ANTI-HCV (4th Generation)
ELISA

Intended Use:
Fortress HCV kit is an enzyme-linked immunosorbent assay for qualitative detection of antibodies to hepatitis C virus in human serum or plasma. It is intended for screening blood donors and diagnosing patients related to infection with hepatitis C virus.

Summary:
Hepatitis C virus (HCV) is an envelope, single stranded positive sense RNA (19.5 kb) virus belonging to the family Flaviviridae. Six major genotypes and series of subtypes of HCV have been identified. Isolated in 1989, HCV is now recognized as the major cause for transfusion associated non-A, non-B hepatitis. The virus is characterized by acute and chronic form although more than 50% of the infected individuals develop severe, life threatening chronic hepatitis with liver cirrhosis and hepatocellular carcinomas. Since the introduction in 1990 of anti-HCV screening of blood donations, the incidence of this infection in transfusion recipients has been significantly reduced. The first generation of HCV ELISAs showed limited sensitivity and specificity and was produced using recombinant proteins complementary to the NS5 of different subtypes.

Additional Materials And Instruments Required But Not Provided:
- Freshly distilled or deionized water.
- Disposable gloves and timer.
- Appropriate waste containers for potentially infectious materials.
- Disposable V-shaped troughs.
- Dispensing system and/or pipette (single or multichannel), disposable pipette tips.
- Absorbent tissue or clean towel.
- Dry incubator or water bath, 37±0.5°C.
- Microtiter plate reader, single wavelength 450nm or dual wavelength 450nm and 630nm.
- Microwell aspiration/wash system.

Specimen Collection, Transportation And Storage:
1. Sample Collection: Either fresh serum or plasma samples can be used in this assay. Blood collected by venipuncture should be allowed to clot naturally and completely. The specimen should be stored frozen (-20°C) before, and return to 2-8°C (18-20°C) before use. Samples should be packaged and labelled correctly and precisely matched as to achieve optimal performance.

2. Transportation And Storage: Store samples at 2-8°C. Samples for testing should be kept refrigerated when transported. All samples collected into EDTA, sodium citrate or heparin should be tested, but highly lipemic, icteric, or haemolyzed samples should not be used as they could give erroneous results in the assay. Do not heat inactivate samples. This can cause sample deterioration.

Special Instructions For Washing:
1. A good washing procedure is essential to obtain correct and precise analytical data. It is therefore recommended to use a good quality ELISA microplate washer, maintained at the best level of washing performances. In general, no less than 5 automatic wash cycles are recommended. To avoid incorrect result, strictly follow the test procedure steps and do not modify them. The components of the kit are precisely matched as to achieve optimal performance. The system on the kit itself in order to match the declared analytical performances. Assure that the microplate washer's liquid dispensing channels are not blocked or contaminated, and that sufficient volume of Wash Buffer is dispensed each time into the wells.

5. In case of manual washing, we suggest to perform at least 5 cycles, dispensing 350-400μl/well and aspirating the liquid for 5times. If poor results (high background) are observed, increase the washing cycles or soaking time per well.

6. In any case, the liquid aspirated out the strips should be treated with a sodium hypochlorite solution (final concentration of 2.5%) for 24 hours, before liquids are disposed in an appropriate way.

Storage And Stability:
The ELISA assay is a time and temperature sensitive method. To avoid incorrect result, strictly follow the test procedure steps and do not modify them.
1. To avoid cross-contamination of the plate with different lots, or use reagents from other commercially available kits.

2. Make sure that all reagents are within the validity indicated on the kit box and are of the same lot. Never use reagents beyond the expiry date stated on reagents labels or on the kit box.

3. CAUTION - CRITICAL STEP: Allow the reagents and samples to stabilize at room temperature (18-30°C) before use. Shake reagent gently before, and return to 2-8°C immediately after use.

4. Use only sufficient volume of sample as indicated in the procedure steps. Failure to do so may cause sample dilution and inaccuracy.

5. Do not touch the bottom exterior of the wells; fingerprints or scratches may interfere with microwell reading.

6. Wiping the results, ensure that the plate bottom is dry and there are no air-bubbles inside the wells.

7. Never allow the microplate wells to dry after the
washing step. Immediately proceed to the next step. Avoid the formation of air-bubbles when adding the reagents.
8. Avoid assay steps long time interruptions. Assure tandem that all reagents are added.
9. Calibrate the pipette frequently to assure the accuracy of samples/reagents dispensing. Always use different disposable pipette tips for each specimen to avoid cross-contaminations. Never pipette solutions by mouth.
10. The use of automatic pipettes is recommended.
11. Avoid the use of the incubation temperature is 37°C inside the incubator.
12. When adding samples, avoid touching the well's bottom with the pipette tip.
13. When dispensing liquids with a plate reader, it is recommended to determine the absorbance at 450nm or at 450nm with reference at 630nm.
14. All specimens from human origin should be considered as potentially infectious.
15. Materials from human origin may have been used in the kit. These materials have been tested with tests kits with accepted performance and found to be free of HIV-1, HIV-2, HBV, HCV and HBSAg. However, there is no analytical method that can assure that infectious agents in the specimen or reagents are completely absent. Therefore, handle reagents and specimens with extreme caution as if capable of transmitting infectious diseases. Strict adherence to GLP (Good Laboratory Practice) regulations can ensure the personal safety. Never eat, drink, smoke, or apply cosmetics in the assay laboratory.

