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1 INTENDED USE

HepAK Dot is used for the qualitative determination of IgG autoantibodies to M2, LKM1, LC1 and SLA in human serum or plasma in for the differential determination of autoimmune liver diseases.

For in vitro use only.

The group of primary autoimmune liver disease (PAL) comprises autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC).

The clinical picture of PAL is in most cases not different from other chronic liver diseases. About 15% of all cases with chronic liver diseases show an autoimmune pathogenesis (1). Therefore, after exclusion of infectious etiology especially by viruses, the determination of different autoantibodies is recommended.

Patients with PSC show intestine related symptoms and with regard to serological diagnosis atypical ANCA patterns in indirect immune-fluorescence on neutrophile. PBC is characterized by occurrence of antibodies to mitochondrial antigens. Antibody M2 seems to be the most specific out of 9 described so far for PBC. M2 antibodies react with epitopes of the E2 component of the pyruvate-dehydrogenase complex found in mitochondria. M2 has been found with 95% of all PBC patients.

Patients suffering from AIH show a variety of autoantibodies. Due to the appearance of different antibody specificities classification of AIH into different subgroups is discussed. Type I is characterized by the occurrence of antinuclear antibodies (ANA) and antibodies to smooth muscles (ASMA). For type II a high prevalence of antibodies to liver and kidney microsomal antigens (LKM) has been described. LKM1 antibodies recognize epitopes of cytochrome P450 2D6. LC1 antibodies are specific for type II hepatitis, too. The respective antigen is formiminotransferase / cyclodeaminase located in the cytosol of liver cells. Patients with type III autoimmune hepatitis exhibit antibodies to the soluble liver antigen (SLA). This type III is not yet fully accepted as independent subgroup for autoimmune hepatitis by the "International Hepatitis Group" (2).

1. Czaja AL. Natural history, clinical features and treatment of autoimmune hepatitis. *Semin Liver Dis* (1987) 4: 1 - 12
2. Johnson PJ, McFarlane IA. Meeting Report: International Autoimmune Hepatitis Group. *Hepatology* (1993) 18: 998 - 1005

2 PRINCIPLE OF THE TEST

HepAK Dot is a sensitive immunodot assay for the determination of IgG antibodies to M2, LKM1, LC1 and SLA in human serum or plasma, respectively.

HepAK Dot includes 24 numbered test strips with 6 dots fixed on a plastic support: Dots are coated with purified M2, LKM1, LC1 and SLA. Two test dots serve as positive and cut-off controls.

Patient sera and strips are incubated in the test tray. During the first incubation antibodies of the patient sample bind to the target antigens immobilized on the solid-phase of the strips. Following an incubation period of 30 min unbound serum components are removed by a washing step.

Bound antibodies react specifically with anti-human-IgG conjugated to alkaline phosphatase. Following an incubation period of 30 min excessive conjugate is separated from the solid-phase immune complexes by an additional washing step.



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Alkaline phosphatase converts the colorless substrate solution into a dark purple precipitating dot. After 10-12 minutes while shaking the reaction is stopped by a washing step.

Strips are dried for at least 30 min by pressing the reactive side onto absorbent paper. Results are regarded to be positive if the coloration of the test dot is more intense than the coloration of the cut-off control.

3 PATIENT SAMPLES

3.1 Specimen collection and storage

Blood is taken by venipuncture. Serum is separated after clotting by centrifugation. Plasma can also be used.

The samples may be kept at 2-8°C for up to three days. Long-term storage requires -20°C.

Repeated freezing and thawing should be avoided. If samples are to be used for several assays, initially aliquot samples and keep at -20°C.

3.2 Preparation before use

Allow samples to reach room temperature prior to use in the assay. Take care to agitate serum samples gently in order to ensure homogeneity.

