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Instruction For Use
2012-11

ORG 721 Liver-9-Line

NAME AND INTENDED USE

Liver-9-Line Immunoblot assay is a membrane based enzyme immunoassay for the semi-quantitative measurement of IgG class autoantibodies to AMA-M2, Sp100, gp210, SLA/LP, LKM-1, LC1, F-Actin, desmin and myosin in human serum or plasma. The assay is intended for professional in vitro diagnostic use only.

SYMBOLS USED

IVD	In vitro diagnostic medical device		
	Manufacturer		
REF	Catalogue number	BLOT STRIPS	Blot strips
Σ	Sufficient for	DILUENT	Sample Buffer
LOT	Batch code	CONJUGATE	Enzyme Conjugate
	Use by	WASH	Wash Buffer
2°C 8°C	Temperature limitation	BCIP	BCIP Substrate
	Consult instructions for use	RTU	Ready to use
	Keep away from sunlight		
	Do not reuse		
	Date of manufacture		

SUMMARY AND EXPLANATION OF THE TEST

Autoimmune hepatitis (AIH) is a chronic liver disease of unclear aetiology. AIH is a very rare disease (estimate: 50 to 200 infected per 1 million). Elevated transaminase levels, hypergammaglobulinemia, and elevated titres of antinuclear antibodies (ANA) and/or smooth muscle antibodies (SMA) are typical of this disease. Histologically, the disease is characterised by interface hepatitis and plasma cell infiltrates in the portal fields. This disease occurs in both sexes and in all age groups, although women are more often affected than men. Untreated, this disease progresses to cirrhosis and/or esophageal varices. At this point, mortality is high. AIH often begins stealthily. The patient first notices nausea, fatigue and joint and muscle pain. Physical symptoms of chronic liver disease, up to and including cirrhosis of the liver, may also be present.

In a substantial fraction of patients, this disease is often diagnosed in the process of investigating elevated liver values in a clinically asymptomatic stage of the disease. Because the prognosis can be significantly improved with the latest treatments, the timely diagnosis of autoimmune hepatitis is of great importance. Based on the differentiated detection of autoantibodies, type I AIH (AIH-1) and type 2 AIH (AIH-2) can be distinguished. Whereas AIH-1 affects all age groups, AIH-2 is typically found in children and young adults. AIH-2 represents only a small fraction of all AIH cases, but progression of this disease is very grave.

Liver-9-Line from ORGENTEC is an immunoblot for the serological detection of autoimmune hepatitis (AIH). The simultaneous detection of autoantibodies against nine different antigens also makes it possible to differentiate the AIH and to rule out other liver diseases.

AMA-M2

Anti-mitochondrial antibodies (AMA) are a heterogeneous group of autoantibodies directed against various proteins of the outer and inner mitochondrial membrane. AMA of the M2 subtype are directed against epitopes of the pyruvate dehydrogenase complex. The high sensitivity and specificity of M2 autoantibodies makes them excellent for the detection of primary biliary cirrhosis (PBC). AMA are detected in 90 to 95 % of PBC patients; the detection of a significantly raised AMA titre (>1:40 in indirect immunofluorescence) is a significant indicator of PBC. AMA are also detected in up to 25 % of AIH patients, though in these cases the titres are usually low5.

Sp100-Antibodies

There is mounting of evidence underlining, that Sp100-antibodies (displaying a clear nuclear dot staining pattern by IFA) are highly specific (specificity 97%) for primary biliary cirrhosis (PBC), and can be found in 31% of PBC patients. In addition, Sp100 can be found in almost every second (48%) AMA-negative patient with clinical and histological confirmed PBC. For this reason, Sp100 is accepted to represent an additional important marker beside AMA. Therefore, the simultaneous detection of both, AMA and Sp100, is highly specific for PBC. In contrast, in autoimmune hepatitis (AIH) type 1, 2, and 3 or primary sclerosing cholangitis (PSC), Sp100 antibodies are rarely existent. Finally, appearance of Sp100 in rheumatoid arthritis (3%), systemic lupus erythematosus (up to 10%), systemic scleroderma (5%), and finally in patient with Sjörgen Syndrom (2%) have been observed.

gp210-Antibodies

It is widely accepted that gp210 (displaying a nuclear membrane staining pattern in the IFA) are highly specific for PBC and are detectable in 21-41% of PBC patients. Furthermore, in 21-47% cases of AMA- negative and clinical confirmed PBC, gp210 is observed. Specificity ranged up to 99,5%, whereas in autoimmune hepatitis, rheumatoid arthritis, Polymyositis, or in Sjörgen Syndrom patients gp210 is rarely observed. At the end, gp210-antibodies are associated with extra hepatic manifestations, such as arthritis, and once determined, an unfavorable course of PBC can be expected.

