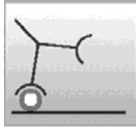
LIMITATION OF THE PROCEDURE

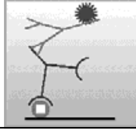
All highly sensitive immunoassay systems have a potential for nonspecific reactions therefore the specificity of repeatable reactive specimens should be verified using appropriate test methods.

OVERVIEW OF PROCEDURE

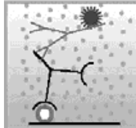
- Wash once with washing solution
- Dispense 80 µl of specimen diluent and 20 µl both control and investigated sera in wells
- Incubate for 60 min at 37°C (forming complex antigen-antibody)
- Wash 4 times with washing solution



- Dispense 100 µl of conjugate solution in wells
- Incubate for 30 min at 37°C (forming complex antigen-antibody with conjugate)
- Wash 6 times with washing solution



- Dispense 100 µl TMB substrate in wells
- Incubate for 30 min at room temperature (colouring)
- Stop the reaction by adding 100 µl stop-reagent
- Read the optical density at 450/620 nm



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Diaproph Med
Diagnostics Prophylaxis Medicine

DIA-HCV

The enzyme immunoassay test system for the detection of antibodies to the hepatitis C virus

96 tests **Product code: T-0307C**

EXAMPLE FORM

INTENDED USE

Enzyme immunoassay kit is intended for the detection of antibodies to hepatitis C virus in human serum and plasma.

INTRODUCTION

At the present moment hepatitis C is an actual problem in medicine. The hepatitis C gravity is caused by serious disease consequences. In most cases acute hepatitis C is taking minimum clinical symptomatology. However 50-80% HCV-infectious persons get chronical hepatitis C with high risks of cirrhosis and hepatocellulaire carcinome development.

The parenteral way of infecting and asymptomatic infection are an important reason for revealing of infected persons among different groups of population and first of all blood donors, bone marrow, organs and tissues.

Current laboratory diagnostics of hepatitis C is based on revealing of specific infecting HCV markers such as antibodies to viral proteins, HCV RNA. The viral genome codes 3 structural and 6 nonstructural proteins. Among first three ones are nucleocapsid protein – core protein and two glycoprotein of viral envelope (E1 and E2/NS1 – envelope protein). There is defined proteins with a fermentative activity as NS2, NS3, NS4a, NS4b, NS5a, NS5b in the nonstructural region of HCV genome. There is produced antibodies to each of structural and nonstructural proteins of viral polyprotein. The test kits of third generation allow to detect antibodies to antigens of the following regions core, NS3, NS4 and NS5. Application of such test kits for screening of donor blood minimizes a frequency of transfusion infecting with hepatitis C.

PRINCIPLE OF PROCEDURE

DIA-HCV is based on an indirect ELISA procedure.

There is used a solid phase (microelisa stripplate) coated with a mixture of recombinant HCV-specific proteins (NS3, NS4 and Core) and conjugate (monoclonal antibodies against human IgG bound to a horseradish peroxidase).

When investigated specimens of human plasma or serum are placed into wells, HCV-specific antibodies presented in specimen bind to recombinant antigens on solid phase

forming antigen-antibodies complexes. Formed complexes are detected using the specific conjugate.
The substrate buffer (hydrogen peroxide) and TMB solution is added to wells after washing non-bound components. Solution is coloured in case of presence of peroxidase conjugate in complexes.
To stop the colour reaction it is added stop-reagent and then determined the absorbance at 450/620 nm.

STORAGE CONDITIONS AND TRANSPORTATION

The kit must be stored and transported at 2-8°C. The kit must not be frozen. Shelf life of the kit is 14 months.

KIT REAGENTS

For *in vitro* diagnostic use. Each kit contains:

№	Reagents	Presentation
1	**Stock washing solution (№1)** (concentrate 45x) Phosphate buffer, containing 2.2% Triton X100.	2 bottles 2 × 25 ml
2	**Microelisa strips** 12 strips per plate each with 8 wells coated with mixture of recombinant HCV antigens: NS3, NS4 and Core.	1 plate
3	**Solution for sera dilution (№3)** Phosphate buffer, containing skimmed powdered milk. Preservatives: 0.01% thimerosal.	1 bottle 1 × 15 ml
4	**Solution for conjugate dilution (№4)** Phosphate buffer, containing skimmed powdered milk. Preservatives: 0.01% thimerosal.	1 bottle 1 × 15 ml
5	**Solution for chromogen preparation (№5T)** Citrate-phosphate buffer, containing 0.016% hydrogen peroxide.	1 bottle 1 × 8 ml
6	**Chromogen TMB** Solution containing 0.03% 3,3',5,5'-tetramethylbenzidine.	1 bottle 1 × 8 ml
7	**Positive control (C+)** Human serum reactive for antibodies to HCV. Inactivated by heating and treated with β-propiolacton and chloroform. Preservatives: 0.04 % MIT, 0.02% BND.	1 vial 1 × 0.5 ml
8	**Negative control (C-)** Heating inactivated human serum nonreactive for hepatitis B surface antigen (HBsAg) and antibodies to HIV and HCV. Preservatives: 0.1% sodium azide.	1 vial 1 × 1 ml
9	**Conjugate** (concentrate x11) Monoclonal antibodies against human IgG bound to a horseradish peroxidase (HRP). Preservatives: 0.01% thimerosal.	1 vial 1 × 1.5 ml
10	**Stop-reagent** 0.5 M sulphuric acid solution.	1 bottle 1 × 15 ml
11	**Adhesive film**	3 items

ADDITIONAL MATERIALS AND INSTRUMENTS REQUIRED

- distilled or deionized water;
- disposable gloves;

Grey zone is the zone with sample absorbance within the range

Cut-off – 10% ≤ OD ≤ Cut-off.

Interpretation of the results

The result is evaluated as **the nonreactive** one if the specimen absorbance is below the grey zone.
The result is evaluated as **the reactive** one if the specimen absorbance is equal or greater than the cut-off.
Specimens with absorbance values of the grey zone range are considered **indeterminate** and should be retested with caution in duplicate.
Specimens that show an initially reactive or indeterminate result should be retested in two or more wells:
- specimens reactive in one or more wells are considered as reactive ones;
- specimens nonreactive in two or more wells are considered as nonreactive ones.

Performance characteristics of the test

Sensitivity

The sensitivity of DIA-HCV was evaluated using panel of sera derived from chronical hepatitis C patients and panels of characterized sera manufactured by Boston Biomedica Inc. (USA), Bio Clinical Partners Inc. (USA), State Standardization and Control Institute of biological medications (SSCI, Russia):

Panel	*Characteristics of panel sera*
6212 (BCPI)	Anti-HCV seroconversion (9)
PHV105 (BBI)	Anti-HCV low titre (14), negative (1)
SSCI	Anti-HCV low titre (16), negative (8)
PHV 906 (BBI)	Anti-HCV seroconversion (7)
PHV 907 (BBI)	Anti-HCV seroconversion (7)
PHV 910 (BBI)	Anti-HCV seroconversion (5)

The test kit DIA-HCV under testing on seroconversion panel 6212 (BCPI) detects HCV-infection on 26 day, on seroconversion panel PHV906 – since 7th day, on panel PHV907 – on 18th day, on panel PHV 910 – on 11th day after HCV-RNA detection by PCR that corresponds to the third generation enzyme immunoassay sensitivity.
The results of determined sensitivity of DIA-HCV using above low titre panels are summarized in the table below.

Sensitivity of DIA-HCV in serum panels

Panel	Number of positive specimens from total quantity	Number of positive results in DIA-HCV
PHV105	14 of 15	14
SSCI	16 of 24	16

It was obtained no false negative results while investigating 347 sera derived from chronical hepatitis C patients.

Specificity

During investigating 8869 random donor samples it was shown 99.7% specificity of DIA-HCV.
The specificity of DIA-HCV was evaluated using samples from hospitalize patients, pregnant women, samples positive for markers of CMV-infection, HSV-1, HSV-2, tuberculosis, rubella, syphilis (interfering samples).
It was obtained at the most 0.5% false positive results while investigating interfering samples.

- aspirate completely.

Make sure that no fluid remains on the top and the bottom of the strips and stripholder after the last aspiration (e. g. by blotting with absorbent tissue).

∗ Contact our company for further information on the different types of washers validated by our technical services.

Test procedure

- Fit the stripholder with required number of **strips**.
- Wash once the strips with **washing solution** as recommended (section *Wash procedure*). If necessary, dry the plate by slight tapping upside-down on absorbent paper.
- Pipette 80 μl of the **solution for sera dilution (№3)** into each well.
- Distribute in the wells as follows:
  - well A1: 20 μl of **positive control (C+)**.
  - wells B1, C1, D1: 20 μl of **negative control (C-)**.
  - the rest wells : 20 μl of **specimens**.

