CE **RecombiLISA**

Leptospira IgM ELISA

IVD | REF E0330

Instructions for Use

- 96-well ELISA kit for the qualitative detection of anti-*Leptospira interrogans* IgM in human serum or plasma
- For export only, not for re-sale in the USA
- Store at 2-8°C upon receipt

INTENDED USE

The *RecombiLISA* Leptospira IgM ELISA is a solid-phase enzyme-linked immunosorbent assay for the qualitative detection of anti-*Leptospira interrogans* (*L. interrogans*) IgM in human serum or plasma. It is intended to be used by professionals as an aid in the diagnosis of acute infection with *L. interrogans*.

INTRODUCTION

Leptospirosis occurs worldwide and is a common mild to severe health problem for humans and animals, particularly in areas with a hot and humid climate. The natural reservoirs for leptospirosis are rodents as well as a large variety of domesticated mammals. Human infection is caused by *L. interrogans*, the pathogenic member of the genus of *Leptospira*[1,2]. The infection is spread via urine from the host animal.

After infection, leptospires are present in the blood until they are cleared after 4 to 7 days following the production of anti-*L. interrogans* antibodies, initially of the IgM class. Culture of the blood, urine and cerebrospinal fluid is an effective means of confirming the diagnosis during 1st to 2nd weeks after exposure. Serological detection of anti-*L interrogans* antibodies is also a common diagnostic method. Tests available under this category include: 1) The microscopic agglutination test (MAT)[3], 2) ELISA[4-5], and 3) Indirect fluorescent antibody tests (IFATs)[6]. However, all above mentioned methods require a sophisticated facility and well-trained technicians.

Anti-*L. interrogans* IgM become detectable in the first week of illness, allowing treatment to be initiated while it is likely to be most effective[7]. Anti-*L. interrogans* IgM generally disappears after recovery but may stay in circulation for several months[2].

TEST PRINCIPLE

The *RecombiLISA* Leptospira IgM ELISA is a solid-phase enzyme-linked immunosorbent assay based on the principle of the indirect immunoassay technique for the qualitative detection of IgM anti-*L. interrogans* in human serum or plasma.

The *RecombiLISA* Leptospira IgM ELISA is composed of two key components:
1) Solid microwells pre-coated with *L. interrogans* antigen
2) Liquid conjugates composed of monoclonal anti-human IgM reagent conjugated with horseradish peroxidase (HRP-anti-human IgM Conjugate)

During the assay, the test specimen is first incubated with the coated microwells. The anti-*L. interrogans* IgM, if present in the specimen, binds to the antigen coated on the microwell surface, and any unbound specimen is then removed by a wash step.

In the second incubation with the HRP-anti-human IgM Conjugate, the anti-*L. interrogans* IgM antibody adsorbed on the surface of the microwells binds to the anti-human IgM in the HRP conjugate, forming a conjugate complex.

Unbound conjugate is then removed by washing. TMB Substrate is then added to the microwells, and the presence of the conjugate complex is shown by a blue color resulting from the reaction between the enzyme and substrate. The reaction is then quenched upon addition of the Stop Solution and the absorbance value for each microwell is determined using a spectrophotometer at 450/620-690 nm.

MATERIALS AND REAGENTS

Materials and reagents provided with the kit

Item	Description	Quantity	Catalog
1	*L. interrogans* Ag Coated Microwells	8 wells x12 strips	E0330W
2	HRP–anti-human IgM Conjugate	11 mL	E0330H
3	Leptospira IgM Positive Control	1 mL	E0330P
4	Leptospira IgM Negative Control	1 mL	E0330N
5	Sample Treatment Solution	2 x 30 mL	E0330ST
6	Wash Buffer (30X Concentrate)	20 mL	WE3001
7	Sample Diluent	11 mL	E0330SD
8	TMB Substrate A	6 mL	TME2000A
9	TMB Substrate B	6 mL	TME2000B
10	Stop Solution	6 mL	SE1000
11	ELISA Working Sheet	2	E0001ES
12	Instructions for Use	1	PI-E0330
Others	3 x Microplate Sealers and 1 x Resealable Bag		

Materials and reagents required but not provided in the kit

1. Pipette capable of delivering 5 µL, 10 µL, 50 µL and 100 µL volume
2. 1 mL plastic tubes
3. Microplate reader with a bandwidth of 10 nm or less and an optical density range of 0-3 OD or greater at 450 nm wavelength is acceptable
4. Vortex mixer or equivalent
5. Absorbent paper for blotting the microwells
6. Graph paper
7. Timer
8. Distilled or de-ionized water

STORAGE AND STABILITY

All reagents except the concentrated wash buffer are ready to use as supplied. Store all components at 2-8°C. Do not freeze. Avoid strong light. Ensure that reagents are brought to room temperature before opening. Reseal the microwells after removing the desired number of wells. Place unused wells in resealable plastic bag provided and return to 2-8°C. All the reagents are stable through the expiration date printed on the label if not opened.

