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**User's Manual**

# Phospholipid-8-Profile IgG/IgM ELISA Kit

REF **DEIABL361**

Σ **96T**

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

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# PRODUCT INFORMATION

## Intended Use

The Phospholipid-8-Profile IgG/IgM ELISA Kit is used for the separate quantitative determination of IgG and / or IgM antibodies to phospholipides (cardiolipin, phosphatidyl-serine, -inositol, -ethanolamine, phosphatidic acid and $\beta_2$ glycoprotein I) in human serum for the diagnosis of anti-phospholipid antibody syndrome (APAS).

## General Description

APAS is an autoimmune disorder comprising such clinical symptoms like arterial or venous thrombosis, thrombocytopenia and recurrent fetal loss. Primary APAS as well as systemic lupus erythematosus (SLE) are characterized by the appearance of autoantibodies to negatively charged phospholipids. 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.

Unlike phospholipid antibodies which appear in some infectious disease patients autoimmune patients exhibit phospholipid antibodies that seem to recognize phospholipids in association with plasma protein cofactors such as $\beta_2$ glycoprotein-I ($\beta_2$ GP-I) (apolipoprotein H). $\beta_2$ GP-I, a serum protein with a molecular weight of 50 kDa, affects platelet aggregation and coagulation. The positively charged fifth domain of $\beta_2$ GP-I interacts with negatively charged phospholipids such as Cardiolipin. This interaction results in conformational changes of the protein and the creation of new epitopes apparently recognized by autoimmune phospholipid autoantibodies.

## Principles of Testing

The Phospholipid-8-Profile IgG/IgM ELISA Kit is an enzyme immunoassay for the quantitative determination of IgG and / or IgM antibodies to negatively charged phospholipids.

The antibodies of the standards, controls and diluted patient samples react with an antigen complex consisting of cardiolipin, phosphatidylserine, -inositol, -ethanolamine, phosphatidic acid and the cofactor $\beta_2$ GP-I immobilized on the solid phase of microtiter plates. The use of highly purified human $\beta_2$ GP-I guarantees the specific binding of autoimmune related phospholipid antibodies of the specimen under investigation. Following an incubation period of 60 min at room temperature, unbound sample components are removed by a wash step.

The bound antibodies react specifically with antihuman-IgG or anti-human-IgM conjugated to horseradish peroxidase (HRP) within the incubation period of 30 min at room temperature (RT). Excessive conjugate is separated from the solidphase immune complexes by the following wash step.

HRP converts the colorless substrate solution of 3,3',5,5'-tetramethylbenzidine (TMB) added into a blue product. The enzyme reaction is stopped by dispensing an acidic solution into the wells after 15 min at room temperature turning the solution from blue to yellow.

The optical density (OD) of the solution at 450 nm is directly proportional to the amount of specific antibodies bound. The standard curve is established by plotting the antibody concentrations of the calibrators (x-axis) and their corresponding OD values (y-axis) measured. The concentration of antibodies of the specimen is directly read off the standard curve. Evaluating the test by a semi-quantitative method using a cut-off

calibrator is also possible.

## Reagents And Materials Provided

| | | |
|---|---|---|
| **A** Ag 96 | **Microtiter plate**, 12 breakable strips per 8 wells coated with cardiolipin, phosphatidyl-serine, -inositol, -ethanolamine, phosphatidic acid (bovine) and $\beta_2$ glycoprotein I (human), on each well | 1 vacuum sealed with desiccant |
| **B** BUF WASH | **Concentrated wash buffer** sufficient for 1000 ml solution 10x | 100 ml concentrate capped white |
| **C** DIL | **Sample diluent** | 100 ml concentrate capped black |
| **D** CONJ G | **Conjugate** containing anti-human-IgG- (sheep) coupled with horseradish peroxidase | 15 ml ready for use capped red |
| **E** CONJ M | **Conjugate** containing anti-human-IgM- (sheep) coupled with HRP | 15 ml ready for use capped green |
| **F** SOLN TMB | **Substrate** 3,3',5,5'-tetramethylbenzidine in citrate buffer containing hydrogen peroxide | 15 ml ready for use capped blue |
| **G** H2SO4 | **Stop solution** 0.25 sulfuric acid 0.25 M | 15 ml ready for use capped yellow |
| **0 - 4** CAL | **Calibrators** (diluted sera) conc.: 1, 10, 30, 100, 300 U/ml | 1 ml each ready for use capped white |
| **P** CONTROL | **Positive control** (diluted serum) conc.: see leaflet enclosed + | 1 ml ready for use capped red |
| **N** CONTROL | **Negative control** (diluted serum) conc.: see leaflet enclosed – | 1 ml ready for use capped green |

## Materials Required But Not Supplied

1. micropipettes.
2. multi-channel pipette or multi-pipette trough for multi-channel pipette.
3. 8-channel wash comb with vacuum pump and waste bottle or microplate washer.
4. microplate reader with wavelength for 450 nm and 620 nm or 690 nm.

