ab178622 –
anti-Phospholipid screen IgG and IgM ELISA Kit

Instructions for Use

For the quantitative measurement of IgG and IgM class antibodies against Phospholipids in Human serum and plasma.

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

Abcam’s anti-Phospholipid screen Human in vitro ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for the accurate quantitative measurement of IgG and IgM class antibodies against β2-glycoprotein mediated anionic phospholipids including cardiolipin, phosphatidyl serine, phosphatidyl inositol and phosphatidic acid in Human serum and plasma.

A 96-well plate has been precoated with phospholipid antigens to bind cognate antibodies. Controls or test samples are added to the wells and incubated. Following washing, a horseradish peroxidase (HRP) labelled anti-Human IgG conjugate or anti-Human IgM conjugate is added to the wells, which binds to the immobilized phospholipid-specific IgG or IgM class antibodies respectively. TMB is then catalyzed by the HRP to produce a blue color product that changes to yellow after adding an acidic stop solution. The intensity of yellow coloration is directly proportional to the amount of phospholipid IgG (or IgM) sample captured in plate.

The first study on the anti-phospholipid antibodies began in 1906 when Wasserman introduced a serological test for syphilis. In 1942 it was found the active component that is a phospholipid indicated by the name of cardiolipin. In the 50’s it was observed that a large number of people appeared to be positive for syphilis tests but did not show any evidence of disease. At the beginning the phenomenon was classified as a series of false positive syphilis test, then a more accurate analysis revealed, for this group of patients, a high prevalence of autoimmune disorders including systemic lupus erythematosus (SLE) and Sjögrens syndrome.

The term lupus anticoagulant (LA), used for the first time in 1972, derives from experimental observations in which it was observed an increased risk of thrombosis. Paradoxically, with the presence of some anticoagulants factors; the term LA is not totally correct. In fact the disease is present more frequently in patients without lupus and it is associated with thrombosis rather than to abnormal bleeding.

Some years later the role of a cofactor has been investigated, the β2-glycoprotein I (apolipoprotein H) also said β2GPI, and its interactions with anionic phospholipid in human serum / plasma. This cofactor is a
β-globulin with a molecular weight of 50 kDa that has a concentration of 200 μg/mL in plasma. The β2GPI is involved in the regulation of blood coagulation, inhibiting the intrinsic way. β2GPI in vivo is associated with negatively charged substances such as anionic phospholipids, heparin and lipoproteins. The region that binds phospholipids is in its fifth domain.

The acronym "aPL" (anti-phospholipid antibodies) indicates improperly antibodies directed against phospholipids negatively charged like cardiolipin (CL), phosphatidyl serine (PS) phosphatidyl inositol (PI) and phosphatidic acid (PA); more correctly the term anti-phospholipid antibodies indicate those antibodies directed against the complex between β2GPI and anionic phospholipids that can bind to the fifth domain of β2GPI.

Among these, the cardiolipin is the most commonly used phospholipid as an antigen for determining the aPL with ELISA method. Diagnostic laboratories measure the antibodies directed against the complex between β2GPI and negatively charged phospholipids, as phosphatidyl serine (PS) phosphatidyl inositol (PI) and phosphatidic acid (PA).

Some researchers suggest the use of PS instead of cardiolipin in ELISA assays, for a more precise diagnosis. However, these antibodies against phospholipids are less commonly used, even if their use may increase the clinical sensitivity of patients’ samples with suspected anti-phospholipid syndrome (APS), but it cannot replace the determination of autoantibodies anti-cardiolipin.
2. ASSAY SUMMARY

Prepare all reagents, samples and controls as instructed.

Add samples and controls to wells used. Incubate at 37°C.

Wash each well and add prepared labeled HRP-Conjugate. Incubate at room temperature.

After washing, add TMB substrate solution to each well. Incubate at room temperature. Add Stop Solution to each well. Read immediately.
3. **PRECAUTIONS**

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. **STORAGE AND STABILITY**

Store kit at 2-8°C immediately upon receipt.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in section 9. Reagent Preparation.

