ELISA-VIDITEST anti-HHV-6 IgG (CSF)

ODZ-344

Instruction manual

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1. TITLE
ELISA-VIDITEST anti-HHV-6 IgG (CSF) – ELISA kit for detection of IgG antibodies to HHV-6 in human serum (plasma) and for estimation of the intrathecal antibody production.

2. INTENDED USE
The kit is intended for serological diagnosis of diseases associated with HHV-6 infection, such as exanthema subitum, acute respiratory illnesses, diarrhoea with fever and febrile seizures in infants, heterophile antibody-negative infectious mononucleosis in children, also interstitial pneumonia, encephalitis, meningitis, hepatitis and aplastic anemia in immunodeficient patients. The presence of IgG anti-HHV-6 antibody reveals the immune status of the patient. Significant rise in anti-HHV-6 IgG antibodies in paired serum samples, taken in acute and convalescent phase of the infection, is indicative of the active infection. The test does not differentiate between HHV-6 subtype A and B. The test can be used for the anti HHV-6 antibody determination in human sera or cerebrospinal fluids (CSFs). Using quantitative evaluation, the test can be applied for intrathecal antibody synthesis assessment. The test should be supplemented with determination of anti-HHV-6 IgM antibody in human serum (ELISA-VIDITEST anti-HHV-6 IgM).

3. TEST PRINCIPLE
ELISA-VIDITEST anti-HHV-6 IgG (CSF) assay is a solid-phase immunoanalytical test. The strips are coated with native HHV6 antigen. The anti-HHV-6 antibodies, if present in the sera tested, bind to the immobilized antigens and the antibodies being in complexes with antigen are later on recognised by animal anti-human IgG antibodies labelled with horseradish peroxidase. The attached labelled antibodies are revealed by an enzymatic reaction with a chromogenic substrate. Negative sera do not react - a mild change in colour, if present, may be attributed to the reaction background.

4. KIT COMPONENTS
ELISA strips coated with native antigen \[ \text{STRIPS [Ag]} \]
1 microplate
1.3 mL Standard A=negative control serum, r.t.u. \[ \text{STA/CTRL-} \]
1 vial
1.3 mL Standard B, r.t.u. \[ \text{ST B} \]
1 vial
1.3 mL Standard C, r.t.u. \[ \text{ST C} \]
1 vial
1.3 mL Standard D=Standard 1, r.t.u. \[ \text{STD/ST1} \]
1 vial
1.3 mL Standard E=Standard 2, r.t.u. \[ \text{STE/ST2} \]
1 vial
13 mL Anti-human IgG antibodies labelled with horseradish peroxidase (Px-conjugate) r.t.u. \[ \text{CONJ} \]
1 vial
55 mL Wash buffer 10x concentrated \[ \text{WASH 10x} \]
1 vial
60 mL Dilution buffer anti-HHV6 r.t.u. \[ \text{DIL} \]
1 vial
13 mL Chromogenic substrate (TMB substrate) r.t.u. \[ \text{TMB-O} \]
1 vial
13 mL Stop solution r.t.u. \[ \text{STOP} \]
1 vial
Sealable pouch for unused strips
Instruction manual
Quality control certificate
1) r.t.u. ready to use
2) The antibody concentration for each Standard (A-E) is mentioned in enclosed Quality control certificate (AU/ml (Artificial units/ml)

| Dilution Buffer [DIL] is intended only for ELISA-VIDITEST anti-HHV-6 IgG, IgM kits. | Chromogenic substrate [TMB-O] is compatible and interchangeable between ELISA-VIDITEST kits which contain [TMB-O] and not with other Chromogenic substrates [TMB, TMB-BF]. |

5. MATERIAL REQUIRED BUT NOT PROVIDED WITH THE KIT
Distilled or deionised water for dilution of the Wash buffer concentrate.
Appropriate equipment for pipetting, liquid dispensing and washing.
Spectrophotometer/colorimeter (microplate reader – wavelength 450 nm).