## Storage

The kit should be stored at 2-8°C upon receipt. After opening all kit components are stable for at least 2 months, provided proper storage.

## Specimen Collection And Preparation

Blood is taken by venipuncture. Serum is separated after clotting by centrifugation. Lipaemic, hemolytic or contaminated samples should not be run. Repeated freezing and thawing should be avoided. If samples are to be used for several assays, initially aliquot samples and keep at -20°C.

**Preparation before use**

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

**Note:** Patient samples have to be diluted 1 + 100 (v/v), e.g. 10 µl sample + 1.0 ml sample diluent (C), prior to assay.

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

## Reagent Preparation

**Preparation before use**

1. Allow all components to reach room temperature prior to use in the assay.
2. The microtiter plate is vacuum-sealed in a foil with desiccant. The plate consists of a frame and strips with breakable wells. Allow the sealed microplate to reach room temperature before opening. Unused wells should be stored refrigerated and protected from moisture in the original cover carefully resealed.
3. Prepare a sufficient amount of wash solution by diluting the concentrated wash buffer 10 times (1 + 9) with de-ionized or distilled water. For example, dilute 8 ml of the concentrate with 72 ml of distilled water. The wash solution prepared is stable up to 30 days at 2-8°C.
4. Make sure the soak time of the wash buffer in the wells is at least 5 seconds per wash cycle.
5. Avoid exposure of the TMB substrate solution to light!

## Assay Procedure

• Dilute patient sera with sample diluent (C) 1+100 (v/v), e.g. 10 µl serum + 1.0 ml sample diluent (C).

• Avoid any time shift during pipetting of reagents and samples.

1. Bring all reagents to room temperature (18-25°C) before use. Mix gently without causing foam.
2. Dispense 100 µl calibrators (0 optional) 1-4 (quantitative) or 100 µl calibrator 1 (semi-quantitative) 100 µl control P (N optional) 100 µl diluted patient samples into the respective wells.
3. Incubate 60 min at room temperature (18-25°C).
4. Decant, then wash each well three times using 300 µl wash solution (made of B).
5. Add 100 µl of conjugate (D or E) solution to each well.
6. Incubate 30 min at room temperature (18-25°C).

7. Decant, then wash each well three times using 300 µl wash solution (made of B).
8. Add 100 µl of substrate (F) to each well.
9. Incubate 15 min protected from light at room temperature (18-25°C).
10. Add 100 µl of stop solution (G) to each well and mix gently.
11. Read the OD at 450 nm versus 620 or 690 nm within 30 min after adding the stop solution.

## Quality Control

The test run is valid if:

• The mean OD of the calibrators 4 is ≥ 1.2

• Concentration of Control P see leaflet enclosed

• Control N is negative

If the above mentioned quality criteria are not met, repeat the test and make sure that the test procedure is followed correctly (incubation times and temperatures, sample and wash buffer dilution, wash steps etc.). In case of repeated failure of the quality criteria contact your supplier.

## Interpretation Of Results

The ELISA Kit allows both the quantitative (4 + 1 calibrators) and semi-quantitative (calibrator 1 for cut-off determination) evaluation of the results.

**Quantitative evaluation**

The standard curve is established by plotting the mean OD-values of the calibrators 1-4 (CAL 0 optionally) on the ordinate, y-axis, (lin. scale) versus their respective anti-phospholipid concentrations on the abscissa, x-axis, (log. scale). Anti-phospholipid concentrations of the unknown samples are directly read off in U/ml against the respective OD values. Using the recommended dilution of 1 + 100 (v/v) for patient's sera, no correction factor is necessary, as all other components of the kit are supplied accordingly.

**Semi-quantitative evaluation**

Results are interpreted by calculating the binding index (BI) using **calibrator 1 (10 U/ml)** as **cut-off calibrator**. The BI is the ratio of the OD-value of a sample to the cut-off OD-value (CAL 1).

$$\mathbf{BI = OD_{sample} / (OD_{calibrator\ 1})}$$

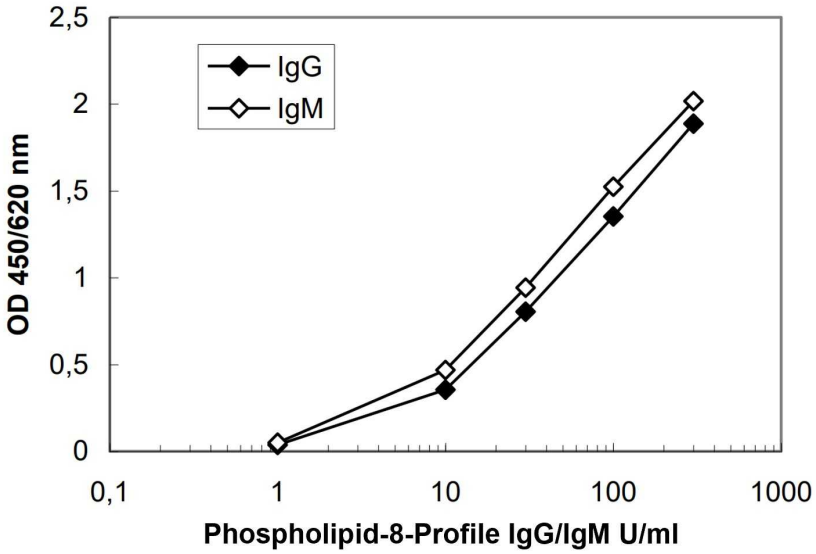
Both evaluation variants of Anti-Phosphatidyl-Serin may be achieved also with computer assisted analysis software integrated in the photometers.

