ELISA-VIDITEST anti-CMV IgG and IgG avidity

ODZ-177

Instruction manual

PRODUCER: VIDIA spol. s r.o., Nad Safinou II 365, 252 42 Jesenice, Czech Republic, Tel.: +420 261 090 565, www.vidia.cz

1. TITLE:
ELISA-VIDITEST anti-CMV IgG and IgG avidity – ELISA kit for the semiquantitative determination of IgG antibodies to human cytomegalovirus (CMV) in serum and their avidity evaluation.

2. INTENDED USE:
The kit is designed for differentiation of primary and recurrent CMV infection. After primary infection human body produces mainly low avidity (bind with low affinity) IgG antibodies to viral antigens. Later, antibodies with high avidity prevail. The predominance of low avidity IgG antibodies indicates recent primary infection. Otherwise, individuals with predominance of high avidity antibodies underwent the primary infection in the past. If such individuals have the symptoms of active CMV infection it means the reactivation of the infection.

3. TEST PRINCIPLE:
ELISA-VIDITEST anti-CMV IgG and IgG avidity assay is a solid-phase immunoanalytical test. The strips are coated by a mixture of specific antigens that bear immunodominant CMV epitopes. Each serum sample is applied into two wells in parallel (eventually, into four wells) and the anti-CMV antibodies present in serum bind to the immobilized antigens. The next step is the incubation of one well with a wash buffer, the second respective well with urea solution. Antibodies with low and high avidity are bound to the antigen in the first well, whereas in the second well the low avidity antibodies are released due to the high concentration of urea and only the high avidity antibody-antigen complexes remain. The bound antibodies are recognized by animal anti-human IgG antibodies labelled with horseradish peroxidase. The amount of the bound labelled antibodies is revealed by an enzymatic reaction that leads to a colour change. The presence of the low avidity antibodies is indicated by a drop of absorbance in wells where urea solution was added. The ratio between the optical density of the well without urea (the one with the wash buffer) and the corresponding well with urea represents relative avidity index (RAI).

4. KIT COMPONENTS:

<table>
<thead>
<tr>
<th>Description</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA 8-well break-away strips coated with a mixture of CMV antigens</td>
<td>1 microplate</td>
</tr>
<tr>
<td>1.3 mL Standard 1, r.t.u.</td>
<td>1 vial</td>
</tr>
<tr>
<td>1.3 mL Negative control serum, r.t.u.</td>
<td>1 vial</td>
</tr>
<tr>
<td>1.3 mL High avidity control serum, r.t.u.</td>
<td>1 vial</td>
</tr>
<tr>
<td>1.3 mL Low avidity control serum, r.t.u.</td>
<td>1 vial</td>
</tr>
<tr>
<td>13 mL Anti-human IgG antibodies labelled with horseradish peroxidase, r.t.u.</td>
<td>1 vial</td>
</tr>
<tr>
<td>125 mL Wash buffer, 10x concentrated</td>
<td>1 vial</td>
</tr>
<tr>
<td>13 mL Urea solution, r.t.u.</td>
<td>1 vial</td>
</tr>
<tr>
<td>100 mL Dilution buffer, r.t.u.</td>
<td>1 vial</td>
</tr>
</tbody>
</table>
5. MATERIAL REQUIRED BUT NOT PROVIDED WITH THE KIT:

1. Distilled or deionised water for dilution of the Wash buffer concentrate.
2. Appropriate equipment for pipetting, liquid dispensing and washing.

6. PREPARATION OF REAGENTS AND SAMPLES:

a. Allow all kit components to reach room temperature.

b. Vortex samples, the Standards and the Controls in order to ensure homogeneity and mix all solution well prior use.

c. **Dilute serum samples 1:100 in Dilution buffer** and mix (e.g. 5 μL of serum sample + 500 μL of Dilution buffer). Do not dilute the Controls and the Standards, 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. 100 mL of the concentrated Wash buffer + 900 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 week if stored at +2 to +10°C.

e. Do not dilute Px-conjugate, TMB substrate and Stop solution, they are ready to use.

7. ASSAY PROCEDURE FOR MEASURING AVIDITY OF IgG ANTIBODIES:

a. Allow the vacuum-closed aluminium bag with strips to reach a room temperature. Withdraw the adequate number of strips and put unused strips into the provided pouch and seal it carefully with the desiccant kept inside.

b. Pipette 100 μL of Dilution buffer, Controls, Standards and serum samples to the wells according to the pipetting scheme in Figure 1 (page 3). First, fill wells in the first raw of the first two strips with Dilution buffer (DIL) to determine the reaction background. Then fill two wells with High avidity control serum (CONTROL HIGH AVID) and two wells with Low avidity control serum (CONTROL LOW AVID). If you want to use qualitative interpretation for serum samples, fill next two wells with STANDARD 1. Then pipette the diluted serum samples (S1, S2, S3…) in doublets into the remaining wells. It is satisfactory to use the “simple doublettes”, however, if you want to minimize a pipetting error, apply serum samples as quadruplettes, i.e. two wells for each of the respective strip.