6. PREPARATION OF REAGENTS AND SAMPLES
a. Allow all kit components to reach room temperature.
b. **Mix samples and Standards in order to ensure homogeneity** and mix all solution well prior use.
c. Dilute serum samples 1:100 in Dilution buffer and mix (5 µL of serum sample + 500 µL of Dilution buffer). Dilute cerebrospinal fluid samples 1:2 in Dilution buffer (e.g. 75 ul of cerebrospinal fluid sample + 75 ul of Dilution buffer).
   - **Do not dilute** Controls (Standards), Px-conjugate, TMB substrate and Stop solution, they are ready to use.
d. Prepare Wash buffer by diluting the Wash buffer concentrate 10 times with an appropriate volume of distilled or deionised water (e.g. 50 mL of the concentrated Wash buffer + 450 mL of distilled water). If there are crystals of salt present in the concentrated Wash buffer, warm up the vial to +32 to +37°C in a water bath. Diluted Wash buffer is stable for one month at laboratory temperature.
e. **Do not dilute** Px-conjugate, TMB substrate and Stop solution, they are ready to use.

7. ASSAY PROCEDURE
7.1. Qualitative and semiquantitative antibody determination
a. Allow the microwell strips sealed inside the aluminium bag to reach room temperature. Withdraw an adequate number of strips and put the unused strips into the provided pouch and seal it carefully with the desiccant kept inside.
b. Choose the proper method for data interpretation (qualitative, semiquantitative or quantitative, see below and/or paragraph 8) and pipette Standards and samples according to the pipetting scheme (Figure 1). Start with filling the first well with 100 µl of Dilution buffer [DIL] to estimate the reaction background. In case of choosing the qualitative or semiquantitative method, fill two wells with 100 µl/well of Standard D [ST D/ST 1], next one well with 100 µL of Standard A [ST A/CTRL-]. In case of quantitative method, pipette 100 µl all Standards (A, B, C, D, E) – see paragraph 7.2. Fill the remaining wells with 100 µl of diluted serum samples (S1, S2, S3, ...). It is sufficient to apply samples as singlets, however, if you wish to minimize the laboratory error apply the samples and Standards in doublets.
c. Incubate **60 minutes (+5 min) at room temperature**.
d. Aspirate the liquid from the wells into a collecting bottle containing appropriate disinfectant (see Safety Precautions). Wash and aspirate the wells four times with 250 µl/well of Wash buffer. Avoid cross-contamination between wells! If some liquid remains in the wells, invert the plate and tap it on an adsorbent paper to remove the last remaining drops.
e. Add 100 µL of Px-conjugate r.t.u. **CONJ** into each well.

f. Incubate **60 minutes (±5 min)** at room temperature.

g. Aspirate and wash four times with 250 µl/well of Wash buffer.

h. Dispense 100 µl of **TMB-O** substrate into each well.

i. Incubate for **20 minutes (+/-30 seconds)** at room temperature. The time measurement must be started at the beginning of TMB dispensing. Cover the strips with an aluminium foil or keep them in the dark during the incubation with TMB substrate.

j. Stop the reaction by adding 100 µL of Stop solution **STOP**. Use the same pipetting rhythm as with the TMB substrate to ensure the same reaction time in all wells. Tap gently the microplate for a few times to ensure complete mixing of the reagents.

k. Read the absorbance at 450 nm with a microplate reader within **20 minutes**. It is recommended to use reference reading at 620-690 nm.

Fig 1: Pipetting scheme for qualitative and semiquantitative method

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7.2. Quantitative antibody determination

a. Allow the antigen-coated strips to reach room temperature before opening sealed the aluminium bag in order to prevent water condensation within the wells. Withdraw the adequate number of strips and put the remaining strips in the sealable pouch and keep the desiccant inside.

b. Pipette Standards and samples according to the pipetting scheme (Fig. 2). Start with filling the first well with 100 µl of Dilution buffer **DIL** to estimate the reaction background. Fill the next wells with 100 µl of Standard A-E (**STA/CTRL-**), **ST B**, **ST C**, **STD/ST1**, **STE/ST2**. Fill the remaining wells with 100 µl of serum samples (S1, S2, S3,...). It is sufficient to apply samples as singlets, however, if you wish to minimize laboratory error apply the samples in doublets. We recommend to include positive reference serum sample (your in-house internal control) into each run to follow the sequence, variability and accuracy of calibration.

c. Incubate for **60 (+/- 5) minutes at room temperature**.

d. Aspirate the liquid from the wells into a collecting bottle containing appropriate disinfectant (see Safety Precautions). Wash and aspirate the wells 4x with 250 µl/well of Wash buffer. Avoid cross-contamination between wells!

e. Add 100 µL of Px-conjugate r.t.u. into each well.

