Anti-Phosphatidyl Inositol IgG / IgM

ORG 536

96 Tests

Immunometric Enzyme Immunoassays for the quantitative determination of IgG and IgM class Autoantibodies against Phosphatidyl Inositol

Instruction for use
# CONTENTS

CONTENTS ................................................................................................................ 2  
WARNINGS AND PRECAUTIONS ............................................................................. 3  
MATERIALS SUPPLIED ............................................................................................. 3  
CONTROLS ................................................................................................................ 4  
TECHNICAL DATA ..................................................................................................... 4  
PRINCIPLE OF THE PROCEDURE ........................................................................... 4  
CLINICAL RELEVANCE ............................................................................................. 5  
NORMAL VALUES ...................................................................................................... 5  
SPECIFICITY .............................................................................................................. 6  
CALIBRATION ............................................................................................................ 6  
REFERENCES ............................................................................................................ 6  
MATERIALS REQUIRED ............................................................................................ 7  
SPECIMEN COLLECTION AND PREPARATION ...................................................... 7  
PREPARATION AND STORAGE OF REAGENTS ..................................................... 8  
NOTES ON TECHNIQUE ........................................................................................... 8  
IMMUNOASSAY PROCEDURE ................................................................................. 9  
CALCULATION OF RESULTS .................................................................................. 10  
CALCULATION EXAMPLE ....................................................................................... 10  
ASSAY CHARACTERISTICS .................................................................................... 11  
INCUBATION SCHEME ............................................................................................. 11
WARNINGS AND PRECAUTIONS

All reagents of this test kit are strictly intended for in vitro use only.
Please adhere strictly to the sequence of pipetting steps provided in this protocol. Observe the
guidelines for performing quality control in medical laboratories by assaying controls and/or pooled
sera.

All reagents should be stored refrigerated at 2 - 8 °C in their original container.
Do not interchange kit components from different lots. The expiration dates stated on the labels of
the shipping container and all vials have to be observed. Do not use kit components beyond their
expiration dates.
Allow all kit components and specimen to reach room temperature prior to use and mix well.
During handling of all kit reagents, controls and serum samples observe the existing legal
regulations. The following precautions should be taken handling potentially infectious materials:
- do not eat, drink or smoke in areas where specimens or kit reagents are handled
- do not pipette by mouth
- wear disposable gloves while handling specimens or kit reagents and wash hands thoroughly
  afterwards.

The test kit contains components of human origin which, when tested by FDA-licensed methods,
were found negative for hepatitis B surface antigen and for HIV antibody. No known test can
guarantee, however, that products derived from human blood will not be infectious. Handle,
therefore, all reagents and human blood derivatives, like plasma or serum samples, as if capable
of transmitting infection.
Avoid contact with the TMB (3,3’,5,5’-Tetramethyl-benzidine). If TMB comes into contact with skin
wash thoroughly with water and soap.
The stop solution contains hydrochloric acid. If it comes into contact with skin, wash thoroughly with
water and seek medical attention.
Avoid contact between the buffered Peroxide Solution and easily oxidized materials; extreme
temperatures may initiate spontaneous combustion.

MATERIALS SUPPLIED

Package size  96 determ.
divisible microplate consisting of 12 modules of 8 wells each, ............ 1
coated with highly purified Phosphatidyl Inositol, the microplate is
saturated with human β2-Glycoprotein I
combined calibrators with IgG and IgM class anti-Phospholipid .......... 6 vials, 1.5 ml each
antibodies in a PBS/BSA matrix containing
IgG:  0; 6.3; 12.5; 25; 50; 100  GPL U/ml  and
IgM:  0; 6.3; 12.5; 25; 50; 100  MPL U/ml
anti-Phospholipid controls in a PBS/BSA matrix (positive .............. 2 vials, 1.5 ml each
and negative), for the respective concentrations
see the enclosed package insert
Anti-Cardiolipin sample buffer, yellow, Concentrate ..................... 1 vial, 20 ml
enzyme conjugate solution (light red), containing polyclonal .......... 1 vial, 15 ml
rabbit anti-h-IgG-IgG, labelled with horseradish peroxidase
enzyme conjugate solution (light red), containing polyclonal .......... 1 vial, 15 ml
rabbit anti-h-IgG-IgM, labelled with horseradish peroxidase
TMB substrate solution ............................................................... 1 vial, 15 ml
stop solution (1 M hydrochloric acid) ........................................... 1 vial, 15 ml
buffered wash solution, Concentrate ....................................... 1 vial, 20 ml
CONTROLS

A set of two controls is provided with the kit.

