

Revised 16 Sept. 2010 rm (Vers. 2.1)**USA: RUO****INTENDED USE**

Third generation Enzyme ImmunoAssay (ELISA) for the determination of antibodies to Hepatitis C Virus in human plasma and sera.

For Research Use Only.

PRINCIPLE OF THE TEST

Microplates are coated with HCV-specific antigens derived from “core” and “ns” regions encoding for conservative and immunodominant antigenic determinants (Core peptide, recombinant NS3, NS4 and NS5 peptides).

The solid phase is first treated with the diluted sample and HCV Ab are captured, if present, by the antigens.

After washing out all the other components of the sample, in the 2nd incubation bound HCV antibodies, IgG and IgM as well, are detected by the addition of polyclonal specific anti hIgG&M antibodies, labelled with peroxidase (HRP).

The enzyme captured on the solid phase, acting on the substrate/chromogen mixture, generates an optical signal that is proportional to the amount of anti HCV antibodies present in the sample. A cut-off value let optical densities be interpreted into HCV antibody negative and positive results.

COMPONENTS

Each kit contains reagents for 96 tests.

1. Microplate MICROPLATE

n° 1 microplate

12 strips of 8 microwells coated with Core peptide, recombinant NS3, NS4 and NS5 peptides. Plates are sealed into a bag with desiccant.

2. Negative Control CONTROL -

1x2.0ml/vial. Ready to use control. It contains 1% goat serum proteins, 10 mM Na-citrate buffer pH 6.0 +/-0.1, 0.5% Tween 20, 0.09% Na-azide and 0.1% Kathon GC as preservatives. The negative control is olive green colour coded.

3. Positive Control CONTROL +

1x2.0ml/vial. Ready to use control. It contains 1% goat serum proteins, human antibodies positive to HCV, 10 mM Na-citrate buffer pH 6.0 +/-0.1, 0.5% Tween 20, 0.09% Na-azide and 0.1% Kathon GC as preservatives. The Positive Control is dark green colour coded.

4. Calibrator CAL

n° 1 vials. Lyophilized calibrator. To be dissolved with the volume of EIA grade water reported on the label. It contains foetal bovine serum proteins, human antibodies to HCV whose content is calibrated on the NIBSC Working Standard code 99/588-003-WI, 10 mM Na-citrate buffer pH 6.0 +/-0.1, 0.3 mg/ml gentamicine sulphate and 0.1% Kathon GC as preservatives. ***Note: The volume necessary to dissolve the content of the vial may vary from lot to lot. Please use the right volume reported on the label.***

5. Wash buffer concentrate WASHBUF 20X

1x60ml/bottle. 20x concentrated solution. Once diluted, the wash solution contains 10 mM phosphate buffer pH 7.0 +/-0.2, 0.05% Tween 20 and 0.05% Kathon GC.

Revised 16 Sept. 2010 rm (Vers. 2.1)

USA: **RUO**

6. Enzyme Conjugate CONJ

1x16ml/vial. Ready to use and red colour coded reagent. It contains Horseradish Peroxidase conjugated goat polyclonal antibodies to human IgG and IgM, 5% BSA, 10 mM Tris buffer pH 6.8+/-0.1, 0.1% Kathon GC and 0.02% gentamicine sulphate as preservatives.

7. Chromogen/Substrate SUBS TMB

1x16ml/vial. Ready-to-use component. It contains 50 mM citrate-phosphate buffer pH 3.5-3.8, 4% dimethylsulphoxide, 0.03% tetra-methyl-benzidine or TMB and 0.02% hydrogen peroxide or H₂O₂. **Note: To be stored protected from light as sensitive to strong illumination.**

8. Assay Diluent DILAS

1x8ml/vial. 10 mM tris buffered solution pH 8.0 +/-0.1 containing 0.1% Kathon GC for the pre-treatment of samples and controls in the plate, blocking interference. **Note: Use all the content of one vial before opening a second one. The reagent is sensitive to oxidation.**

9. Sulphuric Acid H₂SO₄ 0.3 M

1x15ml/vial. It contains 0.3 M H₂SO₄ solution. Attention: Irritant (Xi R36/38; S2/26/30).