f. Incubate for **60 (+/-5) minutes at room temperature**.

g. Aspirate and wash 4x with 250 µl/well of Wash buffer. Tap the plate on an adsorbent paper.

h. Dispense 100 µL of the **TMB-O** substrate into each well; pipette in a regular rhythm or use an appropriate dispensing instrument. Incubate for **20 minutes (+/- 30 sec.) at room temperature**. The time measurement must be started right at the beginning of TMB dispensing. Cover the strips and keep them in the dark during the enzymatic reaction.
i. Stop the reaction by adding 100 µL of Stop solution STOP. Use the same pipetting rhythm as with the TMB substrate to ensure the same reaction time in all wells. Tap gently the microplate for a few times to ensure complete mixing of the reagents.

j. Read the absorbance at 450 nm with a microplate reader within 20 minutes. It is recommended to use reference reading at 620 - 690 nm.

Figure 2: Pipetting scheme – quantitative detection of serum samples

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7.3. Procedure for serum and cerebrospinal fluid samples (detection of intrathecal antibodies)

a. Allow the antigen-coated strips to reach room temperature before opening sealed the aluminium bag in order to prevent water condensation within the wells. Withdraw the adequate number of strips and put the remaining strips in the sealable pouch and keep the desiccant inside.

b. Pipette Standards and samples according to the pipetting scheme (Fig 3). Start with filling the first well with 100 µl of Dilution buffer DIL to estimate the reaction background. Fill the next wells with 100 µl of Standard A-E (STA/CTRL-, ST B, ST C, STD/ST1, STE/ST2). Fill the remaining wells with 100 µl of serum samples (S1, S2, S3, ...) and cerebrospinal fluid samples (CSF1, CSF2, ...). We recommend testing each serum sample in two different dilutions: 1:100 and 1:400 and each cerebrospinal fluid sample in one dilution 1:2. If you wish to minimize laboratory error apply the samples in doublets. We recommend to include positive reference serum sample (your in-house internal control) into each run to follow the sequence, variability and accuracy of calibration.

c. Incubate 60 minutes (+/-5 min) at room temperature.

d. Aspirate the liquid from wells into a waste bottle containing an appropriate disinfectant (see Safety Precautions). Wash and aspirate the wells four times with 250 µl/well of Wash buffer. Avoid cross-contamination between wells! If any liquid stays trapped inside the wells, invert the plate and tap it on an adsorbent paper to remove the remaining drops.

e. Add 100 µL of Px-conjugate r.t.u. CONJ into each well.

f. Incubate 60 minutes (+/-5 min) at room temperature.

g. Aspirate and wash four times with 250 µl/well of Wash buffer.

h. Dispense 100 µl of TMB-0 substrate into each well.

i. Incubate for 20 minutes (+/- 30 seconds) at room temperature. The time measurement must be started at the beginning of TMB dispensing. Keep the strips in the dark during the incubation with TMB substrate.

j. Stop the reaction by adding 100 µL of Stop solution STOP. Use the same pipetting rhythm as with the TMB substrate to ensure the same reaction time in all wells. Tap gently the microplate few times to ensure complete mixing of the reagents.

k. Read the absorbance at 450 nm with a microplate reader within 20 minutes. It is recommended to use a reference reading at 620 - 690 nm.
**Figure 3:** Measurement of intrathecal antibody production (serum and CSF samples)

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<tbody>
<tr>
<td>a</td>
<td>DIL</td>
<td>S1(1:400)</td>
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<td>b</td>
<td>STA/CTRL-</td>
<td>S2(1:400)</td>
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<td>c</td>
<td>ST B</td>
<td>CSF1 (1:2)</td>
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<td>d</td>
<td>ST C</td>
<td>CSF2 (1:2)</td>
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<td>g</td>
<td>S1(1:100)</td>
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<td>S2(1:100)</td>
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**8. PROCESSING OF THE RESULTS**

Begin the processing with subtraction of the absorbance of the DIL well (background absorbance) from the absorbances in all other wells.

**8.1 Qualitative evaluation**

1. Compute the mean absorbance of the two parallels of Standard D (ST D/ST 1). (If the Standard D was applied in three parallels and one the absorbance is different from the mean more than 20%, then exclude the deviating well from the calculation and compute a new absorbance mean using the other two wells).