5. **MATERIALS SUPPLIED**

<table>
<thead>
<tr>
<th>Item</th>
<th>Amount</th>
<th>Storage Condition (Before Preparation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospholipid Coated Microplate (12 x 8 wells)</td>
<td>96 Wells</td>
<td>2-8°C</td>
</tr>
<tr>
<td>IgG Sample Diluent</td>
<td>100 mL</td>
<td>2-8°C</td>
</tr>
<tr>
<td>Stop Solution</td>
<td>15 mL</td>
<td>2-8°C</td>
</tr>
<tr>
<td>10X Washing Solution</td>
<td>50 mL</td>
<td>2-8°C</td>
</tr>
<tr>
<td>Phospholipid anti-IgG HRP Conjugate</td>
<td>15 mL</td>
<td>2-8°C</td>
</tr>
<tr>
<td>Phospholipid anti-IgM HRP Conjugate</td>
<td>15 mL</td>
<td>2-8°C</td>
</tr>
<tr>
<td>TMB Substrate Solution</td>
<td>15 mL</td>
<td>2-8°C</td>
</tr>
<tr>
<td>Phospholipid Standard 0 - 0 U/mL</td>
<td>1.2 mL</td>
<td>2-8°C</td>
</tr>
<tr>
<td>Phospholipid Standard 1 - 5 U/mL</td>
<td>1.2 mL</td>
<td>2-8°C</td>
</tr>
<tr>
<td>Phospholipid Standard 2 - 10 U/mL</td>
<td>1.2 mL</td>
<td>2-8°C</td>
</tr>
<tr>
<td>Phospholipid Standard 3 - 20 U/mL</td>
<td>1.2 mL</td>
<td>2-8°C</td>
</tr>
<tr>
<td>Phospholipid Standard 4 - 80 U/mL</td>
<td>1.2 mL</td>
<td>2-8°C</td>
</tr>
<tr>
<td>Phospholipid Positive Control</td>
<td>1.2 mL</td>
<td>2-8°C</td>
</tr>
<tr>
<td>Phospholipid Negative Control</td>
<td>1.2 mL</td>
<td>2-8°C</td>
</tr>
<tr>
<td>Strip Holder</td>
<td>1 unit</td>
<td>2-8°C</td>
</tr>
<tr>
<td>Cover Foil</td>
<td>1 unit</td>
<td>2-8°C</td>
</tr>
</tbody>
</table>
6. **MATERIALS REQUIRED, NOT SUPPLIED**

These materials are not included in the kit, but will be required to successfully utilize this assay:

- Microplate reader capable of measuring absorbance at 450 nm or 620 nm
- Incubator at 37°C
- Multi and single channel pipettes to deliver volumes between 10 and 1,000 µL
- Optional: Automatic plate washer for rinsing wells
- Vortex tube mixer
- Deionised or (freshly) distilled water
- Disposable tubes
- Timer

7. **LIMITATIONS**

- ELISA kit intended for research use only. Not for use in diagnostic procedures
- All components of Human origin used for the production of these reagents have been tested for anti-HIV antibodies, anti-HCV antibodies and HBsAg and have been found to be non-reactive. Nevertheless, all materials should still be regarded and handled as potentially infectious
- Use only clean pipette tips, dispensers, and lab ware.
- Do not interchange screw caps of reagent vials to avoid cross-contamination
- Close reagent vials tightly immediately after use to avoid evaporation and microbial contamination
- After first opening and subsequent storage check conjugate and control vials for microbial contamination prior to further use
• To avoid cross-contamination and falsely elevated results pipette patient samples and dispense conjugate without splashing accurately to the bottom of wells

8. **TECHNICAL HINTS**

• Avoid foaming or bubbles when mixing or reconstituting components

• Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.

• Ensure plates are properly sealed or covered during incubation steps

• Complete removal of all solutions and buffers during wash steps is necessary for accurate measurement readings

• This kit is sold based on number of tests. A ‘test’ simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions
9. REAGENT PREPARATION

Equilibrate all reagents, samples and controls to room temperature (18-25°C) prior to use.

9.1 1X Washing Solution

Prepare 1X Washing Solution by diluting 10X Washing Solution with deionized water. To make 500 mL 1X Washing Solution combine 50 mL 10X Washing Solution with 450 mL deionized water. Mix thoroughly and gently.