10. Sample Diluent: DILSPE

1x50ml/vial. It contains 1% goat serum proteins, 10 mM Na-citrate buffer pH 6.0 +/-0.1, 0.5% Tween 20, 0.09% Na-azide and 0.1% Kathon GC as preservatives. To be used to dilute the sample. **Note: The diluent changes colour from olive green to dark bluish green in the presence of sample.**

11. Plate sealing foils n° 2

12. Package insert n° 1

MATERIALS REQUIRED BUT NOT PROVIDED

1. Calibrated Micropipettes (200ul and 10ul) and disposable plastic tips.
2. EIA grade water (bidistilled or deionised, charcoal treated to remove oxidizing chemicals used as disinfectants).
3. Timer with 60 minute range or higher.
4. Absorbent paper tissues.
5. Calibrated ELISA microplate thermostatic incubator capable to provide a temperature of +37°C.
6. Calibrated ELISA microwell reader with 450nm (reading) and possibly with 620-630nm (blanking) filters.
7. Calibrated ELISA microplate washer.
8. Vortex or similar mixing tools.

SPECIMEN: PREPARATION AND RECOMMENDATIONS

1. Blood is drawn aseptically by venipuncture and plasma or serum is prepared using standard techniques of preparation of samples for clinical laboratory analysis. No influence has been observed in the preparation of the sample with citrate, EDTA and heparin.
2. Avoid any addition of preservatives to samples; especially sodium azide as this chemical would affect the enzymatic activity of the conjugate, generating false negative results.

Revised 16 Sept. 2010 rm (Vers. 2.1)

USA: **RUO**

3. Samples have to be clearly identified with codes or names in order to avoid misinterpretation of results. When the kit is used for the screening of blood units, bar code labeling and electronic reading is strongly recommended.
4. Hemolyzed (red) and visibly hyperlipemic ("milky") samples have to be discarded as they could generate false results. Samples containing residues of fibrin or heavy particles or microbial filaments and bodies should be discarded as they could give rise to false results.
5. Sera and plasma can be stored at +2°..8°C for up to five days after collection. For longer storage periods, samples can be stored frozen at -20°C for several months. Any frozen samples should not be frozen/thawed more than once as this may generate particles that could affect the test result.
6. If particles are present, centrifuge at 2.000 rpm for 20 min or filter using 0.2-0.8u filters to clean up the sample for testing.

PREPARATION OF COMPONENTS AND WARNINGS

A study conducted on an opened kit has not pointed out any relevant loss of activity up to 6 re-use of the device and up to 6 months.

Microplates: Allow the microplate to reach room temperature (about 1 hr) before opening the container. Check that the desiccant is not turned to dark green, indicating a defect of manufacturing. In this case call DRG's customer service. Unused strips have to be placed back into the aluminum pouch, in presence of desiccant supplied, firmly zipped and stored at +2°..8°C. When opened the first time, residual strips are stable till the indicator of humidity inside the desiccant bag turns from yellow to green.

2. Negative Control: Ready to use. Mix well on vortex before use.

3. Positive Control: Ready to use. Mix well on vortex before use. Handle this component as potentially infective, even if HCV, eventually present in the control, has been chemically inactivated.

4. Calibrator: Dissolve carefully the content of the lyophilized vial with the volume of EIA grade water reported on its label. Mix well on vortex before use.

Handle this component as potentially infective, even if HCV, eventually present in the control, has been chemically inactivated.

Note: When dissolved the Calibrator is not stable. Store in aliquots at -20°C.

5. Wash buffer concentrate:

The whole content of the 20x concentrated solution has to be diluted with bidistilled water up to 1200 ml and mixed gently end-over-end before use.

Once diluted, the wash solution is stable for 1 week at +2..8° C.

In the preparation avoid foaming as the presence of bubbles could give origin to a bad washing efficiency.

Note: Once diluted, the wash solution is stable for 1 week at +2..8° C.

6. Enzyme conjugate:

Ready to use. Mix well on vortex before use.

Be careful not to contaminate the liquid with oxidizing chemicals, air-driven dust or microbes.