SLA/LP-Antibodies

Antibodies against the soluble liver antigen (SLA/LP, soluble liver antigen/liver pancreas antigen) are highly specific markers for AIH-1. The target autoantigen is a cytosolic 50 kD protein, probably a transfer ribonucleoprotein complex.

LKM-1-Antibodies

Antibodies against type 1 liver-kidney mircosomes are serological markers for the diagnosis of AIH-2. The target antigen is cytochrome P450 2D6 (CYP2D6). LKM-1 antibodies are also detected in up to 7 % of patients with chronic hepatitis C3, where they include a further eptitope of CYP2D64.

LC-1-Antibodies

Antibodies against the liver cystolic antigen type 1 (LC-1) are specific markers found in up to 50 % of AIH-2 patients. Anti-LC-1 are detected in about half of all anti-LKM-1 positive patients. In contrast to anti-LKM-1, the LC-1 antibody titre correlates with the activity of the infection2. It has been demonstrated that anti-LC-1 is the only serological marker found in 10 % of AIH patients. The target antigen of antibodies against LC-1 is the enzyme formiminotransferase cyclodeaminase (FTCD).

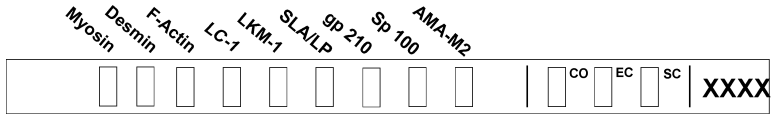
Anti-SMAs

Anti-SMAs are antibodies directed against the smooth muscle. They are typical markers for AIH-1. SMA antibodies are detected in 87 % of AIH patients, either as the only marker of the disease, or in combination with ANA1 (anti-nuclear antibodies). Anti-SMAs can be directed against microfilaments (F-actin or myosin) or against intermediary filaments (desmin) of the smooth muscle. In cases of type 1 AIH, antibodies against F-actin are predominantly detected.

PRINCIPLE OF THE TEST

Highly purified antigens AMA-M2, Sp100, gp210, SLA/LP, LKM-1, LC1, F-Actin, desmin and myosin as well as three control antigens for CO Cut-off Control, EC Enzyme Conjugate Control and SC Serum Control are bound to nitrocellulose membrane blot strips.

Autoantibodies present in serum or plasma bind to the immoblizied antigen. Washing of the blot strips removes unbound antibodies and unspecific sample components. Alkaline phosphatase conjugated anti-human IgG detect the bound sample antibodies forming a conjugate/antibody/antigen complex. Washing of the blot strips removes unbound conjugate. The substrate BCIP/NBT is hydrolized by bound enzyme conjugate to form an insoluble blue-violet product. Washing of the blot strips removes unhydrolyzed substrate and stopps the reaction. The amount of color is directly proportional to the concentration of IgG antibodies present in the original sample.



WARNINGS AND PRECAUTIONS

- All reagents of this kit are intended for professional in vitro diagnostic use only.
- Bovine serum albumin (BSA) used in components has been tested for BSE and found negative.
- Avoid contact with the substrate BCIP/NBT.
- Sample buffer and wash buffer contain sodium azid 0.09% as preservative. This concentration is classified non-hazardous.
- Enzyme conjugate contains 0.05% ProClin as preservative. This concentration is classified as non-hazardous.

During handling of all reagents, controls and serum samples observe the existing regulations for laboratory safety regulations and good laboratory practice:

- First aid measures: In case of skin contact, immediately wash thoroughly with water and soap. Remove contaminated clothing and shoes and wash before reuse. After contact with the eyes carefully rinse the opened eye with running water for at least 10 minutes. Get medical attention if necessary.
- Personal precautions, protective equipment and emergency procedures:

Observe laboratory safety regulations. Avoid contact with skin and eyes. Do not swallow. Do not pipette by mouth. Do not eat, drink, smoke or apply makeup in areas where specimens or kit reagents are handled. When spilled, absorb with an inert material and put the spilled material in an appropriate waste disposal.

