ELITEST CLA

CK105A

Kit for the detection of IgG antibodies in sera from sheep or goats with caseous lymphadenitis (CLA)

For *in vitro* use only

BioHazard

For research use only

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INTENDED USE

ELITEST CLA is an Enzyme Immuno-Assay (EIA) for the detection of IgG antibodies specific for the causative agent of caseous lymphadenitis (CLA) in sheep or goat sera.

CLINICAL BACKGROUND

CLA is a bacterial disease of sheep and goats caused by *Corynebacterium pseudotuberculosis*. This organism belongs to a family of related bacteria, several of which are pathogenic for man and/or animals. *C. pseudotuberculosis* can survive in the environment for several months and is highly infectious. Current treatment of diseased animals is limited. Monitoring of disease status is compounded by the fact that animals may be infected without showing obvious clinical symptoms. This direct enzyme-linked immunosorbent assay (ELISA) utilises a recombinant form of the important, conserved *C. pseudotuberculosis* virulence factor, phospholipase D (PLD) to detect anti-PLD IgG antibodies in sera from sheep and goats with CLA. As PLD is not known to be produced by any other sheep pathogenic bacteria this makes it a very specific test. In cases of CLA, infected animals mount an immune response against PLD; thus, when sera from infected animals are screened with the ELISA, antibodies specific for PLD bind to the recombinant antigen and are quantified. By measuring the levels of anti-PLD antibodies in sheep, the likelihood of infection can be determined. Detection of diseased animals through serodiagnosis has proven effective in eradicating CLA in infected goat herds in The Netherlands.

https://www.moredun.org.uk/research/diseases/caseous-lymphadenitis-cla

TEST PRINCIPLE

The wells of microplate strips have been coated with highly-purified, recombinant PLD (rPLD), which constitutes the solid phase antigen. The test sample is incubated in the well and specific antibodies to rPLD, if present in the sample, bind to the solid phase immobilized antigen.

Subsequently, a horseradish peroxidase (HRP)-labelled mouse monoclonal anti-goat/sheep IgG antibody is added (secondary antibody). When anti-PLD antibodies are present they bind to the coated antigen and are detected by the secondary antibody. Subsequent incubation with HRP substrate produces a blue colour in the test wells; the colourimetric assay is further enhanced (and stopped) by the addition of sulphuric acid, resulting in a yellow colour change. If a serum sample contains no anti-PLD antibodies, the HRP-labelled secondary antibody does not bind, and no colour develops.

REAGENTS SUPPLIED

Each pack contains:

1. 5 sachets, each containing a strip-holder with 12×8 **coated test wells**, with a desiccant.
2. 1 vial containing 200 µl of **negative control** (**NC**; sheep serum containing 0.1% sodium azide as preservative).
3. 1 vial containing 200 µl of **positive control** (**PC**; sheep serum containing 0.1% sodium azide as preservative).
4. 3 vials, each containing 125 ml of **Sample Diluent** (**SD**; phosphate buffer containing sodium chloride, protein stabilizers and 0.05 % Kathon CG as preservative).
5. 1 vial containing 125 ml of **Conjugate Diluent** (**CD**; phosphate buffer containing protein and enzyme stabilizers and 0.05 % Kathon CG as preservative).
6. 1 vial containing 1.2 ml of 100-fold concentrated **Immuno-Conjugate** (**C**; mouse monoclonal anti goat/sheep IgG labelled with horseradish peroxidase), to be diluted **1:100** before use.
7. 1 vial containing 250 ml of concentrated **Wash Solution** (**WS**; phosphate buffer containing 2% Tween 20), to be diluted 1:20 before use.
8. 5 vials, each containing 25 ml of ready to use **TMB substrate solution** (peroxidase substrate: 3,3',5,5' **– Tetramethylbenzidine** containing hydrogen peroxide).
9. 5 vials, each containing 6 ml of **0.45M Sulfuric Acid Stop solution** (**SA**); ready to use.
10. 1 plastic **minigrip bag** for storage of unused strips.

MATERIALS REQUIRED BUT NOT PROVIDED

- Distilled or deionised water.
- Precision pipettes with disposable tips to deliver volumes in the ranges 1-10 µl, 20-200 µl; and 200-1000 µl, respectively.
- Optionally, a multichannel pipette to deliver 200 µl can be used together with disposable V-shaped troughs for addition of immuno-conjugate, substrate and sulphuric acid.
- Microplate shaker (optional but recommended).
- Microplate washer (alternatively, washing can be performed manually, *e.g.* by using a repeating syringe delivering 0.3 ml volumes and an aspirating device).
- Absorbent tissues.
- Microplate photometric reader, equipped with 450 nm and 620 nm filters (690 nm may be used instead 620 nm).