If this component has to be transferred use only plastic, possibly sterile disposable containers.

7. Chromogen/Substrate:

Ready to use. Mix well on vortex before use.

Be careful not to contaminate the liquid with oxidizing chemicals, air-driven dust or microbes.

Revised 16 Sept. 2010 rm (Vers. 2.1)**USA: RUO**

Do not expose to strong illumination, oxidizing agents and metallic surfaces.

If this component has to be transferred use only plastic, possible sterile disposable container.

8. Assay Diluent:

Ready to use. Mix well on vortex before use.

9. Sulphuric Acid:

Ready to use. Mix well on vortex before use.

Attention: Irritant (Xi R36/38; S2/26/30)

Legenda: R 36/38 = Irritating to eyes and skin.

S 2/26/30 = In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.

10. Sample Diluent:

Ready to use. Mix well on vortex before use.

INSTRUMENTS AND TOOLS USED IN COMBINATION WITH THE KIT

1. Micropipettes have to be calibrated to deliver the correct volume required by the assay and must be submitted to regular decontamination (household alcohol, 10% solution of bleach, hospital grade disinfectants) of those parts that could accidentally come in contact with the sample. They should also be regularly maintained in order to show a precision of 1% and a trueness of +/-2%. Decontamination of spills or residues of kit components should also be carried out regularly.
2. The ELISA incubator has to be set at +37°C (tolerance of +/-0.5°C) and regularly checked to ensure the correct temperature is maintained. Both dry incubators and water baths are suitable for the incubations, provided that the instrument is validated for the incubation of ELISA tests.
3. The ELISA washer is extremely important to the overall performances of the assay. The washer must be carefully validated and correctly optimized using the kit controls and reference panels, before using the kit for routine laboratory tests. Usually 4-5 washing cycles (aspiration + dispensation of 300ul/well of washing solution = 1 cycle) are sufficient to ensure that the assay performs as expected. A soaking time of 20-30 seconds between cycles is suggested. In order to set correctly their number, it is recommended to run an assay with the kit controls and well characterized negative and positive reference samples, and check to match the values reported below in the sections "Validation of Test" and "Assay Performances". Regular calibration of the volumes delivered by, and maintenance (decontamination and cleaning of needles) of the washer has to be carried out according to the instructions of the manufacturer.
4. Incubation times have a tolerance of +5%.
5. The ELISA microplate reader has to be equipped with a reading filter of 450nm and ideally with a second filter (620-630nm) for blanking purposes. Its standard performances should be (a) bandwidth < 10 nm; (b) absorbance range from 0 to > 2.0; (c) linearity to > 2.0; repeatability > 1%. Blanking is carried out on the well identified in the section "Assay Procedure". The optical system of the reader has to be calibrated regularly to ensure that the correct optical density is measured. It should be regularly maintained according to the manufacturer 's instructions.
6. When using an ELISA automated work station, all critical steps (dispensation, incubation, washing, reading, data handling) have to be carefully set, calibrated, controlled and regularly serviced in order to match the values reported in the sections "Validation of Test" and "Assay Performances". The assay protocol has to be installed in the operating system of the unit and validated as for the washer and the reader. In addition, the liquid handling part of the station (dispensation and washing) has to be validated and correctly set. Particular attention must be paid to avoid carry over by the needles used for dispensing and for washing. This must be studied and controlled to minimize the possibility of contamination of adjacent wells. The use of ELISA automated work stations is recommended for blood screening when the number of samples to be tested exceed 20-30 units per run.

7. DRG's customer service offers support to the user in the setting and checking of instruments used in combination with the kit, in order to assure compliance with the requirements described. Support is also provided for the installation of new instruments to be used with the kit.

