Enterovirus ELISA
IgG Testkit / IgA Testkit / IgM Testkit

Order No.:
EC116G00 (IgG Testkit)
EC116A00 (IgA Testkit)
EC116M00 (IgM Testkit)

Color Coding:
IgA: colorless
IgG: brown/dark brown
IgM: teststrips brown
IgM: reference strips brown/transparent

FOR IN VITRO DIAGNOSIS ONLY

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1. **Intended Use**

The ELISA is intended for the detection of group-specific IgG-, IgA- and IgM-antibodies against Enteroviruses in human serum.

2. **Diagnostic Relevance**

Human enteroviruses are small RNA viruses belonging to the Picornaviridae family (Italian pico = small). The enterovirus group includes the Coxsackie viruses with subgroups A (23 serotypes) and B (6 serotypes), the polioviruses (3 serotypes), the echoviruses (31 serotypes) and the enteroviruses type 68-71 (1,3).

Infections due to enteroviruses occur worldwide. In temperate climates, there is an increased seasonal incidence in late summer and autumn. Children are most often affected. Transmission is mainly by the faecal-oral route, but droplet infection is also possible. The portal of entry is usually the respiratory tract and the intestine. The incubation period can last from 12 hours to up to 35 days. Although enteroviruses cause asymptomatic infections in 90-95% of cases, they are the cause of a range of clinically relevant diseases. These include aseptic meningitis, herpangina, hand, foot and mouth disease, haemorrhagic meningitis and diseases of the respiratory tract and also of internal organs. All enteroviruses can cause uncharacteristic, febrile infections that are known as "summer flu". They are responsible for about 15% of infections in the upper respiratory tract. Treatment is symptomatic and depends on the affected organ system (1,2,3).

In the diagnosis of enteroviruses, detection of antibodies has become accepted alongside detection of antigen. The serological test is performed routinely with CBR and ELISA. Specific antibodies are usually present in the serum during the acute phase. A definite conclusion about acute infection is possible only if there are significant increases in titre (IgG/IgM) (2). It has been shown that an acute coxsackie B (CVB) infection can also be diagnosed by measuring specific IgA (4,5). IgM antibodies can usually be detected in CVB infections over a period of 6 to 8 weeks. In rare cases, CVB IgM can also persist in otherwise healthy persons for 6 months after aseptic meningitis. The finding of CVB IgM in otherwise healthy persons for months or years does not automatically exclude the persistence of the pathogen. In some cases, particularly with repeated enterovirus infections, only the simultaneous measurement of specific CVB IgM, IgA and IgG permits a diagnostic conclusion (1,5).

3. **Test Principle**

The antibody (IgG, IgA) searched for in the human serum forms an immune complex with the antigen coated on the microtiter-plate. Unbound immunoglobulins are removed by washing processes. The enzyme conjugate attaches to this complex. Unbound conjugate is again removed by washing processes. After adding the substrate solution (TMB), a blue dye is produced by the bound enzyme (peroxidase). The color changes to yellow when the stopping solution is added.

The antibody (IgM) searched for in the human serum reacts as described for IgG and IgA. Additionally to the antigen-coated microtiter plate (teststrips) a second microtiter plate (reference strips) is tested. The difference in color intensity between teststrips and reference strips is an indicator for the number of antibodies bound.

