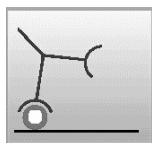
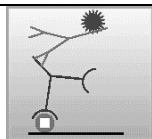


OVERVIEW OF PROCEDURE

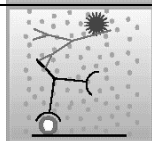
- Wash once with washing solution
- Dispense 90 µl of specimen diluent and 10 µl both controls and investigated sera in wells
- Incubate for 60 min at 37°C (forming complex antigen-antibody)
- Wash 4 times with washing solution



- Dispense 100 µl of conjugate solution in wells
- Incubate for 30 min at 37°C (forming complex antigen-antibody with conjugate)
- Wash 6 times with washing solution



- Dispense 100 µl TMB substrate in wells
- Incubate for 30 min at room temperature (colouring)
- Stop the reaction by adding 100 µl stop-reagent
- Read the optical density at 450/620 nm



Diaproph Med
Diagnostics Prophylaxis Medicine

DIA-Leptospirosis -V

**Diagnostic immunoenzyme test system for
the detection of antibodies against
pathogen leptospira in cattle serum**

96 tests

Product code: T-10407

INTENDED USE

EXAMPLE FORM

Enzyme immunoassay kit is intended for the detection of antibodies to pathogenic serovars *Leptospira interrogans* in bovine serum.

INTRODUCTION

Leptospirosis is the most spread antropozoonotic illness caused by *Leptospira interrogans*. Today more than 200 morphologic similar serovars of *Leptospira* have been consolidated in 23 serological groups. The following serovars as *Icterohaemorrhagiae*, *Hebdomadis*, *Grippotyphosa*, *Sejroe*, *Pomona*, *Tarassovi* mostly cause leptospirosis among cattle population.

Clinical diagnostics of leptospirosis is difficult enough at the early stages of the disease, when it may be confused with many other common febrile illnesses. Besides the typical clinical presentation of leptospirosis among bovine population is rare in occurrence. The most widespread state is the latent one, which lasts up to 6 months and among certain animals - for term of life. In connection with it serological diagnostics of leptospira get a special importance. The most applicable method for screening is enzyme-linked immunosorbent assay (ELISA). The assay is suitable for use in the routine diagnostics laboratories as a rapid screening test for leptospira infections in cattle-breeding.

PRINCIPLE OF PROCEDURE

The main kit components are an immunosorbent and an enzyme-containing conjugate. The immunosorbent is a polystyrene plate whose wells are coated with purified antigens of pathogenic serovars *Leptospira interrogans*. The conjugate contains horse radish peroxidase (HRP) bound to anti-bovine IgG antibodies.

The sera samples to be investigated are put into plate wells. The antibodies against the pathogenic serovars *Leptospira interrogans* present in the sera bind to the coated

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antigens of the solid phase forming antigen-antibody complexes. These complexes are detected using the specific enzyme-containing conjugate. Non-bound components are to be washed out. Following washing procedure, a developer solution containing enzyme substrate and chromogen (tetramethylbenzidine, TMB) are added to wells. The reaction is stopped by a stop-reagent (0,5 M sulfuric acid). The optical density (OD) values in wells are determined at 450 nm, this values being correlated with specific antibody concentration in investigated blood serum.

STORAGE CONDITIONS AND TRANSPORTATION

The kit must be stored and transported at 2-8°C. The kit must not be frozen. Shelf life of the kit is 12 months.

KIT REAGENTS

Only for *in vitro* diagnostic use.

Each kit contains:

Label	Reagents	Presentation
1	Microelisa strips 6 strips per plate each with 16 wells coated with purified antigens of pathogenic serovars of <i>Leptospira interrogans</i> .	1 plate
2	Stock washing solution (No.1) (45x) Phosphate buffer, containing 2.2% Triton X100.	2 bottle 2 × 25 ml
3	Solution for sera dilution (No.3) Phosphate buffer, containing powdered milk. Preservatives: 0.01% mertiolat and 0,01% benzoic acid.	1 bottle 1 × 15 ml
4	Positive control (C+) Bovine serum containing antibodies to pathogenic serovars of <i>Leptospira interrogans</i> Preservatives: 0.1% sodium azide and 0,01% parnitrofenol	1 vial 1 × 0.2 ml
5	Negative control (C-) Bovine negative serum Preservatives: 0.1% sodium azide. and 0,01% parnitrofenol	1 vial 1 × 0.35 ml
6	Conjugate (concentrate 10x) Monoclonal antibodies against bovine IgG bound to a horseradish peroxidase (HRP). Preservatives: 0.01% thimerosal.	1 vial 1 × 1.5 ml
7	Solution for conjugate dilution (No4) Phosphate buffer, containing powdered milk. Preservatives: 0.01% thimerosal and 0,01% benzoic acid.	1 bottle 1 × 15 ml

Results

Calculation of the results

NC – absorbance of the negative control

PC – absorbance of the positive control

\overline{NC} – mean absorbance of the negative control

- Calculate the mean absorbance of the negative control.