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 4x 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 Wash buffer into wells of even-numbered strips (i.e. column 2, 4, 6, 8, 10 and 12) and 100 µL of Urea solution into each well of odd-numbered strips (i.e. column 1, 3, 5, 7, 9 and 11).

f. Incubate 10 minutes (±5 sec) at room temperature.

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

h. Add 100 µL of Px-conjugate r.t.u. (CONJ) into each well.

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

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

k. Dispense 100 µl of TMB substrate (TMB-O) into each well.

l. Incubate for 10 minutes (+/-5 seconds) in dark at room temperature.

m. Stop the reaction by adding 100 µL of Stop solution. 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.

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

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**Figure 1. Pipetting scheme for measuring avidity of IgG antibodies:**

<table>
<thead>
<tr>
<th></th>
<th>UREA</th>
<th>WASH</th>
<th>UREA</th>
<th>WASH</th>
<th>…</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>DIL</td>
<td>2</td>
<td>DIL</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>B</td>
<td>CONTROL HIGH AVID</td>
<td>CONTROL HIGH AVID</td>
<td>S5</td>
<td>S5</td>
<td>…</td>
</tr>
<tr>
<td>C</td>
<td>CONTROL LOW AVID</td>
<td>CONTROL LOW AVID</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>STANDARD 1</td>
<td>STANDARD 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>S1</td>
<td>S1</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>F</td>
<td>S2</td>
<td>S2</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>S3</td>
<td>S3</td>
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<td></td>
<td></td>
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<tr>
<td>H</td>
<td>S4</td>
<td>S4</td>
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<td></td>
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</tr>
</tbody>
</table>

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**8. ASSAY PROCEDURE FOR THE DETECTION OF IgG ANTIBODIES:**

a. Allow the vacuum-closed aluminium bag with strips to reach a room temperature. Withdraw the adequate number of strips and put unused strips into the provided pouch and seal it carefully with the desiccant kept inside.

b. Pipette 100 µL of Dilution buffer, Standards, Control sera and serum samples to the wells according to the pipetting scheme in Figure 2 (page 4): fill first well with Dilution buffer (DIL) to determine reaction background. Fill the next two wells with STANDARD 1 (it serves as a calibrator). Fill the next well with Negative control serum (CONTROL -). The remaining wells fill with diluted tested samples (S1...). It is also suitable to apply positive control serum (CONTROL HIGH AVID) for the test control. It is satisfactory to apply one serum into one well (S1, S2, S3...). However, if you want to minimize a laboratory error, apply control sera and tested sera as doublets, STANDARD 1 as triplet. We also recommend applying internal reference positive control sample for verification of the calibration, continuity and variability of the test.

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

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

   i. **Incubate for 10 minutes (+/- 5 seconds) in dark 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. 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 620-690nm.

**Figure 2. Pipetting scheme for the detection of IgG antibodies:**

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
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<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>DIL</td>
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<td></td>
<td></td>
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<tr>
<td>b</td>
<td>STANDARD 1</td>
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<tr>
<td>c</td>
<td>STANDARD 1</td>
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<td>d</td>
<td>CONTROL -</td>
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<tr>
<td>f</td>
<td>S2</td>
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<td>g</td>
<td>S3</td>
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<tr>
<td>h</td>
<td>S…</td>
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</tr>
</tbody>
</table>

9. **PROCESSING OF RESULTS FOR MEASURING AVIDITY OF IgG ANTIBODIES:**

   a. First, subtract the absorbance of the background (absorbance of the DIL well) from the absorbancies of all other wells. Use the respective background value for wells incubated with the Urea solution and for wells incubated without the Urea solution.

   **Important note: Avidity evaluation is possible in anti-CMV IgG-positive serum samples only.** If the tested serum sample is negative or indifferent, the relative avidity index (RAI) cannot be assessed. IgG-positivity can be checked using following calculation:

   Calculate cut-off value of the test: Multiply the absorbance value of STANDARD 1 in the wells with Wash buffer (WASH) with correction factor. The **correction factor value determined for this lot of the kit is written in the Quality control certificate.**

   Samples incubated with Wash buffer with absorbance (OD) lower than the 90% cut-off value are considered negative and samples (incubated with WASH) with absorbance higher than the 110% cut-off value are considered positive.
b. If you applied two duplicates, compute the mean absorbance of serum from wells on the same strip.

c. Calculate the relative avidity index value (RAI): divide the absorbance of a sample well incubated with the Urea solution by the absorbance of the sample well incubated with the Wash buffer, express in percent (i.e. multiply by 100).

