Anti-Cardiolipin IgG/IgM

ORG 515

96 Tests

Immunometric Enzyme Immunoassay for the quantitative determination of Anti-Cardiolipin (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 Cardiolipin and saturated with human β2-Glycoprotein I
combined calibrators with IgG and IgM class Anti-Cardiolipin .......... 6 vials, 1.5 ml each
antibodies in a PBS/BSA matrix containing:
IgG:  0; 7.5; 15; 30; 60; 120 GPL U/ml and
IgM:  0; 5; 10; 20; 40; 80 MPL U/ml
Anti-Cardiolipin controls in a PBS/BSA matrix........................................ 2 vials, 1.5 ml each
(positive 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
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 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: 7.5 - 120 GPL U/ml
5 - 80 MPL U/ml
Sensitivity: 1 GPL U/ml resp. 0.5 MPL 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
The Anti-Cardiolipin 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 Cardiolipin. The microplate is coated with highly purified cardiolipin and saturated with human β2-Glycoprotein I (β2GPI). β2-Glycoprotein I is known as a cofactor for the binding of Anti-Cardiolipin antibodies. This new coating procedure guarantees reproducible results independent of endogeneous β2GPI.
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 immunised 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 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
Cardiolipin (diphosphatidylglycerine) belongs to the group of negatively charged phospholipids. Antibodies to these phospholipids are described to be often - but not necessarily - identical to the Lupus anticoagulants (LA), a class of antibodies responsible for a prolonged clotting process.
Autoantibodies to cardiolipin are described for various autoimmune diseases. The presence of anti-cardiolipin antibodies in systemic lupus erythematosus (SLE) can be related to the development of thrombosis and thrombocytopenia, in gynaecology they are supposed to cause intrauterine death or recurrent abortion. Furthermore, anti-cardiolipin antibodies have been found in some non-thrombotic neurological disorders like cerebrovascular insufficiency, cerebral ischemia or chorea and in myocardial infarction.
Anti-Cardiolipin autoantibodies are found in the immunoglobulin classes IgG, IgM and/or IgA. The determination of IgM antibodies is a valuable indicator in the diagnosis of beginning autoimmune diseases, whereas IgG antibodies will be found in progressive stages of manifested autoimmune disorders. Anti-Cardiolipin IgG antibodies show a good correlation to the clinical status of the patient in thrombosis, thrombocytopenia, fetal loss and some neurological disorders. Anti-Cardiolipin IgA antibodies are often associated with IgG antibodies. The determination of IgA antibodies seems to have a greater validity in thrombosis and fetal loss.
Major diseases associated with anti-Cardiolipin antibodies are deep vein thrombosis and other thrombotic disorders. Anti-Cardiolipin antibodies will be found in the sera of approx. 60 percent of the SLE patients. The presence of these antibodies is a high risk factor for the development of thromboembolism and thrombocytopenia.
In gynaecology, anti-Cardiolipin antibodies seems to be a good indicator in the prognosis of recurrent abortion or intrauterine death. Furthermore, routine determination of anti-Cardiolipin antibodies in risky pregnancies of women with SLE or thrombosis is recommended. Quantitative measurements of anti-Cardiolipin antibodies, especially IgG, is an important parameter with high specificity in therapy-monitoring.
The presence of anti-Cardiolipin antibodies is also described in neurological diseases. Therefore, cerebral thrombosis may cause ischemia. Non-thrombotic diseases of the central nervous system, like chorea or epilepsy, are also associated with anti-Cardiolipin antibodies.
The anti-Cardiolipin antibody test shows no correlation to the VDRL antibody test (Reagins Test) in the diagnosis of Syphilis. Also no cross-reactivities with anti-DNA antibodies are observed, because both types of antibodies belong to separate antibody populations.
Recent studies have shown that anti-Cardiolipin antibodies require a plasma/serum cofactor for binding to cardiolipin. The cofactor has been identified as β2-glycoprotein I (β2GPI) also called apolipoprotein H, a 50 kDa β2-globulin which occurs in plasma at a level of 200 µg/ml. β2GPI is associated in vivo with lipoproteins, anionic phospholipids, platelets and mitochondria. It has been found that β2GPI inhibits the intrinsic coagulation pathway and the ADP-mediated platelet aggregation. Therefore, it is suggested that β2GPI plays a role in the regulation of blood coagulation.
The presence of \( \beta_2 \text{GPI} \) is absolutely required for the binding of anti-Cardiolipin antibodies to cardiolipin. Recent studies have shown that anti-Cardiolipin antibodies either recognise cardiolipin on its own or the complex of cardiolipin and \( \beta_2 \text{GPI} \).