TECHNICAL DATA

| Sample material: | serum or plasma |
| Required sample volume: | 10 µl of sample to be diluted 1:100 with sample buffer |
| | 100 µl prediluted sample per single determination |
| Total incubation time: | 60 minutes at room temperature (20 - 28 °C) |
| Calibration range: | IgG and IgM: 6.3 - 100 U/ml |
| Sensitivity: | IgG and IgM: 0.5 U/ml |
| Storage: | refrigerated at 2 - 8 °C |
| Shelf life: | 12 months after manufacturing or until the expiration date printed on the labels |
| Package size: | 96 tests |

PRINCIPLE OF THE PROCEDURE

Anti-Phosphatidyl Inositol is an indirect solid phase enzyme immunometric assays (ELISA). It is designed for the quantitative measurement of IgG or IgM class autoantibodies directed against negatively-charged phospholipids. The microplate is coated with highly purified Phosphatidyl Inositol.

Anti-Phospholipid autoantibodies require β2-Glycoprotein I as a co-factor for binding. The microplate is therefore saturated with human β2-Glycoprotein I.

The microplates can be divided into 12 modules of 8 wells each or can be used completely for 96 determinations. Each well can be separated from the module ("break-away").

The binding of present autoantibodies, formation of the sandwich complexes and enzymatic colour reaction take place during three different reaction phases:

**Phase 1:**
Calibrators, controls and prediluted patient samples are pipetted into the wells of the microplate. Any present antibodies bind to the inner surface of the wells. After a 30 minutes incubation the microplate is washed with wash buffer for removing non-reactive serum components.

**Phase 2:**
An anti-human-IgG (or anti-human-IgM) horseradish peroxidase conjugate solution is pipetted into the wells of the microplate to recognise IgG class autoantibodies (or IgM class autoantibodies) bound to the immobilized antigens. After a 15 minutes incubation any excess enzyme conjugate, which is not specifically bound is washed away with wash buffer.

**Phase 3:**
A chromogenic substrate solution containing TMB (3,3',5,5'-Tetramethylbenzidine) is dispensed into the wells. During 15 minutes of incubation the colour of the solutions change into blue. Colour development is stopped by adding 1 M hydrochloric acid as stop solution. The solutions colour change into yellow. The amount of colour is directly proportional to the concentration of IgG resp. IgM antibodies present in the original sample.

To read the optical density a microplate reader with a 450 nm filter is required. Bi-chromatic measurement with a 600-690 nm reference is recommended.
CLINICAL RELEVANCE

The first study of anti-Phospholipid antibodies began in 1906, when Wasserman introduced a serological test for Syphilis. In 1942, the active component was found to be a phospholipid, which was designated Cardiolipin. In the 1950s it became clear that a number of people had positive tests for syphilis without any evidence of the disease. This phenomenon was referred to as the biological false positive serological test for syphilis. A high prevalence of autoimmune disorders, including systemic lupus erythematosus (SLE) and Sjögrens Syndrome occurred in this group of patients.

The presence of circulating anticoagulants in patients with SLE was first documented in 1952 and was associated with increased risk of paradoxical Thrombosis in 1963. The term Lupus anticoagulant (LA), first used in 1972, is clearly a misnomer, because LA is more frequently encountered in patients without lupus and is associated with thrombosis rather than abnormal bleeding.

During the last years it became clear that the optimal binding of anti-Phospholipid antibodies is depending on a cofactor termed β2-Glycoprotein I (apolipoprotein H) (β2GPI). β2GPI is a 50 kDa β2-globulin occurring in plasma at a level of 200 µg/ml. It has been found that β2-Glycoprotein I inhibits the intrinsic coagulation pathway and, therefore, it is involved in the regulation of blood coagulation. β2GPI is associated in vivo with negatively-charged substances, e.g. anionic phospholipids, heparin and lipoproteins. The phospholipid binding region is located on its fifth domain.

