IVERMECTIN ELISA

5141IVER[15]04.20

A competitive enzyme immunoassay for screening and quantitative analysis of ivermectin in various matrices

EUROPROXIMA IVERMECTIN ELISA

A competitive enzyme immunoassay for screening and quantitative analysis of Ivermectine (IVER) in various matrices

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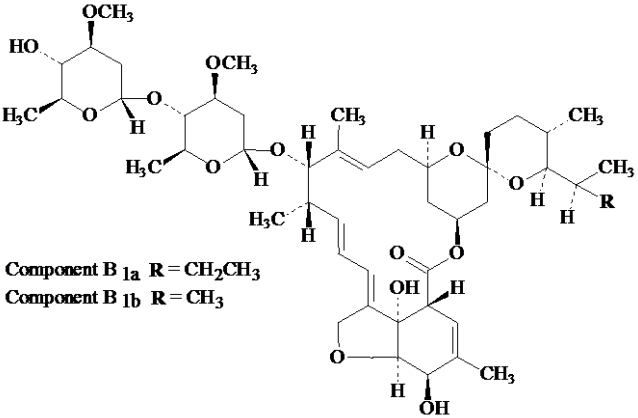
BRIEF INFORMATION

The ivermectin ELISA is a competitive enzyme immunoassay for the screening of various samples. The test is based on antibodies directed against ivermectin. The ELISA kit contains a 96 well microtiter plate as well as all essential reagents including ready-to-use standards to perform the test.

1. INTRODUCTION



Chemical structure of ivermectin.
22,23 dihydroavermectin B1a (R = C_2H_5) and 22,23 dihydroavermectin B1b (R = CH_3)

Ivermectin, also named dihydroavermectin, is a member of a group of naturally occurring macrocyclic lactones known as avermectins. The following compounds belong to the avermectines: abamectin, ivermectin, doramectin and eprinomectin. Ivermectin is frequently used to control parasites in many food producing animal species. It is a mixture of two homologues containing not less than 80% 22,23-dihydroavermectin B1a and not more than 20% 22,23-dihydroavermectin B1b. It is an exceptionally potent drug which exhibits a broad spectrum activity against parasites in many species.
During the early 1980s the first analytical techniques capable of detecting tissue ivermectin residues in the parts per billion range were reported. These were largely based on high performance liquid chromatography. In subsequent years many different approaches have been employed including chemical ionisation/mass spectrometry and planar chromatography. Although some of these techniques have been described as rapid or for use as screening methods, e.g. the liquid chromatography techniques developed by Reising and by Reuvers , it is generally accepted that less expensive immunoassay procedures are preferred.

A method for the detection and quantification of ivermectin residues in bovine liver has recently been developed.
Within the European Union Maximum Residue Limits (MRL) for ivermectine have been established ; based on the 22,23-dihydro-avermectin B1a marker residue.

2. PRINCIPLE OF THE IVERMECTIN ELISA

The microtiter plate based ivermectin ELISA kit consists of one plate (12 strips, 8 wells each) precoated with sheep antibodies to rabbit IgG. Antibody, horseradish peroxidase (-HRP) labeled ivermectin and ivermectin standard solution or sample are added to the wells. Free ivermectin from the samples or standards and ivermectin-HRP conjugate compete for the specific antibody binding sites (competitive enzyme immunoassay).

After an incubation time of one hour, the non-bound reagents are removed in a washing step. The amount of bound ivermectin-HRP conjugate is visualized by the addition of a substrate/chromogen solution (H_2O_2/TMB). Bound ivermectin-HRP conjugate transforms the colourless chromogen into a coloured product.
The substrate reaction is stopped by the addition of sulfuric acid. The colour intensity is measured photometrically at 450 nm. The optical density is inversely proportional to the ivermectin concentration in the sample.

