ELISA *Clostridium chauvoei* flagellum kit

For detection of *Clostridium chauvoei* protective antigen

Reference #EC001 *extended version*

Instructions for use

RUO **This product is for research use only and is not intended for diagnostic use.**

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INTRODUCTION

This protocol describes the ELISA method used to detect *Clostridium chauvoei* flagellin in production cultures, for the formulation of vaccines against this pathogen. The present kit has the unique property of using the camelid monodomain recombinant antibody technology. This confers unmatched characteristics, such as inter-lot reproducibility, 100% specificity and ultra-sensitivity. The combination of this new technology and the ease of use, allow generating an agile test with reliable results.

The disease generated by *C. chauvoei* (Black leg) spreads worldwide, manifesting as fulminant myonecrosis in ruminants, which generally leads to the death of the animal in a short time. Due to high mortality, *C. chauvoei* causes significant losses in livestock production (Hatheway, 1990; Groseth et al., 2011; Frey and Falquet, 2015).

Commercial and locally produced vaccines successfully control the infection in ruminants (Uzal, 2012; Frey and Falquet, 2015). Traditionally, flagellar antigens are considered important virulence attributes and protective factors in vaccines (Chandler and Gulasekharam, 1974; Tamura and Tanaka, 1987; Tanaka et al., 1987; Tamura et al., 1995). Therefore, if these antigens play a role in the protective immunity induced by vaccines, an evaluation of them in vaccine preparations presents great relevance in the design and composition of vaccines and tests of their efficacy.

Commercially vaccines commonly found, consist of chemically inactivated bacteria, mostly of relatively old strains, which provide outer membrane proteins and flagellar proteins, which have been proposed as immunogens; and bacterial culture supernatants that are expected to contain the main toxins (Mattar et al., 2002; Uzal, 2012).

GENERAL INFORMATION

1. Precautions

Please read the instructions carefully before starting the test.

- Store all reagents at the temperature indicated in point 3.1.
- Modifications to kit components or procedures may result in loss of performance.
- Do not change or substitute reagents with other lots or origins.

2. Storage and stability

Store kit immediately upon receipt at +2 to +8°C

- Check the storage conditions of each individual reagent in the list of materials below.
- Accelerated stability tests ensure the stability of each reagent for 12 months after the production date, under the indicated conditions.
- All reagents are stable +2 to +8°C for at least 72 hours to ensure transportation.

3. Materials and Equipment

3.1 Supplementary materials

Item	Quantity	Storage conditions	Notes
96-well microtiter plate (12x8 strips)	5	+2 to +8°C	-
PBS 1X	15 tubes x 1 g	+2 to +8°C	See point 4.1 for its preparation
Streptavidin (5 mg/mL)	2 x 18 µL	-20°C ⚠	See point 4.2 for its preparation
Wash solution Tween-20	6 envelopes 6 x 1 mL	+2 to +8°C *	See point 4.3 for its preparation
Bovine seroalbumin	2 x 1.5 g	+2 to +8°C *	See point 4.4 for its reparation
Flagellin Standard (1.0 µg/mL)	5 x 650µL	-20°C ⚠	-

Sample diluent	60 mL	+2 to +8°C	Ready to use
Detection antibody	60 mL	+2 to +8°C	Ready to use
HRP-conjugated anti-rabbit antibody	650µL	-20°C ⚠	See point 4.1 for its preparation
Conjugate diluent	60 mL	+2 to +8°C	Ready to use
Wash solution Tween-20	Five envelopes 5 x 1mL	+2 to +8°C *	See point 4.2 for its preparation
Substrate solution	60 mL	+2 to +8°C	Ready to use
Stop solution	30 mL	+2 to +8°C	Ready to use
Illustrative brochure	-	-	-
Instructions for use	-	-	-
Analysis certificate	-	-	-

***Once the solution is prepared, store at +2- 8°C, for a maximum of 3 months.**

3.2 Materials required, not supplied

The following materials are not included in the kit, but are necessary for the correct performance of the assay

- Micropipettes (20-1000-µL) and multichannel pipette for 100 µL
- Microplate reader (wavelength 450 nm)
- Microplate shaker
- Refrigerator, +2 to +8°C
- Freezer, -20°C
- Distilled water

TEST PROTOCOL

4. Reagents Preparation

4.1 HRP-conjugated anti-rabbit antibody

The reagent must be used in a final dilution of 1/100. Calculate the amount of diluted anti-rabbit-HRP antibody solution to use, based on the number of strips to be processed. Use the following ratio: 1000 uL of diluted antibody is needed for one strip (10 µL anti-rabbit-HRP antibody in 990 µL of conjugate diluent). Homogenize avoiding foam formation. Keep cold +2 to +8°C until use. Once the preparation has been used, discard the remainder.

Attention: This reagent is stable for 2 hours.

4.2 Wash solution

Prepare the washing solution by dissolving all the salts contained in one sachet in 1 L of distilled water. Once complete dissolution is verified, add 1 mL of Tween-20 and mix; this volume is sufficient for washing a 96-well plate. Unused volume should be stored at +2 to +8°C until next use. Do not use again if precipitate of salts or turbidity is observed, nor for a period greater than 3 months.