- All other solutions are supplied ready to use

10. SAMPLE COLLECTION AND STORAGE

- Use Human serum or plasma (citrate) samples with this assay. If the assay is performed within 5 days of sample collection, the specimen should be kept at 2-8°C; otherwise they should be aliquoted and stored deep-frozen (-20 to -80°C). If samples are stored frozen, mix thawed samples well before testing. Avoid repeated freezing and thawing.
  Heat inactivation of samples is not recommended

11. SAMPLE PREPARATION

- Before assaying, all samples should be diluted 1:100 with IgG Sample Diluent. Add 10 µL sample to 990 µL IgG Sample Diluent to obtain a 1:100 dilution. Mix gently and thoroughly.
12. PLATE PREPARATION

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents.
- Unused well strips should be returned to the plate packet and stored at 4°C.
- For each assay performed, a minimum of 1 well must be used as a blank, omitting sample and conjugate from well addition.
- For statistical reasons, we recommend each standard and sample should be assayed with a minimum of two replicates (duplicates).
13. ASSAY PROCEDURE

- Equilibrate all materials and prepared reagents to room temperature prior to use.

- All controls (Phospholipid Positive and Phospholipid Negative) must be included with each assay performed to determine test results.

- Please read the test protocol carefully before performing the assay. Reliability of results depends on strict adherence to the test protocol as described.

- If performing the test on ELISA automatic systems we recommend increasing the washing steps from three to five and the volume of washing solution from 300 µL to 350 µL to avoid washing effects.

- Assay all standards, controls and samples in duplicate.

  13.1. Prepare all reagents, working standards, and samples as directed in the previous sections.

  13.2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal and return to 4°C storage.

  13.3. Add 100 µL of each standard or sample into appropriate wells. Leave one well for substrate blank.

  13.4. Cover wells with the foil supplied in the kit and incubate for 60 minutes at room temperature.

  13.5. Remove the foil, aspirate the contents of the wells and wash each well three times with 300 µL of 1X Washing Solution. Avoid spill over into neighboring wells. The soak time between each wash cycle should be >5 seconds. After the last wash, remove the remaining 1X Washing Solution by aspiration or decanting. Invert the plate and blot it against clean paper towels to remove excess liquid.

  **Note:** Complete removal of liquid at each step is essential for good assay performance.

  13.6. Add 100 µL Phospholipid anti-IgG HRP Conjugate into all wells except for the blank well. Cover with foil.
13.7. Incubate for 30 minutes at room temperature. Do not expose to direct sunlight.

13.8. Repeat step 13.5.

13.9. Add 100 µL TMB Substrate Solution into all wells

13.10. Incubate for exactly 15 minutes at room temperature in the dark.

13.11. Add 100 µL Stop Solution into all wells in the same order and at the same rate as for the TMB Substrate Solution.

**Note:** Any blue color developed during the incubation turns into yellow.

13.12. Highly positive samples can cause dark precipitates of the chromogen. These precipitates have an influence when reading the optical density. Predilution of the sample with PBS for example 1:1 is recommended. Then dilute the sample 1:100 with IgG Sample Diluent and multiply the results in Standard Units by 2 (See Section 14. Calculations.)

13.13. Measure the absorbance of the specimen at 450 nm within 5 minutes of addition of the Stop Solution.

*Dual wavelength reading using 620 nm as reference wavelength is recommended.*
14. **CALCULATIONS**

**Calculation of Results**
Calculate the mean background subtracted absorbance for each point of the standard curve and each sample. Plot the mean value of absorbance of the standards against concentration. Draw the best-fit curve through the plotted points. (e.g.: Four Parameter Logistic).
Interpolate the values of the samples on the standard curve to obtain the corresponding values of the concentrations expressed in U/mL.

**Interpretation of Results**
Normal value ranges for this ELISA should be established by each researcher.
The following values should be considered as a guideline only:

<table>
<thead>
<tr>
<th></th>
<th>IgG (GPL U/mL)</th>
<th>IgM (MPL U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>&lt; 10</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Elevated</td>
<td>≥ 10</td>
<td>≥10</td>
</tr>
</tbody>
</table>
15. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for demonstration purposes only. A new standard curve must be generated for each assay performed.

<table>
<thead>
<tr>
<th>Conc. (U/mL)</th>
<th>O.D</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.03</td>
</tr>
<tr>
<td>5</td>
<td>0.39</td>
</tr>
<tr>
<td>10</td>
<td>0.77</td>
</tr>
<tr>
<td>20</td>
<td>1.48</td>
</tr>
<tr>
<td>80</td>
<td>3.05</td>
</tr>
</tbody>
</table>
16. TYPICAL SAMPLE VALUES

PRECISION –

Precision and reproducibility are evaluated by eight reply of two positive samples by two different runs with two different lots. Dispensing and washing operations were performed manually by an operator.