3. SPECIFICITY SENSITIVITY

The ivermectin ELISA utilizes antibodies raised in rabbits against protein conjugated ivermectin.
The reactivity pattern of the antibody is:

Cross-reactivity:	Ivermectin	100%
	Emamectin	112%
	Eprinomectin	58%
	Abamectin	60%
	Doramectin	9%
	Milbemycin	<1%

The cross-reactivities are determined in a buffer system. The reported values may be different in samples due to matrix effects.
The test cannot discriminate between analytes and cross-reactive substances.

The Limit of detection (LOD) and the detection capability (CCβ) are determined under optimal conditions. Cut-off criteria need critical consideration.

Matrix	Procedure	LOD (ppb)	CCβ (ppb)
Milk	8.2	5	-
Tissue	8.1 Method I	3	6
Tissue	8.1 Method II	4	-
Tissue	8.2	3	-
Serum	8.2	1	-
Serum	8.3	2	-
Urine	8.2	4	-
Urine	8.3	2	-
Liver	8.4	8	12.5
Corned beef	8.4	5	5

If the sample is found to be non-compliant, the results shall be verified by re-analysis of the sample using a confirmatory method.

4. HANDLING AND STORAGE

- Kit and kit components should be stored at 2°C to 8°C in a dark place. For repeated use store kit components as specified under chapter 9.
- After the expiry date of the kit and/or components has passed, no further quality guarantee is valid.
- Bring all kit components including the microtiter plate to ambient (room) temperature before use.
- Dilute the kit components immediately before use, but after the components are brought to ambient temperature.
- Avoid condensation in the wells of the plate. Bring the sealed plate to ambient temperature before opening the plate sealing.
- The substrate chromogen solution can be stored in a refrigerator (2ºC to 8ºC) until the expiry date stated on the label.
- Exposure of the chromogen solution to light should be avoided.

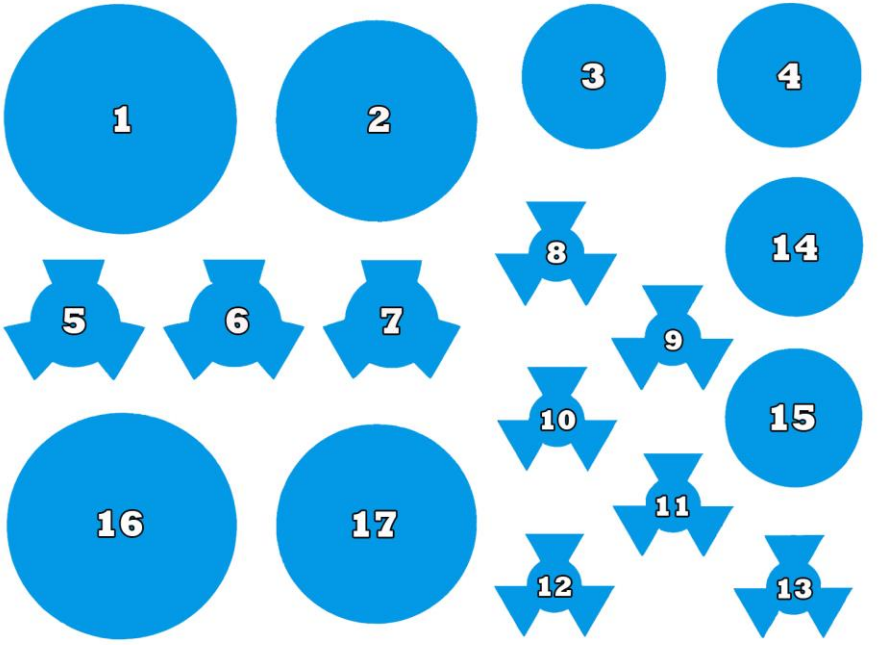
Degeneration of the reagents may have occurred when the following phenomena are observed:

- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

5. KIT CONTENTS

Manual
One sealed (96-wells) microtiter plate (12 strips, 8 wells each), coated with antibody. Plate is ready-to-use.

Position of the reagents in the kit. For preparation of the reagents see Chapter 9.