2. Compute the cut-off value by multiplying the Standard D mean with a Correction factor. **The Correction factor of Standard D is indicated on Quality Control Certificate.**

3. Assign the samples with absorbances less than the 90% of the cut-off value as negative and the samples with absorbances higher than 110% of the cut-off value as positive.

**8.2. Semiquantitative evaluation**

Determination of sample Positivity Index:

1. Compute the cut-off value (see previous paragraph 8.1)

2. Compute the Positivity Index for each sample according to the following formula:

   \[
   \text{Sample Positivity Index} = \frac{\text{sample absorbance}}{\text{cut-off value}}
   \]

3. Express serum reactivity in accordance with data in Table 1 (Evaluation of results)

**Table 1 Evaluation of results**

<table>
<thead>
<tr>
<th>Index value</th>
<th>Interpretation</th>
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<tr>
<td>&lt; 0.90</td>
<td>Negative</td>
</tr>
<tr>
<td>0.90 - 1.10</td>
<td>+/-</td>
</tr>
<tr>
<td>1.11 - 2.00</td>
<td>+</td>
</tr>
<tr>
<td>2.01 - 4.00</td>
<td>++</td>
</tr>
<tr>
<td>&gt; 4.00</td>
<td>+++</td>
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</table>

*Note! Indifferent sample reactivity, i.e. interpreted as +/-, requires repetition of the sample testing. If the result is repetitively indifferent then it is recommended to use an alternative testing method or to obtain another sample from the patient withdrawn 1-2 weeks later.*
Example of calculation:

<table>
<thead>
<tr>
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<th>Value</th>
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<tbody>
<tr>
<td>Standard 1 absorbances</td>
<td>1.407; 1.377</td>
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<tr>
<td>Standard 1 mean</td>
<td>1.392</td>
</tr>
<tr>
<td>Correction factor</td>
<td>0.205</td>
</tr>
<tr>
<td>Cut-off value</td>
<td>1.392*0.21 = 0.292</td>
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<tr>
<td>Sample absorbance</td>
<td>1.200</td>
</tr>
<tr>
<td>Sample Positivity Index</td>
<td>1.200 / 0.292 = 4.11</td>
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8.2. Quantitative antibody determination in serum samples

Compute the sample antibody concentration in artificial units (AU/ml) as follows:

1. Construct the calibration curve by plotting the absorbance of Standards (y-axis – in linear scale) to antibody concentration in artificial units (AU/ml) (x-axis – may have logarithmic scale). The antibody concentration in each Standard (A-E) is mentioned in Quality control certificate.

2. Find the place where the absorbance of tested samples intersect calibration curve and find the corresponding values on the axis x. It is possible to use various softwares for the standard curve fitting and for the calculation of the unknowns, e.g. Winliana, KimQ. For better fitting, the polynomic (four-parameter) function is the most convenient.

3. Result interpretation:

<table>
<thead>
<tr>
<th>Concentration (AU/mL)</th>
<th>Evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 10.50</td>
<td>Negative</td>
</tr>
<tr>
<td>10.50 - 12.50</td>
<td>+/-</td>
</tr>
<tr>
<td>&gt; 12.50</td>
<td>Positive</td>
</tr>
</tbody>
</table>

Note 1: Sample in the range between 10.50 – 12.50 AU/ml is equivocal. In such case it is recommended to repeat the assay. If the result of the sample is in the grey zone again use an alternative diagnostic method or initiate taking another blood sample 1-2 weeks later.

Note 2: The quantification is accurate only within the linear part of the calibration curve. If the sample OD is outside the linear interval, it is necessary to repeat the test with more diluted serum samples to obtain the precise quantification.

8.3 Processing of results for estimation of the intrathecal antibody production

An automatic data processing is possible when purchasing the VIDITAB software available from VIDIA.

1) Calculate the anti-HHV6 IgG antibody concentrations (AU/mL) for all tested samples by multiplying the AU of the sample subtracted from the calibration curve with the sample dilution. For sera AU x101 and AU x 404, for cerebrospinal fluid AU x 2.