*Both Controls (CONTROL HIGH AVID and CONTROL LOW AVID) are used for the internal validity test and must be involved in each run of the assay.*

**Formula:**

\[
\text{absorbance with urea solution} \times 100 = \text{RAI (\%)}
\]

\[
\frac{\text{absorbance with urea solution}}{\text{absorbance with wash buffer}} \times 100 = \text{RAI (\%)}
\]

**INTERPRETATION OF RESULTS:**

<table>
<thead>
<tr>
<th>RAI value in %</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 40 %</td>
<td>Presence of low avidity antibodies</td>
</tr>
<tr>
<td>40 % - 60 %</td>
<td>Indifferent result</td>
</tr>
<tr>
<td>&gt; 60 %</td>
<td>Presence of high avidity antibodies</td>
</tr>
</tbody>
</table>

*Example:*

Absorbances of High avidity control in wells with Urea solution = 2.310; 2.215
Mean absorbance of High avidity control in wells with Urea solution = 2.263
Absorbances of High avidity control in wells with Wash buffer = 2.526; 2.406
Mean absorbance of High avidity control in wells with Wash buffer = 2.466
RAI (%) = \((2.263/2.466) \times 100 = 92 \%\)

10. **PROCESSING OF RESULTS FOR THE DETECTION OF IgG ANTIBODIES:**

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

**10.1 Processing of results for Qualitative interpretation**

1. Compute the mean absorbance of STANDARD 1. If you applied STANDARD 1 into 3 wells and if any of the three STANDARD 1 absorbances falls out of the range +/-20% of the mean absorbance then exclude the deviating well from the calculation and compute a new STANDARD 1 mean using the values from the other two wells.

2. **Compute the Cut-off value** of the test by multiplication the STANDARD 1 mean by the Correction factor. **The correction factor value determined for this lot of the kit is stated in the Quality control certificate.**

3. Sera that have absorbance value < 90% cut-off are negative and sera with absorbance value > 110% cut-off are considered to be positive.

**10.2. Semiquantitative evaluation**

Determine the Positivity Index for each serum sample as follows:

1. Compute the cut-off value (see previous paragraph 10.1).

2. Compute the Positivity Index according to the following formula:

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

3. Express a serum reactivity according to Table 1 (Semiquantitative interpretation of results).
Table 1: Semiquantitative interpretation of results:

<table>
<thead>
<tr>
<th>Index value</th>
<th>Evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 0.90</td>
<td>Negative</td>
</tr>
<tr>
<td>0.90 - 1.10</td>
<td>+/-</td>
</tr>
<tr>
<td>1.11 - 4.50</td>
<td>+</td>
</tr>
<tr>
<td>4.51 - 8.00</td>
<td>++</td>
</tr>
<tr>
<td>8.01 - 12.00</td>
<td>+++</td>
</tr>
<tr>
<td>&gt; 12.00</td>
<td>++++</td>
</tr>
</tbody>
</table>

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

Example of calculation:

STANDARD 1 absorbancies = 1.140; 1.112; 1.145
Mean STANDARD 1 absorbance = 1.132
Sample absorbance = 0.800
Correction factor = 0.15
Cut-off value = 1.132*0.15 = 0.170
Sample Positivity Index = 0.800/0.170 = 4.71

11. CHARACTERISTICS OF THE TEST:

11.1 Validity of the test
The test is valid if:

a. The background of the reaction (the absorbance of the Dilution buffer) is less than 0.100.
b. RAI of High avidity control serum is > 60 %.
c. RAI of Low avidity control serum is < 40 %.
d. The mean STANDARD 1 absorbance should be in range that is written in enclosed Quality control certificate.
e. OD values of control sera should be: CONTROL HIGH AVID > STANDARD 1 > CONTROL -.

11.2. Precision of the test
Tests were done with samples of various OD and RAI values when performing the interassay and intraassay variability evaluation.

