room temperature for ~30 minutes prior to starting the test procedure. Return all unused specimens and reagents to the refrigerator promptly after use.
- Proper washing technique is critical to the satisfactory performance of the assay.
- Manipulate test strips with clean forceps only. Do not touch with bare hands.
- Strips are individually numbered at the bottom of each strip. Assign specimen identification numbers to the respective strips on the Report Form.
- Complete all other relevant information on the Report Form prior to starting the assay.

Test Method

Step 1 Using blunt forceps, place required number of **Strips** labeled side up into individual wells of the assay tray.

Step 2 Pipet **1.0 ml** of Serum Diluent into each well.

Step 3 Pipet **10 µl** of Positive and Negative Control and patient sample into appropriate wells to obtain a **1:101 dilution**. Incubate **60 minutes** (±5 min.) at room temperature on a rocker or rotating shaker.

Step 4 Aspirate sample solution into waste container. Thoroughly wash strips with Wash Buffer by squirting approximately 2ml of solution directly onto strips. Wash strips with gentle agitation for **5 minutes** and aspirate solution into waste container. **Repeat 2x**. *Caution: Complete washing of the strips between incubations is crucial to obtain valid results. Improper washing will result in high background staining.*

Step 5 Pipet **1.0 ml** of Serum Diluent followed by **10 µl** of Conjugate A into each well. Incubate **30 minutes** (±5 min) at room temperature on rocker or rotating shaker.

Step 6 Repeat **Step 4**.

Step 7 Pipet **1.0 ml** of Serum Diluent followed by **10 µl** of Conjugate B into each well. Incubate **30 minutes** (±5 min.) at room temperature on rocker or rotating shaker.

Step 8 Repeat **Step 4**.

Step 9 Pipet **1.0 ml** Substrate into each well and incubate with gentle shaking **10 minutes** (±5 min) at room temperature and reduced light.

Step 10 Stop development with deionized water (2 x 1minute).

Step 11 Using blunt forceps, remove strips from assay tray and place them gently onto absorbent paper. Handle strips only at the ends and let them dry **15-20 minutes**.

Quality Control

Though the control cards are lot specific, negative and positive controls must be included in each test run to ensure proper performance of the assay.

Positive Control Reaction: A dense and diffused blue-violet band should appear at 35-40 kD, representing the anti-Hu reaction. A dense, sharp blue-violet band should appear at 62kD representing the anti-Yo reaction. The positive control strip will usually develop the anti-Hu and anti-Yo bands along with the internal markers at 125 kD and a doublet at 70-77 kD. These are internal reference bands. In addition to these markers the positive control strip will also show a blue 116 kD marker (*refer to Figure 1*).

Negative Control Reaction: Typically, the negative control reaction will cause only the internal molecular weight markers at 125 kD and 70-77 kD to appear. Similarly, the negative control strip also shows a blue 116 kD marker (*refer to Figure 2*).

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RESULTS

Reading and Interpretation Guidelines

The ImmuBlot™ anti-Neuronal antibody strips contain 125, 70, and 77 kD molecular weight markers. The 116 kD alignment marker serves as a guide to align the strips on the control card (*refer to Figure 2*).

Step 1 Hold test specimen strip between the positive and negative control strips on the provided, laminated Control Card and align test strip using the 116 kD molecular weight marker as the reference point (*refer to Figure 2*).

Step 2 Align test strip in the center of the control card and compare reactions of test strip with those of the controls on either side.

Figure 1

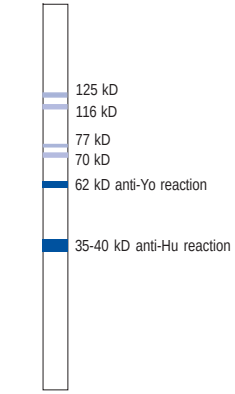
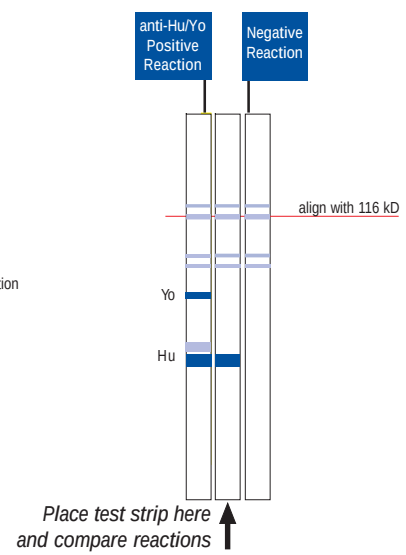


Figure 2

Immublot™ anti-Neuronal ab.
Western Blot



Step 3 If the band on the test strip is slightly below the anti-Yo control line and is approximately 55kD it should be considered an anti-Ri reaction. A band on the test strip that aligns with the anti-Yo control line should be considered an anti-Yo reaction. A band on the test strip that aligns with the lower-most (darkest) anti-Hu control line should be considered an anti-Hu reaction. Positive reactions can also occur in varying intensities, from weak to strong. Weak reactions should be compared with baseline reaction intensities at the corresponding position on the negative control strip. Figure 2 provides an example of a properly aligned positive result.

Step 4 If there are no reaction bands visible and/or bands not corresponding to either anti-Hu (35-40 kD), anti-Yo (62kD) or anti-Ri (55 kD) bands these should be considered anti-neuronal nuclear antigen negative specimens.