2) Calculate the Specific antibody quotient as follows:

\[ Q_{\text{spec}} = \frac{\text{concentration of IgG anti-HHV6 (AU/mL) in cerebrospinal fluid}}{\text{concentration of IgG anti-HHV6 (AU/mL) in serum}} \]

3) Compute the Total antibodies quotient as follows:

\[ Q_{\text{total}} = \frac{\text{concentration of total IgG (mg/mL) in cerebrospinal fluid}}{\text{concentration of total IgG (mg/mL) in serum}} \]

4) Compute the Albumine quotient as follows:

\[ Q_{\text{alb}} = \frac{\text{concentration of albumin (mg/mL) in cerebrospinal fluid}}{\text{concentration of albumin (mg/mL) in serum}} \]
5) Calculate the Limite quotient $Q_{\text{lim}}$ that shows status of the hematoencephalic barrier

Compute $Q_{\text{lim}}$ using the equation:

$$Q_{\text{lim}} (\text{IgG}) = 0.93 * \sqrt{(Q_{\text{ab}})^2 + 6*10^{-6}} - 1.7 * 10^{-3}$$

6) Compute the Antibody Index AI:

a) If $Q_{\text{total \ IgG}} < Q_{\text{lim}}$, then calculate AI using the formula:

$$AI = \frac{Q_{\text{spec}}}{Q_{\text{total}}}$$

b) If $Q_{\text{total \ IgG}} > Q_{\text{lim}}$ compute AI using the formula:

$$AI = \frac{Q_{\text{spec}}}{Q_{\text{lim}}}$$

9. RESULTS INTERPRETATION

9.1. Interpretation of the anti-HHV-6 antibodies in serum.

Anti-HHV-6 IgG antibodies are anamnestic. They persist for the whole life after the primary infection. The kit can detect cross-reactive antibodies to HHV-7. Significant increase of IgG antibodies can be caused by reactivation of the infection, but could not be always proved due to the recurrent character of the reactivations. For final diagnosis, the clinical symptoms of the patient should be taken in consideration. Results from immunosuppressed patients should be interpreted with caution.

9.2. Evaluation of intrathecal antibody synthesis

- $AI < 1.5$ intrathecal antibody production not found
- $AI 1.5 - 2.0$ suspect intrathecal antibody production
- $AI > 2.0$ intrathecal antibody production proven

10. VALIDITY, SPECIVITY AND SENSITIVITY OF THE TEST

10.1 Validity of the test (qualitative and semiquantitative evaluation)

The test is valid if:

The background absorbance (the absorbance of the Dilution buffer) is less than 0.100.

The ratio of Standard A $\text{ST A/CTRL}$ absorbance / cut-off is lower than 0.9.

The mean absorbance values of $\text{ST D/ST 1}$ is in the range stated in the Quality control certificate for this kit lot.

The Standards absorbance values keep the order that:

$$\text{ST A/CTRL} < \text{ST B} < \text{ST C} < \text{ST D/ST 1} < \text{ST E/ST 2}$$

10.2 Validity of the test (quantitative evaluation)

The test is valid if:

The mean absorbance values of standards/ control sera are in the ranges stated in the Quality control certificate for this kit lot.
10.2 Precision of the test
The intra-assay and the inter-assay reproducibility were determined using samples with different absorbance values. In anti-HHV-6 IgG-positive samples intra-assay and inter-assay variability coefficients (CV) did not exceed 5% and 15% of mean absorbance values, respectively.

An example of **intra-assay** variability (n= number of parallel determinations in the same test)

<table>
<thead>
<tr>
<th>n</th>
<th>A</th>
<th>±δ</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>1.536</td>
<td>0.036</td>
<td>2.3%</td>
</tr>
<tr>
<td>16</td>
<td>1.891</td>
<td>0.042</td>
<td>2.2%</td>
</tr>
</tbody>
</table>

An example of **inter-assay** variability (n= number of determinations in several independent tests)

<table>
<thead>
<tr>
<th>n</th>
<th>A</th>
<th>±δ</th>
<th>Min.- max.</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1.601</td>
<td>0.145</td>
<td>1.425-1.747</td>
<td>9.1%</td>
</tr>
<tr>
<td>5</td>
<td>1.162</td>
<td>0.075</td>
<td>1.086-1.238</td>
<td>6.5%</td>
</tr>
</tbody>
</table>

10.3 Sensitivity and specificity of the test
The diagnostic sensitivity of the test is 99% and the specificity is 95%. Evaluation was performed by comparing the ELISA-VIDITEST anti-HHV-6 kit with other commercial ELISA tests.