5. Procedure

Notes prior to the start of the test

- All reagents must be at room temperature (+18 to +25 °C) prior to use; except those where storage at -20 °C is indicated, that must be thawed on ice at the time of use (see point 5.1 and 5.5).
- The amount of reagents provided is just the necessary for the development of the kit, measure the right amount for each test (1 mL per strip).
- Reagents should be well mixed before use, and observed for salt precipitation or turbidity.
- The reagents must be protected against any contamination by using disposable tips for dosing.
- Once it has been thermostated, open the envelope with the pre-sensitization strips, remove only those that will be used. The rest of the strips must be stored immediately, keeping the silica gel envelope, closing the aluminum wrapper.
- It is recommended that each sample be assayed at least in duplicate.

- It is recommended to diagram the assay, to facilitate the dosing of controls and samples, as well as the preparation of reagents.

5.1 Dilution of samples/flagellin standard

Both the samples and the flagellin standard should be dispensed in a ½ dilution in sample diluent. Follow the instructions below:

A. Add 100 µL of sample diluent to wells A to H in the number of strips needed (nº of strips needed= nº *of C. chauvoei samples* x2 + 2 strips for the Flagellin standard).
B. Add 100 µL of the Flagellin standard or samples in the first well of the corresponding column, and homogenize with the 100 µL of sample diluent present in the well.
   Attention: Shake the samples and the flagellin standard before plating. Keep the Flagellin cold and freeze immediately after using.
C. Transfer 100 µL of each dilution to the next well (from A to G) and homogenize 10 times each before transferring again; once the dilutions are complete, discard 100 µL from well G. Avoid bubble formation.
   Well H corresponds to the sample blank (sample diluent only).
D. Incubate the microplate for 60 min at room temperature, with shaking; keep cover.

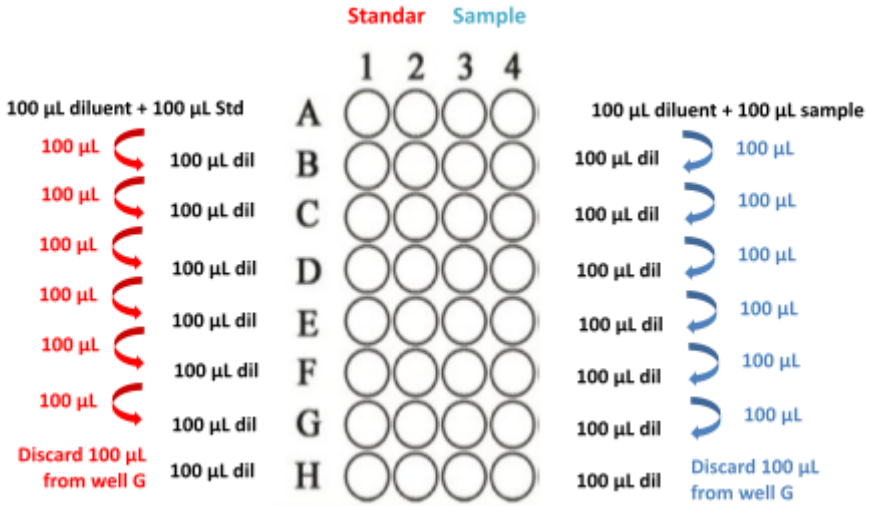


Figure 1. Scheme of serial dilutions: diluent: sample diluent; standard: flagellin standard (1 µg/mL). Well H corresponds to the zero or blank of each strip.

5.2 Washing

Wash the plate 10 times (200 µL/well) with a washing solution. Dry the microplate on absorbent paper.

5.3 Detection Antibody Incubation

Add 100 µL of detection antibody per well (ready to use).
Cover the plate and incubate for 60 minutes at room temperature, while shaking.

5.4 Washing

Wash the plate 5 times (200 µL/well) with a washing solution. Dry the plate on absorbent paper.

5.5 Incubation of anti-rabbit-HRP antibody

A. Prior to use, dilute the anti-rabbit-HRP antibody 1/100 in the conjugate diluent, as described in section 4.1. Keep the dilution cold until use.
B. Add 100 µL of the diluted conjugate to each well. Cover and incubate for 45 minutes at room temperature, while shaking. Once used, discard.

Attention: In this step, warm the substrate solution, protecting it from light.

5.6 Washing

Wash the plate 5 times (200 µL/well) with the washing solution. Dry the plate on absorbent paper.

Attention: Remaining liquid residues in the wells after the washing process can influence the conversion of the substrate and lead to falsely low values. On the other hand, insufficient washing (eg fewer washing cycles or insufficient volume of washing solution) can lead to falsely high values.

5.7 Substrate incubation

Add 100 µL of the substrate solution to each well. Incubate for **15 minutes** at room temperature (protect from direct exposure to light).

5.8 Stop

Add 50 µL of the Stop Solution to each well, in the same order that the Substrate Solution was added previously.

