Rheumatoid Factor IgA

ORG 522A

96 Tests

Immunometric Enzyme Immunoassay for the quantitative measurement of IgA Rheumatoid Factors in serum

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'-Tetramethylbenzidine). 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 the FC fragment of human Immunoglobulin G

calibrators with Rheumatoid Factors of IgA class in a PBS/BSA ........ 5 vials, 1.5 ml each
matrix, containing: 0; 15; 50; 150; 500 U/ml
Rheumatoid Factor controls in PBS/BSA matrix (positive and ............ 2 vials, 1.5 ml each
negative), for the respective concentrations see the enclosed package insert

sample buffer, yellow, Concentrate ............................................... 1 vial, 20 ml
enzyme conjugate solution (light red), containing polyclonal ............ 1 vial, 15 ml
rabbit anti-h-IgA-IgG, labelled with horseradish peroxidase in 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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample material</td>
<td>serum or plasma</td>
</tr>
<tr>
<td>Required sample volume</td>
<td>10 µl of sample to be diluted 1:100 with sample buffer</td>
</tr>
<tr>
<td></td>
<td>100 µl prediluted sample per single determination</td>
</tr>
<tr>
<td>Total incubation time</td>
<td>60 minutes at room temperature (20 - 28 °C)</td>
</tr>
<tr>
<td>Calibration range</td>
<td>15 - 500 U/ml</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>1 U/ml</td>
</tr>
<tr>
<td>Storage</td>
<td>refrigerated at 2 - 8 °C</td>
</tr>
<tr>
<td>Shelf life</td>
<td>12 months after manufacturing or until the expiration date printed on the labels</td>
</tr>
<tr>
<td>Package size</td>
<td>96 tests</td>
</tr>
</tbody>
</table>

PRINCIPLE OF THE PROCEDURE

Rheumatoid Factor is an indirect solid phase enzyme immunometric assay (ELISA). It is designed for the quantitative measurement of IgA rheumatoid factors. The microplate is coated with the FC fragment of highly purified human Immunoglobulin G.

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 are pipetted into the wells of the microplate. Any present rheumatoid factors bind to the FC fragment coated on 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:**
Anti-h-IgA-horseradish peroxidase conjugate solution is pipetted into the wells of the microplate to recognise rheumatoid factors of the respective class bound to the immobilized antigen. 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 rheumatoid factors 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 presence of IgM Rheumatoid Factor (RF) in the serum is the sole serological indicator included in the ACR list of criteria for the diagnosis of RA. RFs are a subset of antiglobulins directed against the FC region of IgG. We do not include in this definition antibodies to the IgG Fab region and pepsin agglutinators, directed against neoantigens on IgG exposed by pepsin cleavage. It is claimed that the majority of antiglobulin activity in normal serum is Fab-specific, whereas antiglobulin from RA patients is mostly FC-specific. RFs are present in the serum of 75-80% of patients with RA at some time during the disease course. However, RFs are also found in the serum of patients with infectious and autoimmune diseases, hyperglobulinemias, B-cell lymphoproliferative disorders and in the aged population. This suggests that RF may be a finding associated with B-cell hyperactivity.

Rheumatoid Factors which have been found among the IgM, IgG and IgA classes of immunoglobulins, reacting only with xenogeneic FC are not autoantibodies and are unlikely to be of pathological significance. However, RFs can bind IgG from many species, including autologous IgG, when immobilized on surfaces. Autologous binding is of higher affinity than xenogeneic binding. The here presented test systems for the determination of rheumatoid factors uses only human FC-fragments as coated antigen.

It is generally considered that high titer RFs are associated with more severe disease and the presence of extra-articular features and rheumatoid nodules. This conclusion may depend on the disease duration. Serum IgM RF may precede the onset of RA by several years. A high titer of RF in non-RA individuals is associated with increased risk of developing RA. In the first 2 years of RA (early RA), serum levels of IgM, IgG and IgA RF do not correlate with disease activity. Serum IgG and IgA RF in these years are prognostic of erosive joint disease.

In established RA, high titer serum IgM RF correlates with the presence of articular disease and nodules but not with systemic disease activity. The presence of either IgG or IgA RF in patients with longstanding RA may be a good prognostic indicator of systemic manifestations. IgG and IgM RF are associated with extra-articular RA including rheumatoid vasculitis and nodules. The presence of IgM RF - containing immune complexes with bound complement (C1q) - is also associated with extra-articular RA.

NORMAL VALUES

In a normal range study with serum samples from healthy blood donors the following ranges have been established with the Rheumatoid Factor test:

<table>
<thead>
<tr>
<th>Rheumatoid Factor IgA [U/ml]</th>
</tr>
</thead>
<tbody>
<tr>
<td>normal:</td>
</tr>
<tr>
<td>elevated:</td>
</tr>
<tr>
<td>&lt; 20</td>
</tr>
<tr>
<td>&gt; 20</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 Rheumatoid Factors.