Test run is valid if \overline{NC} is not higher than 0.100.

Exclude any NC, which is higher than 0.100 or if it is more than twice exceed the \overline{NC} , and use remaining NC that is not higher than 0.100.

Test run is valid if PC is not lower than 0.600.

- Calculate **Cut-off** value:

$$\text{Cut-off} = \overline{NC} + 0.12$$

- Determine the grey zone.

Grey zone is the zone with sample absorbance within the range:

$$\text{Cut-off} - 10\% \leq OD \leq \text{Cut-off.}$$

Interpretation of the results

The result is considered as **nonreactive** if the specimen absorbance is below the grey zone.

The result is considered as **reactive** if the specimen absorbance is equal or greater than the cut-off.

Specimens with absorbance values within the grey zone are considered **indeterminate** and should be retested in duplicate.

- specimens reactive in one or more wells are considered as reactive ones;
- specimens nonreactive in two or more wells are considered as nonreactive ones.

Wash procedure

Washing must be performed strictly according to the instructions, as insufficient plate washing leads to incorrect results.

Use automatic washer*, as recommended; in case of its absence or faulty work – use multi-channel pipette for washing.

Follow this procedure in each washing:

- aspirate the wells contents completely into a waste flask;
- then fill the wells completely with washing solution (not less than 350 µl per well) avoiding overflow of buffer from one well to another;
- aspirate completely.

Make sure that no fluid remains on the top and the bottom of the strips and stripholder after the last aspiration (e. g. by blotting with absorbent tissue).

* Contact our company for further information on the different types of washers validated by our technical services.

Test procedure

- Fit the stripholder with required number of **Microelisa strips**.
- Wash once the strips with **washing solution** as recommended (section *Wash procedure*). If necessary, dry the plate by slight tapping upside-down on absorbent paper.
- Pipette 90 µl of the **solution for sera dilution** into each well.
- Distribute in the wells as follows:
 - well: A1: 10 µl of **positive control (C+)**.
 - wells: C1, D1: 10 µl of **negative control (C-)**.
 - the rest wells: 10 µl of **specimens**.

Carefully repipette mixture in wells.

- Cover the plate with adhesive film and incubate at 37°C for 60 minutes.
- Aspirate the contents of all wells and wash the plate with **washing solution** 4 times (according the section *Wash procedure*). If necessary, dry the plate by slight tapping upside-down on absorbent paper.
- Pipette 100 µl of the **conjugate solution** into each well.
- Cover the plate with adhesive film and incubate at 37°C for 30 minutes.
- Aspirate the contents of all wells and wash the plate with **washing solution** 6 times (according the section *Wash procedure*). If necessary, dry the plate by slight tapping upside-down on absorbent paper.
- Pipette into each well 100 µl of the **TMB substrate**.
- Cover the plate with adhesive film and incubate at 18-25°C for 30 minutes in the dark.
- Add into each well 100 µl of **stop-reagent** to stop colour reaction (maintain the same pipetting sequence and rate used for TMB substrate addition).
- Read the absorbance at 450/620 nm within 5 minutes after stopping the reaction using a dual wavelength microplate reader.

Absorbance may be measured at 450 nm (single wavelength) against a blank well; for that include an empty well in the run.

8	Chromogen (TMB) Solution containing 0.03% 3,3',5,5'-tetramethylbenzidine.	1 bottle 1 × 8 ml
9	Substrate buffer Citrate-phosphate buffer, containing 0.016% hydrogen peroxide.	1 bottle 1 × 8 ml
10	Stop-reagent 0.5 M sulphuric acid solution.	1 bottle 1 × 15 ml
	Adhesive film	3 items

ADDITIONAL MATERIALS AND INSTRUMENTS REQUIRED

- distilled or deionized water;
- disposable gloves;
- disposable V-shaped troughs;
- vial for reagents preparation (glass or plastic);
- graduated cylinder (1000 ml);
- absorbent paper;
- sodium hypochlorite solution or other accepted disinfectant;
- sodium bicarbonate;
- ethanol, 70°;
- automatic single-channel pipettes (e.g. 5-40, 20-200, 200-1000 µl) with disposable tips;
- automatic multi-channel pipettes (50-300 µl) with disposable tips;
- incubator, 37±1°C;
- microwell wash system*;
- microwell reader* (with dual wavelength 450/620);
- biohazard waste containers for potentially contaminated materials.