SPECIMEN COLLECTION AND PREPARATION

- Serum or plasma should be prepared from a whole blood specimen obtained by acceptable venipuncture technique.
- This kit is designed for use with serum or plasma specimen without additives only.
- If a specimen is not tested immediately, the specimen can be stored at 2°C-8°C for up to 7 days. The specimens should be frozen at -20°C for longer storage. Avoid multiple freeze-thaw cycles. If a specimen is to be shipped, pack in compliance with federal regulations covering the transportation of etiologic agents.
- Specimens containing precipitants may give inconsistent test results. Clarify such specimens by centrifugation before testing.
- Do not use serum specimens demonstrating gross lipemia, gross hemolysis or turbidity.
- Specimens containing sodium azide may interfere with test results.

PREPARATION OF THE REAGENTS

1. Bring all reagents, controls to room temperature (18-28°C).
2. **Preparation of working Wash Buffer:**
   If precipitants are visible, warm up the Wash Buffer (30X concentrate) at 37˚C. Dilute concentrated Wash Buffer 30 fold with water as follows:

Plate	DI water	Wash buffer (30X)	Final volume
1 strip	58 mL	2 mL	60 mL
2 strips	116 mL	4 mL	120 mL
3 strips	174 mL	6 mL	180 mL
4 strips	232 mL	8 mL	240 mL

3. **Specimens must be treated prior to testing:**
   Dilute the specimens with the Sample Treatment Solution at 1:101 dilution, i.e. 5 µL of test serum or plasma into 500 µL of the Sample Treatment Solution. Mix well. The specimens are ready for test.
4. Mix each reagent before adding to the test wells.
5. Determine the number of strips needed and mark on the ELISA Working Sheet with the appropriate information. Positive and Negative Controls require to be run in duplicate to ensure accuracy.

ASSAY PROCEDURE

1. Calculate the desired number of microwells. Remove the remaining microwells and place them with desiccant into the resealable plastic bag, seal and store at 2-8°C for later use.
2. Add treated specimens according to the designation on the ELISA Working Sheet.

2.1 Blank Well: Do not add any reagents.

2.2 Control Wells: Add 100 µL of Leptospira IgM Positive or Negative Control into the designated control wells, respectively.

2.3 Test Wells: Add 100 µL of Sample Diluent to all the test wells, then transfer 10 µL of each treated test specimen to the corresponding test well, respectively.

3. Gently shake the plate wells for 20 seconds, then cover the plate with a sealer.
4. Incubate the wells at 37°C for 60 minutes.
5. Wash Step (Can be performed manually or with automated washing):
   Manual washing: Carefully remove the incubation mixture by disposing the solution into a waste container. Fill each well with diluted 350 µL of Wash Buffer and shake gently for 20-30 seconds. Discard the wash solution completely. Repeat 4 more times. After completing the last wash step, tap the plate on absorbent paper to remove residual liquid.

   Automatic washing: Automatic plate washer must be calibrated to ensure efficient washing. Aspirate incubation mixture from all wells completely. Fill each well with 350 µL of diluted wash buffer. Aspirate all wells completely. Repeat 4 more times.
6. Add 100 µL of HRP-anti-human IgM Conjugate into each well except the Blank Wells, cover the plate with a sealer.
7. Incubate at 37°C for 30 minutes.
8. Wash the plate 5 times as described at step 5.
9. Add 50 µL of TMB Substrate A and 50 µL of TMB Substrate B into each well including the Blank Well.
10. Incubate at 37˚C in the dark for 10 minutes.
11. Stop the reaction by adding 50 µL of Stop Solution to each well. Gently mix for 30 seconds**. It is important to ensure that all the blue color completely changes to a yellow color**.
12. Set the microplate reader wavelength at 450 nm. Measure the absorbance (OD) of each well against the Blank Well immediately after adding Stop Solution. Do not exceed 5 minutes. A filter of 620-690 nm can be used as a reference wavelength to optimize the assay result.