4. **Package Contents**

4.1 **IgG Testkit**

1. 1 Microtiter-Plate consisting of 96 wells with antigen coated, breakable single wells, lyophilised
2. PBS-Dilution Buffer (blue, ready to use) 2x50ml, pH 7.2, with preservative and Tween 20
3. PBS-Washing Solution (20x concentrated) 50ml, pH 7.2, with preservative and Tween 20
4. IgG negative Control, 2000µl, human serum with protein-stabilizer and preservative, ready to use
5. IgG cut-off Control, 2000µl, human serum with protein-stabilizer and preservative, ready to use
6. IgG positive Control, 2000µl, human serum with protein-stabilizer and preservative, ready to use
7. IgG-Conjugate (anti-human), 11ml, (sheep or goat)-horseradish-peroxidase-conjugate with protein-stabilizer and preservative in Tris-Buffer, ready to use
8. Tetramethylbenzidine substrate solution (3,3',5,5'-TMB), 11ml, ready to use
9. Citrate-Stopping Solution, 6ml, contains an acid mixture
4.2 IgA Testkit
1. Microtiter-Plate consisting of 96 with antigen coated, breakable single wells, lyophilised
2. PBS-Dilution Buffer (blue, ready to use) 2x50ml, pH 7.2, with preservative and Tween 20
3. PBS-Washing Solution (20x concentrated) 50ml, pH 7.2, with preservative and Tween 20
4. IgA negative Control, 2000µl, human serum with protein-stabilizer and preservative, ready to use
5. IgA cut-off Control, 2000µl, human serum with protein-stabilizer and preservative, ready to use
6. IgA positive Control, 2000µl, human serum with protein-stabilizer and preservative, ready to use
7. IgA-Conjugate (anti-human), 11ml, (sheep or goat)-horseradish-peroxidase-conjugate with FCS and preservative in Tris-Buffer, ready to use
8. Tetramethylbenzidine substrate solution (3,3',5,5'-TMB), 11ml, ready to use
9. Citrate-Stopping Solution, 6ml, contains an acid mixture

4.3 IgM Testkit
Box 1
1. Microtiter-Plate (teststrips) consisting of 96 with antigen coated, breakable single wells, lyophilised
2. PBS-Dilution Buffer (blue, ready to use) 3x50ml, pH 7.2, with preservative and Tween 20
3. Tetramethylbenzidine substrate solution (3,3',5,5'-TMB), 2x11ml, ready to use

Box 2
1. Microtiter-Plate (reference strips) consisting of 96 coated, breakable single wells, lyophilised
2. PBS-Washing Solution (20x concentrated) 2x50ml, pH 7.2, with preservative and Tween 20
3. IgM negative Control, 4000µl, human serum with protein-stabilizer and preservative, ready to use
4. IgM cut-off Control, 4000µl, human serum with protein-stabilizer and preservative, ready to use
5. IgM positive Control, 4000µl, human serum with protein-stabilizer and preservative, ready to use
6. IgM-Conjugate (anti-human), 2x11ml, (sheep or goat)-horseradish-peroxidase-conjugate with FCS and preservative in Tris-Buffer, ready to use
7. Citrate-Stopping Solution, 2x6ml, contains an acid mixture

5. Storage and Shelflife of the Testkit and the ready to use reagents
Store the testkit at 2-8°C. The shelf life of all components is shown on each respective label; for the kit shelf life please see Quality Control Certificate.

1. Microtiter strips/single wells are to be resealed in package after taking out single wells and stored with desiccant at 2-8°C. Reagents should immediately be returned to storage at 2-8°C after usage.
2. The ready to use conjugate and the TMB-substrate solution are sensitive to light and have to be stored in dark. Should there be a color reaction of the substrate dilution due to incidence of light, it is not useable anymore.
3. Take out only the amount of ready to use conjugate or TMB needed for the test insertion. Additional conjugate or TMB taken out may not be returned but must be dismissed.

