HCV Ab
ELISA TEST SYSTEM

INTENDED USE

The Reactiva Search.'s HCV Ab ELISA Test System is a third generation enzyme immunoassay for the qualitative detection of antibody to Hepatitis C Virus (HCV) in human serum or plasma samples. The assay is intended for the screening of blood units and for the diagnosis and monitoring of HCV infection.

SUMMARY AND EXPLANATION

The HCV is a member of Flaviviridae family, Hepacivirus genus. It is a spherical enveloped virus with single strand linear RNA of positive sense genome. The RNA of HCV consists of a 5' and 3' noncoding regions and of a single large open reading frame (ORF) encoding a large polyprotein precursor of approximately 3000 amino acids (1). Co- or post-traslationally cleavage produce a capsid protein and two envelope glycoproteins (gp33 and gp72). At least five non-structural proteins (NS1-NS5) are assayed in the 3' portion of ORF. The 5' end of E2 gene is the most heterogeneous region named "first hypervariable region". Based on genetic heterogeneity, the HCV strains are divided in types (a, b, c, d, e), subtypes (1, 2, 3, 4, 5, 6) and quasispecies indicating the heterogeneous populations of isolate subtype genome (2-4). The HCV infection is spread through sexual intercourse, contaminated needles, blood transfusion, maternal/new-born route (5,6). In the majority of cases, HCV infection gives rise to an acute illness; 80% of such cases develop into chronic hepatitis. Almost all patients develop a vigorous antibody and cell-mediated immune response which fails to clear the infection but may contribute towards liver damage. Spontaneous resolution of chronic liver disease is very rare (<2%) and patients with chronic disease are at risk to developing severe liver damage as cirrhosis and hepatocellular carcinoma (7). Currently, third-generation enzyme immunoassay for antibody to HCV is the most practical screening test for HCV infection. The diagnosis of HCV infection is supported or confirmed by the recombinant immunoblot assay (RIBA) or tests for HCV RNA (RT-PCR) (811).

PRINCIPLE OF THE TEST

The kit HCV Ab is based on "indirect sandwich" Elisa principle. The Microplate wells are coated with HCV synthetic peptides (Core, NS4, NS5) and with recombinant antigen (NS3). These antigens are derived from "Core" and "NS" conserved regions encoding for immunodominant antigenic determinants. The sample, dispensed into the well, reacts with the solid phase and the antibodies to HCV, if present, are captured by the antigens. After washing out all the other components of the sample, in the second incubation bound antibodies to HCV are detected by the addition of goat antihuman IgA, IgG and IgM antibody, labelled with horse radish peroxidase (HRP). The enzyme captured on the solid phase, acting on the Chromogen/Substrate solution, generates an optical signal that is proportional to the amount of antibodies to HCV present in the sample.

MATERIALS AND COMPONENTS PROVIDED

• **Strip Microplate** – Microplate of 8 x 12 strips of breakable wells activated with synthetic and recombinant HCV antigens. The microplates are sealed in an aluminium pouch in presence of desiccant bag. no. of microplates: 1

• **Positive Control** – Ready to use. Buffered solution of serum base highly reactive for antibodies to HCV. It contains 0.09 % sodium azide, 0.09 % Kathon as preservatives and Coomassie brilliant blue as coloring agent. Volume: 0.6 ml

Note – The Positive Control has been inactivated with 1% tri (n-buthyl) phosphate and 1% Triton X-100 at 30 °C for 4 hours by the manufacturer.

• **Negative Control** – Ready to use. Buffered solution of serum base not reactive for antibodies to HCV that contains 0.09 % sodium azide, 0.09 % Kathon as preservatives and Coomassie brilliant blue as coloring agent. Volume: 1.2 ml

• **Sample Diluent** – Proteic solution for the dilution of samples that contains stabilizers, 0.09 % sodium azide, 0.09 % Kathon as preservatives and Coomassie brilliant blue as colouring agent. Volume: 50.0 ml

• **Washing Solution** – To dilute before use. Solution 25x concentrated that contains Imidazole buffer and surfaceactive agent. Volume: 50.0 ml