11.2.1. Intraassay variability
The coefficient of intraassay variability is max. 5%. It is measured for each particular Lot at least on 12 parallels of the same microtitration plate.
Example: (N = number of parallels of the same microtitration plate, SD = standard deviation)

<table>
<thead>
<tr>
<th>N</th>
<th>Mean absorbance</th>
<th>SD</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>1.335</td>
<td>0.050</td>
<td>3.8%</td>
</tr>
<tr>
<td>16</td>
<td>0.614</td>
<td>0.023</td>
<td>3.7%</td>
</tr>
</tbody>
</table>

11.2.2. Interassay variability
The coefficient of interassay variability is max. 15%. It is measured for each particular Lot as comparison of the OD values of the same serum sample in several consecutive tests.
Example: (N = number of an independent examination of the same serum sample, SD = standard deviation):
11.2.3 Intraassay and interassay variability of RAI determination

**Example of evaluation with samples containing high avidity antibodies (n=8):**
RAI range: 79% - 100%.

**Example of evaluation with samples low avidity antibodies (n=8):**
RAI range: 20% - 35%

**Example of intraassay variability**
The coefficient of intraassay variability is max. 5% (n = the number of parallels):

<table>
<thead>
<tr>
<th>n</th>
<th>RAI in %</th>
<th>±δ</th>
<th>% of mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>76</td>
<td>2%</td>
<td>2.6%</td>
</tr>
<tr>
<td>16</td>
<td>43</td>
<td>2%</td>
<td>4.7%</td>
</tr>
</tbody>
</table>

**Example of interassay variability (n = 6):**
The coefficient of interassay variability is max. 15%.
Mean RAI in %: 23 ± 2.0% min – max 20 – 26%
Mean RAI in %: 93 ± 3.5% min – max 87 – 98%

**Recovery test**
Measured values of recovery test for every Lot are between 80-120% of expected values.

11.3. Diagnostic sensitivity and specificity of the test
Diagnostic sensitivity of the test is 99.8%. Diagnostic specificity is 100%.
Diagnostic sensitivity and specificity were determined by a multicentric evaluation study of this test. Serum samples of 544 CMV-IgG negative and 1101 CMV-IgG positive people were tested by the kit and the results were confirmed by other commercially available diagnostic tests.

11.4. Interference
Haemolytic and lipemic samples have no influence on test results to minimal concentration 50 mg/mL of haemoglobin, 5 mg/mL of bilirubin and 50 mg/mL triglycerides.

12. SAFETY PRECAUTIONS:
All ingredients of the kit are intended for laboratory use only.
The Standards and the 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 1 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.
The waste from strip washing disinfect in waste container using appropriate disinfection solution (e.g. Incidur, Incidin, chloramin,...) in concentrations recommended by the producer.
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.

13. HANDLING PRECAUTIONS:

Manufacturer guarantees performance of the entire ELISA kit. Follow the assay procedure indicated in the Instruction manual. Wash buffer, chromogenic substrate TMB, Stop solution and Dilution buffer are interchangeable between ELISA-VIDITEST kits unless otherwise stated in the instruction manual. Controls, Standards, TMB substrate, Dilution buffer, Urea solution 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

14. 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 serum 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.
15. FLOW CHART:
Note: If you carry out the test only due to determination of IgG antibodies (and not due to determination of their avidity), please omit the Step 3.

<table>
<thead>
<tr>
<th>Step 1</th>
<th>Prepare reagents and samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Step 2</td>
<td>Dispense 100 µL/well of DIL, standards, controls and samples</td>
</tr>
<tr>
<td></td>
<td>Incubate 60 minutes at room temperature</td>
</tr>
<tr>
<td></td>
<td>Wash 4 x (250 µL/well), aspirate</td>
</tr>
<tr>
<td>Step 3</td>
<td>Dispense 100 µL/well of Wash buffer into the even-numbered strips and 100 µL/well of Urea solution into the odd-numbered strips</td>
</tr>
<tr>
<td></td>
<td>Incubate 10 minutes (± 5 sec.) at room temperature</td>
</tr>
<tr>
<td></td>
<td>Wash 4 x (250 µL/well), aspirate</td>
</tr>
<tr>
<td>Step 4</td>
<td>Dispense 100 µL/well of Px-conjugate</td>
</tr>
<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>
<tr>
<td>Step 5</td>
<td>Dispense 100 µL/well of TMB substrate</td>
</tr>
<tr>
<td></td>
<td>Incubate 10 minutes in dark at room temperature</td>
</tr>
<tr>
<td>Step 6</td>
<td>Dispense 100 µL/well of Stop solution</td>
</tr>
<tr>
<td>Step 7</td>
<td>Read the absorbance at 450/ 620-690 nm within 20 minutes</td>
</tr>
</tbody>
</table>
References:


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

Date of the last revision of this manual: 05/2013