



Liver Immunoblot

Westernblot for the detection of anti-mitochondrial (AMA),
anti-LP and anti-LKM antibodies

Code:

IB 150 **8 Tests**

IB 150 C **8 Tests** (only for AID-SCAN)

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CONTENTS

Theory	Page
Precautions	3
Materials provided	3
Technical data	3
Principle of the test	3
Summary and explanation of the test	4
References	4
Instructions for use	
Materials required but not provided	4
Specimen collection and preparation	4
Instructions for reagent preparation and storage	4
Technical notes and QC-criteria	5
Assay procedure	5
Analysis of band pattern and interpretation of the results	6
Limitations of use	7
Summary of assay procedure	7

PRECAUTIONS

The reagents supplied in this pack are for in vitro diagnostic use only. Do not change anything of the recommended protocol!

Store all kit components at 2 – 8 °C in a refrigerator! After first opening the vial with the strips, store remaining strips at room temperature protected from light.

Do not touch the nitrocellulose-strips with fingers. Always use clean tweezers.

The components supplied in this kit are intended for use as an integral unit. Therefore, the components of different lots should not be mixed.

Do not use kits or components after the expiry date printed on the outer carton.

Do not cross-contaminate reagents. Always use fresh pipette tips when drawing from stock reagent bottles. Always use clean, preferably disposable, glassware for all reagent preparations.

The chromogen is potentially cancerogenic and can irritate skin and mucous membranes. Any substrate coming into contact with skin should be rinsed off with running tap water immediately.

MATERIALS PROVIDED

Test unit	8 Tests
Nitrocellulose-strips loaded with mitochondrial, liver-pancreas (LP/SLA) and LKM antigens	8 strips
Blocking buffer (cloudy) (mix well prior to use!) <i>ready for use</i>	1 vial 20 ml
Goat-anti-human-IgG-Conjugate (conjugated with alkaline phosphatase), <i>concentrated</i>	1 vial 0,1 ml
Substrate (BCIP), <i>ready for use</i>	1 vial 10 ml
Stop solution (3 % acetic acid), <i>ready for use</i>	1 vial 20 ml
Serum dilution- and washingbuffer (SDW-buffer), <i>10x concentrated</i>	1 vial 20 ml

TECHNICAL DATA

Specimen:	Serum
Required sample size:	20 µl per test
Incubation time:	approx. 2,5 hours at room temperature
Storage:	at 2 to 8 °C (refrigerator)
Testunit	8 strips

PRINCIPLE OF THE TEST

Mitochondrial, microsomal and cytosolic antigens are separated by SDS-PAGE electro-phoresis. According to their molecular weight, the antigens migrate through the gel as fine bands. Finally the antigens are transferred to nitrocellulose membranes.

The required number of strips is prepared in the respective channel of the incubation tray. To rehydrate and to block free binding sites for non-specific binding, the strips are incubated with the proteins of the blocking buffer. After discarding the blocking buffer, the membrane strips are incubated with prediluted serum samples. According to their specificity, autoantibodies present in the sample will bind to the antigens during the 40 minutes incubation. Unbound serum components are eliminated by a washing step.

During the next incubation step all specifically bound autoantibodies are traced by alkaline phosphatase conjugated anti-human-IgG antibodies. After a second wash to remove excess enzyme label, specifically bound autoantibodies are detected by the use of the chromogenic reaction with BCIP.

Labelled antigens and their respective autoantibodies occur as blue stained bands on the strip. Using the evaluation template the specificity of the autoantibodies is identified.

SUMMARY AND EXPLANATION OF THE TEST

Autoimmune diseases are serologically characterized by the detection of several autoantibodies and typical antibody profiles. The majority of these autoantibodies is directed against antigens localized in the nucleus, the nuclear plasma, the nuclear matrix and the nucleolus, but also against mitochondrial and microsomal antigens in the cytoplasm.

In patients with autoimmune liver diseases mainly anti-cytoplasmatic antibodies are found, whereas in cases of lupoid hepatitis also anti-nuclear antibodies may occur.

M2-type anti-mitochondrial antibodies (AMA) react with proteins of the α ketoacid dehydrogenase complex of the mitochondria and are characteristic markers for primary biliary cirrhosis (PBC), a chronic cholestatic liver disease.

The so-called 'liver-kidney-microsomal' antibodies (anti-LKM) are directed against proteins of the cytochrom-P-450 system and are characteristic disease-specific markers for the subgroup of autoimmune chronic active hepatitis (aCAH).

Disease-specific anti-LP (liver-pancreas / anti-SLA) antibodies are associated with chronic active hepatitis and are found in approx. 25 percent of CAH-patients. These antibodies are directed against a cytoplasmatic antigen with a molecular weight of 51 kD. It is not identical with a cytokeratin.

