INSTRUCTION MANUAL

REF 5006

September 09, 2014

Anti-Phospholipid Dot

- 20 x 4 determinations -

IVD *In-vitro*-diagnostic device

CE

Enzyme immunodot for the determination of IgG- or IgM-antibodies to phospholipids and ß2-glycoprotein I in human serum

REF	Catalogue number	**LOT**	Batch code
	Consult accompanying documents		Manufactured by
	Temparuture limitations		Use by
	Consult operating instruction		Biological risk

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INTENDED USE

Anti-Phospholipid Dot is used for the qualitative detection of IgG or IgM antibodies to phospholipids and ß2-glycoprotein I in human serum for the diagnosis of anti-phospholipid antibody syndrome (APAS).

APAS is an autoimmune disorder comprising such clinical symptoms like arterial or venous thrombosis, thrombocytopenia and recurrent foetal loss. Primary APAS as well as systemic lupus erythematosus (SLE) are characterized by the appearance of autoantibodies to negatively charged phospholipids including Cardiolipin antibodies (1). Although significance and pathological relevance of phospholipid antibodies are not completely revealed yet, the detection of such autoantibodies is widely established and plays an important role in the diagnostics of systemic autoimmune diseases.

Antibodies to Cardiolipin, Phosphatidyl-Serine and Phosphatidyl-inositol are considered as anti-phospholipids antibodies. Anti-Cardiolipin antibodies are most frequently use for the diagnosis of APAS. Antibodies to the other phospholipids are especially of importance for the differential diagnosis if anti-Cardiolipin antibodies are negative. Anti-phospholipid antibodies of APAS patients bind preferentially to a complex of phospholipids and the co-factor ß2-glycoprotein I (ß2-GP I, Apolipoprotein H) (2, 3), whereas antibodies only directed against phospholipids can also be detected in patients suffering from infectious diseases. ß2-GP-I, a serum protein with a molecular weight of 50 kDa, affects platelet aggregation and coagulation. The positively charged fifth domain of ß2 GP-I interacts with negatively charged phospholipids. This interaction results in conformational changes of the protein and the creation of new epitopes apparently recognized by autoimmune phospholipid autoantibodies.

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(1) Harris EN, Gharavi AE, Boey ML, Patel BM, Mackworth-Young GG, Loizou S and Hughes GRV: Anticardiolipin antibodies: detection by radioimmunoassay and association with thrombosis in systemic lupus erythematosus. Lancet 1983 11:1211

(2) Galli M, Comfurius P, Maassen C, Hemker HC, DeBaets MHVan Breda-Vriesman PJC, Barbui T, Zwaal RFA, Bevers EM: Anticardiolipin antibodies (ACA) directed not to cardiolipin but to a plasma protein factor. Lancet 1990 335:1544-1547

(3) McNeil HP, Simpson RJ, Chesterman CN, Krilis SA: Anti-phospholipid antibodies are directed against a complex antigen that includes a lipid-binding factor of coagulation: beta 2-glycoprotein I (apolipoprotein H). Proc Natl Acad Sci USA 1990 87:4120-4124

PRINCIPLE of the TEST

Anti-Phospholipid Dot is an immunodot assay used for the qualitative detection of IgG or IgM antibodies to phospholipids and ß2-glycoprotein I in human serum.

Each Anti-Phospholipid Dot kit includes 20 numbered strips. The strips consist of a membrane where 4 different autoantigen lines and a positive control line are sprayed on. Each line contains highly purified antigen preparation.

During the first incubation autoantibodies of the patient sample bind to the target antigen immobilized on the solid phase (membrane). Unbound sample components are removed by a washing step after an incubation period of 30 minutes at room temperature (RT) while shaking.

During a second step bound antibodies react specifically with anti-human-IgG or anti-human-IgM antibodies which are conjugated to horse radish peroxidase (POD). Excessive conjugate is removed from the solid-phase immune complex by an additional washing step after an incubation period of 15 minutes at RT while shaking.

The horse radish peroxidase converts the colourless substrate solution into a dark purple precipitating line on the membrane. After 10 minutes at RT while shaking the reaction is stopped by two washing steps.

After the strips have been dried the results can be read by eye.

PATIENT SAMPLES

Specimen collection and storage

Blood is taken by venipuncture. Serum is separated after clotting by centrifugation.

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 them at – 20 °C.