The results in terms of standard deviation and coefficient of variation were below:

<table>
<thead>
<tr>
<th></th>
<th>IgG</th>
<th></th>
<th>IgM</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>SD</td>
<td>CV%</td>
<td>SD</td>
<td>CV%</td>
</tr>
<tr>
<td>Intra-assay</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample 1</td>
<td>1.03</td>
<td>5.9</td>
<td>1.31</td>
<td>7.4</td>
</tr>
<tr>
<td>Sample 2</td>
<td>0.26</td>
<td>9.2</td>
<td>5.25</td>
<td>11.7</td>
</tr>
<tr>
<td>Inter-assay</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample 1</td>
<td>0.61</td>
<td>7.6</td>
<td>1.97</td>
<td>5.9</td>
</tr>
<tr>
<td>Sample 2</td>
<td>0.15</td>
<td>7.1</td>
<td>2.98</td>
<td>6.6</td>
</tr>
</tbody>
</table>
17. ASSAY ANALYTICAL SPECS

SPECIFICITY –
The specificity of anti-phospholipids screen IgG assay is 84.6%.
The specificity of anti-phospholipids screen IgM assay is 100%.
This is defined as the probability of the assay scoring negative in the presence of the specific analyte.

SENSITIVITY –
The sensitivity of anti-phospholipids screen IgG assay is 92.3%.
The sensitivity of anti-phospholipids screen IgM assay is 68.8%.
This is defined as the probability of the assay scoring positive in the presence of the specific analyte.

The lowest concentration of anti-phospholipid IgG that can be distinguished from zero standard is 0.3 U/mL.
The lowest concentration of anti-phospholipid IgM that can be distinguished from zero standard is 0.16 U/mL.
18. INTERFERENCES

The presence of immune complexes or other immunoglobulin aggregates in the patient sample may cause an increased level of non-specific binding and produce false positives in this assay.

19. TROUBLESHOOTING

<table>
<thead>
<tr>
<th>Problem</th>
<th>Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low signal</td>
<td>Incubation time to short</td>
<td>Try overnight incubation at 4 °C</td>
</tr>
<tr>
<td></td>
<td>Precipitate can form in wells upon substrate addition when concentration of target is too high</td>
<td>Increase dilution factor of sample</td>
</tr>
<tr>
<td></td>
<td>Using incompatible sample type (e.g. serum vs. cell extract)</td>
<td>Detection may be reduced or absent in untested sample types</td>
</tr>
<tr>
<td></td>
<td>Sample prepared incorrectly</td>
<td>Ensure proper sample preparation/dilution</td>
</tr>
<tr>
<td>Large CV</td>
<td>Bubbles in wells</td>
<td>Ensure no bubbles present prior to reading plate</td>
</tr>
<tr>
<td></td>
<td>All wells not washed equally/thoroughly</td>
<td>Check that all ports of plate washer are unobstructed/wash wells as recommended</td>
</tr>
<tr>
<td></td>
<td>Incomplete reagent mixing</td>
<td>Ensure all reagents/master mixes are mixed thoroughly</td>
</tr>
<tr>
<td></td>
<td>Inconsistent pipetting</td>
<td>Use calibrated pipettes &amp; ensure accurate pipetting</td>
</tr>
<tr>
<td></td>
<td>Inconsistent sample preparation or storage</td>
<td>Ensure consistent sample preparation and optimal sample storage conditions (e.g. minimize freeze/thaws cycles)</td>
</tr>
<tr>
<td>Problem</td>
<td>Cause</td>
<td>Solution</td>
</tr>
<tr>
<td>------------------</td>
<td>------------------------------------------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>High background</td>
<td>Wells are insufficiently washed</td>
<td>Wash wells as per protocol recommendations</td>
</tr>
<tr>
<td></td>
<td>Contaminated wash buffer</td>
<td>Make fresh wash buffer</td>
</tr>
<tr>
<td></td>
<td>Waiting too long to read plate after adding stop solution</td>
<td>Read plate immediately after adding stop solution</td>
</tr>
<tr>
<td>Low sensitivity</td>
<td>Improper storage of ELISA kit</td>
<td>Store all reagents as recommended. Please note all reagents may not have identical storage requirements.</td>
</tr>
<tr>
<td></td>
<td>Using incompatible sample type (e.g. Serum vs. cell extract)</td>
<td>Detection may be reduced or absent in untested sample types</td>
</tr>
</tbody>
</table>
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