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4 TEST COMPONENTS

For 24 x 4 determinations

A Ag	Dot strips 24 numbered strips coated with 4 specific antigens (M2 (porcine), LKM1 (human recombinant), LC1 (human recombinant), SLA (murine) - Positive dot-control - Cut-off dot-control	24 dot strips for the determination of four antibody specificities each
B BUF WASH	Wash buffer sufficient for 500 ml solution 10x	50 ml concentrate capped blue
C DIL	Sample diluent (colored yellow)	40 ml capped yellow
D CONJ	Conjugate anti-human-IgG (goat) coupled with alkaline phosphatase (colored red)	40 ml ready for use capped red
E SOLN NBT/BCIP	Substrate nitroblue tetrazolium with bromo-chloro-indolyl-phosphate (black bottle)	40 ml ready for use capped black
F	Incubation tray for 8 strips	3 x

4.1 Materials required

- micropipette 100 - 1000 µl
- micropipette 10 - 100 µl
- pipette tips
- graduated cylinders
- distilled or de-ionized water
- plate shaker
- plastic pincers
- paper towel

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4.2 Size and storage

HepAk Dot has been designed for 24 x 4 determinations.

The expiry date of each component is reported on its respective label, that of the complete kit on the box label.

Upon receipt, all components of the HepAk Dot have to be kept at 2 - 8°C, preferably in the original kit box.

After opening all kit components are stable for at least 2 months, provided proper storage.

4.3 Preparation before use

Allow all components to reach room temperature prior to use in the assay.

Prepare a sufficient amount of **wash solution** by diluting the concentrated washing buffer 10 times (**1 + 9**) with de-ionized or distilled water.

For example, dilute 10 ml of the concentrate with 90 ml of distilled water.

For each test strip 15 ml of washing buffer are requested.

The wash solution prepared is stable at 2-8°C up to 30 days.

All other components are ready for use and so stable until the expiry date.

Avoid exposure of the substrate solution to light.

After each filling of wells with solution, agitate the incubation tray manually to ensure strips are completely immersed and to remove air bubbles which may be trapped under the strip.

5 ASSAY PROCEDURE

1. Bring all reagents to room temperature (RT) (18-25°C) before use. Mix gently without causing foam.
2. Place the strips with the reactive side up (labels on top) into the respective well.
Dispense **2 ml of wash solution** (made of B) into the respective wells.
3. Cover tray, incubate **10 min** at RT (18-25°C) while shaking.
4. Discard wash solution. (Discard the solution in the wells by slowly inverting the plate. Dry the edges of the tray with absorbent paper in order to remove the remaining fluid.)
5. Add **1.5 ml sample diluent** (C) and **10 µl patient serum** or plasma to the respective wells.
6. Cover tray and incubate **30 min** at RT (18-25°C) while shaking.
7. Decant or aspirate, wash each well **three times three minutes with 1.5 ml wash solution** (made of B) while shaking. (Discard the solution in the wells by slowly inverting the plate. Dry the edges of the tray with absorbent paper in order to remove the remaining fluid.)
8. Add **1.5 ml conjugate** (D) to each well
9. Cover tray and incubate **30 min** at RT (18-25°C) while shaking.
10. Decant or aspirate, wash each well **three times three minutes with 1.5 ml wash solution** (made of B) while shaking. (Discard the solution in the wells by slowly inverting the plate. Dry the edges of the tray with absorbent paper in order to remove the remaining fluid.)

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11. Add **1.5 ml of substrate** (E) to each well.
12. Cover plate, incubate **10-12 minutes** while shaking.
13. Decant or aspirate, wash each well **once three minutes** with wash solution (made of B) while shaking to stop the reaction. (Discard the solution in the wells by slowly inverting the plate. Dry the edges of the tray with absorbent paper in order to remove the remaining fluid.)
14. Collect the strips from the wells and dry the membranes by pressing briefly the reactive side of the strip onto absorbent paper. After approximately 30 min the strips are to be interpreted.

6 DATA PROCESSING

Evaluation:

Results should be interpreted only after strips have been dried for at least 30 minutes.






The **POSITIVE DOT CONTROL** must be positive in all cases. The coloration of the dot ensures that the test has been run correctly and the kit components are not degraded. If the positive control dot shows no coloration the results cannot be interpreted.

The **CUT-OFF CONTROL** demonstrates the extent of non-specific antibody binding of the sample in the test. The coloration of the dot corresponds to the minimal intensity above which a sample is considered positive.

