Anti-Annexin V
IgG/IgM

ORG 643

96 Tests

Immunometric Enzyme Immunoassay for the quantitative determination of Anti-Annexin V (IgG and IgM) antibodies

Instruction for use
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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 human recombinant annexin V
combined calibrators with IgG and IgM class anti-annexin V ............6 vials, 1.5 ml each antibodies in a PBS/BSA matrix containing:
IgG and IgM:  0; 6.3; 12.5; 25; 50; 100 in U/ml
Anti-annexin V controls in a PBS/BSA matrix ........................................3 vials, 1.5 ml each
Control 1: positive on IgG
Control 2: positive on IgM
Control 3: negative on IgG and IgM
Anti-annexin V 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
in a PBS/BSA matrix
Enzyme conjugate solution (light red), containing polyclonal ..............1 vial, 15 ml
rabbit anti-h-IgG-IgM, labelled with horseradish peroxidase
in a PBS/BSA matrix
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 three 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: 0 - 100 U/ml
Sensitivity: 1 U/ml
Storage: refrigerated at 2 - 8 °C
Shelf life: 12 months after manufacturing or until the expiration date
print on the labels
Package size: 96 tests

**PRINCIPLE OF THE PROCEDURE**

The Anti-annexin V IgG/IgM is an indirect solid phase enzyme immunometric assay (ELISA). It is designed for the quantitative measurement of IgG or IgM class autoantibodies directed against annexin V. The microplate is coated with highly purified human recombinant annexin V.

The microplate 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 will be 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 immobilised 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'-Teramethylbenzidine) 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 or IgM antibodies present in the original sample, respectively.
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**

Anti-phospholipid binding protein antibodies represent a family of immunoglobulins which recognise phospholipids, alone or bound to plasma protein cofactor(s), or the cofactors themselves (1). A variety of proteins have been implicated including: prothrombin, β2-glycoprotein I, protein S and annexin V (13). Autoimmune anti-phospholipid-protein antibodies are associated with a variety of thromboembolic complications involving both arterial and venous sites, as well as recurrent miscarriages and thrombocytopenia (3). The pathophysiologic mechanisms of this antiphospholipid syndrome (APS) have not yet been identified (10).

Annexin V, also known as placental anticoagulant protein I (PAP I) (8), not only belongs to a family of proteins that bind to phospholipids in a calcium dependent manner (5), but is also a potent vascular anticoagulant protein (9). Annexin V is necessary to maintain placental integrity and may have a thromboregulatory effect at the materno-fetal interface, as shown in a murine model (14).

It has been suggested, that interference of anti-annexin V antibodies with the annexin V shield on the placental villi leads to the exposure of anionic procoagulant phospholipids. Anionic procoagulant phospholipids are known to enhance blood coagulation processes in the placental vasculature, and may be an important contributory factor of thrombosis and recurrent loss of pregnancy in the APS (9).

Anti-annexin V antibodies are not only highly specific for APS, but also elevated levels of antibodies are detected in patients with systemic lupus diseases (SLE) (4, 6). It has been suggested that anti-annexin V autoantibodies may influence in the clinical course of rheumatoid arthritis (2, 11). A significantly high frequency of arterial or venous thrombosis is found in patients with anti-annexin V antibodies (12). And are a significant risk factor for recurrent miscarriages in SLE patients (7).

**NORMAL VALUES**

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

<table>
<thead>
<tr>
<th>Anti-Annexin V IgG and IgM</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>normal:</td>
<td>&lt; 5 U/ml</td>
</tr>
<tr>
<td>elevated:</td>
<td>5-8 U/ml</td>
</tr>
<tr>
<td>positive:</td>
<td>&gt; 8 U/ml</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-annexin V. The values below should be regarded as guidelines only.

**SPECIFICITY**

The microplate is coated with highly purified human recombinant annexin V. Special coating processes, developed by the manufacturer guarantee for the native immunogenic structure of annexin V after immobilisation on the solid phase. The Anti-Annexin V test kits are specific only for autoantibodies directed against annexin V.

**CALIBRATION**

Since no International reference preparation for annexin V autoantibodies is available, the assay system is calibrated in relative arbitrary units.

**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**
- destilled water
- graduated cylinder for 100 and 1000 ml
- plastic container for storage of the wash solution

**Optional**
- Multi-Chanel Dispenser
- or repeatable pipet for 100 µl
- data reduction software
SPECIMEN COLLECTION AND PREPARATION

For determination of anti-annexin V 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. No interference has been observed with haemolytic, lipemic or bilirubin containing sera. Nor have any interfering effects been noticed with the use of anticoagulants.

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 distilled 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 & C2: positive controls
C3: 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.

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

For Anti-Annexin V IgG and IgM a 4-Parameter-Fit with lin-log coordinates for optical density and concentration is the data reduction method of choice. Smoothed Spline Approximation and log-log coordinates 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-Annexin V IgG. These data are intended for illustration only and should not be used to calculate results from another run.

<table>
<thead>
<tr>
<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</th>
<th>decl.Conc.</th>
<th>CV %</th>
</tr>
</thead>
<tbody>
<tr>
<td>STA</td>
<td>A 1/B 1</td>
<td>0.076</td>
<td>0.074</td>
<td>0.075</td>
<td>0.09</td>
<td>0.09</td>
<td>0.0</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>STB</td>
<td>C 1/D 1</td>
<td>0.286</td>
<td>0.288</td>
<td>0.287</td>
<td>5.5</td>
<td>5.6</td>
<td>5.6</td>
<td>6.3</td>
<td>0.5</td>
</tr>
<tr>
<td>STC</td>
<td>E 1/F 1</td>
<td>0.610</td>
<td>0.629</td>
<td>0.620</td>
<td>12.0</td>
<td>12.4</td>
<td>12.2</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td>STD</td>
<td>G 1/H 1</td>
<td>1.199</td>
<td>1.223</td>
<td>1.211</td>
<td>25.8</td>
<td>26.5</td>
<td>26.2</td>
<td>25.0</td>
<td>1.4</td>
</tr>
<tr>
<td>STE</td>
<td>A 2/B 2</td>
<td>1.803</td>
<td>1.743</td>
<td>1.773</td>
<td>49.3</td>
<td>46.2</td>
<td>47.7</td>
<td>50.0</td>
<td>2.4</td>
</tr>
<tr>
<td>STF</td>
<td>C 2/D 2</td>
<td>2.381</td>
<td>2.318</td>
<td>2.350</td>
<td>109.8</td>
<td>98.0</td>
<td>103.9</td>
<td>100.0</td>
<td>1.9</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>No</th>
<th>Position</th>
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<th>OD 2</th>
<th>Mean</th>
<th>Conc. 1</th>
<th>Conc. 2</th>
<th>Mean</th>
<th>decl.Conc.</th>
<th>CV %</th>
</tr>
</thead>
<tbody>
<tr>
<td>STA</td>
<td>A 1/B 1</td>
<td>0.046</td>
<td>0.045</td>
<td>0.046</td>
<td>0.09</td>
<td>0.09</td>
<td>0.0</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>STB</td>
<td>C 1/D 1</td>
<td>0.272</td>
<td>0.264</td>
<td>0.268</td>
<td>6.0</td>
<td>5.8</td>
<td>5.9</td>
<td>6.3</td>
<td>2.1</td>
</tr>
<tr>
<td>STC</td>
<td>E 1/F 1</td>
<td>0.586</td>
<td>0.570</td>
<td>0.578</td>
<td>12.5</td>
<td>12.1</td>
<td>12.3</td>
<td>12.5</td>
<td>1.7</td>
</tr>
<tr>
<td>STD</td>
<td>G 1/H 1</td>
<td>1.107</td>
<td>1.151</td>
<td>1.129</td>
<td>25.1</td>
<td>26.4</td>
<td>25.8</td>
<td>25.0</td>
<td>2.7</td>
</tr>
<tr>
<td>STE</td>
<td>A 2/B 2</td>
<td>1.723</td>
<td>1.648</td>
<td>1.686</td>
<td>50.6</td>
<td>46.2</td>
<td>47.7</td>
<td>50.0</td>
<td>3.2</td>
</tr>
<tr>
<td>STF</td>
<td>C 2/D 2</td>
<td>2.205</td>
<td>2.206</td>
<td>2.206</td>
<td>102.6</td>
<td>102.8</td>
<td>102.7</td>
<td>100.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>
ASSAY CHARACTERISTICS

**Sensitivity**
The lower detection limit for Anti-Annexin V IgG and IgM was determined at 1.0 U/ml.

**Parallelism**
In dilution experiments sera with high antibody concentrations were diluted with sample buffer and assayed in the Anti-Annexin V kit. The assay shows linearity over the full measuring range.

**Precision**
Statistics for Coefficients of variation (CV) were calculated for each of three samples from the results of 24 determinations in a single run for Intra-Assay precision. Run-to-run precision was calculated from the results of 6 different runs with 8 determinations each:

<table>
<thead>
<tr>
<th>Sample No</th>
<th>Mean [U/ml]</th>
<th>CV [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11</td>
<td>6.7</td>
</tr>
<tr>
<td>2</td>
<td>27</td>
<td>3.8</td>
</tr>
<tr>
<td>3</td>
<td>67</td>
<td>4.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample No</th>
<th>Mean [U/ml]</th>
<th>CV [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9</td>
<td>4.1</td>
</tr>
<tr>
<td>2</td>
<td>23</td>
<td>4.2</td>
</tr>
<tr>
<td>3</td>
<td>64</td>
<td>5.7</td>
</tr>
</tbody>
</table>
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