Flow chart of assay procedure

1.	Secure strips in microwell frame	Number of strips
2.	Add controls into Control Wells, and add Sample Diluent and specimens into Test Wells, respectively	100 µL 100 µL + 10 µL
3.	Gently shake	20 seconds
4.	Incubate	37°C, 60 minutes
5.	Wash: manual or automatic	5 times
6.	Add HRP-anti-human IgM Conjugate, except Blank Wells	100 µL
7.	Incubate	37°C, 30 minutes
8.	Wash: manual or automatic	5 times
9.	Add TMB Substrate A and B, respectively	50 µL + 50 µL
10.	Incubate in dark	37°C, 10 minutes
11.	Add Stop Solution. Gently mix	50 µL 30 seconds
12.	Read result	450/620-690 nm within 5 minutes

INTERPRETATION OF RESULTS

A. Set up the cut-off value
The cut-off value = 0.244 + N
N: Mean OD of the negative control. Use N=0.05 for calculation of the cut-off value if the mean OD is less than 0.05.

B. Calculation of specimen OD ratio
Calculate an OD ratio for each specimen by dividing its OD value by the cut-off value as follows:

$$\text{Specimen OD ratio} = \frac{\text{Specimen OD}}{\text{Cut-off Value}}$$

C. Assay Validation
The mean OD value of the Leptospira IgM positive controls should be ≥ 0.50.
The mean OD value of the Leptospira IgM negative controls should be ≤ 0.10.

Check the procedure and repeat assay if above conditions are not met.

D. Interpretation of the results

Specimen OD ratio	
Negative	< 1.00
Positive	≥ 1.00

1. The negative result indicates that there is no detectable anti-*L. interrogans* IgM antibody in the specimen.

2. Specimens with OD ratio ≥ 1.00 are initially considered to be positive by the *RecombiLISA* Leptospira IgM ELISA. They should be retested in duplicate before a final interpretation is made.
3. Results just below the cut-off value (OD ratio between 0.9 and 1) should be interpreted with caution. It is advisable to retest in duplicate the corresponding specimens when it is applicable.
   If after retesting the absorbance of one of the duplicates is equal or greater

   than the cut-off value, the initial result is repeatable and the specimen is considered to be positive with the *RecombiLISA* Leptospira IgM ELISA, subject to the limitations of the procedure, described below.
   If after re-testing of a specimen, the absorbance value of the 2 duplicates are less than the cut-off value, the initial result is non-repeatable and the specimen is considered to be negative with the *RecombiLISA* Leptospira IgM ELISA.
   Non-repeatable reactions are often caused by:
   - Inadequate microwell washing
   - Contamination of negative specimens by serum or plasma with a high antibody titer
   - Contamination of the substrate solution by oxidizing agents (bleach, metal ions, etc.)
   - Contamination of the stopping solution

PERFORMANCE CHARACTERISTICS

1. Clinical Performance

A total of 30 Leptospira positive specimens determined by MAT method and 95 non-Leptospira specimens were tested by the *RecombiLISA* Leptospira IgM ELISA. The test results for all subjects are shown in the following table:

	RecombiLISA Leptospira IgM ELISA		
Leptospira	**Positive**	**Negative**	**Total**
Positive	26	4	30
Negative	3	92	95
Total	29	96	125

Relative Sensitivity: 86.7%, Relative Specificity: 96.8%, Overall Agreement: 94.4%

2. Cross Reactivity

No false positive test results from *RecombiLISA* Leptospira IgM ELISA were observed on 2-7 specimens from each of the following disease states or special conditions, respectively:

Dengue	HIV	HBsAg	HCV	*H. pylori*
Malaria	Syphilis	Typhi	ANA	HAMA
RF (up to 8,400 IU/mL)				

3. Interference

Common substances (such as pain and fever medication and blood components) may affect the performance of the *RecombiLISA* Leptospira IgM ELISA. Interference was studied by spiking these substances into 3 Leptospira clinical specimens: negative, low positive and high positive. The results demonstrate that at the concentrations tested, the substances studied do not affect the performance of the *RecombiLISA* Leptospira IgM ELISA.