The test dots are coated with autoantigens and detect specific antibody binding of the sample in the test. The color intensity of the test dot depends on the titer of specific antibody binding in the sample.

The patient sample is positive concerning a certain antibody if the test dot coloration is stronger (more intense) than the cut-off control.

7 REFERENCE VALUES

1	HepAK Dot	result:
	- positive dot control	positive
	- M2	negative
	- LKM1	
	- LC1	positive
	- SLA	

Positive result:

A sample is considered to be positive for autoantibodies to M2, LKM1, LC1 or SLA if the coloration of the test dot is more intense than the coloration of the cut-off control.

The color intensity of the cut-off dot depends on the test conditions (e.g. incubation times, temperature, washing efficiency) and on the composition of each individual sample. It might be uncolored even if the test has been run in optimal conditions.

Negative result:

A sample is considered to be negative for autoantibodies to M2, LKM1, LC1 or SLA if the coloration of the test dot is less intense than the coloration of the cut-off control.

7.1 Limitations of Method

Healthy individuals should be tested negative by the HepAK Dot. However, M2, LKM1, LC1 or SLA autoantibody positive apparently healthy individuals do occur.

Any clinical diagnosis should not be based on the results of in vitro diagnostic methods alone. Physicians are supposed to consider all clinical and laboratory findings possible to state a diagnosis.

8 CHARACTERISTIC ASSAY DATA

8.1 Comparison to reference methods

The evaluation of the kit was performed in collaboration with the Laboratoire d'Immunopathologie du Centre Hospitalier du Luxembourg (CHL, Prof. Dr. R.L. Humbel).

negative sera (n=20): sera of asymptomatic individuals, negative for respective autoantibodies in ELISA, IFA, Western Blot and immunodiffusion

M2 positive sera (n=15): positive in indirect immunofluorescence (IFA) on cryostat sections of rat liver / kidney / stomach and HEp-2 cells as well as in ELISA (CHL, Anti-PDH ELISA)

		ELISA		IFA	
		+	-	+	-
Dot	+	15	0	15	0
	-	0	20	0	20

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LKM1 positive sera (n=23): sera of 15 patients with autoimmune hepatitis type 2 (AIH-2) and 8 patients with AIH-2 and associated chronic hepatitis C (HC),
all sera positive in IFA on rat liver / kidney sections and in ELISA with recombinant P450-2D6

		ELISA		IFA	
		+	-	+	-
Dot	+	23	0	23	0
	-	0	20	0	20

LC1 positive Sera (n=10): positive in IFA on rat liver sections and in Ouchterlony immunodiffusion (ID) using rat liver extract as antigen

		IFA		ID	
		+	-	+	-
Dot	+	10	0	10	0
	-	0	20	0	20

SLA positive Sera (n=5): sera from patients with autoimmune hepatitis, positive in ELISA (INOVA, USA) and Western Blot (WB) using rat liver extract

		ELISA		WB	
		+	-	+	-
Dot	+	5	0	5	0
	-	0	20	0	20

9 SAFETY PRECAUTIONS

- This kit is for in vitro use only. Follow the working instructions carefully. DRG and its authorized distributors shall not be liable for damages indirectly or consequentially brought about by changing or modifying the procedure indicated.
- The expiration dates stated on the respective labels are to be observed. The same relates to the stability stated for re-constituted reagents.
- Do not use or mix reagents from different lots.
- Do not use reagents from other manufacturers.

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- Avoid time shift during pipetting of reagents.
- All reagents should be kept at 2 - 8 °C prior use in the original shipping container.
- Some of the reagents contain small amounts of bromonitrodioxane (< 0.01 % w/w), methylisothiazolones (< 20 ppm) or sodium azide (< 0.05 %) as a preservative. They must not be swallowed or allowed to come into contact with skin or mucosa.
- Since the kit contains potentially hazardous materials the following precautions should be observed:
 - Do not smoke, eat or drink while handling kit material,
 - Always use protective gloves,
 - Never pipette material by mouth,
 - Wipe up spills promptly, washing the affected surface thoroughly with a decontaminant.
- In any case GLP should be applied with all general and individual regulations to the use of this kit.