<table>
<thead>
<tr>
<th>Material</th>
<th>Status</th>
<th>Storage</th>
<th>Shelflife</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test Samples</td>
<td>Diluted</td>
<td>+2 to +8°C</td>
<td>max. 6h</td>
</tr>
<tr>
<td></td>
<td>Undiluted</td>
<td>+2 to +8°C</td>
<td>1 week</td>
</tr>
<tr>
<td>Controls</td>
<td>After Opening</td>
<td>+2 to +8°C</td>
<td>3 months</td>
</tr>
<tr>
<td>Microtitreplate</td>
<td>After Opening</td>
<td>+2 to +8°C (storage in the provided bag with desiccant)</td>
<td>3 months</td>
</tr>
<tr>
<td>Rheumatoid factor - Absorvent</td>
<td>Undiluted, After Opening</td>
<td>+2 to +8°C</td>
<td>3 months</td>
</tr>
<tr>
<td></td>
<td>Diluted</td>
<td>+2 to +8°C</td>
<td>1 week</td>
</tr>
<tr>
<td>Conjugate</td>
<td>After Opening</td>
<td>+2 to +8°C (protect from light)</td>
<td>3 months</td>
</tr>
<tr>
<td>Tetramethylbenzidine</td>
<td>After Opening</td>
<td>+2 to +8°C (protect from light)</td>
<td>3 months</td>
</tr>
<tr>
<td>Stop Solution</td>
<td>After Opening</td>
<td>+2 to +8°C</td>
<td>3 months</td>
</tr>
<tr>
<td>Washing Solution</td>
<td>Final Dilution (ready-to-use)</td>
<td>+2 to +25°C</td>
<td>4 weeks</td>
</tr>
</tbody>
</table>
6. Precautions and Warnings

1. Only sera which have been tested and found to be negative for HIV-1 antibodies, HIV-2 antibodies, HCV antibodies and Hepatitis-B surface-antigen are used as control sera. Nevertheless, samples, diluted samples, controls, conjugates and microtiter strips should be treated as potentially infectious material. Please handle products in accordance with laboratory directions.

2. Those components that contain preservatives, the Citrate Stopping Solution and the TMB have an irritating effect to skin, eyes and mucous. If body parts are contacted, immediately wash them under flowing water and possibly consult a doctor.

3. The disposal of the used materials has to be done according to the country-specific guidelines.

7. Material required but not supplied

1. Aqua dest./demin.
2. Eight-channel pipette 50µl, 100µl
3. Micropipettes: 10µl, 100µl, 1000µl
4. Test tubes
5. Paper towels or absorbent paper
6. Cover for ELISA-plates
7. Disposal box for infectious material
8. ELISA handwasher or automated EIA plate washing device
9. ELISA plate spectrophotometer, wavelength = 450nm, reference length = 620nm (Reference Wavelength 620-690nm)
10. Incubator

8. Test Procedure

Working exactly referring to the Sekisui Virotech user manual is the prerequisite for obtaining correct results.

8.1 Examination Material

Either serum or plasma can be used as test material, even if only serum is mentioned in the instructions. Any type of anticoagulant can be used for plasma. Always prepare patient-dilution freshly. For a longer storage the sera must be frozen. Repeated defrosting should be avoided.

1. Only fresh non-inactivated sera should be used.
2. Hyperlipaemic, haemolytic, microbially contaminated and turbid sera should not to be used (false positive/negative results).

8.2 Preparation of Reagents

The Sekisui Virotech System Diagnostica offers a high degree of flexibility regarding the possibility to use the dilution buffer, washing solution, TMB, citrate stopping solution as well as the conjugate for all parameters and for all different lots. The ready to use controls (positive control, negative control, cut-off control) are parameter specific and only to use with the plate lot indicated in the Quality Control Certificate.

1. Set incubator to 37°C and check proper temperature setting before start of incubation.
2. Bring all reagents to room temperature before opening package of microtiter strips.
3. Shake all liquid components well before use.
4. Make up the washing solution concentrate to 1 L with distilled or demineralised water. If crystals have formed in the concentrate, please bring the concentrate to room temperature before use and shake well before use.
5. High IgG-titer or rheumatoid factors may disturb the specific detection of IgM-antibodies and may lead to false positive resp. false negative results. For a correct IgM-determination it is therefore necessary to pre-treat the sera with RF-SorboTech (VIROTECH adsorbent). For IgM-controls a pre-absorbent treatment is not necessary.