List of potentially inferring substances and concentrations tested:

1. Salicylic acid	4.34 mmol/L	5. Glucose	55 mmol/L
2. Sodium citrate	1.3 %	6. Heparin	3,000 U/L
3. Creatinine	0.5 mmol/L	7. Bilirubin	10 mg/dL
4. EDTA	3.4 µmol/L		

WARNING AND PRECAUTIONS

For *In Vitro* Diagnostic Use

1. Read these Instructions for Use completely before performing the test. Failure to follow the instructions could lead to inaccurate test results.
2. Do not use expired kits.
3. Bring all reagents to room temperature (18-28°C) before use.
4. Do not use the components of any other type of test kit as a substitute for the components in this kit.
5. Do not use serum derived from hemolyzed blood specimens for testing.
6. Do not ingest the reagents. Avoid contacting with eyes, skin and mouth. Wear protective clothing and disposable gloves while handling the kit reagents and clinical specimens. Wash hands thoroughly after performing the test.
7. Do not smoke, drink, or eat in areas where specimens or kit reagents are being handled.
8. Users of this test should follow the US CDC Universal Precautions for prevention of transmission of HIV, HBV and other bloodborne pathogens.
9. Dispose of all specimens and materials used to perform the test as bio-hazardous waste.
10. Gently tap the microwell plate to ensure thorough mixing as mentioned in the procedure. Avoid the formation of air bubbles which results in inaccurate absorbance values. Avoid splashing liquid while rocking or shaking the wells.
11. Do not allow the microwells to dry between the end of the washing operation and the reagent distribution.
12. The enzyme substrate reaction is very sensitive to metal ions. Thus, do not allow any metal elements to come into contact with the conjugate or TMB Substrate.
13. The enzyme-substrate is temperature dependent. Ensure that the room temperature for TMB incubation falls between 18-28°C.
14. The TMB Substrate must be colorless. The appearance of color indicates that the reagent cannot be used and must be replaced. The TMB Substrate must be stored in the dark.
15. Use a new dispensing tip for each specimen. Never use the specimen container to distribute conjugate and TMB Substrate.
16. **The wash procedure is critical. Wells must be aspirated completely before adding the Wash buffer or liquid reagents. Automatic washers must be validated with the test kit prior to use. Insufficient washing will result in poor precision and falsely elevated absorbance values.**
17. **Microplate reader must be calibrated per manufacturer's instruction to ensure accurate determination of absorbance. Non-calibrated reader often leads to invalid test results.**
18. Avoid exposure to strong light during color development.

LIMITATIONS OF TEST

1. The Assay Procedure and the Interpretation of Results must be followed closely when testing the presence of antibodies to *L. interrogans* in serum or plasma. Failure to follow the procedure may give inaccurate results.
2. The *RecombiLISA* Leptospira IgM ELISA is limited to the qualitative detection of IgM anti- *L. interrogans* in human serum or plasma.
3. A negative result indicates absence of detectable anti-*L. interrogans* antibodies. However, a negative test result does not preclude the possibility of exposure to or infection with *L. interrogans*.
4. A negative result can occur if the quantity of the anti-*L. interrogans* virus antibodies present in the specimen is below the detection limit of the assay, or the antibodies that are to be detected are not present during the stage of the disease in which a specimen is collected, particularly very early during the course of the infection.
5. A negative result may also occur due to geological variation of different serovars.
6. Infection may progress rapidly. If the symptom is highly suspicious or persists, while the result from the *RecombiLISA* Leptospira IgM ELISA is negative, it is recommended to test with an alternative test method.
7. As with all diagnostic tests, a definitive clinical diagnosis should not be based on the results of a single test, but should only be made by the physician after all clinical and laboratory findings have been evaluated.
8. The results obtained with this test should only be interpreted in conjunction with other diagnostic procedures and clinical findings.

REFERENCES

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Index of Symbols

See instructions for use		Use by	
IVD	For *in vitro* diagnostic use only	CONJ	Conjugates
REF	Catalog #	MICROWELLS	Coated microwells
LOT	Lot number	CONTROL +	Positive control
Σ N	Tests per kit	CONTROL -	Negative control
	Do not reuse	SAMP DILT	Sample diluent
	Manufacturer	SAMP SOLN	Sample treatment solution
	Date of manufacture	TMB SUBS	TMB substrate
EC REP	Authorized representative	WASH BUFF	Wash buffer
8°C 2°C	Store between 2-8°C	STOP SOLN	Stop solution

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