HCV

ELISA 3ª generación

Enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to the Hepatitis C virus

SUMMARY

Hepatitis C is the most common form of post-transfusional hepatitis. Its etiological agent is the hepatitis C virus (HCV) and is mainly transmitted parenterally. Other forms of transmission, such as mother-to-fetus and sexual, are less efficient. Approximately 80% of the individuals infected with hepatitis C virus develop a chronic infection and in a 30% of the cases the disease develops to cirrhosis and liver cancer. Most of the persons with hepatitis C do not present symptoms and, therefore, the disease is rarely diagnosed before the onset of chronic complications.

The HCV genome is formed by a simple positive ARN chain encoding a polyprotein, which may originate at least 9 functional proteins. The first hepatitis C assays where 1st generation and used only NS4 protein. However, they lacked sensitivity and specificity. Currently, there are 3rd generation tests available which incorporate proteins from the core (structural) and non-structural regions (NS3, NS4 and NS5). Serological tests for the diagnosis of the HCV infection detect anti-HCV antibodies. They are used in the diagnosis of the infection and the control of donor units in blood banks.

PRINCIPLE

The microtitration wells are coated with recombinant antigens derived from the structural (core) region and the non-structural regions (NS3, NS4 and NS5) of hepatitis C virus. The diluted sample is incubated in the wells. If the serum samples contain the specific antibodies, these will form a complex with the antigens and will remain bound to the support. The unbound fraction is eliminated by washing, after which human monoclonal anti-IgG antibodies conjugated to peroxidase are added. The conjugate will bound to the previously formed antigen-antibody complexes. The unbound conjugate is removed by washing. Tetramethylbenzidine and hydrogen peroxide solution is then added. The reactive samples develop a light blue color that changes to yellow when the reaction is stopped by adding sulfuric acid.

PROVIDED REAGENTS

Coated microtitration plate: microtitration plate with cutout strips and 96 wells coated with HCV recombinant antigens.
Sample Diluent: saline buffer with surfactant. Violet color.
Concentrated Conjugate: human anti-IgG monoclonal antibody conjugated to peroxidase (10x). Red color.
Conjugate Diluent: saline buffer with proteins.
Substrate: tetramethylbenzidine (TMB) and hydrogen peroxide solution.
Stopper: 2 N sulfuric acid.
Concentrated Wash Buffer: saline buffer with surfactant (25x). Green color.
Positive Control: inactivated human serum containing antibodies to HCV. Orange color.
Negative Control: inactivated non-reactive human serum. Yellow color.

NON-PROVIDED REAGENTS

Distilled or deionized water.

REQUIRED MATERIAL (non-provided)

- Micropipettes for measuring stated volumes
- Disposable tips
- Volumetric material to prepare stated dilutions
- 37ºC incubator
- Absorbent paper
- Disposable gloves
- Timer or stopwatch
- Sodium hypochlorite
- Microtitration plate wash system (manual or automatic)
- Spectrophotometer for microtitration plate reading

WARNINGS

- The reagents are for “in vitro” diagnostic use.
- All patient samples should be handled as capable of transmitting infection.
- The control sera have been tested for Hepatitis B Surface Antigen (HBsAg) and antibodies to Hepatitis C Virus (HCV) and Human Immunodeficiency Virus (HIV) and found to be non-reactive. However, because no test method can offer complete assurance that infectious agents are absent, they should be handled as potentially infectious material.
- All materials used to perform the test must treated to ensure the inactivation of pathogenic agents. The recommended method is autoclaving for one hour at 121ºC. Liquid waste may be disinfected with sodium hypochlorite (5% final concentration) for at least 60 minutes.
- Avoid contact of strips with hypochlorite fumes from biohazards disposal containers or other sources, since hypochlorite affects the reaction.
- Avoid liquid spilling and aerosol formation.
- Do not use the reagents after the expiration date.
- Do not exchange reagents from different lots and do not modify assay procedures.
- Do not use reagents from other origin.
- Avoid touching the walls of the wells with the tips.
- Avoid using metal objects that may be in contact with the reagents.