16. Bovine derived sera may have been used in this kit. Bovine serum albumin (BSA) and fetal calf sera (FCS) are derived from animals from BSE/TSE geographical areas.
17. The pipette tips, vials, strips and sample containers should be collected and autoclaved – disposed solutions.
18. The pipette tips, vials, strips and sample containers should be collected and autoclaved – disposed solutions.
19. Containers should be collected and autoclaved – disposed solutions.
20. The pipette tips, vials, strips and sample containers should be collected and autoclaved – disposed solutions.

Assay Procedure:
Step 1 - Reagents preparation:
Allow the reagents and samples to reach room temperature (18-30°C) for at least 15-30 minutes. Check the pH of the samples.
21. Using fully automated microplate processing system, during incubation, do not cover the plates with the plate cover. The tapping out of the remains inside the plate after washing, can also be omitted.

Step 1 - Reagents preparation:
Allow the reagents and samples to reach room temperature (18-30°C) for at least 15-30 minutes. Check the pH of the samples.

Step 2 - Numbering Wells:
Set the strips needed in strip-holer and number sufficient number of wells including the Negative control (e.g. B1, C1, D1), two positive control (e.g. E1, F1) and one blank (A1, neither samples nor HRP-Conjugate should be added into the Blank well). If the number of samples is to be determined, use a dual wavelength plate reader, the requirement for use of Blank well could be omitted. Use only number of strips required for the test.

Step 3 - Adding Diluent:
Add 100μl specimen Diluent into each well except the blank.

Step 4 - Adding Sample:
Add 10μl of Positive control, Negative control, and Specimen into their respective wells. Note: Use a separate disposable pipette tip for each specimen, Negative and Positive control as to avoid cross contaminations. Carefully tap the plate gently. Incubate the plate at 37°C for 30 minutes.

Step 5 - Incubating (1):
Cover the plate with the plate cover and incubate for 30 minutes at 37°C. It is recommended to use a water tank to assure the temperature stability and humidity during the incubation. If dry incubator is used, do not open the door frequently.

Step 6 - Washing (1):
After the end of the incubation, remove and discard the plate cover. Wash each well 5times with diluted Wash buffer. Each time, allow the microwells to soak for 30-60 seconds. Collect all washing liquid and wash the strips plate onto blotting paper or clean towel, and tap it to remove any remains.

Step 7 - Adding HRP-Conjugate:
Add 100μl HRP-Conjugate to each well except the Blank.

Step 8 - HRP-Conjugate Incubating (2):
Cover the plate with the plate cover and incubate for 30 minutes at 37°C.

Step 9 - Washing (2):
At the end of the incubation, remove and discard the plate cover. Wash each well 5times with diluted Wash buffer in Step6.

Step 10 - Colouring:
Dispense 50μl of Chromogen A and 50μl Chromogen B solution into each well including the Blank and mix by tapping the plate gently. Incubate the plate at 37°C for 15 minutes avoiding light. The enzymatic reaction between the Chromogen A/B solutions produces blue colour in Positive control and anti-HCV positive sample wells.
Step 11 - Stopping Reaction:
Using a multichannel pipette or manually, add 50μl Stop Solution into each well and mix by tapping the plate gently. Intensive yellow colour develops in Positive control and anti-HCV positive sample wells.

Step 12 - Measuring the Absorbance:
Calibrate the plate reader with the Blank well and read the absorbance at 450nm. If a dual filter instrument is used, use the reference wavelength at 630nm. Calculate the Cut-off value and evaluate the results (Note: read the absorbance within 5 minutes after stopping the reaction).

Interpretation of Results and Quality Control:
Each microplate should be considered separately when calculating and interpreting results of the assay, regardless of the number of plates concurrently processed. The results are calculated by relating each sample’s optical density (OD) value to the Cut-off value (C.O.) of the plate. If the Cut-off reading is based on single filter plate reader, the results should be calculated by subtracting the Blank well OD value from the print report values of samples and controls. In case the reading is based on dual filter plate reader, do not subtract the Blank well OD from the print report values of samples and controls.

1. Calculation of Cut-off value (C.O.) = \text{Nc} \times 0.12

\text{Nc} = \text{the mean absorbance value for three negative controls.}

*Important:* If the mean OD value of the negative control is lower than 0.02, take it as 0.02. If higher than 0.02 see the Quality control range.