- Exposure controls / personal protection: Wear protective gloves of nitril rubber or natural latex. Wear protective glasses. Used according to intended use no dangerous reactions known.
- Conditions to avoid: Since substrate solution is light-sensitive. Store subtrate solution in the dark.
- For disposal of laboratory waste the national or regional legislation has to be observed.

Observe the guidelines for performing quality control in medical laboratories by assaying control sera.

SPECIMEN COLLECTION, STORAGE AND HANDLING

- Collect whole blood specimens using acceptable medical techniques to avoid hemolysis.
- Allow blood to clot and separate the serum by centrifugation.
- Test serum should be clear and non-hemolysed. Contamination by hemolysis or lipemia is best avoided, but does not interfere with this assay.
- Specimens may be refrigerated at 2-8 °C for up to five days or stored at -20 °C up to six months.
- Avoid repetitive freezing and thawing of serum samples.
- Testing of heat-inactivated sera is not recommended.

CONTENTS OF THE KIT

▽16	ORG 721-16	Sufficient for 16 determinations
▽8	ORG 721-08	Sufficient for 8 determinations
BLOT STRIPS	1x/2x	8 antigen coated nitrocellulose strips. Ready to use. 1 pre-developed calibration strip (coded CAL) for semiquantitative evaluation. Ready to use. Product code on strip: ***721*** Code on Calibration strip: ***CAL***
DILUENT	1x 20 ml	Sample Buffer PB, containing PBS, BSA, detergent, preservative sodium azide 0.09%, yellow. Ready to use.
CONJUGATE	1x 20 ml	Enzyme Conjugate containing anti-human IgG antibodies, alkaline phosphatase labelled; PBS, BSA, detergent, preservative ProClin 0.05%, light red. Ready to use.
WASH	1x 20 ml	Wash Buffer WB, containing Tris, detergent, preservative sodium azide 0.09%; 50 x conc.
BCIP	1x/2x 10 ml	BCIP Substrate; containing BCIP/NBT. Ready to use.
		Incubation tray
	1x	Instruction for Use: ELISA Mini-CD0
	1x	Certificate of Analysis

MATERIALS REQUIRED

- Pipettes for 10 µl and 1000 µl
- Distilled or deionised water
- Graduated cylinder for 1000 ml
- Laboratory timing device
- Rocking platform
- Tweezers

STORAGE AND STABILITY

- Store the kit at 2-8 °C.
- Keep nitrocellulose strips carefully sealed in the original plastic tube with desiccants provided.
- Important: The calibration strip is very light-sensitive. Store in the dark!
- Do not expose test reagents to heat, sun or strong light during storage and usage.
- The unopened test kit is stable for 18 months from day of production. See expiry date on outer labels for individual batches.
- Diluted wash buffer is stable for at least 30 days when stored at 2-8°C. We recommend consumption on the same day.

PROCEDURAL NOTES

- Do not use kit components beyond their expiration dates.
- Do not interchange kit components from different lots.
- All materials must be at room temperature (20-28 °C).
- Have all reagents and samples ready before start of the assay. Once started, the test must be performed without interruption to get the most reliable and consistent results.
- Perform the assay steps only in the order indicated.
- Always use fresh sample dilutions
- To avoid carryover contamination, change the tip between samples.
- All incubation steps must be accurately timed.
- Control sera should routinely be assayed as unknowns to check performance of the reagents and the assay.
- Nitrocellulose strips must be handled with gloves or tweezers.
- It is important to make sure, that air-bubbles do not interfere with the strip during incubation. This could cause irregularities in coloration of developing bands and can lead to wrong results.

PREPARATION OF REAGENTS

WASH

Dilute the contents of one vial of the buffered wash solution concentrate (50x) with distilled or deionised water to a final volume of 1000 ml prior to use.

DILUENT

Ready to use.

Preparation of samples

Sample dilution see test procedure. Effective dilution during test is 1:101.