* Contact our company for further information on the equipment validated by our technical services.

SAFETY PRECAUTIONS AND WARNINGS

- Use a new tip for pipetting specimens in wells.
- All reagents included in the kit are intended for "in vitro" diagnostic use.
- Wear disposable gloves when handling reagents and samples and thoroughly wash hands after handling them.
- Do not pipette with mouth.
- all liquid biohazardous wastes are to be treated by hydrogen peroxide 6% at room temperature during 3 hours;
- all solid wastes are to be stored in a special container and autoclaved for 1 hour at 120°C;
- instruments, equipment and also working surfaces are to be treated with 70° ethanol.

Handling precautions:

- do not use the kit beyond the expiration date; do not mix kit components from different lots;
- mix thoroughly the reagents during preparation and procedure of analysis;
- to prepare the reagents use the glassware washed and rinsed by distilled water;
- do not allow the drying of wells during ELISA procedure;
- to check dosing precision, working pipettes and other equipment state;
- prevent the direct light to fall on the working surface during ELISA procedure.

SPECIMEN PREPARATION

Serum specimens are to be stored at 2-8°C during 72 hours. If necessary these specimens may be frozen (not more than two freezing-thawing procedures are allowed) at temperature below -20°C.

All specimens containing aggregates and visible suspended particles are to be clarified by centrifugation.

Specimens with sodium azide, hemolysis, hyperlipidemiae or bacterial contamination may not be used in the ELISA procedure.

ASSAY PROCEDURE

Reagents and specimens should be at room temperature (18-25°C) before beginning the assay and can remain at room temperature during testing. Return reagents to 2-8°C after use.

Reagents preparation

Microelisa strips

Open the pack and remove the plate. Return unused strips in the pack. Reseal the pack and return to 2-8°C.

The strips are stable for 4 weeks at 2-8°C after opening the pack.

Washing solution

Check **Stock washing solution (№1)** for the presence of salt crystals. If crystals are seen in the solution, dissolve them by heating at 35-37°C.

Dilute the washing solution concentrate 1:45 with distilled or deionised water (see chart below), shake intensively.

Washing solution is stable for 10 days at 2-8°C.

Number of wells	Stock washing solution (№1)	Distilled or deionised water
16	6 ml	270 ml
32	8 ml	350 ml
48	12 ml	540 ml
64	16 ml	720 ml
80	20 ml	900 ml
96	25 ml	1130 ml

Conjugate solution

Dilute (1:10) 100 µl of the **conjugate** (concentrate) with 1 ml of the **solution for conjugate dilution (№4)** in a clean vial. Mix well avoiding foaming.

Conjugate solution has to be prepared before use.

TMB substrate

To prepare **TMB substrate**, combine the required amount of **chromogen (TMB)** in a clean vial in equal parts with **substrate buffer** according to the number of wells being run (see chart below). Mix well. TMB substrate must be colourless before use.

The TMB substrate must be kept away from light and no solutions contact with metals or metal ions is allowed.

The solution has to be prepared before use.

TMB substrate is stable for 2 weeks at room temperature (18-25°C) if kept in the dark.

number of wells	chromogen (TMB)	solution for chromogen preparation (№5T)
16	1 ml	1 ml
32	2 ml	2 ml
48	3 ml	3 ml
64	4 ml	4 ml
80	5 ml	5 ml
96	6 ml	6 ml

Procedural notes

Authenticity of results depends on correct execution following instructions:

- Reagents should not be used beyond the expiry date shown on the package label.
- Reagents should not be mixed from different lots during performing test.
- Reagents and samples should be at room temperature (18-25°C) before testing begins. Return the reagents to 2-8°C after use.
- The temperature in room where performing analysis should be in the range 18-25°C.
- It should accurately dissolve reagents avoiding its contamination.
- Do not perform the test in the presence of reactivity vapours (for example, from sodium hypochlorite, acids, alkalis, or aldehydes) or dust because the enzymatic activity of the conjugate may be affected.
- Use glass vessels thoroughly washed and rinsed with deionized water or use disposable ones.
- Do not allow drying contents of wells on all stages of procedure.
- Enzyme reaction is sensitive to metal ions, so avoid contacting with metal elements.
- TMB substrate (substrate buffer + TMB solution) is to be colourless. Appearance of colouring after dilution is evidence of unavailability for using and solution is to be replaced. The solution is to be prepared in clean plastic ware or clean glassware. The reagent is to be kept in dark.
- Prevent the direct light to fall on the working surface during ELISA procedure.
- Use a new tip for brining specimens in wells.
- Never use the same trough for distribution conjugate and TMB substrate.
- Check the pipettes and other equipment for accuracy and correct operation.
- Do not change the assay procedure.