Under the acronym "aPL" (anti-Phospholipid antibodies) antibodies against negatively-charged phospholipids, such as CL (Cardiolipin), LA (Lupus Anticoagulant), PS (Phosphatidyl Serine), PI (Phosphatidyl Inositol) and PA (Phosphatidic Acid) are summarised. Of these, Cardiolipin is the phospholipid most commonly used as antigen to test for aPL by ELISA. Some Antisera which bind cardiolipin-coated ELISA plates can also bind to plates coated with other negatively-charged phospholipids, such as Phosphatidyl Serine (PS), Phosphatidyl Inositol and Phosphatidic Acid (PA).

Some investigators have suggested that the use of PS in place of cardiolipin in ELISA tests enables more specific diagnosis. These antigens are less commonly used and their additional use can improve the clinical sensitivity in patient samples with suspected APS (Anti-Phospholipid-Syndrome), but they can't replace the measurement of autoantibodies against Cardiolipin.

NORMAL VALUES

In a normal range study with serum samples from healthy blood donors the following ranges have been established with the anti-Phospholipid tests:

<table>
<thead>
<tr>
<th>anti-Phosphatidyl Inositol-Ab</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>

Positive results should be verified concerning the entire clinical status of the patient. Also every decision for therapy should be taken individually.

It is recommended that each laboratory establishes its own normal and pathological ranges of serum anti-Phospholipid.
SPECIFICITY

The microplate is coated with highly purified Phosphatidyl Inositol and saturated with human $\beta_2$-Glycoprotein I. Special coating processes, developed by the manufacturer guarantee for the native immunogenic structure of the phospholipids after immobilization on the solid phase. The elisa kit is specific for autoantibodies directed against the respective phospholipid or the complex of the negatively-charged phospholipid and $\beta_2$-Glycoprotein I.

No cross-reactivity was observed to anti-DNA antibodies and those types of antibodies occurring in Syphilis.

CALIBRATION

The assay system is calibrated against the internationally recognized reference sera from E.N. Harris, Louisville, since no other international standards are available.

REFERENCES

MATERIALS REQUIRED

Equipment
- Microplate reader capable for endpoint measurements at 450 nm
- Vortex mixer
- Pipets for 10 µl, 100 µl and 1000 µl

Preparation of reagents
- distilled water
- graduated cylinder for 100 and 1000 ml
- plastic container for storage of the wash solution

Optional
- Multi-Channel Dispenser
- or repeatable pipet for 100 µl
- data reduction software

SPECIMEN COLLECTION AND PREPARATION

For determination of anti-Phosphatityl Inositol antibodies serum or plasma are the preferred sample matrixes. All serum and plasma samples are prediluted 1 : 100 with sample buffer. Therefore 10 µl of sample may be diluted with 1000 µl of sample buffer.

The patients need not to be fasting, and no special preparations are necessary. Collect blood by venipuncture into vacutainers and separate serum or plasma from the cells by centrifugation after clot formation. Samples may be stored refrigerated at 2 - 8 °C for at least 5 days. For longer storage of up to six months samples should be stored frozen at -20 °C. To avoid repeated thawing and freezing the samples should be aliquoted.

Neither Bilirubin nor Hemolysis have significant effect on the procedure.
PREPARATION AND STORAGE OF REAGENTS

All components of this test kit are supplied in a liquid format and ready to use, except the sample buffer and wash buffer. When stored refrigerated at 2 - 8 °C the components are stable for at least 30 days after opening or until the expiration date printed on the labels.

Remaining modules of the microplate should be stored refrigerated at 2 - 8 °C protected from moisture; store together with desiccant and carefully sealed in the plastic bag.

Preparation of sample buffer
Dilute the contents of each vial of the sample buffer concentrate (5x) with destilled water to a final volume of 100 ml prior to use. Store refrigerated: stable at 2 - 8 °C for at least 30 days after preparation or until the expiration date printed on the label.