For ELISA-tests sufficient concentrations of \( \beta_2 \text{GPI} \) could be achieved either by addition of ABS/FCS or by coating with \( \beta_2 \text{GPI} \). The latter version provides constant quality and quantity for maintaining reproducible results.

Indication for determination of anti-Cardiolipin antibodies:

- SLE  - Thrombosis
- Thrombocytopenia  - Cerebral Ischemia
- Chorea  - Epilepsy
- Recurrent Abortion  - Intrauterine Death

**NORMAL VALUES**

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

<table>
<thead>
<tr>
<th>Anti-Cardiolipin-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; 7</td>
</tr>
<tr>
<td>elevated:</td>
<td>&gt; 10</td>
<td>&gt; 7</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-Cardiolipin. The values below should be regarded as guidelines only.

**SPECIFICITY**

The microplate is coated with highly purified Cardiolipin and human \( \beta_2 \)-Glycoprotein I. Special coating processes, developed by the manufacturer guarantee for the native immunogenic structure of Cardiolipin after immobilisation on the solid phase. The Anti-Cardiolipin test kits are specific only for autoantibodies directed against Cardiolipin or to the complex of Cardiolipin and \( \beta_2 \)-Glycoprotein I.

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

**CALIBRATION**

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

1. Hughes, G.R.V., Harris, E.N. and Gharavi, A.E.
   The Anticardiolipin Syndrome.

2. Domke, N. and Siegert, G.
   Phospholipidantikörper and ihre klinische Bedeutung.

3. Triplett, D.A.
   Coagulation Assays for the Lupus Anticoagulant: Review and Critique of Current Methodology.

4. Harris, E.N.
   Serological Detection of Antiphospholipid Antibodies.

   Detection of Beta-2-Glycoprotein-I-Dependend Antiphospholipid Antibodies and Anti-Beta-2-Glycoprotein-I Antibody in Patients with Systemic Lupus Erythematosus and in Patients with Syphilis.

   Clinical Significante of a Single Test for Anti-Cardiolipin Antibodies in Patients with Systemic Lupus Erythematosus.

7. Khalili, A. and Cooper, R.C.

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-Cardiolipin 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, excep 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 accomodate 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>
</tr>
<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 Anti-Cardiolipin 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-Cardiolipin 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>0.050</td>
<td>0.050</td>
<td>0.050</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>STB</td>
<td>C 1/D 1</td>
<td>0.322</td>
<td>0.300</td>
<td>0.311</td>
<td>7.8</td>
<td>7.0</td>
<td>7.4</td>
<td>7.5</td>
<td>7</td>
</tr>
<tr>
<td>STC</td>
<td>E 1/F 1</td>
<td>0.520</td>
<td>0.519</td>
<td>0.520</td>
<td>15.2</td>
<td>15.2</td>
<td>15.2</td>
<td>15.0</td>
<td>0</td>
</tr>
<tr>
<td>STD</td>
<td>G 1/H 1</td>
<td>0.803</td>
<td>0.824</td>
<td>0.814</td>
<td>29.2</td>
<td>30.5</td>
<td>29.9</td>
<td>30.0</td>
<td>3</td>
</tr>
<tr>
<td>STE</td>
<td>A 2/B 2</td>
<td>1.226</td>
<td>1.191</td>
<td>1.209</td>
<td>61.6</td>
<td>58.2</td>
<td>59.9</td>
<td>60.0</td>
<td>3</td>
</tr>
<tr>
<td>STF</td>
<td>C 2/D 2</td>
<td>1.640</td>
<td>1.637</td>
<td>1.638</td>
<td>120.5</td>
<td>119.9</td>
<td>120.2</td>
<td>120.0</td>
<td>0</td>
</tr>
</tbody>
</table>
ASSAY CHARACTERISTICS