Recently, AMA have mainly been detected by immunofluorescence tests (IFT) using cells or cryostatic tissue sections. Distinct immunofluorescence patterns allow for the discrimination of autoantibody specificities. Furthermore, most of today's known ANA specificities to soluble cellular antigens have been defined by the use of radial immunodiffusion (ID) in the Ouchterlony tests. The characterization of the specificity of mitochondrial antibodies has been carried out by the aid of complement binding reaction (KBR) and immunoblotting.

Using this immunoblot technique (with electrophoretically separated antigens), the detection and characterization of CAH- and PBC-specific autoantibodies is possible within one and the same test.

REFERENCES

1. Manns, M.
Autoantibodies and antigens in liver diseases -updated
Journal of Hepatology 1989; 9: 272-280
2. Stechemeser, E., Strienz, J., Berg, P.A.
Serological definition of a new subgroup of patients with autoimmune chronic active hepatitis.
Lancet 1987; Vol. I 683
3. Tan, E.M.
Antinuclear Antibodies: Diagnostic Markers for Autoimmune Diseases and Probes for Cell Biology.
Adv. Immunol. 1989; 44: 93 - 151.

INSTRUCTIONS FOR USE

MATERIALS REQUIRED BUT NOT PROVIDED

- Disposable tip micropipettes to dispense volumes of 10, 20 und 1000 μ l
- Range of standard, clean volumetric laboratory glassware
- Freshly distilled water
- Whirlmix
- Clean disposable glass or plastic tubes for serum dilution
- Absorbent paper towels
- Shaker
- Tweezers

SPECIMEN COLLECTION AND PREPARATION

Autoantibody detection with the Liver-Autoantibody - Immunoblot is carried out in serum.

All serum samples are prediluted 1:50 with Serum-Dilution- and Washingbuffer (SDW-buffer). Therefore 20 μ l of serum sample may be diluted with 1.000 μ l SDW-buffer.

Serum samples may be stored refrigerated at 2 – 8 °C for up to 5 days. For longer storage the samples should be stored frozen at – 20 °C. To avoid repeated thawing and freezing the samples should be aliquoted.

INSTRUCTIONS FOR REAGENT PREPARATION AND STORAGE

All unopened components can be used up until the date printed on the outer box, provided they are stored at 2 - 8 °C. The strips should be stored at room temperature after first opening.

Reconstitution of the serum-dilution-washingbuffer (SDW)

Add 20 ml concentrated washingbuffer to 180 ml distilled water (= 200 ml). Mix well. Working strength solution is stable up to two weeks at 2 – 8 °C.

Conjugate

While the first incubation is in progress dilute the conjugate:

number of strips needed	serum-dilution-wash-buffer (ml)	concentrated conjugate (µl)
1	1,0	10
2	2,0	20
3	3,0	30
4	4,0	40
5	5,0	50
etc.	etc.	etc.

It is not necessary to bring the conjugate to room temperature prior to dilution. It is recommended to take the conjugate out of the refrigerator, draw the needed amount from the vial and put the remaining conjugate back into the refrigerator immediately. Working strength solution is stable for one hour at room temperature (22 °C).

TECHNICAL NOTES AND QC-CRITERIA

Do not change anything of the recommended protocol! Temperatures below 18 °C and above 28 °C may affect the development of the bands. In the case of a too high temperature, the substrate incubation should be watched with care and stopped sooner than in the recommended protocol to avoid an over dyeing of the bands. The opposite applies in the case of too low temperatures. The recommended time for substrate incubation is a guideline only.

Store all kit components at 2 – 8 °C in a refrigerator!

The components supplied in this kit are intended for use as an integral unit. Therefore, the components of different lots should not be mixed. **This is especially true for the reference (template) delivered in the kit. This reference (template) can be used for the strips of the kit only.**

Do not use kits or components after the expiry date printed on the outer box.

Efficient rinsing of uncomplexed serum components is a fundamental requirement of the immunoblot procedures. Therefore, the washing procedure should be performed particularly carefully. Insufficient washing will cause aberrant results and high background staining.

QC-criteria

These immunoblots are manufactured in lots of 10 (20) strips. Two of these strips are developed with control sera. One of the developed strips is delivered in each kit. The other strip is documented at the manufacturer's. Using these two marker strips, the reference (template) is made. The reference (template) indicates the exact positions of the antigens on the undeveloped strips. Therefore, the running of controls is not necessary to analyse the band pattern.

To control the proper performance of each strip, a control band is dotted 1 cm below the upper end. This band **must** develop in any case. If this band does not develop, or only very faintly, one of the reagents was not added or is deteriorated.