Preparation of buffered wash solution
Dilute the contents of each vial of the buffered wash solution concentrate (50x) with distilled water to a final volume of 1000 ml prior to use. Store refrigerated: stable at 2 - 8 °C for at least 30 days after preparation or until the expiration date printed on the label.

NOTES ON TECHNIQUE

Control sera or pools should routinely be assayed as unknowns to check performance of the reagents and the assay.

For all controls, the respective concentrations are provided on the labels of each vial. Using these concentrations a calibration curve may be calculated to read off the patient results semi-quantitatively.

Pipetting and Sample Handling
Use a disposable-tip micropipette to dispense sera and plasma samples. Pipet directly to the bottom of the wells. To avoid carryover contamination change the tip between samples.

Patient samples expected to contain high concentrations should be additionally diluted with sample buffer before. Additional dilutions must be considered during calculation.
**IMMUNOASSAY PROCEDURE**

Do not interchange components of different lots.
All components should be at room temperature before use.

Dilute all patient samples 1:100 with sample buffer before assay. Therefore combine 10 µl of sample with 1000 µl of sample buffer in a polystyrene tube. Mix well. Calibrators and controls are ready to use and need not to be diluted.

1. Prepare a sufficient number of microplate modules to accommodate calibrators, controls and prediluted patient samples in duplicates.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>SA</td>
<td>SE</td>
<td>P1</td>
<td>P5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>SA</td>
<td>SE</td>
<td>P1</td>
<td>P5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>SB</td>
<td>SF</td>
<td>P2</td>
<td>P..</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>SB</td>
<td>SF</td>
<td>P2</td>
<td>P..</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>SC</td>
<td>C1</td>
<td>P3</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>F</td>
<td>SC</td>
<td>C1</td>
<td>P3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>SD</td>
<td>C2</td>
<td>P4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>SD</td>
<td>C2</td>
<td>P4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SA - SF: standards A to F
P1, P2... patient sample 1, 2...
C1: positive control
C2: negative control

2. For the determination of one class of autoantibodies **pipette 100 µl of calibrators, controls and prediluted patient samples** into the wells.
For determination of both IgG and IgM autoantibodies calibrators, controls and patient samples have to be pipetted in two attempts.

3. Incubate for **30 minutes** at room temperature (20 - 28 °C).
4. Discard the contents of the microwells and wash **3 times with 300 µl of wash solution**.
5. Dispense **100 µl of enzyme conjugate** solution into each well.
6. Incubate for **15 minutes** at room temperature.
7. Discard the contents of the microwells and wash **3 times with 300 µl of wash solution**.
8. Dispense **100 µl of TMB substrate solution** into each well.
9. Incubate for **15 minutes** at room temperature protected from light.
10. Add **100 µl of stop solution** to each well of the modules and leave untouched for 5 minutes.
11. Read the optical density at **450 nm** and calculate the results. Bi-chromatic measurement with reference at 600-650 nm is recommended.

The developed color is stable for at least 30 minutes.
Read optical densities during this time.
CALCULATION OF RESULTS

For the anti-Phosphatidyl Inositol test a 4-Parameter-Fit with lin-log co-ordinates for optical density and concentration is recommended. Smoothed Spline approximation and log-log co-ordinates are also suitable.

Recommended Lin-Log Plot

First calculate the averaged optical densities for each calibrator well. Use lin-log graph paper and plot the averaged optical density of each calibrator versus the concentration. Draw the best fitting curve approximating the path of all calibrator points. The calibrator points may also be connected with straight line segments. The concentration of unknowns may then be estimated from the calibration curve by interpolation.

CALCULATION EXAMPLE

The figures below show typical results for anti-Phospholipid IgG and IgM. These data are intended for illustration only and should not be used to calculate results from another run.