TEST PROCEDURE

Using tweezers insert one nitrocellulose strip into one chamber of the incubation tray:

- Add **1.0 ml sample buffer** to the strip in the chamber.
- Allow to equilibrate for 5 minutes with gentle bobbing.
- Add **10 µl of patient sample** directly to the chamber.
- Incubate for **60 minutes** at room temperature (20-28 °C) with gentle bobbing.
- Remove the diluted sample completely from the chamber.
- Add 2.0 ml wash buffer to the chamber, incubate for 5 minutes.
- Remove wash buffer completely. Repeat this procedure twice.

- Add **1.0 ml enzyme conjugate** to each strip in the chamber of the incubation tray.
- Incubate for **30 minutes** at room temperature with gentle bobbing.
- Remove the conjugate completely from the chamber.
- Add 2.0 ml wash buffer to the chamber, incubate for 5 minutes.
- Remove wash buffer completely. Repeat this procedure twice.

- Add **1.0 ml substrate** to each strip in the chamber of the incubation tray.
- Incubate for **10 minutes** at room temperature with gentle bobbing.
- Remove the substrate completly.
- Add 1.0 ml distilled water to the chamber, incubate for 5 minutes.
- Remove water completely. Repeat this procedure twice.

Carefully blot the strips with a tissue paper. Allow strips to air dry before evaluating with the calibration strip.

VALIDATION

The assay is valid if the all three control lines (**CO** Cut-off Control, **EC** Enzyme Conjugate Control and **SC** Serum Control) show a turn-over of substrate in terms of blue-violet lines! If this criteria is not met the assay is invalid and should be repeated.

Note: Borderline samples should be repeated or tested using an alternative procedure. Samples from patients diagnosed with autoimmune diseases often show multiple autoantibody specificities. Such samples may show a positive reaction with more than one antigen line.

CALCULATION OF RESULTS

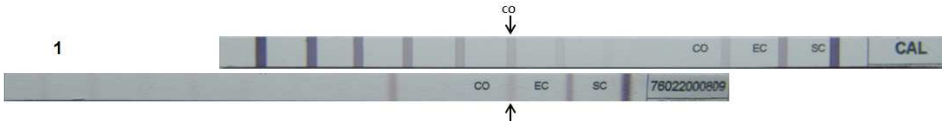
The intensity of a **blue-violet line** at the position of the coated antigen is directly proportional to the concentration of IgG antibodies present in the sample tested.

Semi-quantitative evaluation of sample strip:

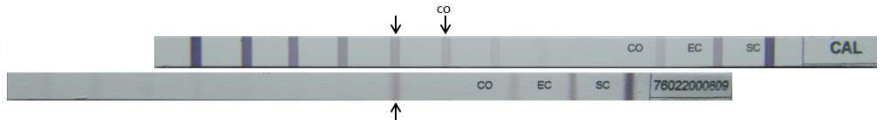
negativ	intensity of patient sample line weaker than intensity of CO-line
borderline	intensity of patient sample line eqivalent to intensity of CO-line
weak positive	intensity of patient sample line up to 1 level stronger than intensity of CO-line
positive	intensity of patient sample line up to 2 levels stronger than intensity of CO-line
strong positive	intensity of patient sample line ≥3 levels stronger than intensity of CO-line

Interpretation of the intensity of blue-violet lines:

(1) Compare intensity of the **CO-line of the sample strip** to the intensity of the lines of the calibration strip.
Example:



(2) Compare the intensity of the **patient sample line** to the intensity of the lines of the calibration strip.
Example: Interpretation of intensity of patient sample line is "weak positive".



PERFORMANCE CHARACTERISTICS

CALIBRATION

The sensitivity, specificity and dose response of the Liver-9-Line immunoblot was evaluated using clinically defined in house quality control sera containing varying relative amounts of sera with known specificity.

Measuring range

The evaluation of the intensity of the blue lines as described above allows a semi-quantitative determination of IgG class autoantibodies in the sample tested into quantification ranges:

negative, borderline, weak positive, positive, strong positive

Expected values

In a normal range study with samples from healthy blood donors the following ranges have been established with this assay. Cut-off: borderline

Interpretation of results

normal:	negative
elevated:	weak positive, positive, strong positive

Linearity

Patient samples containing high levels of specific antibody were serially diluted in sample buffer. Activity of each dilution step was determined using the calibration strip.