PRE ASSAY CONTROLS AND OPERATIONS

1. Check the expiration date of the kit printed on the external label of the kit box. Do not use if expired.
2. Check that the liquid components are not contaminated by naked-eye visible particles or aggregates. Check that the Chromogen/Substrate is colorless or pale blue by aspirating a small volume of it with a sterile transparent plastic pipette. Check that no breakage occurred in transportation and no spillage of liquid is present inside the box. Check that the aluminum pouch, containing the microplate, is not punctured or damaged.
3. Dilute all the content of the 20x concentrated Wash Solution as described above.
4. Dissolve the Calibrator as described above.
5. Allow all the other components to reach room temperature (about 1 hr) and then mix as described.
6. Set the ELISA incubator at +37°C and prepare the ELISA washer by priming with the diluted washing solution, according to the manufacturers instructions. Set the right number of washing cycles as found in the validation of the instrument for its use with the kit.
7. Check that the ELISA reader has been turned on at least 20 minutes before reading.
8. If using an automated workstation, turn it on, check settings and be sure to use the right assay protocol.
9. Check that the micropipettes are set to the required volume.
10. Check that all the other equipment is available and ready to use.
11. In case of problems, do not proceed further with the test and advise the supervisor.

ASSAY PROCEDURE

The assay has to be carried out according to what reported below, taking care to maintain the same incubation time for all the samples in testing.

Automated assay:

In case the test is carried out automatically with an ELISA system, we suggest to make the instrument aspirate 200 ul Sample Diluent and then 10 ul sample.

All the mixture is then carefully dispensed directly into the appropriate sample well of the microplate. Before the next sample is aspirated, needles have to be duly washed to avoid any cross-contamination among samples.

Do not dilute controls/calibrator as they are ready to use.

Dispense 200 ul controls/calibrator in the appropriate control/calibration wells.

Important Note: *Visually monitor that samples have been diluted and dispensed into appropriate wells. This is simply achieved by checking that the colour of dispensed samples has turned to dark bluish-green while the colour of the negative control has remained olive green.*

For the next operations follow the operative instructions reported below for the Manual Assay.

It is strongly recommended to check that the time lap between the dispensation of the first and the last sample will be calculated by the instrument and taken into consideration by delaying the first washing operation accordingly.

Manual assay:

1. Place the required number of Microwells in the microwell holder. Leave the 1st well empty for the operation of blanking.

Revised 16 Sept. 2010 rm (Vers. 2.1)

USA: **RUO**

2. Dispense 200 ul of Negative Control in triplicate, 200 ul Calibrator in duplicate and 200 ul Positive Control in single in proper wells. Do not dilute Controls and Calibrator as they are pre-diluted, ready to use !
3. Add 200 ul of Sample Diluent (DILSPE) to all the sample wells; then dispense 10 ul sample in each properly identified well. Mix gently the plate, avoiding overflowing and contaminating adjacent wells, in order to fully disperse the sample into its diluent.

Important note: Check that the colour of the Sample Diluent, upon addition of the sample, changes from light green to dark bluish green, monitoring that the sample has been really added.

4. Dispense 50 ul Assay Diluent (DILAS) into all the controls/calibrator and sample wells. Check that the color of samples has turned to dark blue.
5. Incubate the microplate for **45 min at +37°C**.

Important note: Strips have to be sealed with the adhesive sealing foil, supplied, only when the test is carried out manually. Do not cover strips when using ELISA automatic instruments.

6. Wash the microplate with an automatic washer by delivering and aspirating 300ul/well of diluted washing solution as reported previously (section I.3).
7. Pipette 100µl Enzyme Conjugate into each well, except the 1st blanking well, and cover with the sealer. Check that this red coloured component has been dispensed in all the wells, except A1.

Important note: Be careful not to touch the plastic inner surface of the well with the tip filled with the Enzyme Conjugate. Contamination might occur.

8. Incubate the microplate for **45 min at +37°C**.
9. Wash microwells as in step 6.
10. Pipette 100µl Chromogen/Substrate mixture into each well, the blank well included. Then incubate the microplate at **room temperature (18-24°C) for 15 minutes**.

Important note: Do not expose to strong direct illumination. High background might be generated.

11. Pipette 100µl Sulphuric Acid into all the wells using the same pipetting sequence as in step 10 to stop the enzymatic reaction. Addition of acid will turn the positive control and positive samples from blue to yellow.
12. Measure the colour intensity of the solution in each well, as described in section I.5, at 450nm filter (reading) and possibly at 620-630nm (background subtraction), blanking the instrument on A1.

