Anti-alpha-Fodrin IgG/IgA

ORG 642

96 Tests

Immunometric Enzyme Immunoassay for the quantitative determination of anti-alpha-Fodrin autoantibodies

Instruction for use
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, .....................1
coated with human alpha-Fodrin
Calibrator combined IgG/IgA class anti-alpha-Fodrin
antibodies in a PBS/BSA matrix containing ........................................6 vials, 1.5 ml each
0; 6.3; 12.5; 25; 50 and 100 U/ml
Anti-alpha-Fodrin controls in a PBS/BSA matrix...................................3 vials, 1.5 ml each
Control 1 positive on IgG
Control 2 positive on IgA
Control 3 negative on IgG and IgA
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-IgA-IgG; 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 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 printed on the labels
Package size: 96 tests

PRINCIPLE OF THE PROCEDURE

The Anti-alpha-Fodrin IgG/IgA is an indirect solid phase enzyme immunometric assay (ELISA). The microplate is coated with human alpha-Fodrin. And the ELISA is designed for the quantitative measurement of IgG and IgA class autoantibodies directed against alpha-Fodrin.

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 horseradish peroxidase conjugate solution is pipetted into the wells of the microplate to recognise IgG or IgA 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 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

Alpha-Fodrin is an intracellular, actin-binding, organ-specific protein of the cytoskeleton. It is a dimer composed of an alpha- and a beta-subunit. The network of actin and fodrin situated below the plasma membrane of secretorial cells, is important for the alignment of secretorial vesicles to the plasma membrane during secretorial processes.

During apoptosis the alpha-fodrin dimer is cleaved into a 120 kDa breakdown product, which is found abundantly in the salivary gland [2, 6]. This proteolysis of fodrin may be a consequence of protease activation during apoptosis [11]. The cleavage product of 120 kDa alpha-fodrin was found to be an important autoantigen in the pathogenesis of organ-specific autoimmune response [8]. Clinical studies have shown, that in patients with Sjögren Syndrome alpha-fodrin is involved in the stimulation of peripheral blood T-cells [2]. These findings suggest, that an increase in protease activity and the stimulation of T-cells play an important role in the alpha-fodrin proteolysis during the development of primary Sjogren's Syndrome [7].

The Sjogren's Syndrome is an autoimmune disorder affecting lachrymal and salivary glands (Sicca symptomatic). It is characterised by keratoconjunctivitis and dryness of eyes and of the mouth. Sjogren's syndrome is elicited by lymphocytic infiltration of the lachrymal and salivary glands [9]. Sicca syndrome frequently affects patients with Grave's ophthalmopathy [13].

The diagnostic markers for Sjogren's syndrome are anti-SS-A/Ro and anti-SS-B/La antibodies directed against intracellular antigens [1, 3]. Juvenile Sjogren's syndrome cases have been reported to be negative for both anti-SS-A and anti-SS-B antibodies. Therefore, it is necessary to define highly specific autoantibodies characteristic for Sjogren's syndrome.

Antibodies directed against alpha-fodrin have been described in articles published in 1997 and 1999 to be present in adults with primary (95% and 78%) as well as secondary Sjogren's syndrome (62.5% and 60%) [2, 5, 12]. Furthermore, they are found in children with Sjogren's syndrome [5]. Takahashi et al. described these antibodies in children with Rheumatoid Arthritis (JRA; n=5/9) and with SLE (n=5/6) [10].

During an international congress in 1999 controversial data was presented regarding the prevalence of IgG and IgA antibodies directed against alpha-fodrin. A more recent study demonstrates a significantly higher prevalence of immunoglobulins class IgG [13]. In patients with SLE or Grave's ophthalmopathy and antibodies directed against alpha-fodrin, no correlation was found to antibodies directed against SS-A or SS-B [5, 13].

Kobayashi et al. detected anti-alpha-fodrin autoantibodies before anti-SS-A or anti-SS-B antibodies became positive [4]. Thus, the authors conclude, that anti-alpha-fodrin antibodies could be a useful marker for the early diagnosis of SS.

Antibodies directed against alpha-fodrin are likely to be a reliable diagnostic marker for Sjogren's syndrome, particularly during the early stages of the pathogenesis of this disorder [4, 5]. According to the latest findings, routine screening for antibodies directed against alpha-fodrin can be a useful tool in diagnosing Sjogren's syndrome in an early stage.

NORMAL VALUES

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

<table>
<thead>
<tr>
<th>Anti-alpha-Fodrin</th>
<th>[U/ml]</th>
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<tbody>
<tr>
<td>normal:</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>elevated:</td>
<td>&gt; 10</td>
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</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.

**SPECIFICITY**

The microplate for Anti-alpha-Fodrin is coated with human alpha-Fodrin.
The test kit is specific only for autoantibodies directed to the respective antigen. No crossreactivities have been observed.

**CALIBRATION**

Since no international reference preparation for anti-alpha-Fodrin 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
- distilled 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-alpha-Fodrin 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 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.
2. Pipet **100 µl of calibrators, controls and prediluted patient samples** into the wells.
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 the Anti-alpha-Fodrin antibody test a 4-Parameter-Fit with lin-log coordinates for optical density and concentration is recommended. 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.
ASSAY CHARACTERISTICS

Sensitivity
The lower detection limits for Anti-alpha-Fodrin IgG/IgA 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-alpha-Fodrin 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 24 determinations each:

<table>
<thead>
<tr>
<th>Sample No</th>
<th>Mean [U/ml]</th>
<th>CV [%]</th>
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<tbody>
<tr>
<td>1</td>
<td>15.9</td>
<td>4.0</td>
</tr>
<tr>
<td>2</td>
<td>59.0</td>
<td>4.0</td>
</tr>
<tr>
<td>3</td>
<td>137.0</td>
<td>2.2</td>
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</table>

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<th>Sample No</th>
<th>Mean [U/ml]</th>
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</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15.7</td>
<td>2.9</td>
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<tr>
<td>2</td>
<td>58.1</td>
<td>1.1</td>
</tr>
<tr>
<td>3</td>
<td>144.0</td>
<td>3.4</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