8.3 Virotech ELISA Test Procedure

Each sample has to be tested on the teststrip (Enterovirus antigen) as well as on the reference strip (control antigen) for IgM. The respective number of antigen- and control antigen strips have to be put into the frame next to each other before carrying
Please use test strips and reference strips only with lot numbers indicated in the Quality Control Certificate. IgG and IgA are tested each with one plate only.

1. For each test run, pipette 100µl each of ready to use dilution buffer (blank), IgG-, IgM- and IgA-positive, negative and cut-off controls as well as diluted patient sera. We propose a double insertion (blank, controls and patient sera); for cut-off control a double insertion is absolutely necessary. Working dilution of patient sera: 1+100; e.g. 10µl serum + 1ml dilution buffer.

2. After pipetting start incubation for 30 min. at 37°C (with cover).

3. End incubation period by washing microtiter strips 4 times with 350–400µl washing solution per well. Do not leave any washing solution in the wells. Remove residues on a cellulose pad.

4. Pipette 100µl of ready to use conjugate into each well.

5. Incubation of conjugates: 30 min. at 37°C (with cover).

6. Stop conjugate incubation by washing 4 times ( pls. refer to point 3 above).

7. Pipette 100µl of ready to use TMB into each well.

8. Incubation of substrate solution: 30 min. at 37°C (with cover, keep in dark).

9. Stopping of substrate reaction: pipette 50µl of citrate stopping solution into each well. Shake plate carefully and thoroughly until liquid is completely mixed and a homogeneous yellow color is visible.

10. Measure extinction (OD) at 450/620nm (Reference Wavelength 620-690nm). Set your photometer in such a way that the blank value is deducted from all other extinctions. Extinctions should be measured within 1 hour after adding the stopping solution!

Please refer to last page for Test Procedure Scheme

8.4 Usage of ELISA processors

All Sekisui Virotech ELISAs can be used on ELISA processors. The user is bound to proceed a validation of the devices (processors) on a regular basis.

Sekisui Virotech recommends the following procedure:

1. Sekisui Virotech recommends to proceed the validation of device referring to the instructions of the device manufacturer during the implementation of the ELISA processor respectively after bigger reparations.

2. It is recommended to check the ELISA-processor with the Validationkit (EC250.00) afterwards. A regular check using the Validationkit shall be proceeded minimum once a quarter to test the accuracy of the processor.

3. The release criteria of the Quality Control Certificate of the product must be fulfilled for each test run.

With this procedure, your ELISA processor will function properly and this will support quality assurance in your laboratory.

9. Test Evaluation

The ready to use controls serve for a semiquantitative determination of specific IgG-, IgA- and IgM-antibodies. Their concentration can be expressed in Virotech units = VE. Fluctuations resulting from the test procedure can be balanced with this calculation method and a high reproducibility is achieved in this way. Use the means of the OD values for calculation of the VE.

9.1 Test function control (IgG and IgA)

a) OD-values

The OD of the blank should be < 0.15.

The OD-values of the negative controls should be lower than the OD-values mentioned in the Quality Control Certificate. The OD-values of the positive controls as well as of the cut-off controls should be above the OD-values mentioned in the Quality Control Certificate.

b) Virotech Units (VE)

The Virotech Units (VE) of the cut-off controls are defined as 10 VE. The calculated VE of the positive controls should be within the ranges mentioned in the Quality Control Certificate.

If those requirements (OD-values, VE) are not fulfilled, the test has to be repeated.
9.2 Test function control (IgM)

1. The blank value on the test strip has to be deducted from all extinctions of positive control, cut-off control, negative control and patient sera measured on the test strips (= "test values").

2. The blank value on the reference strip has to be deducted from all extinctions of positive control, cut-off control, negative control and patient sera measured on the reference strips (= "reference values").

3. Now the "differences test values minus reference values" have to be calculated for all positive controls, negative controls and patient sera, by substracting the reference values from the test values.

a) OD-values

The difference between test value minus reference value of the negative controls should be lower than the OD-values mentioned in the Quality Control Certificate. The difference between test value minus reference value of the positive controls as well as the cut-off controls should be above the OD-values mentioned in the Quality Control Certificate.

b) Virotech units

The Virotech units (VE) of the cut-off controls are defined as 10. The calculated VE of the positive controls should be within the ranges mentioned in the Quality Control Certificate.

