I. TECHNICAL BASIS

The kit has been designed to detect antibodies specific of *Micobacterium bovis*, being able to detect very low titters of antibodies in serum of infected animals.

INgezim TB DR kit is based on a novel immunoenzymatic assay called double recognition ELISA which is described below:

Plates are coated with MPB83 protein of *M.bovis*. After adding the sample to the well, if it contains specific antibodies, they will bind to the antigen coating the plate. After a washing step to remove unbound material, peroxidase conjugated MPB83 protein is added. In case of a positive sample, antibodies are capable of binding the MPB83-HRPO while still attached to the MPB83 protein coating the plate

Presence or absence of labelled MPB83 will be detected by addition of substrate (TMB) which, in presence of peroxidase, will develop a colorimetric reaction. This is a very sensitive assay recommended for early detection of antibodies specific of TB since IgM detection is favoured. The assay is also useful for negative herd certifications and to test new animals before be introduced in the herd.

IMPORTANT: Not suitable for cattle. It can be used for pigs, although INGENASA has a specific test for both pork and wild boar, which is the recommended.

II. . PRECAUTOINS AND WARNINGS FOR USERS:

1. Read the instructions of use carefully.
2. Bring all reagents to room temperature (20°-25°C) prior to use.
3. Do not mix instructions or reagents from different kits.
4. Avoid any contamination of the reagents of the Kit.
5. Do not use components after expiration dates and do not mix components from different lots.
6. There should be no eating, drinking, or smoking where specimens or Kit reagents are being handled.
7. Do not pipette by mouth.
8. Use a new tip for each serum sample.
9. For each utilisation of the Kit, control positive, negative and CUT OFF must be tested in a systematic way.
10. Stop solution is a strong acid solution that must be used with precaution. In case of accidental contact with skin, wash gently with water.
11. Substrate must by handle whit care, it is very sensible to light and contamination.

III. STORAGE OF COMPONENTS

All reagents and plates must be stored at +4ºC

IFORMATION ABOUT THE WASHING STEPS

'ashing steps could be done an automatic washing machine multichannel pipetting device e for dispensing 300 µl on each

the incubation periods, the ıg steps must be done following xt instructions:
pense a volume of 300 µl of shing solution on each well.
ake delicately the plate, ›iding the contamination ween wells.

- Turn over the plate brusquely to empty the wells.
- Repeat the process as much times as is indicated on the instructions of the Kit.
- Prior to empty the content of the last washing step, verify that the next reagent to be added to the plate is ready to use. Do not maintain the plate on dry more time than strictly needed
- After the last step of washing shake the plate turned over an absorbent filter paper.

REPARATION OF REAGENTS

ıshing solution:

ute one part of the concentrate shing solution provided in the Kit h 24 parts of distilled or onised water. Once prepared, s solution remains stable at °C.

- **Serum diluent:** It is ready to use
- **Positive and Negative controls:** Controls are ready to use
- **Preparation of the conjugate:** Conjugate is ready to use

REPARATION OF SAMPLES:

ierum samples
ierum samples must be tested at 1/25 dilution in diluent DE01-01 (i.e.: 8 µl of erum + 192 µl of serum diluent DE01-01).

MPORTANT: Pig and wild boar serum samples must be tested at 1/250 dilution ı diluent DE01-01.

Milk (individual and tank)
Milk samples must be tested undiluted (100 µl/well). Fresh, refrigerated or previously frozen milk may be tested. To eliminate interference of lipids, milk nust be partially skimmed by either centrifugation (15 min at 2000xg) or naintaining overnight at 4°C till the formation of a lipid layer on the surface can ›e detected. Then, sample should be collected under the layer of lipids to be ısed in the assay. To obtain whey samples, freeze/thaw the milk sample.

Blood spot on filter samples
See annexe

VII. TEST PROCEDURE

1. Prior to starting the test, bring all reagents to room temperature (22-25ºC).
2. Add 100 µl of positive control to two wells of the plate, 100 µl of the negative control to other 2 wells and 100 µl of each sample prepared according to previous instructions. Seal the plate and incubate for **1 hour at room temperature** (22-25ºC).
3. Wash 6 times following the procedure previously described.
4. Add 100 µl of ready to use conjugate to each well. Seal the plate and incubate for **1 hour at room temperature** (18-25º C).
5. Wash 6 times following the described procedure.
6. Add 100 µl of substrate solution to each well. Keep the plate at room temperature for **10 minutes**. In order to speed up this process, it is advisable to use a multichannel pipette. Add 100 µl of stop solution to each well. We recommend adding this reagent following the same order as the substrate was added
7. Read the OD of each well at 450 nm.

VIII. READING AND RESULT INTERPRETATION

Determine the mean absorbance of controls and samples, in case of assayed the last ones in duplicate.

1 VALIDATION OF THE RESULTS

OD of Positive Control > 1.2
OD of Negative Control < 0.25

2 CUT OFF CALCULATION

Positive Cut Off = OD of negative control +0.35
Negative Cut off = OD of negative control + 0.30

3 RESULTS INTERPRETATION

Samples will be considered **POSITIVE**, if the OD value at 450nm is higher than the positive cut off.

Samples will be considered **NEGATIVE** if the OD value at 450nm is equal or lower than the negative cut off.

Sample will be considered **DOUBTFUL** if the OD value at 450 nm is within the range positive and negative cut off