<table>
<thead>
<tr>
<th>HHV-6 status</th>
<th>Negative</th>
<th>Equivocal</th>
<th>Positive</th>
<th>Sum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seronegative</td>
<td>40</td>
<td>1</td>
<td>2</td>
<td>43</td>
</tr>
<tr>
<td>Seropositive</td>
<td>3</td>
<td>0</td>
<td>245</td>
<td>248</td>
</tr>
</tbody>
</table>

10.4 Accuracy of the test
10.4.1 Spiking recovery test
Measured values of recovery test for every Lot are between 80-120% of expected values.

10.4.2 Interference
Haemolytic and lipemic samples have no influence on the test results up to concentration of 50 mg/mL of haemoglobin, 5 mg/mL of bilirubin and 50 mg/mL of triglycerides. However, examination of such samples is not recommended.

10.5. The limit of quantification
The limit of quantification is defined as the lowest measurable concentration which can be 95% sure distinguished from zero. This value is 2.95 AU/mL.

11. SAFETY PRECAUTIONS
All ingredients of the kit are intended for laboratory use only. Controls contain human sera that has been tested negative for HBsAg, anti-HIV-1,2 and anti-HCV. However they should be regarded as contagious and handled and disposed of according to the appropriate regulations.
Autoclave all reusable materials that were in contact with human samples for 0.5 hour at 121°C, burn disposable ignitable materials, decontaminate liquid wastes and nonignitable materials with 3% chloramine. Liquid wastes containing acid (Stop solution) should be neutralized in 4% sodium bicarbonate solution.
Handle Stop solution with care. Avoid contact with skin or mucous membranes. In case of contact with skin, rinse immediately with plenty of water and seek medical advice.
Do not smoke, eat or drink during work. Do not pipette by mouth. Wear disposable gloves while handling reagents or samples and wash your hands thoroughly afterwards. Avoid spilling or producing aerosol.

12. HANDLING PRECAUTIONS

Manufacturer guarantees performance of the entire ELISA kit

Follow the assay procedure indicated in the Instruction manual.

Controls (Standards), Dilution buffer, Chromogenic substrate and Px-conjugate contain preservative ProClin 300®.

Avoid microbial contamination of serum samples and kit reagents.

Avoid cross-contamination of reagents.

Avoid contact of the TMB substrate with oxidizing agents or metal surfaces.

Variations in the test results are usually due to:

* Insufficient mixing of reagents and samples
* Inaccurate pipetting and inadequate incubation times
* Poor washing technique or spilling the rim of well with sample or Px-conjugate
* Use of identical pipette tip for different solutions

13. STORAGE AND EXPIRATION

Store the kit and the kit reagents at +2 to +10°C, in a dry place and protected from the light. Store unused strips in the sealable pouch and keep the desiccant inside. Store serum samples at +2 to +10°C up to one week. For longer period make aliquots and keep them at -20°C. Avoid repeated thawing and freezing. Do not store diluted samples. Always prepare fresh.

Kits are shipped in cooling bags, the transport time of 72 hours have no influence on expiration. If you find damage at any part of the kit, please inform the manufacturer immediately.

Expiration date is indicated at the ELISA kit label and at all reagent labels.

References:


De Bolle L., Naesens L., De Clercq E., Update on Human Herpesvirus 6 Biology, Clinical Features and Therapy, Clinical Microbiology Reviews, 217-245, 2005


14. FLOW CHART

<table>
<thead>
<tr>
<th>Step 1</th>
<th>Prepare reagents and samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Step 2</th>
<th>Dispense 100 µL/well standards and samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Incubate 60 minutes at room temperature</td>
</tr>
<tr>
<td></td>
<td>Wash 4 times (250 µL/well), aspirate</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Step 3</th>
<th>Dispense 100 µL/well of Px-conjugate r.t.u.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Incubate 60 minutes at room temperature</td>
</tr>
<tr>
<td></td>
<td>Wash 4 times (250 µL/well), aspirate</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Step 4</th>
<th>Dispense 100 µL/well of TMB-O substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Incubate 20 minutes at room temperature</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Step 5</th>
<th>Dispense 100 µL/well of Stop solution</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Step 6</th>
<th>Read the absorbance at 450 / 620-690 nm within 20 minutes</th>
</tr>
</thead>
</table>

Date of the last revision of this manual: 09/2014

The development of this kit was supported by grant from the Ministry of Industry and Trade of the Czech Republic.