ASSAY PROCEDURE

1. Take the required number of strips out of the tube with tweezers and put them the right side up in their respective channels of the incubation tray. The upper side of the strips (with the strip number and the front marker line) must never turn down during all incubations. If that happens while adding reagents or buffers, turn the strip back the right way up immediately with tweezers.
2. Add 2 ml blocking buffer (mix well prior to use) to each strip. Take care that all strips are completely covered with fluid. That is important for all other steps of the procedure, too.
3. Incubate for 15 min. on a shaker. Take care that the fluid is mixed well by the shaking but cannot contaminate the adjacent channel. That is especially important for the following serum incubation!
4. Dilute serum specimens 1 : 51 with SDW buffer (add 20 µl specimen to 1.000 µl SDW buffer and mix well).
5. Discard the blocking buffer by decanting from the incubation tray. The strips are completely rehydrated now and will stay adhesive to the bottom of the channels during decanting. Add 1 ml diluted serum specimen to their respective channels and incubate for 40 minutes on the shaker.
6. Rinse all strips 4 times. Sufficient washing is very important:
 1. Inclining the tray carefully, aspirate the channel contents using a vacuum line fitted with a trap. Avoid cross-contamination!

2. Add 1 ml SDW buffer, shake gently for some seconds and aspirate.
 3. Add 2 ml SDW buffer, shake for 3 minutes and aspirate. Repeat this step two times. For the last two steps, aspirating can be replaced by decanting.
 4. Invert the tray and tap firmly on absorbent paper towels.
 5. Check that there is no residual wash-buffer in the channels.
7. Add 1 ml freshly prepared conjugate to each strip.
 8. Incubate for 30 minutes on the shaker.
 9. Rinse all strips 4 times as described in step 6. Aspirating can be replaced by decanting.
 10. Rinse all strips with distilled water (don't incubate). This extra washing improves the development of the bands and reduces background staining.
 11. Add 1 ml substrate to each strip.
 12. Incubate for 10 – 15 minutes on the shaker.
 13. Add 1 ml stop solution to each channel, shake gently for some seconds and decant. Add 1 ml stop solution again, shake for 3 minutes and decant.
 14. Remove the strips with tweezers from the tray and dry them on absorbent paper. Analyse the band pattern according to the procedure on the next page. For documentation store the strips protected from light.

ANALYSIS OF BAND PATTERN AND INTERPRETATION OF THE RESULTS

After the complete drying of the strips (please note, minimum drying 1 hour) put them on white paper with the front line and the number at the bottom. Put the template above the strip so placed that the white rim with lot number etc. is at the top. Fit the strip into the long blank space marked on the template. The bottom end line of the blank space must be fitted exactly upon the front line of the strip. At the rim of the blank space the expected band pattern and the position of the important antigens are marked. Inspect the ready developed strip to see whether there are bands **at least overlapping** with the markers on the template. There may be bands on the strip not fitting to marked antigen positions on the template. Because nothing is known about their diagnostic relevance they must be ignored.

Specificity of the antigens

AMA (M2-Type)

Mitochondrial antibodies are directed against proteins of the inner and outer membrane of the mitochondria. These autoantibodies are a highly specific and highly sensitive marker for primary biliary cirrhosis (PBC).

M2 is the specific marker for PBC. The antigen preparation used in this immunoblot is derived from heart and contains all typical PBC-specific marker antigens.

⇒ In M2-positive samples the **major band** against the 74 kD Antigen **must be present!!**

When this major band at 74 kD of molecular weight is demonstrated, all further bands found in the region of 50 kD to 54 kD are to be interpreted as M2 subtypes.

In general, the occurrence of a single band at 74 kD supports the suspicion of autoimmune primary biliary cirrhosis. This finding has to be verified and interpreted in combination with the results of other tests, such as immunofluorescence.

Other additional bands at 54 kD and 52 kD are rather close to the LP- and LKM antigens. So, if the exact definition is difficult, it could be helpful to know that, according to existing reports, anti-LP and anti LKM antibodies could not in the past be demonstrated parallel to anti M2 (74 kD) in the same patient.

LKM

The antibodies recognize the so-called liver-kidney-microsomal antigen, which is the microsomal antigen of liver and kidney. They are a marker for autoimmune chronic active hepatitis.

Anti-LKM autoantibodies are demonstrated as a characteristic band against an antigen with 50 kD of molecular weight. These antibodies are specific for a subgroup of the autoimmune hepatitis and are mainly detected in younger patients, aging from childhood up to less than 30 years of age.

LP

Anti-LP autoantibodies recognize a 51 kD cytoplasmatic antigen demonstrated as a single band in the Western Blot. They are directed against the Liver-Pancreas antigen (LP / SLA) and are found in patients with autoimmune chronic active hepatitis. Their incidence is approximately 25 percent.

LIMITATIONS OF USE

As for many other serological tests the results of this immunoblots should only be interpreted in context with all available clinical and laboratory dates.

SUMMARY OF THE PROCEDURE

Liver-Autoantibody-Immunoblot