- The microtitration plates should be incubated in incubator. Do not use water bath. Do not open the incubator during this process.
- Avoid contact of the sulfuric acid (Stopper) with the skin, mucous membranes and eyes. R36/38: irritates eyes and skin. R34 causes burns. S24/25: avoid contact with the eyes and skin. S26: if splashing onto skin occurs, rinse the affected area with copious quantities of water and seek medical attention. S28: after contact with skin, wash immediately with plenty of water. S37/39: wear suitable gloves and eye/face protection.
- Avoid the spill of liquids and the formation of sprays.
- Do not pipette by mouth. Use disposable gloves and eye protection during handling of samples and reagents.
- TMB is sensitive to light. Keep the bottle capped when unused.
- All reagents and samples should be discarded according to the regulations in force.

REAGENTS PREPARATION

All the material used for reagent preparation should be clean and free from detergent and hypochlorite.

Wash Buffer: constituents of the concentrated reagent may precipitate at low temperature. In such case, bring the solution to 37ºC until complete dissolution. To obtain a ready-to-use wash buffer (1x), dilute 1 part Concentrated Wash Buffer (25x) with 24 parts distilled or deionized water. Example: 20 ml with 480 ml for one microtitration plate.

Conjugate: To obtain a ready-to-use conjugate (1x), dilute 1 part Concentrated Conjugate (10x) with 9 parts Conjugate Diluent. Example: see table with concentrated Conjugate and Conjugate Diluent required volumes.

Nº of wells	Concentrated Conjugate	Conjugate Diluent
8	100 ul	0.9 ml
16	200 ul	1.8 ml
24	300 ul	2.7 ml
32	400 ul	3.6 ml
96	1200 ul	10.8 ml

Sample Diluent, Conjugate Diluent, Substrate, Stopper, Negative and Positive Controls: ready to use.

STABILITY AND STORAGE INSTRUCTIONS

Provided Reagents are stable in refrigerator (2-10ºC) until expiration date stated on the box. Do not freeze.

Concentrated Wash Buffer and Stopper: they may be stored at room temperature (2-25ºC).

Wash Buffer (1x): store in sealed container. It is stable for 3 months at room temperature (2-25ºC).

Conjugate (1x): it is stable for 6 hours at room temperature (2-25ºC).

Coated microtitration plate: do not open the pouch until performing the test and until it has reached room temperature. Otherwise, the well strip surface could get moistened. Unused well strips should be kept in the pouch with the desiccant, sealed stored at 2-10ºC. Test strips stored in this manner are stable for 4 months if it does not exceed the date printed on the pouch label.

SAMPLE

Serum or plasma

a) Sample collection: obtain in the usual way.

b) Additives: not required for serum. Employ plasma collected using EDTA, heparin or sodium citrate as anticoagulants.

c) Known interfering substances: no interference has been observed with bilirubin up to 25 mg/dl, ascorbic acid up to 50 mg/dl, triglycerides up to 1,500 mg/dl, hemoglobin up to 300 mg/dl. Samples containing particles should be clarified by centrifugation.

d) Stability and storage instructions: sample should be stored at 2-10ºC up to 3 days. If extended storage periods are required, samples should be frozen at -20ºC (or less). Samples should not be repeatedly frozen and thawed. This may lead to erroneous results. In case of using frozen samples, they should be homogenized and centrifuged before use.

Heat inactivation may affect the result.

Do not use samples with microbial contamination.

If samples are to be transported, they should be packaged according to local regulations for biohazard material shipment.

TEST PROCEDURE

1- Bring the reagents and samples to room temperature before opening.

2- Prepare the necessary volume of diluted Wash Buffer (1x).

3- Place the required wells in the strip holder for the number of determinations to be used, including 2 wells for Positive Control (PC) and 3 for Negative Control (NC).