1. Not in use
2. **Rinsing buffer** (30 ml, 20 times concentrated)
3. **Substrate solution** (12 ml, Ready-to-use)
4. **Stop solution** (15 ml, Ready-to-use)
5. **Conjugate** (lyophilized, blue cap)
6. **Antibody** (lyophilized, yellow cap)
7. **Zero Standard solution** (2ml, Ready-to-use)
8. **Standard solution 1** (1ml, Ready-to-use) **1.25 ng/ml**
9. **Standard solution 2** (1ml, Ready-to-use) **2.5 ng/ml**
10. **Standard solution 3** (1ml, Ready-to-use) **5 ng/ml**
11. **Standard solution 4** (1ml, Ready-to-use) **10 ng/ml**
12. **Standard solution 5** (1ml, Ready-to-use) **25 ng/ml**
13. **Standard solution 6** (1ml, Ready-to-use) **50 ng/ml**
14. Not in use
15. Not in use
16. **Ivermectin Extraction buffer** (60 ml, ready to use)
17. **Dilution buffer** (20 ml, 4 times concentrated)

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- Gloves
- Fume hood
- Homogeniser (vortex, mixer)
- Automated microtiter plate washer or 8-channel micropipette 100 – 300 µl
- Water bath (37ºC)
- Microtiter plate shaker
- Microtiter plate reader with 450 nm filter
- Micropipettes, 10 – 100 µl
- Micropipettes, 100 – 1000 µl
- Multipipette with 2.5 ml combitips
- Centrifuge (4000 x g)
- Rotation mixer
- Glass or polypropylene tubes 4 ml
- Glass tube 10 ml
- 15 ml tubes polypropylene
- Evaporation equipment
- Acetonitril
- Methanol 100%
- Ethylacetate
- Hexane
- Ethanol 100%
- Sodium chloride
- Distilled water
- Columns, NH2 500 mg, 3 ml
- Tertiaire butylmethylether (TBME)

7. PRECAUTIONS

- This kit may contain hazardous substances. For hazard notes please refer to the appropriate safety data sheets (SDS).
- Avoid contact of all biological materials with skin and mucous membranes.
- Do not pipette by mouth.
- Do not eat, drink, smoke, store or prepare foods, or apply cosmetics within the designated work area.
- Do not use components past expiration date and do not use components from different lots.
- Each well is ultimately used as an optical cuvette. Therefore, do not touch the under surface of the wells, prevent damage and dirt.
- All components should be completely dissolved before use. Take special attention to the substrate and rinsing buffer, which crystallize at +4ºC.
- Optimal results will be obtained by strict adherence to this protocol. Careful pipetting and washing throughout this procedure are necessary to maintain good precision and accuracy.

8. SAMPLE PREPARATION

8.1 Liquid – Liquid extraction (LLE) procedure for tissue samples

Method I

- Transfer 1 g of homogenized sample into a 15 ml polypropylene tube.
- Add 0.75 ml of Ivermectin extraction buffer.
- Vortex for 30 seconds.
- Add 1 ml of acetonitril.
- Vortex for 30 seconds.
- Mix head over head for 15 minutes.
- Centrifuge for 10 minutes at 2000 x g.
- Transfer 0.5 ml of the upper layer into a glass tube.
- Evaporate to dryness under a mild stream of nitrogen at 50°C.
- Dissolve the residue in 0.75 ml of sample dilution buffer (see chapter 9).
- Vortex vigorously.
- Use 50 µl of the sample in the ELISA.