• **Conjugate** – To dilute before use. Solution of proteic buffer, 20x concentrated, that contains goat anti-human IgA, IgG and IgM antibodies, labelled with HRP, proteic stabilizers, 0.02% gentamicin sulfate and 0.09% Kathon as preservatives. Volume: 0.6 ml

• **Conjugate Diluent** – Buffered proteic solution, for the dilution of the concentrated Conjugate that contains proteic stabilizers, 0.02% gentamicin sulphate, 0.09 % Kathon as preservatives and Ponceau red as colouring agent. Volume: 12.0 ml

• **Chromogen** – To mix with Substrate. Solution of 3,3',5,5' tetramethylbenzidine (TMB), activators and stabilizers, in a phosphate/citrate buffer.
Warning: Store protected from light. Volume : 7.0 ml

• **Substrate** – To mix with Chromogen. Solution that contains hydrogen peroxide (H2O2), activators and stabilizers, in a phosphate/citrate buffer. Volume: 7.0 ml

• **Stop Solution** – Solution of 0.3 M sulphuric acid.
Note: handle with care. Volume : 10.0 ml

• **Cardboard Sealer** - Transparent plastic sealer to cover microplates during the incubation at 37 °C. no. of sealers: 2

• **Package insert** – The present document.

• **Symbol information sheet** – List of the symbols.

Note - All the materials of human origin have been controlled and certified by the supplier to be negative for HBsAg, HCV Ab and HIV Ab.

MATERIALS REQUIRED BUT NOT PROVIDED

• Micropipettes of 20, 100, 300 and 1000 µl with disposable tips.
• Vortex mixer and adsorbent papers.
• Distilled water.
• Timer.
• Incubator set at 37 ± 1 °C (dry or moist heat).
• Automatic or manual microplate washer able to aspirate and dispense volumes of 300 - 400 µl.
• Photometric microplate reader linear up to at least 2 OD and supplied with filters of 450 nm and 620 - 630 nm.

STORAGE CONDITIONS

• The kit must be stored at 2 – 8 °C and used before the expire date declared on the external label.
• The pouch containing the microplate has to be brought to room temperature before opening. Take out from the frame only the strips necessary for the test programmed and store the remaining strips in the same pouch in presence of the desiccant bag. Close hermetically the pouch and store again at 2 - 8 °C. If stored properly, strips are stable for 2 months from opening.
• The diluted Washing solution, at room temperature, is stable for 1 week.
• The Chromogen/Substrate are stable until the expiration of the kit.
• The other reagents can be used every time, if stored at 2 – 8 °C and handled carefully for avoiding contamination.

PRECAUTIONS

1. All the reagents contained in the kit are for in vitro diagnostic use only.
2. Do not use the kit or reagents after the expiration date stated on labels.
3. Do not mix reagents of different lots.
4. Procedures should be performed carefully in order to obtain reliable results and clinical interpretations.
5. Bring all the reagents to room temperature for at least 60 minutes, before the test is started.
6. Avoid any contamination of reagents when taking them out of vials. We recommend to use automatic pipettes and disposable tips. When dispensing reagents, do not touch the wall of microplate wells with tips, in order to avoid any crosscontamination.
7. In the washing procedure, use only the Washing Solution provided with the kit and follows carefully the indications reported in the "Washing Instructions" Section of this insert.
8. Ensure that the Chromogen/Substrate does not come in contact with oxidizing agents or metallic surfaces; avoid any intense light exposure during the incubation step or the reagent preparation.
9. Put the reagents in a glass or plastic disposable container, washed with sulfuric acid 1N, then with deionized water, before use.
10. Samples and materials potentially infective have to be handled with care as they could transmit infection. All objects come in direct contact with samples and all residuals of the assay should be treated or wasted as potentially infective. Best procedures for inactivation are treatments with autoclave at 121 °C for 30 minutes or withsodium hypochlorite at a final concentration of 2.5 % for 30minutes. This last method can be used for the treatment of theliquid waste after that it has been neutralized with NaOH.
11. Avoid any contact of liquids with skin and mucousmembrane. Use always protective talk-free gloves, glassesand laboratory coats, according to the safety regulations.
12. Some reagents of the kit contain sodium azide which may betoxic if ingested. Sodium azide may react with copper andlead piping to form highly explosive salts. On disposal, flushwith large quantities of water.
13. At least 1 hour before use bring all the reagents necessary to the test to room temperature and mix carefully the liquid reagents supplied on vortex (in particular the Controls, the Conjugate and the Chromogen/Substrate) avoiding foaming. Take out from the frame only the strips necessary for the test programmed and store the remaining strips in the same pouch in presence of the desiccant bag.
14. Distribution and incubation times should be the same for all the wells; avoid long interruptions among the different steps of the assay.
15. It is suggested to eliminate the excess of washing solution from wells by blotting them gently on a paper adsorbent pad.
16. The color developed in the last incubation is stable for maximum 1 hour in the dark.
17. We recommend reading the microplate at 450 nm (reading filter) and subtracting the blank at 620 - 630 nm (blanking filter). Blank the reader on A1 well.

SPECIMEN COLLECTION

Either fresh sera or plasma (EDTA, Heparin, Citrate) can be used for the assay. If not used immediately, they can be stored at 2 - 8 °C for 1 week. In case of longer storage freeze them at – 20 °C. Samples should be clear. If the samples are turbid, could be contaminated by micro-organism, insofar it recommends to centrifuge them at 2000 rpm x 20 minutes at room temperature or filtrate on 0.22 µm filters. The samples that, after the above said procedure, did not became clear, cannot be used.

REAGENT PREPARATION

• **Washing Solution** - The concentrated solution to be diluted 25x with distilled water before use.

• **Conjugate** - Dilute the concentrated Conjugate 1:20 with the Conjugate Diluent. Mix on vortex before use. The diluted Conjugate is stable for 1 week at 2 – 8 °C, when stored in a sterile disposable container.

• **Chromogen/Substrate** - About 5 minutes before use, mix 1 volume of Chromogen with 1 volume of Substrate, in a disposable plastic container, according to needs. This solution is stable for 4 hours at room temperature protected from light.

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WASHING INSTRUCTION

A good washing procedure is essential to get correct and reliable analytical results. In case of manual washing, it is suggested to carry out 5 cycles, first dispensing and then aspirating 300 µl/well per cycle. Usually 5 cycles of automatic washing of 300 µl/well per cycle are sufficient to remove false positives and high background values. It is suggested to use an Elisa automatic microplate washer, qualified and properly serviced. Anyhow, we recommend to calibrate the washing system on the kit itself so to match the declared analytical performances. Any case, potentially infective wastes from microplate washing must be inactivated with Na-hypochlorite at 2.5% final concentration for 30 minutes. All these materials have to be discarded according to the law as potentially infective wastes.

TEST PROCEDURE

At least 1 hour before use bring all the reagents necessary tothe test to room temperature and mix carefully the liquidreagents supplied on vortex (in particular the Controls, theConjugate and the Chromogen/Substrate) avoiding foaming.Do not dilute Controls as they are ready to use.

1. Leave the A1 well empty for blanking operations. Dispense100 µl of Negative Control in triplicate, then 100 µl ofPositive Control. Manually, to dilute all the sample intodisposable dilution vials dispensing 480 µl of Sample Diluent and 20 µl of sample. Mix on vortex, and thendispense 250 µl of diluted sample into each microplate well.In automatic, it is also possible to dispense 240 µl of SampleDiluent and 10 µl of sample directly into each microplatewell.

2. Cover the microplate with the plate sealer and incubate stripsfor 60 minutes at 37°C.

3. Peel out the plate sealer and wash the microplate accordingto instructions. Dilute the quantity of concentrated Conjugateyou need.

4. Add 100 µl of the Conjugate to all the wells, but A1.

5. Cover the microplate with the plate sealer. Then incubate themicroplate sealed for 30 minutes at 37 °C.

6. Peel out the plate sealer and wash the microplate accordingto instructions. Prepare the necessary Chromogen/Substratesolution (1033/1033.X).

7. Add 100 µl of Chromogen/Substrate to all the wells, A1included.

8. Incubate the microplate for 15 minutes at room temperature,protected from light.

9. Block the enzymatic reaction by adding 100 µl Stop Solutionto all the wells, A1 included. Read the microplate at 450 nmand 620 - 630 nm blanking the instrument on A1 well.

Note - Read the microplate within 30 minutes after the dispensing of the Stop Solution.

ASSAY SCHEME

At least 1 hour before use bring all the reagents necessary to the test to room temperature and mix carefully the liquid reagents supplied on vortex (in particular the Controls, the Conjugate and the Chromogen/Substrate) avoiding foaming. Do not dilute Controls as they are ready to use.

Position	Controls/Samples
A1	Blank
B1+C1+D1	Negative Control
E1	Positive Control
F1.....H12	Diluted samples

Reagents	Blank (A1)	Controls	Samples
Controls	-	100 µl	-
Sample Diluent	-	-	240 µl
Samples	-	-	10 µl
Cover with the sealer and incubate for 60 minutes at 37 °C			
Peel out the sealer and wash 5 cycles with 300 µl/well per cycle Dilute the quantity of concentrated Conjugate you need			
Conjugate	-	100 µl	100 µl
Cover with the sealer and incubate for 30 minutes at 37 °C			
Peel out the sealer and wash 5 cycles with 300 µl/well per cycle. Prepare the necessary Chromogen/Substrate solution			
Chromogen/Substrate	100 µl	100 µl	100 µl
Incubate for 15 minutes at room temperature in the dark			
Stop Solution	100 µl	100 µl	100 µl
Blank the reader on A1 well. Read at 620 - 630 nm for measuring the microplate background, then at 450 nm.			
Note - Read the microplate within 30 minutes after thedispensing of the Stop Solution.			

CALCULATION OF RESULTS

If the validity of the assay is confirmed, calculate the Cut-off (Co) value through the following formula:

Cut-off = NC mean + 0.150
Grey-zone = ± 10 %

Example of calculation

Negative Controls 0.050 - 0.060 - 0.070 OD 450 nm
Negative Control mean 0.060 OD 450 nm
Positive Control 2.800 OD 450 nm
Cut-off = 0.060 + 0.150 = 0.210
Grey-zone = 0.189 - 0.231
Sample 1 0.050 negative
Sample 2 0.240 grey zone
Sample 3 1.250 positive

Samples with OD value below the lower limit of the grey-zone are reported as negatives. No further testing is required. Samples with an OD value within or exceeding the upper limit of the grey-zone are reported as initially reactive. The samples should be re-tested in duplicate.

- Initially reactive samples that do not react in both of duplicate repeat tests are reported as negative for antibodies to HCV.
- Initially reactive samples that are confirmed reactive or grey zone have to be submitted to additional more specific tests (confirmatory tests).
- Repeatedly reactive samples not confirmed positive are considered false-reactive samples.
- Repeatedly "grey zone" samples confirmed positives are considered positives for antibodies to HCV.
- Repeatedly "grey zone" samples not confirmed positives are considered indeterminates. In such case the repetition of test with a new sample taken 2-4 weeks is recommended.

VALIDITY OF THE ASSAY

The assay is considered valid if:

1. the OD 450 nm of the A1 blank well is < 0.100. Higher values are index of Chromogen/Substrate contamination;

2. after blanking on A1, the OD 450 nm mean value of the Negative Control (NC) is < 0.200. Abnormal values may be observed when the washing instrument does not work correctly or the washing procedure has not been adapted to the assay as described in the proper section;

3. the OD 450 nm value of the Positive Control (PC) is > 0.800. Lower values can be result when the storage temperature was not optimal or with a not correct operative procedure. In case that the above data do not match the correct values, before repeating the test check carefully the expiration date of the kit, the performances of the instruments used for the assay and the procedure of distribution of Controls and samples.

PERFORMANCE CHARACTERISTICS

The studies were performed in Italy (Milan, Pavia, Legnano, Rome) at laboratories of Hospital's Blood Banks as well at Universities and Hospital microbiological laboratories.