**Example of Typical Assay Results**

| IgG | OD 1 | OD 2 | mean OD | U/ml |
|---|---|---|---|---|
| Calibrator **0** | 0.038 | 0.036 | 0.037 | **1** |
| Calibrator **1** | 0.343 | 0.367 | 0.355 | **10** |
| Calibrator **2** | 0.791 | 0.816 | 0.804 | **30** |
| Calibrator **3** | 1.341 | 1.367 | 1.354 | **100** |
| Calibrator **4** | 1.875 | 1.899 | 1.887 | **300** |
| Patient **1** | 0.661 | 0.677 | 0.669 | **24** |

| IgM | OD 1 | OD 2 | mean OD | U/ml |
|---|---|---|---|---|
| Calibrator **0** | 0.048 | 0.053 | 0.050 | **1** |
| Calibrator **1** | 0.471 | 0.467 | 0.469 | **10** |
| Calibrator **2** | 0.936 | 0.948 | 0.942 | **30** |
| Calibrator **3** | 1.512 | 1.539 | 1.525 | **100** |
| Calibrator **4** | 2.002 | 2.034 | 2.018 | **300** |
| Patient **1** | 1.061 | 1.077 | 1.069 | **40** |

## TYPICAL STANDARD CURVES




Specimens with an OD > calibrator 4 should be diluted with phospholipid antibody negative serum and tested again. Results are multiplied with the dilution factor chosen.

## Reference Values

| Phospholipid IgG/IgM | U/ml | BI |
|---|---|---|
| positive | ≥ 10 | ≥ 1,0 |
| negative | < 10 | < 1,0 |

It is recommended that each laboratory establishes its own normal and pathological reference ranges for serum Anti-Phospholipid levels, as usually done for other diagnostic parameters, too. Therefore, the above mentioned reference values provide a guide only to values which might be expected.

## Precision

**Intra-Assay** coefficient of variation (CV): 20-fold determination:

| | IgG | | | IgM | |
|---|---|---|---|---|---|
| sample | U/ml | CV (%) | sample | U/ml | CV (%) |
| 1 | 187.4 | 4.8 | 1 | 127.4 | 9.0 |
| 2 | 57.2 | 6.7 | 2 | 18.1 | 4.3 |
| 3 | 9.8 | 6.7 | 3 | 5.2 | 4.1 |

**Inter-Assay** coefficient of variation (CV): 5 different runs of 10-fold determinations:

| | IgG | | | IgM | |
|---|---|---|---|---|---|
| sample | U/ml | CV (%) | sample | U/ml | CV (%) |
| 1 | 208.4 | 6.8 | 1 | 127.4 | 12.7 |
| 2 | 60.2 | 3.9 | 2 | 18.5 | 4.5 |
| 3 | 10.9 | 4.9 | 3 | 5.7 | 7.5 |

## Sensitivity

The analytical sensitivity of this assay was determined at 1.0 U/ml.

## Specificity

No cross reactivity to other autoantigens have been found.

## Linearity

Selected positive serum samples have been tested by this assay and found to dilute linearly. However, due to the heterogeneous nature of human autoantibodies there might be sera that do not follow this rule.

## Precautions

1. The kit should be performed by trained technical staff only.
2. The expiration dates stated on the respective labels are to be observed. The same relates to the stability stated for reconstituted reagents.
3. Do not use or mix reagents from different lots.
4. Do not use reagents from other manufacturers.
5. Avoid time shift during pipetting of reagents.
6. All reagents should be kept at 2-8°C before use in the original shipping container.
7. Some of the reagents contain small amounts of Neolone M10 (≤ 1.0 % v/v) as preservative. They must not be swallowed or allowed to come into contact with skin or mucosa.
8. Source materials derived from human body fluids or organs used in the preparation of this kit were tested and found negative for HBsAg and HIV as well as for HCV antibodies. However, no known test guarantees the absence of such viral agents. Therefore, handle all components and all patient samples as if potentially hazardous.
9. Source materials derived from bovine material used in the preparation of this kit were tested and found negative for priones. However, no known test guarantees the absence of such viral agents. Therefore, handle all components and all patient samples as if potentially hazardous.
10. Since the Anti-Phospholipid ELISA Assay 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.

## Limitations

Healthy individuals should be tested negative by the Phospholipid-8-Profile IgG/IgM ELISA Kit. However, phospholipid autoantibody positive apparently healthy persons 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.