4- Dispense the Sample Diluent, then the sample (S) and the controls according to the following scheme:

	S	PC	NC
Sample Diluent	100 ul	100 ul	100 ul
Positive Control	-	20 ul	-
Negative Control	-	-	20 ul
Sample	20 ul	-	-

Homogenize 2-3 times by loading and unloading the micropipette or shaking the plate for 10 seconds. When adding the sample, the Sample Diluent will change color (see table). Control or sample dosing to the wells may be visually or spectrophotometrically verified (at 610/650 nm):

Sample type	Without sample	Serum or plasma	Positive Control	Negative Control
Color	Violet	Light blue	Dark orange	Green

Warning: turbid, icteric or hemolyzed samples may change the final color without affecting the results. Color change may depend on the added sample volume and

its composition. A less intense color change may be due to a lower dispensed sample volume, the sample not being in the appropriate conditions, or a low protein level.
5- To avoid evaporation, cover the plate with the provided adhesive tape and incubate for 60 ± 2 minutes at 37 ± 1ºC. At the same time, prepare the diluted conjugate (see table in REAGENTS PREPARATION).
6- The liquid from each well must be thoroughly removed after incubation. Wash 5 times according to washing instructions (see WASHING PROCEDURE).
7- Add the Conjugate:

Conjugate	100 ul	100 ul	100 ul

To avoid evaporation cover the microplate with adhesive tape.
8- Incubate for 30 ± 2 minutes at 37 ± 1ºC.
9- Wash 5 times according to the washing instructions.
10- Dispense the Substrate. Transfer to a clean recipient only the required Substrate volume. Do not transfer the remaining Substrate back to the original bottle. Avoid reagent contact with oxidizing agents.

Substrate	100 ul	100 ul	100 ul

11- Incubate for 30 ± 2 minutes at room temperature (18-25ºC), protecting from light .
12- Add the Stopper:

Stopper	100 ul	100 ul	100 ul

13- Read absorbance in spectrophotometer monochromatically at 450 nm or bichromatically at 450/620-650 nm.

Note: bichromatic reading is recommended. In case the reading is monochromatic, perform a reagent blank that will have to be subtracted from all sample values.

STABILITY OF THE FINAL REACTION
Reaction color is stable for 10 minutes. Thus, results should be read within this period.

WASHING PROCEDURE
Remove the liquid from the wells by aspiration or inversion. The wells are washed with 350 ul Wash Buffer. When filling the wells make sure not to spill. The wash solution should be in contact with the wells for 30 to 60 seconds. Make sure no residual liquid remains after the final washing step. Perform double aspiration to remove excess buffer. If after such procedure, it still persists, invert the plate onto absorbent paper and tap it several times. Otherwise, erroneous results may be obtained.

Note: the washing procedure is crucial for the test result. If excess wash buffer remains in the wells or if the wells are not completely filled erroneous results may be obtained. Do not let the wells air dry during the procedure. Automatic washers should be rinsed with distilled or deionized water at the end of the day to avoid obstructions due to the presence of salt in the wash buffer.

SUMMARY OF THE PROCEDURE

STAGE	PROCEDURE	WARNINGS/OBSERVATIONS
Dilution	Prepare Wash solution (1x)	Dissolve salt crystals
Sample Diluent	Add 100 ul Sample Diluent in each well	
Samples	Add 20 ul S, PC and NC	Color change is observed when adding the sample and controls
Incubation	Cover the wells and incubate for 60 ± 2 minutes at 37 ± 1ºC	In incubator
Washing step	Wash each well with 350 ul diluted Wash Buffer (5 times)	Time of contact of the Wash solution from 30 to 60 seconds. Completely remove the residual liquid from the wells
Dilution	Conjugate preparation (1x)	During incubation with the sample, dilute the Concentrated Conjugate (10x)
Conjugate	Add 100 ul diluted Conjugate (1x)	
Incubation	Cover the wells and incubate for 30 ± 1 minutes at 37 ± 1ºC	In incubator
Washing step	The same as above	
Substrate	Add 100 ul Substrate	Transfer the required Substrate volume to be used. Do not pipette from the original bottle. Discard the remaining reagent. Avoid contact with oxidizing agents. Do not expose to light.

Incubation	30 ± 2 minutos at 18-25ºC	Mantein the wells protected from light
Stop	Add 100 ul Stopper	
Reading	Read in spectrophotometer	Read within 10 minutes

ASSAY VALIDATION CRITERIA

The assay is considered valid if the following conditions are simultaneously met:

1- The mean absorbance of the Negative Controls should be less than or equal to 0.100.
Example:
Reading 1 = 0.042; Reading 2 = 0.058; Reading 3 = 0.050
Mean = (0.042 + 0.028 + 0.058) / 3 = 0.050

2- Remove any Negative Control with absorbance greater than 0.100.

3- If any Negative Control has been removed, recalculate the Negative Control mean. An assay is valid when at least two of the Negative Controls are accepted.

4- The absorbance mean of the Positive Controls should be greater than 1.000.
Example:
Reading 1 = 1.407; Reading 2 = 1.331
Mean = (1.407+1.331) / 2 = 1.369

5- The absorbance mean difference of the Positive and Negative Controls should be greater or equal to 0.900.

In case one of the above conditions is not met, repeat the assay.
Remember that the obtained readings will depend on the sensitivity of the instrument used.

INTERPRETATION OF RESULTS

The presence or absence of antibodies anti-HCV is determined associating the sample absorbance with the Cut-off value.

Cut-off = NC + 0.150

NC: absorbance mean of the Negative Control

Example: 0.045 + 0.150 = 0.195

Non-reactive samples: samples with absorbances lower than the Cut-off value.
Reactive samples: samples with absorbances greater or equal to the Cut-off value.

If this type of interpretation is selected, every sample not presenting more color than the Negative Controls should be considered non-reactive. Otherwise, an evidently yellow sample is considered Reactive.
All samples initially reactive should be repeated by duplicate. If one or both repetitions are reactive, it should be considered reactive.
A sample initially reactive may be non-reactive in both repetitions. This may be due to:
- Cross contamination of a non-reactive well with a reactive sample.
- Sample contamination during dispensation, lack of precision in sample, conjugate and/or Substrate dispensation into the well.
- Tip re-utilization.
- Well contamination with hypochlorite or other oxidizing agents.

In certain cases a non-reactive sample may produce a falsely reactive reaction, both in the initial analysis as in its repetitions. Some probable causes of this effect may be:
- Sample contamination during collection, processing or storage.
- Presence of interfering substances, such as autoantibodies, drugs, etc.
- Ineffective dispensation and/or aspiration of the wash solution (obstructed system).

PROCEDURE LIMITATIONS

See Known interfering substances under SAMPLE.
Do not use pooled samples.
Do not use other body fluids such as saliva, cerebrospinal fluid or urine.
The presence of antibodies to hepatitis C virus indicates recent or past infection, but it is impossible to differentiate between acute, chronic or resolved infection. Due to the extensive period of time from infection to seroconversion, anti-HCV levels may be undetectable in early stages of the infection. Therefore, a negative result does not exclude the possibility of HCV infection.
Repeatedly reactive samples should be tested using supplemental or confirmatory techniques, such as Immunoblot or PCR, respectively.

SPECIFIC PERFORMANCE FEATURES

a) Sensitivity

Sensitivity in performance panels: in a study performed on different international commercial panels, the following results were obtained:
PHV 103 (Anti-HCV Low Titer Performance Panel, BBI, USA): 14 out of 14 reactive samples were detected.
PHV 105M (Anti-HCV Low Titer Performance Panel, BBI, USA): 12 out of 12 reactive samples were detected.
PHV 205 (Anti-HCV Mixed Titer Performance Panel, BBI, USA): 23 out of 23 reactive samples were detected.
PHV 206 (Anti-HCV Mixed Titer Performance Panel, BBI, USA): 23 out of 23 reactive samples were detected.
PHV 207 (Anti-HCV Mixed Titer Performance Panel, BBI, USA): 21 out of 23 reactive samples were detected.
PP0404 (HCV Performance Panel, Q Panel, Brazil): 16 out of 16 reactive samples were detected.
PP0405 (HCV Performance Panel, Q Panel, Brazil): 16 out of 16 reactive samples were detected.

PP0406 (HCV Performance Panel, Q Panel, Brazil): 16 out of 16 reactive samples were detected.

Sensitivity in Seroconversion Panels: the following international commercial seroconversion panels from BBI (USA) were tested:

Panel	Number of samples	HCV ELISA 3ª generación	RIBA	Seroconversion pattern
PHV 901	11	9 (97)	9 (97)	NS3-NS4
PHV 906	7	5 (7)	7 (0)	NS3-NS4
PHV 910	5	3 (8)	3 (8)	core
PHV 912	3	1 (7)	1 (7)	core
PHV 920	10	7 (13)	7 (13)	core-NS3

The table indicates the number of reactive samples with every method. The number between brackets indicates the number of days from the initial bleeding and the first reactive sample.

Sensitivity to different genotypes: the Worldwide HCV Performance Panel WWHV 302 BBI, USA was tested and 14 out of 14 reactive samples were detected.
In addition, 14 samples from genotype 1, 5 from genotype 2 and 4 from genotype 4 were detected.

Clinical sensitivity in anti-HCV reactive sample panels: in a study performed on 190 samples with HCV infection, confirmed by different methods, all of the samples were found reactive with the HCV ELISA 3ª generación kit.
In a study of 356 reactive samples from diverse hospitals, 355 samples were detected.

b) Specificity
In a study performed on 1364 sera and plasma samples from blood banks, the obtained specificity was 99.41%.
In a further study on 1660 sera and plasma samples from two different health institutions (with high HCV prevalence), a specificity of 99.58% was obtained.
A possible cross-reactivity was evaluated, assaying samples from 556 individuals with different clinical conditions that may be the cause of unspecific reactions for the HCV ELISA 3ª generación test. This group included samples:

- With antibodies to HAV, HBV, EBV, CMV, HSV, VZV, HIV, HTLV and other viruses.
- With different autoantibodies (AGA, AMA, ATA, FAN, rheumatoid factor and others).
- With antibodies to *Treponema pallidum, Mycoplasma pneumoniae, Toxoplasma gondii, Toxocara canis, Trypanosoma cruzi* and other microorganisms.
- From hemodialyzed patients and pregnant women.

The obtained specificity for this population was 98.38%.

c) Precision
The test precision was evaluated following EP5-A protocol recommended by the CLSI (former NCCLS). The assays were performed with samples having different reactivity levels and with controls. Two daily assays were performed testing each sample by duplicate during 20 days.

	Mean absorbance	Intra-assay		Total	
		S.D.	C.V.	S.D.	C.V.
Sample 1	0,330	0,026	7,92%	0,038	11,42%
Sample 2	0,405	0,031	7,73%	0,050	12,37%
Sample 3	0,579	0,044	7,53%	0,075	13,02%
Sample 4	0,976	0,076	7,76%	0,102	10,42%
(+) Control	1,297	0,082	6,30%	0,164	12,64%
(-) Control	0,047	0,004	8,38%	0,007	14,86%

n = 80

WIENER LAB. PROVIDES
Kit for 96 tests (Cat. Nº 1483258).
Kit for 192 tests (Cat. Nº 1483259).

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SYMBOLS EXPLANATION

Policubeta | Sensib.
Coated microtitration plate

Diluyente | Muestra
Sample Diluent

Conjugado | Conc.
Concentrated Conjugate

Conjugado | Diluy.
Conjugate Diluent

Revelador
Substrate

Buf. Lavado | Conc.
Concentrated Wash Buffer

Control | +
Positive Control

Control | -
Negative Control

Stopper
Stopper

The following symbols are used in packaging for Wiener lab. diagnostic reagents kits.

CE This product fulfills the requirements of the European Directive 98/79 EC for "in vitro" diagnostic medical devices

EC REP Authorized representative in the European Community

IVD "In vitro" diagnostic medical device

Contains sufficient for <n> tests

Use by

Temperature limitation (store at)

Do not freeze

Biological risks

Volume after reconstitution

Cont. Contents

LOT Batch code

Manufactured by:

Harmful

Corrosive / Caustic

Irritant

Consult instructions for use

Calibr. Calibrator

CONTROL Control

CONTROL + Positive Control

CONTROL – Negative Control

REF Catalog number

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UR110708