Important notes:

1. If the second filter is not available ensure that no finger prints are present on the bottom of the microwell before reading at 450nm. Finger prints could generate false positive results on reading.
2. Reading has to be carried out just after the addition of the Stop Solution and anyway not any longer than 20 minutes after its addition. Some self oxidation of the chromogen can occur leading to high background.
3. Shaking at 350 ±150 rpm during incubation has been proved to increase the sensitivity of the assay of about 20%.

Revised 16 Sept. 2010 rm (Vers. 2.1)

USA: **RUO**

ASSAY SCHEME

Method	Operations
Controls & Calibrator	200 ul
Samples	200ul dil.+10ul
Assay Diluent (DILAS)	50 ul
1st incubation	45 min
Temperature	+37°C
Wash step	4-5 cycles
Enzyme conjugate	100 ul
2nd incubation	45 min
Temperature	+37°C
Wash step	4-5 cycles
TMB/H ₂ O ₂	100 ul
3rd incubation	15 min
Temperature	r.t.
Sulphuric Acid	100 ul
Reading OD	450nm

An example of dispensation scheme is reported in the table below:

Microplate

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLK	S2										
B	NC	S3										
C	NC	S4										
D	NC	S5										
E	CAL	S6										
F	CAL	S7										
G	PC	S8										
H	S1	S9										

Legenda: BLK = Blank NC = Negative Control
CAL = Calibrator PC = Positive Control S = Sample

CALCULATION OF THE CUT-OFF

The tests results are calculated by means of a cut-off value determined with the following formula on the mean OD450nm value of the Negative Control (NC):

$$NC + 0.350 = \text{Cut-Off (Co)}$$

The value found for the test is used for the interpretation of results as described in the next paragraph.

Important note: When the calculation of results is done by the operative system of an ELISA automated work station be sure that the proper formulation is used to calculate the cut-off value and generate the right interpretations of results.

REFERENCES

1. CDC. Public Health Service inter-agency guidelines for screening donors of blood, plasma, organs, tissues, and semen for evidence of hepatitis B and hepatitis C. MMWR 1991;40(No. RR-4):1-17.
2. Alter MJ. Epidemiology of hepatitis C. Hepatology 1997;26:62S-5S.
3. McQuillan GM, Alter MJ, Moyer LA, Lambert SB, Margolis HS. A population based serologic study of hepatitis C virus infection in the United States. In Rizzetto M, Purcell RH, Gerin JL, Verme G, eds. Viral Hepatitis and Liver Disease, Edizioni Minerva Medica, Turin, 1997, 267-70.
4. Dufour MC. Chronic liver disease and cirrhosis. In Everhart JE, ed. Digestive diseases in the United States: epidemiology and impact. US Department of Health and Human Services, Public Health Service, National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases. Washington, DC: US Government Printing Office, 1994; NIH publication no. 94-1447, 615-45.
5. Alter MJ, Hadler SC, Judson FN, et al. Risk factors for acute non-A, non-B hepatitis in the United States and association with hepatitis C virus infection. JAMA 1990;264:2231-35.
6. Alter HJ, Holland PV, Purcell RH, et al. Posttransfusion hepatitis after exclusion of commercial and hepatitis-B antigen-positive donors. Ann Intern Med 1972;77:691-9.
7. Alter HJ, Purcell RH, Holland PV, Feinstone SM, Morrow AG, Moritsugu Y. Clinical and serological analysis of transfusion-associated hepatitis. Lancet 1975;2:838-41.
8. Seeff LB, Wright EC, Zimmerman HJ, McCollum RW, VA Cooperative Studies Group. VA cooperative study of post-transfusion hepatitis and responsible risk factors. Am J Med Sci 1975;270:355-62.
9. Feinstone SM, Kapikian AZ, Purcell RH, Alter HJ, Holland PV. Transfusion-associated hepatitis not due to viral hepatitis type A or B. N Engl J Med 1975;292:767-70.
10. Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 1989;244:359-62.
11. Kuo G, Choo QL, Alter HJ, et al. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. Science 1989;244:362-4.
12. Alter HJ, Purcell RH, Shih JW, et al. Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A, non-B hepatitis. N Engl J Med 1989;321:1494-1500.
13. Aach RD, Stevens CE, Hollinger FB, et al. Hepatitis C virus infection in post-transfusion hepatitis. An analysis with first- and second-generation assays. N Engl J Med 1991;325:1325-9.
14. Alter MJ, Margolis HS, Krawczynski K, Judson, FN, Mares A, Alexander WJ, et al. The natural history of community-acquired hepatitis C in the United States. N Engl J Med 1992;327:1899-1905.
15. Alter, MJ. Epidemiology of hepatitis C in the west. Semin Liver Dis 1995;15:5-14.
16. Donahue JG, Nelson KE, Muñoz A, et al. Antibody to hepatitis C virus among cardiac surgery donors, homosexual men, and intravenous drug users in Baltimore, Maryland. Am J Epidemiol 1991;134:1206-11.
17. Zeldis JB, Jain S, Kuramoto IK, et al. Seroepidemiology of viral infections among intravenous drug users in northern California. West J Med 1992;156:30-5.
18. Fingerhood MI, Jasinski DR, Sullivan JT. Prevalence of hepatitis C in a chemically dependent population. Arch Intern Med 1993;153:2025-30.
19. Garfein RS, Vlahov D, Galai N, Doherty, MC, Nelson, KE. Viral infections in short-term injection drug users: the prevalence of the hepatitis C, hepatitis B, human immunodeficiency, and human T-lymphotropic viruses. Am J Pub Health 1996;86:655-61.
20. Brettler DB, Alter HJ, Deinstag JL, Forsberg AD, Levine PH. Prevalence of hepatitis C virus antibody in a cohort of hemophilia donors. Blood 1990;76:254-6.
21. Troisi CL, Hollinger FB, Hoots WK, et al. A multicenter study of viral hepatitis in a United States hemophilic population. Blood 1993;81:412-8.