All the tests were performed on human sera or plasma; the sensitivity and the specificity were evaluated in comparison with a licensed reference test.

1. Sensitivity – The clinical sensitivity was assessed examining 17 BBI (Boston Biomedica Inc.) seroconversion panels and serial samples sera collected from 4 subjects during HCV seroconversion. In the table is reported the ability of HCV Ab to detect antibodies to HCV in comparison with a registered assay. In 9 seroconverter subjects a concordance in the detection of the first serum sample HCV positive have been observed between two kits; in 10 subjects HCV Ab detects HCV antibodies before than licensed assay; in 2 subjects licensed kit detects antibodies to HCV before HCV Ab. Generally, the discordances ranging from 7 up to 12 days.

Panel ID	First reactive bleed (days)		Panel ID	First reactive bleed (days)	
	HCV Ab	Ref. Kit		HCV Ab	Ref. Kit
PHV901	97	970	PHV913	0	> 9
PHV904	21	14	PHV914	12	24
PHV905	14	21	PHV915	> 14	12
PHV906	0	0	PHV916	16	23
PHV907	13	21	PHV917	85	85
PHV908	11	19	PHV918	24	> 27
PHV910	4	8	PHV919	28	32
PHV911	14	14	PHV920	7	16
PHV912	7	7	176	90	90
1288	90	90	4127	90	90
6741	90	90			

The sensitivity was also evaluated examining 574 patientswith ongoing HCV infection confirmed by RIBA and PCRpositivity. All samples sera were positive for antibodies toHCV with HCV Ab (sensitivity 100%). The genotypedistribution of 365 out of 574 HCV positive samples hasshown in the following table:

HCV Genotype	No. samples	HCV Genotype	No. samples
1a	25	4b	5
1b	83	4c	5
1c	2	4d	5
2a	40	4e	6
2b	64	5	15
2c	7	6	1
3a	20	Mix 1a + 1b	6
4a	29	Mix 2a + 2c	2

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The HCV Ab shows a high level of sensitivity; the kit was able to detects HCV infection independently from HCV genotype.

2. Specificity – The specificity of HCV Ab testing 5042 samples from unselected blood donors was 99.7% and 99.54% examining 440 hospitalized patients HCV negative with a licensed reference kit.

A total of 156 potentially cross-reactive samples includingHBsAg, HIV, IgM anti-HDV, IgM anti-toxoplasma, IgM antirubella, IgM anti-CMV, antibodies to E. coli positive samplessamples from multiparous females, autoimmune patientslipemic, haemolytic and icteric samples, and subjects RF andVDRL positive have been examined. All samples were negative on HCV Ab (specificity 100%).

Specimen	No. examined	False positive	Specificity
Blood donors sera	5042	17	99.7 %
Hospitalized patients sera	440	2	99.54 %
Potentially crossreactive sera	156	0	100 %

3. Reproducibility – Replicates of HCV antibody negative, low positive and high positive sera have been examined with the same HCV Ab lot and with multiple kit lots on multiple days. The results within and between assays are reported in the table.

Specimen	No. replicates	Intra-assay		No. replicates	Inter-assay	
		SD	CV %		SD	CV %
Negative	24	0.003	7.1	5	0.007	21.0
Low +	36	0.025	6.3	5	0.128	16.6
High +	36	0.095	4.1	5	0.170	7.9

LIMITATION OF THE PROCEDURE

Highly lipemic, icteric, hemolysed samples or repeatedly defrost samples and therefore subject to contamination, should not be used as they can give false results in the assay.

PROCEDURE AUTOMATION

This procedure can be used with an automatic device under customer's responsibility and providing he validates the results with an adequate method. For more information, please contact the automatic device manufacturer.

PRECAUTIONS IN USE

Refer to the Safety Data Sheet. The use of the laboratoryreagents according to Good Laboratory Practice (GLP) isrecommended.

WASTE MANAGEMENT

Please, refer to local legal requirements.

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PRESENTACIÓN:

CONT. 96 TEST CODIGO: RSET064-2