5.9 Measurement

Photometric measurement of color intensity must be performed at a measurement wavelength of 450 nm and a reference wavelength between 620 and 650 nm, **within 30 minutes of adding the stop solution**. Before taking the measurement, shake the microplate slightly to ensure homogeneous distribution.

6. Interpretation of results

6.1 Relative response assessment

Through this assay it is possible to detect *Clostridium chauvoei* flagellin to follow the culture through its production of protective antigen. It is possible to evaluate the relative response in vitro, comparing different samples.

Note: test samples 1 and 2 may correspond to different production batches; at different times from the same batch or samples from different production methods.

Attention: in this case the assay is used for the purpose of studying stability or evaluating different production methodologies or batches. But in no way should it be used to assess potency *in vitro*.

For interpretation, plotting on the y-axis the absorbance values, on a linear scale, against the corresponding dilutions of cultures, on the *x -axis*. Then, compare both curves qualitatively (the one that is furthest to the right represents the sample with the highest concentration of flagellin).

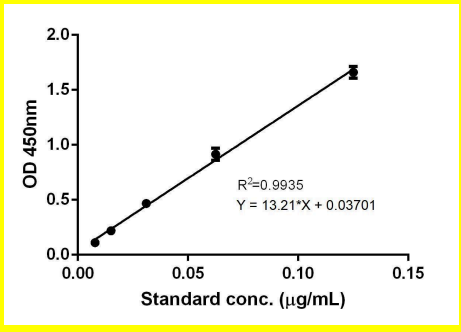
6.2 Theoretical determination of flagellin concentration in samples

Construct the standard curve, plotting on the y-axis the absorbance values, measured for the different points of the flagellin standard, on a linear scale, against the corresponding concentrations, on the *x -axis* .

Attention: A linear graph must be obtained, with an $R^2 \geq 0.95$, so if this criterion is not met with the graphed values, discard saturated points and graph again (keeping at least 3 points).

NOTE: In the case of computer graphing, the evaluation of 4 parameters can be used to make the best fit curve. Take into account that the first point of flagellin corresponds to 0.5 µg/mL.

The following figure represents a typical example of a standard curve. Do not use this curve for concentration determination in samples.



1- Average the duplicates of the absorbances of at least two measured dilutions (eg 1/32 and 1/64). It is advisable to use absorbances around 1 AU.
2- Interpolate the averages obtained in the function of the standard curve, to obtain the theoretical concentration values.
3- Average the obtained theoretical concentration values and report.

6.3 Relative potency

Using this assay it is possible to perform a relative potency *in vitro.* To this end, it is necessary to compare a test sample with a reference sample, the potency of which has been previously tested in vivo.

For this, the following steps must be followed:

1- Plot absorbance as a function of log dilution for both test and reference samples.
2- Carry out a linear fit of each curve to obtain an equation of the line ($R \geq 0.95$).
3- It must be verified that there is parallelism between the reference curve and the problem.

The criterion to consider them parallel is that the quotient of the slopes of both curves must be in a range between 0.8-1.25.

4- If and only if the previous criteria are met, the relative power (PR) is calculated with the following equation:

PR= x of the problem sample/ x of the reference sample. Being x the number that is solved in the zero of the function (0= ax + b).

The test sample passes if $PR \geq 1$ (APHIS_USDA Criteria).

Note: The present interpretation of Relative Power results is a mere summary that was based on the recommendations of the guides provided by APHIS-USDA, for which it is advisable to delve into them.

https://www.aphis.usda.gov/animal_health/vet_biologics/publications/BBPRO0220.pdf

https://www.aphis.usda.gov/animal_health/vet_biologics/publications/CVBSOP0102.pdf

https://www.aphis.usda.gov/animal_health/vet_biologics/publications/memo_800_112.pdf

Attention: according to APHIS_USDA recommendations, to select a sample as a reference sample, it must be evaluated *in vitro* in a large number of replicates. It must be confirmed that dilutions of it lead to sub-potent preparations in order to be used as a reference.

7. Analytical characteristics of the assay

7.1 Analytical sensitivity

The limit of detection (LOD) was defined as the average of an analyte-free sample plus three times the standard deviation, and indicates the minimum detectable concentration.

LOD= 10ng/mL

The limit of flagellin quantification (LOQ) was defined as the minimum concentration of standard measurable and distinguishable from the blank.

LOQ= 15ng/mL

7.2 Precision

To check reproducibility, the intra-assay and inter-assay coefficients of variation (CV) were determined for six concentrations within the linear range of the standard curve.

Intra-assay: CV ≤ 10%
Inter-assay: CV ≤ 20%

7.3 Linearity

The linearity of the ELISA was determined by testing four dilutions of the flagellin standard. The determined linear regression R^2 is > 0.95 for all samples. The ELISA is linear over the concentration range (0.08 to 0.125 µg/mL).

8. Limitations of the kit

- The provided flagellin standard is not a standard certified by a regulatory agent, but is produced *in-house* by Benten Biotech SRL.

- The kit has not been tested for cross-reactivity with other Clostridia.

- The kit has not been evaluated against different types of samples, other than bacterins. It has not been tested with formulated bacterins.

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