Revised 16 Sept. 2010 rm (Vers. 2.1)

USA: RUO

22. Kumar A, Kulkarni R, Murray DL, et al. Serologic markers of viral hepatitis A, B, C, and D in donors with hemophilia. *J Med Virology* 1993;41:205-9.
23. Tokars JJ, Miller ER, Alter MJ, Arduino MJ. National surveillance of dialysis associated diseases in the United States, 1995. *ASAIO Journal* 1998;44:98-107.
24. Osmond DH, Charlebois E, Sheppard HW, et al. Comparison of risk factors for hepatitis C and hepatitis B virus infection in homosexual men. *J Infect Dis* 1993;167:66-71.
25. Weinstock HS, Bolan G, Reingold AL, Polish LB: Hepatitis C virus infection among donors attending a clinic for sexually transmitted diseases. *JAMA* 1993;269:392-4.
26. Thomas DL, Cannon RO, Shapiro CN, Hook EW III, Alter MJ. Hepatitis C, hepatitis B, and human immunodeficiency virus infections among non-intravenous drug-using donors attending clinics for sexually transmitted diseases. *J Infect Dis* 1994;169:990-5.
27. Buchbinder SP, Katz MH, Hessol NA, Liu J, O'Malley PM, Alter, MJ. Hepatitis C virus infection in sexually active homosexual men. *J Infect* 1994;29:263-9.
28. Thomas DL, Zenilman JM, Alter HJ, et al. Sexual transmission of hepatitis C virus among donors attending sexually transmitted diseases clinics in Baltimore--an analysis of 309 sex partnerships. *J Infect Dis* 1995;171:768-75.
29. Thomas DL, Factor SH, Kelen GD, Washington AS, Taylor E Jr, Quinn TC. Viral hepatitis in health care personnel at The Johns Hopkins Hospital. *Arch Intern Med* 1993;153:1705-12.
30. Cooper BW, Krusell A, Tilton RC, Goodwin R, Levitz RE. Seroprevalence of antibodies to hepatitis C virus in high-risk hospital personnel. *Infect Control Hosp Epidemiol* 1992;13:82-5.