If one of the negative control values does not meet the Quality control range specifications, it should be discarded and the mean value is calculated again using the remaining two values. If more than one negative control OD value does not meet the Quality control range specification, the test is invalid and must be repeated.

2. Quality control range:
The test results are valid if the Quality Control criteria are verified. It is recommended that each laboratory must establish appropriate quality control system with quality control material similar to or identical with the patient sample being analyzed.

1. The OD value of the Blank well, which contains only Chromogens and Stop solution, is less than 0.080 at 450 nm.
2. The OD value of the Positive control must be equal to or greater than 0.800 at 450/630nm or at 450nm after blanking.
3. The OD value of the Negative control must be less than 0.100 at 450/630nm or at 450nm after blanking.

Interpretations of the results: (S = the individual absorbance (OD) of each specimen)

Negative Results (S/C.O. < 1): samples giving absorbance less than the Cut-off value are negative for this assay, which indicates that no antibodies to hepatitis C virus have been detected with this anti-HCV ELISA kit, therefore the patient is probably not infected with hepatitis C virus.

Positive Results (S/C.O. ≥1): samples giving an absorbance greater than, or equal to the Cut-off value are considered initially reactive, which indicates that antibodies to hepatitis C virus have probably been detected using this anti-HCV ELISA kit. Retesting in duplicates of any initially reactive sample is recommended. Repeatedly reactive samples could be considered positive for antibodies to HCV and therefore the patient is probably infected with hepatitis C virus. Blood unit positive for HCV antibodies should be immediately discarded.

Bloodline: Samples with absorbance O.D.Cut-off + 2 are considered borderline and retesting of those samples in duplicates is recommended. Repeatedly positive samples could be considered positive for hepatitis C virus infection.

Follow-up and supplementary testing of any anti-HCV positive samples with other analytical system (e.g. RIBA, WB) is required to confirm the diagnosis.

Test Performance And Expected Results:

Clinical Specificity: A blood donor population of 2948 individuals was tested with 3 different kits from different manufacturers. The specificity of this anti-HCV ELISA kit was 99.55%.

Clinical Sensitivity: Among 480 clinical hepatitis C patients confirmed positive 3.0 of 479 were positive when tested with this anti-HCV ESA kit. The sensitivity was 99.77%.

Analytical Specificity:
- No cross reactivity observed with samples from patients infected with HAV, HIV, HBV, CMV, and TP. No interference was observed from rheumatoid factor up to 2000IU/L.
- This antibody performance characteristics are unaffected from elevated concentrations of bilirubin, haemoglobin, and triden.
- Frozen specimens have been tested to check for interference due to collection and storage.

Performance of low and mixed tilters anti-HCV panels
HCV Genotype Antibody Testing:
1. Non-repeatable positive results may occur due to the general biological characteristics of ELISA assays. The assay is designed to achieve very high performance characteristics of sensitivity and specificity, and the "indirect model" minimizes the unspecific reactions, which can occur due to interference between unknown meters in sample and the rabbit anti-human IgG used as a conjugate. Antibodies may be undetectable during the early stages of the disease and in some immunosuppressed individuals.

   **GENOTYPE** | **SAMPLES** | **POSITIVE**
   --- | --- | ---
   1a-b | 15 | 15
   2a-b | 13 | 13
   3a-b | 10 | 10
   4 | 6 | 6
   5 | 12 | 12
   6 | 18 | 18
   TOTAL | 74 | 74

2. Positive results must be confirmed with another available method. Any positive result must be interpreted together with the patient clinical information and other laboratory results.
3. Common sources for mistakes: kits beyond the expiry date, bad washing procedures, contaminated reagents, incorrect assay procedure steps, insufficient aspiration during washing, failure to add samples or reagents, equipment, timing, volumes, sample nature and quality.
4. If, after retesting of the initially reactive samples, the assay results are negative, these samples should be considered as non-repeatable (false positive) and interpreted as negative. As with many very sensitive ELISA assays, false positive results can occur due to the several reasons, most of which are related but not limited to inadequate washing step. The prevalence of the marker will affect the assay's predictive values.
5. This kit is intended ONLY for testing of individual serum or plasma samples. Do not use it for testing of cadaver samples, saliva, urine or other body fluids, or pooled (mixed) blood.

Indications of Instability or Deterioration of the Reagents:
1. Values of the Positive or Negative controls, which are out of the indicated Quality control range, are indicator of possible deterioration of the reagents and/or operator or equipment errors. In such case, the results should be considered as invalid and the samples must be retested. In case of constant erroneous results classified as due to deterioration or instability of the reagents, immediately substitute the reagents with new ones.
2. If after mixing of the Chromogen A and B solutions into the wells, the, the colour of the mixture turns blue within few minutes, do not continue carrying out the testing and replace the reagents with fresh ones.

Reference: