4th GENERATION

HEPALISA ULTRA

Microwell ELISA Test for the Detection of Hepatitis B Surface Antigen (HBsAg) in Human Serum/ Plasma

1. INTRODUCTION

The Viral Hepatitis caused by Hepatitis B Virus is termed as "Serum Hepatitis". 1-5% infected people act as chronic carriers of HBV Virus. Major part of the chronic carriers secrete hepatitis B surface antigen (HBsAg) into blood and other secretions of the body like saliva and vaginal fluid. These chronic carriers are potentially infectious to other seronegative people.

Hepatitis B Virus belongs to a family of enveloped DNA virus, the Hepadnavirus. Related viruses in this group cause chronic hepatitis.

HBsAg has been accepted as a universal and the most reliable seromarker in case of acute HBV infection due to its appearance almost 2-4 weeks before the ALT level becomes abnormal and 3-5 weeks before the onset of symptoms or jaundice as an early detection system for hepatitis infection. In most cases of HBV infection, the incubation period varies from 40 days to 6 months.

Within HBVs, antigenic diversity is recognised in the surface antigens. HBsAg particles contain common "a" antigen, linked to two sets of mutually exclusive determinants, "d" or "y" and "w" or "r" giving the four main types-adw, adr, ayw and ayr.

2. INTENDED USE

4th Generation Hepalisa Ultra is designed for in-vitro qualitative detection of Hepatitis B surface antigen (HBsAg) in human serum or plasma and is used as a screening test for testing of collected blood prior to transfusion.

3. SEROLOGICAL MARKERS OF CLINICAL SIGNIFICANCE FOR HEPATITIS B VIRUS (HBV)

HBsAg : First detected during the incubation period of 6-8 weeks before the appearance of symptoms. It can appear as early as 2 weeks and generally disappears within 3-4 months after exposure. **It is the most reliable & universal marker of HBV infection.** In the carrier and chronic state it persists more than 6 months.

Anti-HBs : Appearance may take several weeks/months after HBsAg clearance causing a 'window' period. Best indicator of recovery & immunity to HBV. Quantitation allows pre-vaccination screening & follow-up of the post-vaccination response.

HBeAg : Appears within 1 week after HBsAg, lasts 3-6 weeks & disappears before HBsAg clearance. Its presence indicates a highly infectious state, except in pre-core mutants. Persistence > 10 weeks suggests progression to chronic carrier state/hepatitis.

Anti-HBe : It appears before clearance of HBsAg, indicates decreasing infectivity and is a good prognosis for the resolution of infection.

IgM anti-HBc : Appears prior to symptoms, found in high titre for a short time during the acute disease stage that covers the serological 'window' period, declines to low levels during recovery. A marker of recent infection. It differentiates between acute & chronic hepatitis.

Anti-HBc total : Appears 4-10 weeks after HBsAg appearance, persists for years/for life, therefore it is a prominent marker of HBV exposure. It is the only indicator of infection in the window period.

HBV-DNA : Detection of low levels by PCR, allows diagnosis of acute/chronic infection/carrier state & also monitors the response to interferon treatment.

All the above antigens and viral DNA polymerase form useful diagnostic markers for HBV.

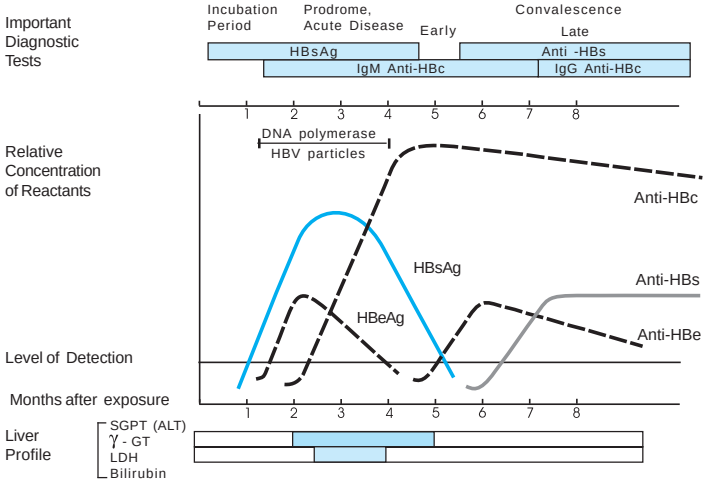

Fig.1: Serological & Clinical pattern during acute HBV infection.

4. PRINCIPLE

4th Generation Hepalisa Ultra is a solid phase enzyme linked immunosorbent assay (ELISA) based on the "Direct Sandwich" principle. The microwells are coated with Monoclonal antibodies with high reactivity for HBsAg. The samples are added in the wells followed by addition of enzyme conjugate (polyclonal antibodies linked to Horseradish Peroxidase (HRPO)). A sandwich complex is formed in the well wherein HBsAg (from serum sample) is "trapped" or "sandwiched" between the antibody and antibody HRPO conjugate. Unbound conjugate is then washed off with wash buffer. The amount of bound peroxidase is proportional to the concentration of HBsAg present in the sample. Upon addition of the substrate buffer and chromogen, a blue colour develops. The intensity of developed blue colour is proportional to the concentration of HBsAg in sample. To limit the enzyme-substrate reaction, stop solution is added and a yellow colour develops which is finally read at 450nm spectrophotometrically.

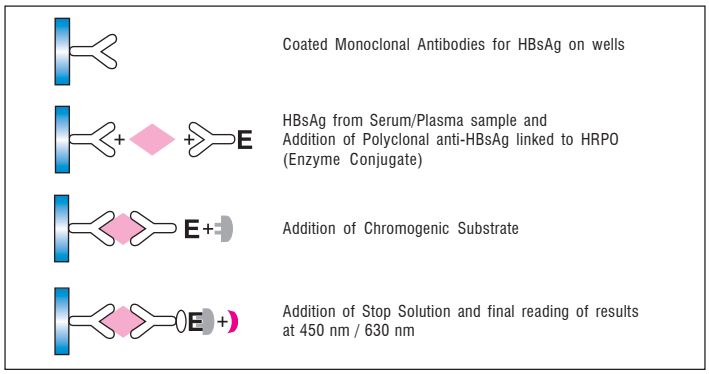

Fig. 2 : Schematic representation of "Hepalisa Ultra"

5. DESCRIPTION OF SYMBOLS USED

The following are graphical symbols used in or found on J. Mitra diagnostic products and packing. These symbols are the most common ones appearing on medical devices and their packing. They are explained in more detail in the European Standard EN ISO 15223-1:2021.

	Manufactured By	IVD	*In vitro* diagnostic medical device
Σ	No. of tests Lot Number		Instruction for use
LOT	Batch Number	8°C 2°C	Temperature Limitation
	Manufacturing Date		Caution See instruction for use
	Expiry Date	REF	Catalogue Number
	Do not use if package is damaged		Keep away from sunlight
BIO	Contains biological Material of Human Origin	BIO	Contains biological Material of Animal Origin
IN	Country of Manufacture		Keep Dry

6. KIT PRESENTATION

- 96 Test Pack

7. KIT & ITS COMPONENTS

PARTICULAR	DESCRIPTION
Microwells	Microwells coated with anti-HBsAg (monoclonal) packed in a pouch provided with desiccant.
Sample Diluent	Buffer containing protein stabilizers and antimicrobial agents as preservative.
Enzyme Conjugate	Containing Polyclonal IgG anti-HBsAg linked to horseradish peroxidase with protein stabilizers.
Wash Buffer Concentrate (25 X)	Concentrated Phosphate buffer with surfactant.
TMB Substrate	TMB dissolved in DMSO.
TMB Diluent	Buffer solution containing H_2O_2 with preservative.
Control -	Ready to use, normal human serum negative for HBsAg, HCV & HIV and with preservative.
Control +	Ready to use, inactivated and diluted human serum, reactive for HBsAg and non-reactive for HIV & HCV and with preservative.
Stop Solution	Ready to use, 1N H_2SO_4.
Plate Sealers	Adhesive sheets to cover the microwells during incubation.

8. STORAGE AND STABILITY

Store all components at 2-8°C when not in use. Expiry date on the kit indicates the date beyond which the kit should not be used.

9. ADDITIONAL MATERIAL AND INSTRUMENTS REQUIRED

- Micropipettes and microtips
- Elisa reader
- Distilled or deionized water
- Graduated Cylinders, for reagent dilution
- Paper towels or absorbent tissue
- Timer
- Elisa washer
- Incubator 37°C
- Disposable gloves

10. SPECIMEN COLLECTION & HANDLING

1. Only human serum or plasma samples should be used for the test. While preparing serum samples, remove the serum from the clot as soon as possible to avoid hemolysis. Fresh serum/plasma samples are preferred.
2. Specimens should be free of microbial contamination and may be stored at 2-8°C for one week, or frozen at -20°C or lower. Avoid repeated freezing and thawing.
3. Do not use heat inactivated samples as their use may give false results. Hemolyzed and Icteric hyperlipemic samples may give erroneous results.

11. WARNING & PRECAUTION

⚠ **CAUTION:** THIS KIT CONTAINS MATERIALS OF HUMAN ORIGIN. NO TEST METHOD CAN OFFER COMPLETE ASSURANCE THAT HUMAN BLOOD PRODUCTS WILL NOT TRANSMIT INFECTION. NEGATIVE CONTROL, POSITIVE CONTROL & ALL THE SAMPLES TO BE TESTED SHOULD BE HANDLED AS THOUGH CAPABLE OF TRANSMITTING INFECTION.

1. The use of disposable gloves and proper biohazardous clothing is STRONGLY RECOMMENDED while running the test.
2. In case there is a cut or wound in hand, DO NOT PERFORM THE TEST.
3. Do not smoke, drink or eat in areas where specimens or kit reagents are being handled.
4. Tests are for *in vitro* diagnostic use only and should be run by competent person only.
5. Do not pipette by mouth.
6. All materials used in the assay and samples should be decontaminated in 5% sodium hypochlorite solution for 30-60 min. before disposal or by autoclaving at 121°C at 15psi for 60 min. Do not autoclave materials or solution containing sodium hypochlorite. They should be disposed off in accordance with established safety procedures.
7. Wash hands thoroughly with soap or any suitable detergent, after the use of the kit. Consult a physician immediately in case of accident or contact with eyes, in the event that contaminated material are ingested or come in contact with skin puncture or wounds.
8. Spills should be decontaminated promptly with Sodium Hypochlorite or any other suitable disinfectant.
9. Stop solution contains sulfuric acid. If sulfuric acid comes in contact with the skin, wash thoroughly with water. In case of contact with eyes, flush with excess of water.
10. All materials used in the assay and samples should be disposed off in the manner that will inactivate virus.
11. ELISA Reader & micropipettes used in testing should be calibrated at regular interval to ensure accurate results.

12. SPECIMEN PROCESSING

(A) FROZEN SAMPLE

4th Generation Hepalisa Ultra test is best used with fresh samples that have not been frozen and thawed. However most frozen samples will perform well if the procedure suggested below is followed.

Allow the frozen sample to thaw in a vertical position in the rack. Do not shake the sample. This allows particles to settle to the bottom. If a centrifuge is available, the sample should be centrifuged. (10,000 rpm for 15 min.)

(B) TRANSPORTATION

If the specimen is to be transported, it should be packed in compliance with the current Government regulations regarding transport of aetiologic agents.

13. PRECAUTIONS FOR USE

Optimal assay performance requires strict adherence to the assay procedure described in the manual.

1. Do not use kit components beyond the expiration date, which is printed on the kit.
2. Avoid microbial contamination of reagents. The use of sterile disposable tips is recommended while removing aliquots from reagent bottles.
3. Prepare working substrate solution just 10 minutes prior to adding in the wells.
4. Mark the test specimen with patient's name or identification number. Improper identification may lead to wrong result reporting.
5. If blue colour or white particles appears in working substrate solution then do not use it. Take fresh containers and tips and prepare it again.
6. Use separate tips for TMB substrate and TMB diluent.
7. Do not allow microwells to dry once the assay has started.
8. Ensure that the microwell strips are levelled in the strip holder. Before reading, wipe the bottom of the microwell strips carefully with soft, absorbent tissue to remove any moisture.
9. If available, a microwell reader which contains a reference filter with settings at 620 or 630 nm should be used. Use of a reference filter minimises interference due to microwells that are opaque, scratched or irregular. However, if a reference filter is unavailable, the absorbance may be read at 450 nm without a reference filter.
10. Distilled or deionised water must be used for wash buffer preparation.
11. Bring all the reagents to room temperature (20-30°C) before use.
12. Do not combine reagents from different batches, as they are optimized for individual batch to give best results.
13. Due to interchange of caps the reagents may get contaminated. Care should be taken while handling the reagents to avoid any sort of contamination.
14. Run negative and positive controls in each assay.
15. Use freshly collected, clean serum samples for assay. Try to avoid Haemolyzed turbid, lipemic serum or plasma samples.
16. Use a separate tip for each sample and then discard it as biohazardous waste.
17. Thorough washing of the wells is critical to the performance of the assay.
18. Avoid strong light exposure during the assay.

14. PRELIMINARY PREPARATIONS

- Pre-warm the incubator to + 37°C.
- Bring foil pack to room temperature (20-30°C) before opening to prevent condensation on the microwell strips.

a. Break-off the required number of strips needed for the assay and place in the strip holder. Take the strip holder with the required number of strips, taking into account that, two negative and two positive control should be included in the run while opening the fresh kit. However for one or two strips two negative and one positive control and for more strips two negative and two positive controls should be included in each subsequent runs.

b. Unused wells should be stored at 2-8°C, with dessicant in a aluminium pouch with clamp & rod. Microwells are stable for 30 days at 2-8°C from the date of opening of sealed pouch, when stored with desicant along with clamp & rod.

Caution: Handle microwell strip with care. Do not touch the bottom exterior surface of the wells.

- **Preparation of Working Wash Buffer:**

a) Check the buffer concentrate for the presence of salt crystals. If crystals are present in the solution, resolubilize by warming at 37°C until all crystals dissolve.

b) Prepare at least 25ml. (1ml. concentrated buffer with 24 ml. water) of buffer for each strip used. Mix well before use.

c) Mix 20 ml. of 25X wash buffer concentrate with 480ml. of distilled or deionized water. Wash buffer is stable for 2 months when stored at 2-8°C.

- **Preparation of working substrate solution :**

Mix TMB substrate and TMB Diluent in 1:1 ratio to prepare working substrate.

No. of Strips	1	2	3	4	5	6	7	8	9	10	11	12
No. of Wells	8	16	24	32	40	48	56	64	72	80	88	96
TMB Susbstrate (ml)	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0
TMB Diluent (ml)	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0

Do not store working substrate. Prepare a fresh dilution for each assay in a clean plastic/glass vessel. Determine the quantity of working substrate solution to be prepared from table. Mix solution thoroughly before use. Discard unused solution. A deep blue color present in the substrate solution indicates that the solution has been contaminated and must be discarded.

15. PROCEDURAL NOTES:

1. Material should not be used after the expiry date shown on the labels. Components and test specimen should be at room temperature (20-30°C) before testing begins. Return the reagents to 2-8°C after use.
2. All reagents must be mixed well before use.
3. To avoid contamination, do not touch the top or bottom of strips or edge of wells.
4. All pipetting steps should be performed with utmost care and accuracy. Cross contamination between reagents and samples will invalidate results.
5. Prevent evaporation during sample incubation by covering the strips with sealer; remove sealer before washing.
6. Routine maintenance of wash system is strongly recommended to prevent carry over from highly reactive specimens to non reactive specimens.

16. TEST PROCEDURE

The instructions of the procedure must be strictly followed.

Fit the stripholder with the required number of Hepalisa Ultra strips. The sequence of the procedure must be carefully followed. Arrange the assay control wells in a horizontal or vertical configuration. Configuration is dependent upon reader software.

1. Add 25 μl sample diluent to each well from A-1 well.
2. Add 100 μl Negative Control in each well No. A-1 and B-1 respectively.
3. Add 100 μl Positive Control in C-1 & D-1 wells.
4. Add 100 μl of sample in each well, starting from E1.
5. Cover the plate and incubate in an incubator at 37°C $\pm$ 1°C for 60 minutes.
6. Add 50μl of Enzyme conjugate to each well. Gently shake the plate for 2-3 seconds to mix the sample & conjugate.
7. Cover the plate and incubate in an incubator at 37°C $\pm$ 1°C for 60 minutes.

8. Dilute the wash buffer concentrate with distilled water to 1:25 dilution.
9. At the end of incubation period, take out the plate from incubator and wash with working wash buffer.

WASHING: Washing can be performed either with ELISA WASHER or manually as follows:

a). Empty the wells

b). Add 300-350µl of working washing solution into each well and give a soak time of 30 seconds.

c). Empty the wells.

d). Wash each well 6 times in total.

e). After the sixth wash, tap dry the Microwells a few times on an absorbent tissue.

10. Tap dry the wells after washing and add 100µl of working substrate solution in all the wells.
11. Cover the plate with an aluminium foil and incubate at room temperature (20-30°C) for 30 minutes in dark.
12. Stop the reaction by adding 100µl of stop solution to each well, mix gently.
13. Read the absorbance of the wells at 450nm in an Elisa Reader. (Use of a reference filter of 630 nm is preferred).

SUMMARY OF PROCEDURE

Addition of sample Diluent		25 µl
Addition of controls & Samples		100 µl
Cover the plate & incubate		60 mins. at 37°C
Add Enzyme Conjugate		50 µl
Cover the plate & incubate		60 mins. at 37°C
Wash		6 Cycles
Prepare Chromogenic Substrate		No of Strips: 1 2 3 4 5 6 7 8 9 10 11 12 TMB Substrate (ml): 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0 5.5 6.0 TMB Diluent (ml.): 0.5 1.0 1.5 2.0 2.5 3.0 3.5 4.0 4.5 5.0 5.5 6.0
Add Substrate		100 µl
Incubate in dark		30 mins. at Room Temp.
Add Stop Solution		100 µl
Read Results		450 nm./630 nm.

17. CALCULATION OF RESULTS

Compute mean of negative and positive control absorbance

Negative Control Acceptance Criteria:

NC must be < 0.150

	0.012	A1 Well
NC	0.010	B1 Well
Total	0.022	

Mean absorbance **NC$\bar{x}$** = 0.022/2 = 0.011

Positive Control Acceptance Criteria:

PC or PCx must be >0.5. If it is not so, the run is invalid and must be repeated.

	1.430	C1 Well
PC	1.500	D1 Well
Total	2.930	

Mean absorbance, PC$\bar{x}$ = 2.930/2 = 1.465

Cut-off Value

Cut-off value can be determined by using the following formula:
Cut-off Value = NC$\bar{x}$ + 0.05
Where NC$\bar{x}$ is mean absorbance (O.D) of Negative Control.
e.g. 0.011 +0.05 = 0.061

18. INTERPRETATION OF RESULTS

The absorbance of the unknown sample is compared with the calculated cut-off value.

a) Test specimens with absorbance (O.D.) value less than cut-off value are non reactive and may be considered as negative for HBsAg.

b) Test specimens with absorbance (O.D.) value greater than or equal to cut off value are reactive for HBsAg by Hepalisa Ultra.

c) Test specimens with absorbance value within 10% below the cut off should be considered suspect for the presence of HBsAg and should be retested in duplicate.

d) Specimens with absorbance value equal to or greater than the cut off value are considered initially reactive by the criteria of Hepalisa Ultra. Original specimen should be retested in duplicate.

e) If both duplicate retest sample absorbance value is less than cutoff value, the specimen is considered non-reactive.

f) If any one of the duplicates retest sample absorbance value is equal to or greater than the cutoff or both duplicate retest value are equal to or greater than the cutoff, the specimen is considered reactive by the criteria of Hepalisa Ultra. Further confirmation by other EIA assays or confirmatory assays are recommended.

g) The O.D. for Crystal clear negative samples can be in minus. However, the minus (-) O.D. does not in any way affect the result interpretation. It rather gives better specificity.

19. PERFORMANCE CHARACTERISTICS

(i) **Analytical Sensitivity** : The sensitivity of the kit has been determined using WHO standard (Second international standard for HBsAg subtype adw2 geno type A) and it is equal to 0.130 IU/ml.

4th Generation Hepalisa Ultra has a sensitivity of 0.050ng/ml for all the 11 subtypes (ad & ay subtype) including mutant strains.

(ii) The performance of **4th Generation Hepalisa Ultra** has been evaluated in house with fresh as well as frozen samples. The testing has been done with clinical samples, samples from random blood donors, cross reacting samples; RA, CRP, ASO and patients with diseases related to HBV. The results of in-house studies are as follows:

No. of Samples	Status	4th Generation Hepalisa Ultra (+ ve)	4th Generation Hepalisa Ultra (- ve)
305	All ELISA +ve	305	-
2508	EIA -ve	1	2507

SENSITIVITY: 100 % ***SPECIFICITY: 99.92%**

Precision : Within-run and between-run precisions have been determined by testing 10 replicates of eight specimens : Three negative and five HBsAg Positive (three weak and two strong HBsAg positive). The C.V.(%) of negative and positive samples were within 10%.

20. LIMITATION OF THE TEST

1. The test should be used for detection of HBsAg in serum or plasma only and not in other body fluids.
2. **This is only a screening test** and will only indicate the presence or absence of Hepatitis B Surface Antigen in the specimen. All reactive samples should be confirmed by confirmatory test. Therefore for a definitive diagnosis, the patients clinical history, symptomatology as well as serological data should be considered. The results should be reported only after complying with the above procedure.
3. False positive results can be obtained due to presence of other antigens or elevated levels of RF factor. This occurs in less then 1% of the sample tested.

21. REFERENCES

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22. TROUBLE SHOOTING CHART

	PROBLEM	POSSIBLE CAUSE	SOLUTION
1.	No colour developed at the end of assay	a) Any one reagent has been added in wrong sequence.	Follow the procedure meticulously & repeat assay.
		b) Inactivated enzyme conjugate, improper storage	Check storage of enzyme conjugate and it should be free of any contamination.
		c) Microplate inactivated, due to improper storage	Keep unused strips in aluminium poly pouch with the dessicant pouch inside and proerly closed with clamp & rod.
		d) Inactivated substrate, improper storage or preparation	Use freshly prepared substrate solution and it should be free of any contamination. Recheck procedure and repeat assay.
		e) Omission of any step in test procedure	Follow the procedure meticulously & repeat assay.
		f) Incorrect (low) incubator temperature, timing or pipetting	Check incubator temperature, procedure & repeat assay
		g) Improper mixing of reagents.	Check procedure & repeat assay
		h) Kit deterioration	Check storage of the kit and should be stored at 2-8ºC.
2.	High O.D. value of Negative control	a) Plate not stopped after 30 minutes of additing stop solution	Follow the procedure meticulously & repeat assay.
		b) Sample microtip used for Positive and negative controls	Change micropipette tips while addition of negative/ positive control
		c) Nonspecific attachment/ binding of other reagent	If plates get scratches/ aberrations during washing, non specifi proteins may bind while addition of next step.
3.	Too much colour in all wells of the plate (high background)	a) Contaminated substrate	Check substrate (TMB Diluent) it should be colourless. If blue in colour then discard and use clean disposable container.
		b) Contaminated washing solution (1X). Poor quality of water used for diluting wash buffer conc.	Check the container and quality of water used for dilution. Use of glass distilled water is preferred.
		c) Over incubation of substrate and delay in addition of stop solution.	Follow the procedure meticulously.
		d) Insufficient washing. i) Washing not consistent	Check wash device, fill the well close to the top.
		ii) Filling volume not sufficient.	After washing, blot the microwells on absorbent
		iii) Insufficient no. of wash cycles.	tissue. Follow wash protocol meticulously
		iv) Contaminated wash device	
		e) Use of wash solution from other manufacturer.	Use only Hepalisa Ultra wash solution.
		f) Working substrate not protected from light	Incubate the plate in dark after addition of substrate.
		g) Improper preparation of working wash buffer (dilution error), improper mixing of reagents.	Check procedure & repeat assay
4.	Poor reproducibility	a) Washing problems.	Check all 8 ports/ manifold for uniform flow of wash buffer. If there are blockage, clen the ports.
		b) Uncalibrated pipettes or tips not well fitted, improper pipetting/ dispensing.	Use only calibrated pipettes with well fitted tips & pipette carefully without bubbling.
		c) Reagent & sera not at room temperature or not well mixed before use.	Equilibrate reagents to room temperature and mix thoroughly before use
		d) Too long time for addition of controls, samples or reagents, Inconsistency in time intervals.	Develop consistent and uniform technique.
		e) Interference in optical pathway due to Air bubbles. and repeat the readings.	Clean or dry the bottom of microwells, check for bubbles
5.	False Positive	Beside 3a, b, c, d, e, f & g incorrect interpretation and calculation of final results	Check the calculation part given in the insert and correctly interpret.
		b) High incubator temperature, incorrect timing or pipetting	Check incubator temperature, procedure & repeat assay.
		c) Use of turbid/ lipaemic or hemolyzed sample.	Centrifuge the sample at 5000 rpm for 30 minutes and re-run the test with clear sample.
6.	False Negative/ low O.D. of Positive control & positive sample	a) Inadequate addition of substrate/conjugate solution.	Follow the procedure meticulously & repeat assay.
		b) Kit expired, reagent of different kit used.	Check the expiry of the kit before use.
		c) White particles in working substrate solution.	Discard the substrate and prepare the working substrate again in fresh tube.
		d) Uncalibrated pipettes, improper pipetting.	Use only calibrated pipettes with well fitted tips & pipette carefully without bubbling.
		e) Deterioration of Enzyme conjugate, TMB Substrate/ TMB Diluent.	Check storage of reagents. They shall be stored at 2-8ºC.
		f) Stop solution is added before 30 minutes. Reaction terminated before 30 minutes.	Follow the test procedure meticulously.
		g) O.D. taken at incorrect wavelength.	Read O.D. values at 450 nm and 630 nm.
		h) Incorrect incubator temperature, timing or pipetting	Check incubator temperature, pipetting & repeat assay.
		i) Kit deterioration	Check storage of kit and it should be stored at 2-8ºC.
		j) Sample deterioration due to improper storage and / or microbial contamination.	Store the sample at 2-8ºC / -20ºC as recommended in the specimen collection & handling.

in vitro diagnostic Reagent, not for medicinal use

VER-01 R-10

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