Sensitivity
The lower detection limit for Anti-Cardiolipin IgG was determined at 1.0 GPL U/ml. Anti-Cardiolipin IgM yielded a sensitivity of 0.5 MPL U/ml.

Parallelism
In dilution experiments sera with high IgG- and IgM-antibody concentrations were diluted with sample buffer and assayed in the Anti-Cardiolipin kit.

<table>
<thead>
<tr>
<th>Anti-Cardiolipin</th>
<th>Sample</th>
<th>Dilution</th>
<th>Observed [U/ml]</th>
<th>Expected [U/ml]</th>
<th>O/E</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>1</td>
<td>1:200</td>
<td>126.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:400</td>
<td>63.7</td>
<td>63.4</td>
<td>100 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:800</td>
<td>32.9</td>
<td>31.7</td>
<td>104 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:1600</td>
<td>14.1</td>
<td>15.8</td>
<td>89 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:3200</td>
<td>7.2</td>
<td>7.9</td>
<td>91 %</td>
</tr>
<tr>
<td>IgG</td>
<td>2</td>
<td>1:100</td>
<td>112.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:200</td>
<td>56.1</td>
<td>56.2</td>
<td>100 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:400</td>
<td>25.0</td>
<td>28.1</td>
<td>89 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:800</td>
<td>12.0</td>
<td>14.0</td>
<td>86 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:1600</td>
<td>6.0</td>
<td>7.0</td>
<td>86 %</td>
</tr>
<tr>
<td>IgM</td>
<td>3</td>
<td>1:100</td>
<td>55.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:200</td>
<td>27.0</td>
<td>27.5</td>
<td>98 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:400</td>
<td>13.0</td>
<td>13.8</td>
<td>94 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:800</td>
<td>6.4</td>
<td>6.9</td>
<td>93 %</td>
</tr>
<tr>
<td>IgM</td>
<td>4</td>
<td>1:200</td>
<td>46.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:400</td>
<td>23.2</td>
<td>23.3</td>
<td>100 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:800</td>
<td>10.9</td>
<td>11.6</td>
<td>94 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:1600</td>
<td>5.2</td>
<td>5.8</td>
<td>90 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1:3200</td>
<td>2.8</td>
<td>2.9</td>
<td>97 %</td>
</tr>
</tbody>
</table>
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 5 different runs with 6 determinations each:

<table>
<thead>
<tr>
<th>anti-Cardiolipin (IgG)</th>
<th>anti-Cardiolipin (IgM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intra-Assay</strong></td>
<td><strong>Intra-Assay</strong></td>
</tr>
<tr>
<td>Sample No</td>
<td>Mean [GPL U/ml]</td>
</tr>
<tr>
<td>1</td>
<td>29.1</td>
</tr>
<tr>
<td>2</td>
<td>62.5</td>
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<tr>
<td>3</td>
<td>109.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Inter-Assay</strong></th>
<th><strong>Inter-Assay</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample No</td>
<td>Mean [GPL U/ml]</td>
</tr>
<tr>
<td>1</td>
<td>32.9</td>
</tr>
<tr>
<td>2</td>
<td>70.9</td>
</tr>
<tr>
<td>3</td>
<td>118.3</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**