If those requirements (OD-values, VE) are not fulfilled, the test has to be repeated.

9.3 Calculation of the Virotech Units (VE) (IgG and IgA)

The extinction of the blank value (450/620nm) has to be subtracted from all other extinctions.

\[
VE_{\text{positive control}} = \frac{OD_{\text{positive control}}}{OD_{\text{cut-off control}}} \times 10
\]

\[
VE_{\text{patient serum}} = \frac{OD_{\text{patient serum}}}{OD_{\text{cut-off control}}} \times 10
\]

9.4 Calculation of the Virotech Units (VE) (IgM)

The extinctions of the blank values (450/620nm) have to be subtracted from all other extinctions.

\[
VE_{\text{positive control}} = \frac{\text{difference (test value - reference value of positive control)}}{\text{difference (test value - reference value of cut off control)}} \times 10
\]

\[
VE_{\text{Patient serum}} = \frac{\text{difference (test value - reference value of patient serum)}}{\text{difference (test value - reference value of cut off control)}} \times 10
\]

Example:

- OD positive control on test strip 0,853
- OD positive control on reference strip 0,107
- Difference of test value to reference value of positive control 0,746
- OD cut-off control on test strip 0,341
- OD cut-off control on reference strip 0,073
- Difference of test value to reference value of cut-off control 0,268

\[
VE_{\text{positive control}} = \frac{0,746}{0,268} \times 10 = 27,8
\]
9.5 Interpretation Scheme IgG, IgM and IgA

<table>
<thead>
<tr>
<th>Result (VE)</th>
<th>Evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 9.0</td>
<td>negative</td>
</tr>
<tr>
<td>9.0 - 11.0</td>
<td>borderline</td>
</tr>
<tr>
<td>&gt; 11.0</td>
<td>positive</td>
</tr>
</tbody>
</table>

1. If the measured values are above the defined borderline range, they are considered to be positive.
2. If the measured VE is within the borderline range, no significant high antibody concentration is present, the samples are considered to be borderline. For the secure detection of an infection it is necessary to determine the antibody concentration of two serum samples. One sample shall be taken directly at the beginning of the infection and a second sample 5 – 10 days later (convalescent serum). The antibody concentration of both samples has to be tested in parallel, that means in one test run. A correct diagnosis based on the evaluation of a single serum sample is not possible.
3. If the measured values are below the defined borderline range, no measurable antigen specific antibodies are present in the samples. The samples are considered to be negative.

10. Limits of the Test

The immune response may be homotypic or heterotypic. Homotypic antibodies are directed against serotype-specific epitopes, while heterotypic antibodies recognize epitopes that are similar or identical in different serotypes.

The VIROTECH ELISA determines cross reacting heterotypical antibodies against Enteroviruses by using antigen preparations denatured by heat. While diagnosing an infection with Enterovirus the following should be considered.

1. There may be quantitative and qualitative differences in the number of cross reacting epitopes to heat inactivated Enteroviruses, depending on which virus type or which isolate has been used for the antigen preparation. Therefore the spectrum of heterotypical antibodies, which can be detected by different test systems, varies.
2. The ratio of homotypical to heterotypical antibodies in patient sera can deviate. Studies from King et al. (6) give hints that in the course of the first Enterovirus infections in child age preferably homotypical antibodies are built. With increasing age and the therefore increasing number of passed Enterovirus infections the proportion of heterotypical antibodies rises. Therefore negative results are possible in the VIROTECH Enterovirus ELISA when heterotypical antibodies are only weakly present compared to the homotypical immune response.
3. As the ELISA detects polio-positive sera too, it cannot be excluded that a vaccination titer present may lead to a positive result.
4. Crossreactions between Enteroviruses and Hepatitis A, Epstein Barr Virus (EBV), Cytomegalovirus and rhinoviruses have been described (7).
5. The interpretation of serological results shall always include the clinical picture, epidemiological data and all further available laboratory results.