Preparation before use

Samples to be measured by the Anti-Phospholipid Dot are used undiluted. Take care to agitate samples gently before pipetting to ensure homogeneity.

TEST COMPONENTS for **20 x 4** determinations

A **Ag**	**Dot strips** 20 strips with 5 test dot lines - 4 test lines coated with highly purified antigens: Phosphatidyl-serine (bovine) Phosphatidyl-inositol (bovine) Cardiolipin (bovine) ß2-GP I (human) - Positive control	20 dot strips vacuum sealed
B **BUF**	**Buffer, 10-fold** sufficient for 150 ml **10x**	**15 ml** concentrate capped white
C **CONJ** **G**	**IgG conjugate, 21-fold** anti-human-IgG (rabbit) coupled with horse radish peroxidase (POD)	**1.2 ml** ready for use capped red
D **CONJ** **M**	**IgM conjugate, 21-fold** anti-human-IgM (rabbit) coupled with horse radish peroxidase (POD)	**1.2 ml** ready for use capped green
E **SOLN** **TMB**	**Substrate** 3,3`,5,5`-Tetramethylbenzidin	**11 ml** ready for use capped blue
F	**Incubation tray for 12 dot strips**	2 x
G	**Lot specific interpretation template**	1 x

Materials required in addition

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

Size and storage

The Anti-Phospholipid Dot has been designed for 20 x 4 determinations of IgG or IgM antibodies.

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 Anti-Phospholipid Dot have to be kept at 2 - 8 °C, preferably in the original kit box.

After opening of the vacuum sealing, the test dot strips are stable for 4 weeks when stored in the plastic foil bag at 2…8°C.

After opening all other kit reagents are stable for at least 4 weeks if properly stored at 2…8°C.

Preparation before use

All wash steps are performed at room temperature (RT). Therefore, the reagents (buffer solution, substrate) must have RT in time.

The dot strips are vacuum sealed. A sufficient number of strips has to be cut off with a scalpel or a cutter from the retaining membrane. Unused dot strips have to be kept dry and stored in the additionally supplied plastic foil bag.

Dilute the 10 fold concentrated buffer with de-ionized or distilled water (1+9).

For each test strip 5 ml of buffer solution are requested

Example:
15 ml concentrated buffer + 135 ml distilled water.
The prepared solution (diluted from B) is stable at 2 - 8 °C up to 30 days.

All other components are ready for use.

Avoid exposure of the substrate to light.

Cleaning procedure of the incubation tray

After application incubate the incubation tray for 30 min with a detergent and rinse with water subsequently.

In the following step fill in any type of alcohol (methanol, propanol or ethanol), incubate on the rocking shaker for 30 min and subsequently rinse with water.

Clean the incubation tray with a cotton bud, rinse with water, and let it dry.

ASSAY PROCEDURE

- **Follow the instruction strictly and avoid any time shift.**
- **The whole assay has to be performed on a shaker (rocking shaker at 40 – 50 per minute is recommended)**
- **The entire assay runs at RT (18…25 °C). Ensure that the required reagents (conjugate, buffer solution, substrate) have RT.**

1. Take the reagents and sufficient number of dot strips out of the box, mix the reagents gently.
2. Dispense **1 ml** of buffer solution (made of B) in each well.
3. Place the strips with the reactive side down into the respective wells.
4. Add **30 µl serum** (final dilution 1 + 33)
5. Incubate **30 minutes** at **RT** while shaking
6. Decant (**Caution:** Turn over carefully the incubation tray and gently decant the buffer solution, any remaining liquid has to be removed with an absorbent paper). Wash **5 min at RT** with **1 ml** buffer solution (made of B) while shaking.
7. Pipette **1 ml** buffer solution (made of B) and add into the respective wells.
   IgG determination: **50 µl** conjugate C
   IgM determination: **50 µl** conjugate D
8. Incubate **15 minutes** at **RT** while shaking.
9. Decant (**Caution:** Turn over carefully the incubation tray and gently decant the buffer solution, any remaining liquid has to be removed with an absorbent paper). Wash **5 min at RT** with **1 ml** buffer solution (made of B) while shaking.
10. Pipette **0.5 ml** substrate (E) in the respective wells.
11. Incubate **10 minutes** at **RT** while shaking.
12. Decant and wash **2 min at RT** with **1 ml** buffer solution (made of B) while shaking.
13. Decant (**Caution:** Turn over carefully the incubation tray and gently decant the buffer solution, any remaining liquid has to be removed with an absorbent paper). Wash **2 min at RT** with **1 ml distilled water** while shaking to stop the reaction.
14. Collect the dot strips from the wells and dry the membranes by pressing the reactive side of the strip onto absorbent paper briefly. After approximately **30 min** the strips are to be interpreted.

DATA PROCESSING

The evaluation of the test results will be performed by means of the provided lot specific evaluation template. For doing this the strips must have been dried and glued onto the template.

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

The test lines are coated with highly purified human antigens and detect specific antibody binding of the sample in the test.
The intensity of the bands given on the evaluation template serves as cut-off of each single band for the decision of positive - negative.

REFERENCE VALUES

Positive control	**strong positive**
Phosphatidyl-serine	**strong positive**
Phosphatidyl-inositol	**weak positive**
Cardiolipin	**strong positive**
ß2-GPI	**strong positive**

Positive result:
A sample is considered to be **positive** in respect to one of the antibodies if the colouration of the test line shows a more intense coloration then the band on the evaluation template.

Negative result:
A sample is considered to be **negative** in respect to one of the antibodies if the colouration of the test line shows the same or less intense coloration then the band on the evaluation template.

Validation:
In order to interpret the results the test line of the positive control has to show a clear colouration.

Limitations of Method

Healthy individuals should be tested negative by the Anti-Phospholipid Dot. However, asymptomatic individuals can show a positive antibody reaction.

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

CHARATERISTIC ASSAY DATA

Specificity and Sensitivity

The diagnostic specificity of the Anti-Phospholipid Dots was determined by measuring 39 normal sera. Regarding IgG all sera were negative which gives 100 %. Regarding IgM a total of 5 sera have been tested positive resulting in a specificity of 87.2 %. The single IgM specificities are as follows: Phosphatidylserine and Cardiolipin 100 % (all 39 samples negative), Phosphatidyl-inositol 94.9 % (2 out of 39 sera positive), ß2-Glycoprotein I 92.3 % (3 out of 39 sera positive).

The diagnostic sensitivity was determined by measuring 50 sera of APAS patients. Regarding IgG the following antibody specific sensitivities have been calculated: Phosphatidyl-serine 72 %, -inositol 34 %, Cardiolipin 92 %, and ß2-GPI 70 %. At least one antibody was detected in 47 out of the 50 sera resulting in a sensitivity for APAS diagnosis of 94 % for IgG. Regarding IgM the following antibody specific sensitivities have been calculated: Phosphatidyl-serine 72 %, -inositol 42 %, Cardiolipin 74 %, and ß2-GPI 92 %. At least one antibody was detected in 49 out of the 50 sera resulting in a sensitivity for APAS diagnosis of 98 % for IgM.

INCUBATION – SCHEME

Anti-Phospholipid Dot (5006)

All steps are performed at RT (18…25°C); the required reagents (dot strips, buffer solution, conjugate) need to have RT in time.

1.	Bring all needed reagents and number of strip to room temperature (18…25 °C) before use	
2.	Pipette **1 ml** buffer solution (diluted from B) for each strip	
3.	Put strips with reaction side to the bottom on the buffer solution	
4.	Add **30 µl** patient serum afterwards (final dilution 1+33)	
5.	Incubate	While shaking, **30 minutes**, RT
6.	Decant, washing of the strips	While shaking, RT, 5 minutes with **1 ml** buffer solution (diluted from B)
7.	Pipette **1 ml b**uffer solution (diluted from B) in each well of the tray, add: for IgG determination: **50 µl** conjugate C for IgM determination: **50 µl** conjugate D	
8.	Incubate	While shaking, **15 minutes**, RT
9.	Decant, washing of the strips	While shaking, RT, 5 minutes with 1 ml buffer solution (diluted from B)
10.	Pipette **0.5 ml** substrate (E) into the respective well	
11.	Incubate	While shaking, **10 minutes**, RT (18…25°C)
12.	Decant, washing of the strips	While shaking, RT, 2 minutes with **1 ml** buffer solution (diluted from B)
13.	Decant, washing of the strips, stop of the reaction	While shaking, RT, 2 minutes with **1 ml** distilled water
14.	Dry test strips on paper towel for 30 minutes, read out results	

SAFETY PRECAUTIONS

- **This kit is for in vitro use only**. Follow the working instructions carefully. GA GENERIC ASSAYS GmbH 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.
- 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 kathon (1% v/v) 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.