<table>
<thead>
<tr>
<th>anti-PL</th>
<th>No</th>
<th>Position</th>
<th>OD 1</th>
<th>OD 2</th>
<th>Mean</th>
<th>Conc. 1</th>
<th>Conc. 2</th>
<th>Mean decl.</th>
<th>Conc.</th>
<th>CV %</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>STA</td>
<td>A 1/B 1</td>
<td>0.085</td>
<td>0.091</td>
<td>0.088</td>
<td>0.0</td>
<td>0.3</td>
<td>0.2</td>
<td>0.0</td>
<td>5</td>
</tr>
<tr>
<td>IgG</td>
<td>STB</td>
<td>C 1/D 1</td>
<td>0.277</td>
<td>0.222</td>
<td>0.225</td>
<td>6.0</td>
<td>5.8</td>
<td>5.9</td>
<td>6.3</td>
<td>2</td>
</tr>
<tr>
<td>IgG</td>
<td>STC</td>
<td>E 1/F 1</td>
<td>0.370</td>
<td>0.376</td>
<td>0.373</td>
<td>12.0</td>
<td>12.3</td>
<td>12.2</td>
<td>12.5</td>
<td>1</td>
</tr>
<tr>
<td>IgG</td>
<td>STD</td>
<td>G 1/H 1</td>
<td>0.687</td>
<td>0.703</td>
<td>0.695</td>
<td>26</td>
<td>27</td>
<td>27</td>
<td>25</td>
<td>2</td>
</tr>
<tr>
<td>IgG</td>
<td>STE</td>
<td>A 2/B 2</td>
<td>1.109</td>
<td>1.113</td>
<td>1.111</td>
<td>48</td>
<td>48</td>
<td>48</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>IgG</td>
<td>STF</td>
<td>C 2/D 2</td>
<td>1.911</td>
<td>1.881</td>
<td>1.896</td>
<td>102</td>
<td>100</td>
<td>101</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>IgM</td>
<td>STA</td>
<td>A 7/B 7</td>
<td>0.031</td>
<td>0.033</td>
<td>0.032</td>
<td>0.0</td>
<td>0.1</td>
<td>0.0</td>
<td>0.0</td>
<td>4</td>
</tr>
<tr>
<td>IgM</td>
<td>STB</td>
<td>C 7/D 7</td>
<td>0.239</td>
<td>0.249</td>
<td>0.244</td>
<td>6.1</td>
<td>6.3</td>
<td>6.2</td>
<td>6.3</td>
<td>3</td>
</tr>
<tr>
<td>IgM</td>
<td>STC</td>
<td>E 7/F 7</td>
<td>0.458</td>
<td>0.465</td>
<td>0.462</td>
<td>12.5</td>
<td>12.7</td>
<td>12.6</td>
<td>12.5</td>
<td>1</td>
</tr>
<tr>
<td>IgM</td>
<td>STD</td>
<td>G 7/H 7</td>
<td>0.791</td>
<td>0.826</td>
<td>0.809</td>
<td>24</td>
<td>26</td>
<td>25</td>
<td>25</td>
<td>3</td>
</tr>
<tr>
<td>IgM</td>
<td>STE</td>
<td>A 8/B 8</td>
<td>1.289</td>
<td>1.299</td>
<td>1.294</td>
<td>50</td>
<td>51</td>
<td>50</td>
<td>50</td>
<td>1</td>
</tr>
<tr>
<td>IgM</td>
<td>STF</td>
<td>C 8/D 8</td>
<td>1.791</td>
<td>1.784</td>
<td>1.788</td>
<td>101</td>
<td>99</td>
<td>100</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>
ASSAY CHARACTERISTICS

Sensitivity
The lower detection limits for the anti-Phosphatidyl Inositol test were determined at 0.5 U/ml for both, IgG and IgM class autoantibodies.

Parallelism
In dilution experiments sera with high IgG- and IgM-antibody concentrations were diluted with sample buffer and assayed in the anti-Phosphatidyl Inositol kits. The assays show linearity over the full measuring range.

INCUBATION SCHEME

1. Pipet 100 µl calibrator, control or diluted patient sample
   Incubate for 30 minutes at room temperature
   Discard the contents of the wells and wash 3 times with 300 µl wash solution

2. Pipet 100 µl enzyme conjugate
   Incubate for 15 minutes at room temperature
   Discard the contents of the wells and wash 3 times with 300 µl wash solution

3. Pipet 100 µl substrate solution
   Incubate for 15 minutes at room temperature

4. Add 100 µl stop solution
   Leave untouched for 5 minutes
   Read at 450 nm