SPECIFICITY

The microplate is coated with the FC fragment of highly purified human Immunoglobulin G. The test kit is specific for all classes of rheumatoid factors.
CALIBRATION

The quantitative test system for Rheumatoid Factor IgA is calibrated in relative arbitrary units. The calibration is related to the 1st British Standard Preparation 64/2. This material tests positive for IgA Rheumatoid Factors.

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 Rheumatoid Factors serum is the preferred sample matrix. All serum 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 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.

<p>| | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>SA</td>
<td>SE</td>
<td>P2</td>
<td>P..</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>SA</td>
<td>SE</td>
<td>P2</td>
<td>P..</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>SB</td>
<td>C1</td>
<td>P3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>SB</td>
<td>C1</td>
<td>P3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>SC</td>
<td>C2</td>
<td>P4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>SC</td>
<td>C2</td>
<td>P4</td>
<td></td>
<td></td>
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<tr>
<td>G</td>
<td>SD</td>
<td>P1</td>
<td>P5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>SD</td>
<td>P1</td>
<td>P5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

2. Pipet 100 µl of calibrators, controls and prediluted patient samples into the wells.
3. Incubate for 30 minutes at room temperature (20 - 28 ºC).
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 Rheumatoid Factor IgA a 4-Parameter-Fit with lin-log co-ordinates 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 Rheumatoid Factor IgA. 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 decl.Conc.</th>
<th>CV %</th>
</tr>
</thead>
<tbody>
<tr>
<td>STA</td>
<td>A 1/B 1</td>
<td>0.029</td>
<td>0.031</td>
<td>0.030</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>STB</td>
<td>C 1/D 1</td>
<td>0.232</td>
<td>0.214</td>
<td>0.223</td>
<td>16.4</td>
<td>14.9</td>
<td>15.6</td>
<td>4</td>
</tr>
<tr>
<td>STC</td>
<td>E 1/F 1</td>
<td>0.563</td>
<td>0.536</td>
<td>0.550</td>
<td>50.2</td>
<td>47.2</td>
<td>48.7</td>
<td>3</td>
</tr>
<tr>
<td>STD</td>
<td>G 1/H 1</td>
<td>1.263</td>
<td>1.181</td>
<td>1.222</td>
<td>160.6</td>
<td>143.8</td>
<td>152.2</td>
<td>5</td>
</tr>
<tr>
<td>STE</td>
<td>A 2/B 2</td>
<td>2.190</td>
<td>2.118</td>
<td>2.154</td>
<td>522.3</td>
<td>474.3</td>
<td>498.4</td>
<td>3</td>
</tr>
</tbody>
</table>
**ASSAY CHARACTERISTICS**

**Sensitivity**
The lower detection limit for Rheumatoid Factor IgA was determined at 1.0 U/ml.

**Parallelism**
In dilution experiments sera with high IgA-antibody concentrations were diluted with sample buffer and assayed in the Rheumatoid Factor IgA kit.

<table>
<thead>
<tr>
<th>Rheumatoid Factor</th>
<th>Sample No.</th>
<th>Dilution</th>
<th>Observed [U/ml]</th>
<th>Expected [U/ml]</th>
<th>O/E</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgA</td>
<td>1</td>
<td>1:100</td>
<td>253.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1:200</td>
<td>132.2</td>
<td>126.5</td>
<td>105 %</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1:400</td>
<td>64.4</td>
<td>63.3</td>
<td>102 %</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1:800</td>
<td>32.2</td>
<td>31.6</td>
<td>102 %</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1:1600</td>
<td>15.7</td>
<td>15.8</td>
<td>99 %</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1:3200</td>
<td>7.5</td>
<td>7.9</td>
<td>95 %</td>
<td></td>
</tr>
<tr>
<td>IgA</td>
<td>2</td>
<td>1:200</td>
<td>223.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1:400</td>
<td>108.4</td>
<td>111.9</td>
<td>97 %</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1:800</td>
<td>56.3</td>
<td>56.0</td>
<td>101 %</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1:1600</td>
<td>24.9</td>
<td>28.0</td>
<td>89 %</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1:3200</td>
<td>13.8</td>
<td>14.0</td>
<td>99 %</td>
<td></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>Intra-Assay</th>
<th>Inter-Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample No</td>
<td>Mean [U/ml]</td>
</tr>
<tr>
<td>1</td>
<td>38.4</td>
</tr>
<tr>
<td>2</td>
<td>93.0</td>
</tr>
<tr>
<td>3</td>
<td>327.2</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