11. Performance Data

11.1 Prevalence (Expected Values)
The following table shows the results of the examination of blood bank sera for IgG (n=119), IgM (n=80) and IgA (n=120):

<table>
<thead>
<tr>
<th></th>
<th>IgG</th>
<th>IgM</th>
<th>IgA</th>
</tr>
</thead>
<tbody>
<tr>
<td>negative</td>
<td>86</td>
<td>80</td>
<td>116</td>
</tr>
<tr>
<td>borderline</td>
<td>13</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>positive</td>
<td>20</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

11.2 Intra-assay-Coefficient of Variation (Repeatability)
In one assay, strips of different plates of one batch have been tested with the same serum sample. The obtained coefficient of variation is for
IgG < 9% (at an average OD-value of 0.72)
IgM < 9% (at an average OD-value of 0.48)
IgA < 9% (at an average OD-value of 0.47)

11.3 Inter-assay-Coefficient of Variation (Reproducibility)

Enterovirus ELISA IgG
Three sera were tested in 10 independent test runs by different persons in different laboratories.

<table>
<thead>
<tr>
<th>Serum</th>
<th>Average Value VE</th>
<th>Coefficient of Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>negative</td>
<td>3,9</td>
<td>11,5%</td>
</tr>
<tr>
<td>negative</td>
<td>4,7</td>
<td>10,8%</td>
</tr>
<tr>
<td>positive</td>
<td>15,0</td>
<td>10,9%</td>
</tr>
</tbody>
</table>

Enterovirus ELISA IgM
Three sera were tested in 14 independent test runs by different persons in different laboratories.

<table>
<thead>
<tr>
<th>Serum</th>
<th>Average Value VE</th>
<th>Coefficient of Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>negative</td>
<td>4,2</td>
<td>8,6%</td>
</tr>
<tr>
<td>borderline</td>
<td>10,6</td>
<td>9,1%</td>
</tr>
<tr>
<td>positive</td>
<td>13,3</td>
<td>8,4%</td>
</tr>
</tbody>
</table>

Enterovirus ELISA IgA
Two sera were tested in 14 independent test runs by different persons in different laboratories.

<table>
<thead>
<tr>
<th>Serum</th>
<th>Average Value VE</th>
<th>Coefficient of Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>negative</td>
<td>4,5</td>
<td>6,3%</td>
</tr>
<tr>
<td>positive</td>
<td>12,5</td>
<td>9,1%</td>
</tr>
</tbody>
</table>

12. Literature

13. Test Procedure Scheme

Preparation of Patient Samples and Washing Solution

▼ Washing Solution: Fill up concentrate to 1 liter with aqua dest./demin.

▼ IgG-/IgA-Samples – Dilution 1:101

e.g.: 10 µl serum/plasma + 1000 µl Dilution Buffer
(Serum Dilution Buffer is ready to use)

▼ IgM-Samples - Dilution 1:101

Rheumafactor-absorption with RF-SorboTech

e.g.: 5 µl serum/plasma + 450 µl Dilution Buffer +
1 drop RF-SorboTech, incubate for 15 min. at room

temperature.

Testprocedure

Samples Incubation 30 minutes at 37°C

100 µl Patient Samples
blank value (Dilution Buffer) and controls

Wash 4times

400 µl Washing Solution
Remove Residues on a Cellulose Pad

Conjugate Incubation 30 minutes at 37°C

100 µl Conjugate
IgG, IgM, IgA

Wash 4times

400 µl Washing Solution
Remove Residues on a Cellulose Pad

Substrate Incubation 30 minutes at 37°C

100 µl Substrate

Stopping

50 µl Stopping Solution
shake carefully

Measure Extinctions

Photometer at 450/620nm
(Reference Wavelength 620-690nm)