Carefully repipette mixture in wells. (During repipetting solution turns its colour).

- Cover the plate with adhesive film and incubate at 37°C for 60 minutes.
- Aspirate the contents of all wells and wash the plate with **washing solution** 4 times (according the section *Wash procedure*). If necessary, dry the plate by slight tapping upside-down on absorbent paper.
- Pipette 100 μl of the **conjugate solution** into each well.
- Cover the plate with adhesive film and incubate at 37°C for 30 minutes.
- Aspirate the contents of all wells and wash the plate with **washing solution** 6 times (according the section *Wash procedure*). If necessary, dry the plate by slight tapping upside-down on absorbent paper.
- Pipette into each well 100 μl of the **TMB substrate**.
- Cover the plate with adhesive film and incubate at 18-25°C for 30 minutes in the dark.
- Add into each well 100 μl of **stop-reagent** to stop colour reaction (maintain the same pipetting sequence and rate used for TMB substrate addition).
- Read the absorbance at 450/620 nm using a dual wavelength microplate reader within 5 minutes after stopping the reaction.

As an exception, absorbance may be measured at 450 nm (single wavelength) against a blank well; for that include an empty well in the run.

Results

Calculation of the results

NC – absorbance of the negative control
PC – absorbance of the positive control
$\overline{NC}$ – mean absorbance of the negative control

- Calculate the mean absorbance of the negative control.

Test run is valid if $\overline{NC}$ is not higher than 0.100 optical units (OU).

Exclude any NC, which is higher than 0.100 OU or if it is more than twice exceed the $\overline{NC}$, and recalculate $\overline{NC}$ of the remaining controls.

Test run is valid if PC is not lower than 0.600 OU.

- Calculate **Cut-off** value.

$$\text{Cut-off} = \overline{NC} + \mathbf{0.120}$$

- Determine the grey zone.

- disposable V-shaped troughs;
- vial for reagents preparation (glass or plastic);
- graduated cylinder (1000 ml);
- absorbent paper;
- sodium hypochlorite solution or other accepted disinfectant;
- sodium bicarbonate;
- ethanol, 70°;
- automatic single-channel pipettes (e.g. 5-40, 20-200, 200-1000 μl) with disposable tips;
- automatic multi-channel pipettes (50-300 μl) with disposable tips;
- incubator, 37±1°C;
- microwell wash system*;
- microwell reader* (with dual wavelength 450/620);
- biohazard waste containers for potentially contaminated materials.

∗ Contact our company for further information on the equipment validated by our technical services.

SAFETY PRECAUTIONS AND WARNINGS

- Use a new tip for pipetting specimens in wells.
- All reagents included in the kit are intended for "in vitro” diagnostic use.
- Wear disposable gloves when handling reagents and samples and thoroughly wash hands after handling them.
- Do not pipette by mouth.
- Human origin material used in the preparation of the negative and positive controls. The positive control has been inactivated by heating and treated with β-propiolacton and chloroform. The negative control has been tested and found nonreactive for hepatitis B surface antigen (HBsAg), antibodies to HCV and antibodies to HIV (HIV-1, HIV-2), however for the purpose of additional protection treated with heating.
- Because no known test method can offer complete assurance that infectious agents are absent, handle reagents and patient samples as if capable of transmitting infectious disease.
- Any equipment directly in contact with specimens and reagents as well as the washing solution be considered as contaminated products and treated as such.
- Avoid spilling samples or solution containing samples.
- Spills must be treated with ethyl alcohol 70°. If the contaminating fluid is an acid, spill must be neutralized with sodium bicarbonate and dried with absorbent paper. The materials used for cleaning must be discarded in a contaminated residue container.
- Samples and reagents of human origin, as well as, contaminated material and products must be discarded after decontamination:
  - Either by immersion solid wastes in sodium hypochlorite at a final concentration of 5%, liquid wastes in sodium hypochlorite at a final concentration of 1% during 30 min.
  - Or by autoclaving at 121°C during 2 hours. The best method of inactivating of HIV, HBV, and HCV is an autoclaving.
  - DO NOT PLACE SOLUTIONS CONTAINING SODIUM HYPOCHLORITE IN THE AUTOCLAVE.
- Do not forget neutralize acid solutions before autoclave.
- Avoid any contacts substrate solution, chromogen and stop-reagent with skin and mucous covers.
- The negative and positive controls contain sodium azide as a preservative. Sodium azide may react with laboratory pluming forming copper or lead azides. Such azides are

explosive. To prevent azide build-up, flush the pipes with a huge quantity of water if solutions containing azide are disposed of the sink after inactivation.

SPECIMEN PREPARATION

Serum or plasma specimens are to be stored at 2-8°C during 72 hours. If necessary these specimens may be frozen (not more than two freezing-thawing procedures are allowed) at temperature below –20°C.
All specimens containing aggregates and visible suspended particles are to be clarified by centrifugation.
Specimens with sodium azide, hemolysis, hyperlipidemiae or bacterial contamination may not be used in the ELISA procedure.

ASSAY PROCEDURE

Reagents and specimens should be at room temperature (18-25°C) before beginning the assay and can remain at room temperature during testing. Return reagents to 2-8°C after use.

Reagents preparation

Microelisa strips

Open the pack and remove the plate. Return unused strips in the pack. Reseal the pack and return to 2-8°C.
The strips are stable for 4 weeks at 2-8°C after opening the pack.

Washing solution

Check **Stock washing solution (№1)** for the presence of salt crystals. If crystals are seen in the solution, dissolve them by heating at 35-37°C.
Dilute the **stock washing solution (№1)** 1:45 with distilled or deionised water (see chart below), shake intensively.
Washing solution is stable for 5 days at 2-8°C.

Number of wells	Stock washing solution (№1)	Distilled water
8	4 ml	180 ml
16	6 ml	270 ml
32	8 ml	360 ml
48	12 ml	540 ml
96	25 ml	1125 ml

Conjugate solution

Dilute to 1:10 the **conjugate** in **solution for conjugate dilution (№4)** (see chart below) in a clean vial. Mix well avoiding foaming.
Conjugate solution has to be prepared before use.
Conjugate solution is stable for 2 weeks at 2-8°C.

Number of wells	Conjugate (concentrate x11)	Solution for conjugate dilution (№4)
8	100 μl	1 ml
16	200 μl	2 ml
32	400 μl	4 ml
48	600 μl	6 ml
96	1000 μl	10 ml

TMB substrate

To prepare **TMB substrate**, mixture the required amount of **chromogen TMB** in a clean vial in equal parts with **solution for chromogen preparation (№5T)** according to the number of wells being run (see chart below). Mix well. TMB substrate must be colourless before use.
The TMB substrate must be kept away from light and no solutions contact with metals or metal ions is allowed.
The solution has to be prepared before use.
TMB substrate is stable for 2 weeks at room temperature (18-25°C) if kept in the dark.

Number of wells	Chromogen TMB	Solution for chromogen preparation (№5T)
8	0.5 ml	0.5 ml
16	1 ml	1 ml
32	2 ml	2 ml
48	3 ml	3 ml
96	6 ml	6 ml

Procedural notes

Authenticity of results depends on correct execution following instructions:

- Reagents should not be used beyond the expiry date shown on the package label.
- Reagents should not be mixed from different lots during performing test.
- Reagents and samples should be at room temperature (18-25°C) before testing begins. Return the reagents to 2-8°C after use.
- The temperature in room where performing analysis should be in the range 18-25°C.
- It should accurately dissolve reagents avoiding its contamination.
- Do not perform the test in the presence of reactivity vapours (for example, from sodium hypochlorite, acids, alkalis, or aldehydes) or dust because the enzymatic activity of the conjugate may be affected.
- Use glass vessels thoroughly washed and rinsed with deionized water or use disposable ones.
- Do not allow drying contents of wells on all stages of procedure.
- Enzyme reaction is sensitive to metal ions, so avoid contacting with metal elements.
- TMB substrate (solution for chromogen preparation + chromogen TMB) is to be colourless. Appearance of colouring after dilution is evidence of unavailability for using and solution is to be replaced. The solution is to be prepared in clean plastic ware or clean glassware. The reagent is to be kept in dark.
- Prevent the direct light to fall on the working surface during ELISA procedure.
- Use a new tip for brining specimens in wells.
- Never use the same trough for distribution conjugate and TMB substrate.
- Check the pipettes and other equipment for accuracy and correct operation.
- Do not change the assay procedure.

Wash procedure

Washing must be performed strictly according to the instructions, as insufficient plate washing leads to incorrect results.
Use automatic washer*, as recommended; in case of its absence or faulty work – use multi-channel pipette for washing.
Follow this procedure in each washing:

- aspirate the wells contents completely into a waste flask;
- then fill the wells completely with washing solution (not less than 350 μl per well) avoiding overflow of buffer from one well to another;