Safety: Avoid contact with and inhalation of TMB solution. If substrate comes into contact with skin, wash thoroughly with water.

Sulphuric acid, although diluted to 0.45M, is caustic. As for any similar chemical, handle sulphuric acid with great care. Avoid any skin and eye contact. Wear protective glasses and gloves when handling.

Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. Flush with large volumes of water when discarding into a sink.

It is possible that any product of biological origin (including the controls) may contain infectious organisms; appropriate care should be taken during handling.

STORAGE AND STABILITY

- If kept at 2° to 8°C, all test reagents, including the coated test wells, are stable until the expiration date indicated on the box.
- All reagents, including the pouch containing the test wells, must be brought to room temperature (18-25°C) approximately 30 minutes before use and must be stored at 2-8°C immediately after use.
- Unused test wells, kept at 2-8°C, are stable for 4 weeks if stored in the plastic minigrip bag with the desiccant. Any presence of moisture adversely affects test performance.
- Diluted wash solution is stable for 7 days, if kept at 2-8°C.
- Diluted conjugate is stable for 4 hours at room temperature (18-25°C) if kept in the dark.
- After using some of the contents of vials containing controls, sample diluent, concentrated conjugate, conjugate diluent, substrate, and concentrated wash solution, the contents are stable until the expiration date if kept at 2-8°C and stored in the closed original vial, provided that any contamination is avoided during use.

SPECIMENS

- ELITEST CLA may be performed with sheep and goat sera.
- Specimens should be free of microbial contamination when tested.
- Insoluble material should be removed from all samples by centrifugation before testing.
- Before storage, sera should be separated from blood cells or clots by centrifugation.
- Store samples at 2-8 °C for up to one week. For longer-term storage maintain aliquots at –20°C.

TEST PERFORMANCE

Preparations:

- Allow all test material to reach room temperature (18-25°C) before use.
- Wash solution should be prepared by diluting concentrated **WS** 1:20 in distilled or deionised water (*e.g.* add 25 ml to 500 ml). Prepare at least 50 ml of diluted wash solution for each strip.

Note: Salt crystals may form in the concentrated **WS** when stored at 2-8°C. These crystals should be completely redissolved by warming the concentrated **WS** at 37°C prior to dilution.
WS must be at room temperature (18-25°C) when used.

- Prepare a 1:50 pre-dilution of test samples and controls in **SD** (*e.g.* 10 µl serum in 500 µl **SD**).
- The conjugate should be prepared by diluting the concentrated immuno-conjugate (**C**) 1:100 in **CD** (*e.g.* by diluting 20 µl to 2 ml per strip or 200 µl to 20 ml per plate).

Directions for washing:

Incomplete washing will adversely affect test outcomes. Operating instructions for washing equipment should be carefully followed. Contamination of wash solution and washer can cause extensive problems. Therefore:

- Store diluted **WS** at 2-8°C, and preferably prepare freshly before use
- Pre-rinse the washer with diluted **WS**

In the case of problems, disinfect wash bottles and washer overnight with a 4 % formalin solution. If no suitable automatic washer is available, washing can be performed manually as follows:

- Completely aspirate the liquid from all wells by lowering an aspiration tip gently to the bottom of each well. **Take care not to scratch the inside of the well surface**.
- After aspiration, fill the wells with diluted **WS** ensuring that the liquid reaches to the top of the wells. Let soak for a minimum of 30 seconds, and then aspirate the liquid. Perform these steps five times.
- After the last aspiration, the washing procedure is completed by inverting the plate and tapping it dry on absorbent tissue.

Remarks and precautions:

1. Do not use the kit beyond the expiration date.
2. Do not mix reagents between kits, unless the components have identical lot numbers.
3. All vessels used to prepare conjugate and use substrate solutions must be cleaned thoroughly and finally rinsed with distilled water.
4. To avoid contamination, do not touch the top of the plates with your fingers.
5. Avoid microbial contamination of reagents.
6. Ensure that the samples and controls are homogeneous solutions before use.
7. Use a new pipette tip for each sample.
8. To avoid contamination, do not touch the edges of the wells with the pipette tips when adding sample or conjugate.
9. Do not expose substrate to strong light during incubation or storage. Substrate solution must be almost colourless when used.
10. To avoid unwanted colour formation, solutions containing TMB, sulphuric acid or peroxide should not be in contact with metals or metal-ions.
11. Ensure no air-bubbles are present in the wells; remove any bubbles by tapping gently.
12. If the wells cannot be filled with conjugate or substrate immediately after washing, the strips may be placed upside down on a wet absorbent tissue for no longer than 15 minutes.

ASSAY PROCEDURE

1. Fill the strip-holder with the required number of strips for the test series planed, taking into account that one negative control and one positive control should be included. If more than 3 strips are used in one run, at least two negative and positive controls should be included in each strip holder. During the processing, strips stay in the strip holder and can be marked on one edge.
2. Make a 1:50 pre-dilution of samples and controls (*e.g.* 10 µl serum in 500 µl of **SD**). Do not use very small volumes of serum (1 µl or less) to avoid pipetting errors.

Reagent addition:

3. Add 200 µl of **SD** to each test well.
4. Add 20 µl of diluted sample or control to each appropriate test well. Make sure samples and controls are completely mixed with the diluent, either on a microplate shaker (at 1,000 rpm) or manually (by pipetting up and down a minimum of five times).
5. Cover the strips with the provided microwell lid. Incubate for 60 minutes at room temperature (18-25°C).

(**Note**: prepare immuno-conjugate solution during incubation; see 'Preparations').

6. Wash each well 5 times (see 'Directions for washing').
7. Add 200 µl prediluted conjugate solution to each well.
8. Cover the strips with the microwell lid. Incubate for 60 minutes at room temperature (18-25°C).
9. Wash each well 5 times (see 'Directions for washing').
10. Add 200 µl of ready to use substrate solution to each well.
11. Incubate for exactly 5 minutes at room temperature (18-25°C).
12. To stop the reaction, add 50 µl **SA** to each well, in the same sequence and at the same time intervals as the substrate solution. Shake the strip holder carefully to ensure thorough mixing.
13. Read (within 10 minutes after step 12) the absorbance of the solution in the wells at 450 nm; subtract absorbance at reference wavelength (this should be between 620 and 690 nm, at Hyphen we use 620nm).

SUMMARY OF ASSAY PROCEDURE

Reagent	Volume	
Sample diluent (SD) (Ready to use)	220 µl	Add to one test well, reserved as blank.
	200 µl	Add to each test well, reserved for specimen and controls.
Diluted samples or control	20 µl (pre-diluted 1:50)	Add to each appropriate test well. Mix manually or on shaker. Cover plate with the provided lid.
Incubate for 60 minutes at room temperature (18-25°C)		
Warning: Prepare the immuno-conjugate before the end of incubation		
Wash solution (**WS**) (to be diluted 1:20 before use in distilled/ deionized water)	5 × 0.3 ml	Wash each well 5 times.
Immuno-conjugate (**C**) (to be diluted 1:100 before use in conjugate diluent **CD**)	200 µl	Add to each well and mix gently. Cover plate with new adhesive sealer.
Incubate for 60 minutes at room temperature (18-25°C)		
Wash solution (to be diluted 1:20 before use in distilled/ deionized water)	5 × 0.3 ml	Wash each well 5 times.
Substrate	200 µl	Add to each well.
Incubate for exactly 5 minutes at room temperature (18-25°C)		
Stop solution (**SA**)	50 µl	Add to each well. Mix by tapping side of plate.
Read absorbance at 450 nm within 10 minutes, subtract absorbance at 620nm,.		

CAUTIONS

1. Variation in room temperature may affect the absorbances obtained; higher temperatures will result in higher absorbances. The assay is optimized for use at 20°C, and performance is stable within the range 18-25°C.
2. The use of a plate shaker is recommended since it allows good mixing of reagents. It is recommended that plates be shaken for 1-2 minutes at the beginning of each step (sample distribution, immuno-conjugate distribution, stop solution distribution). Avoid shaking plates throughout the incubation steps, since this can induce overly-elevated absorbance values.

INTERPRETATION

Abbreviations:

P = the mean of the absorbance of the positive controls
N = the mean of the absorbance of the negative controls
S = the mean of the absorbance of the test sample

Validation:

Check the validity of individual negative and positive controls.

Each of the negative controls should be ≤ 0.200.

- Calculate N (excluding controls >0.200).
- Each of the positive controls should be within the range of 1.00 to 2.00.
- Calculate P (excluding controls outwith the specified range).

If more than half of the controls have to be eliminated the test run should be repeated after careful investigation of the source of possible errors.

Test result:

Calculate the cut-off value as: [(P-N)/4] + N

A sample is NON-REACTIVE if $S < [(P-N)/4] + N$

A sample is REACTIVE if $S \geq [(P-N)/4] + N$

It is useful to establish a 'Grey Zone' which would trigger further testing to confirm the result. If a sample gives a reading in the 'Grey Zone' that sample should be re-tested in duplicate. If still in the 'Grey Zone' the animals should be re-sampled as soon as possible.

When the assay is performed within the range 18-25°C, the following results can be expected:

Blank absorbance value	**< 0.100**
Negative control absorbance value	**< 0.20**
Grey zone	**0.20-0.50**
Positive absorbance value	**> 0.50**