Linearity				
Sample	Dilution	Observed	Expected	O/E
1	1:100	strong positive	strong positive	PASS
.	1:200	positive	positive	PASS
.	1:400	weak positive	weak positive	PASS
.	1:800	borderline	borderline	PASS
.	1:1600	negative	negative	PASS
2	1:100	strong positive	strong positive	PASS
.	1:200	positive	positive	PASS
.	1:400	weak positive	weak positive	PASS
.	1:800	borderline	borderline	PASS
.	1:1600	negative	negative	PASS

Sensitivity

This immunoblot assay is a semi-quantitative assay method. Any reactivity less than borderline is considered

negative and cannot be quantified any further.

Reproducibility

Intra-assay precision: Coefficient of variation (CV) was calculated for each of three samples from the results of 24 determinations in a single run. Results for precision-within-assay are shown in the table below.
Inter-assay precision: Coefficient of variation (CV) was calculated for each of three samples from the results of 6 determinations in 5 different runs. Results for run-to-run precision are shown in the table below.

Intra-Assay		
Sample	Mean	Result
1	negative	PASS
2	weak	PASS
3	positive	PASS

Inter-Assay		
Sample	Mean	Result
1	negative	PASS
2	weak	PASS
3	positive	PASS

Interfering substances

No interference has been observed with haemolytic (up to 1000 mg/dl) or lipemic (up to 3 g/dl triglycerides) sera or plasma, or bilirubin (up to 40 mg/dl) containing sera or plasma. Nor have any interfering effects been observed with the use of anticoagulants (Citrate, EDTA, Heparine). However for practical reasons it is recommended that grossly hemolyzed or lipemic samples should be avoided.

Study results

Study population	n	n pos	%
Autoimmune hepatitis (AIH)	94	92	97.9
Primary biliary cirrhosis (PBC)	41	39	95.1
PBC overlap syndrome	27	12	44.4
Normal human sera	80	4	5.0

			Clinical Diagnosis Pos	Clinical Diagnosis Neg	
ORG 721	Pos		143	4	
Liver-9-Line	Neg		19	76	
.	.		162	80	242
Sensitivity:	88.3	%			
Specificity:	95.0	%			
Overall agreement:	90.5	%			

LIMITATIONS OF THE PROCEDURE

This assay is a diagnostic aid. A definite clinical diagnosis should not be based on the results of a single test, but should be made by the physician after all clinical and laboratory findings have been evaluated concerning the entire clinical picture of the patient. Also every decision for therapy should be taken individually.

REFERENCES

1. Czaja AJ. Behavior and significance of autoantibodies in type 1 autoimmune hepatitis. J Hepatol 1999; 30: 394 -401.
2. Muratore L, Ctaleta M, Muratore P, Lenzi M, Bianchi FB. Liver/kidney microsomal antibody type 1 and liver cytosol antibody type 1 concentrations in type 2 autoimmune hepatitis. Gut 1998; 42: 721-726.
3. Czaja AJ, Homburger HA : Autoantibodies in liver disease. Gastroenterology 2001, 120 : 239-249.
4. Kerkar N et al. Cytochrome P4502D6 (193-212): A new immunodominant epitope and target of virus/self cross-reactivity in liver kidney microsomal autoantibody type I-positive liver disease. J Immunol 2003, 170: 1481-1489.
5. Kenny RP, Czaja AJ, Ludwig J, Dickson ER: Frequency and significance of antimitochondrial antibodies in severe chronic active hepatitis. Dig Dis Sci, 1986, 31: 705-11.

1. Add **blot strip** into the incubation tray
   → Add **1000 µl** sample buffer per strip into the incubation tray
   → Shake **5 minutes** while incubating

2. Add **10 µl** patient sample and resuspend
   → Shake **60 minutes** while incubating
   → Discard content and wash 3 times for **5 minutes** with **2000 µl** wash buffer, discard wash

3. Add **1000 µl** enzyme conjugate solution per strip
   → Shake **30 minutes** while incubating
   → Discard content and wash 3 times for **5 minutes** with **2000 µl** wash buffer, discard wash

4. Add **1000 µl** substrate per strip
   → Shake **10 minutes** while incubating
   → Discard content and wash 3 times for **5 minutes** with **1000 µl distilled water**, dry blot strips. Read after complete drying, only