Method II

- Transfer 1 g of the homogenized sample in a test tube, add 1 ml 0.5 M NaCl (see chapter 9), add 1 ml 100% methanol
- Vortex for 1 minute
- Add 5 ml of ethyl acetate and mix head over head for 10 minutes
- After centrifugation (5 minutes at 2000 x g), 4 ml of the upper layer is pipette into a glass tube and the ethyl acetate is evaporated at 50°C under a mild stream of nitrogen
- Dissolve the residue in 100 µl, acetonitrile, vortex, centrifuge 5 minutes at 2000 x g
- Dilute 10 µl of the supernatant with 190 µl sample dilution buffer (see chapter 9), vortex
- Use 50 µl in the ELISA

8.2 Solid Phase Extraction (SPE) procedure for urine, tissue, milk and serum samples

Pipette 4 ml milk, urine, serum or transfer 4 g of thoroughly homogenised tissue sample into a clean glass tube

- Add 8 ml of acetonitrile to all tubes and vortex well for 2 minutes
- Centrifuge for 10 minutes 2000 x g and at 4ºC
- Transfer 3 ml of the upper layer into another clean glass tube
- Add 0.5 ml 0.25 M NaCl (see chapter 9) to each tube
- Add 2 ml of hexane into each tube
- Vortex gently for 2 minutes and place the tubes in a water bath of 37ºC for two minutes to disperse any emulsions formed
- Remove the upper layer (hexane)
- Add 2 ml of hexane to each tube
- Vortex gently for 2 minutes and place the tubes in a water bath of 37ºC for two minutes to disperse any emulsions formed
- Remove the upper layer (hexane)
- Evaporate the layer underneath (acetonitrile/NaCl) to dryness at 80ºC under a mild

stream of nitrogen
- Reconstitute the residue in 200 µl of distilled water, vortex for 1 minute and allow to cool for another minute
- Add 2.5 ml of ethylacetate, vortex vigorously for 1 minute. This solution is used for further clean-up with solid phase columns
- Enter the SPE procedure

Activate the column
- Add 2 ml of methanol
- Add 2 ml of ethyl acetate

Note: It is important that the cartridge is not allowed to dry completely during activation and prior to sample addition!
If the cartridge runs dry, repeat the activation procedure.

N.B. From this step on all eluents have to be collected in a glass tube.

- Carefully bring 2.7 ml of pretreated sample onto the activated cartridge (flow 1ml/minute)
- Rinse out the tubes with 2 ml of ethyl acetate and apply onto the respective columns
- Add 3 ml of ethylacetate onto each column to ensure complete elution
- All eluents (7.7 ml) are collected, (preferably in conical tubes) and evaporated to dryness under a mild stream of nitrogen at a temperature of 50ºC
- After cooling down the residue is dissolved in 500 µl of ethanol and the tubes are vortexed for 30 seconds. Make sure all residues are dissolved and collect all residues at the bottom of the tube
- Evaporate the ethanol to dryness under a mild stream of nitrogen at 50ºC and dissolve the residue in 50 µl of ethanol
- Add 450 µl of dilution buffer to each tube and vortex
- Pipette 50 µl in the respective wells of the ELISA plate

8.3 Extraction procedure for urine and serum

- Pipette 2 ml of a homogenised urine or serum sample in a glass tube
- Add 2 ml of TBME
- Vortex for 1 minute
- Centrifuge 5 minutes, at 2000 x g and 4ºC
- Pipette 1 ml of the upper layer and transfer into a clean glass tube
- Evaporate to dryness under a mild stream of nitrogen at a temperature of maximally 50ºC
- Dissolve the residue in 50 µl of 100% methanol
- Vortex for 1 minute
- Add 450 µl of dilution buffer
- Vortex for 1 minute
- Pipette 50 µl in the respective wells of the ELISA plate

8.4 Extraction procedure for liver and corned beef samples

- Transfer 1 g of homogenized liver or corned beef sample into a 15 ml Greiner tube.
- Add 0.75 ml of Ivermectin extraction buffer.
- Vortex for 30 seconds.
- Add 1.6 ml of acetonitril.
- Vortex for 30 seconds.
- Mix head over head for 15 minutes.
- Centrifuge for 10 minutes at 2000 x g.
- Transfer 0.5 ml of the upper layer into a glass tube.
- Evaporate to dryness under a mild stream of nitrogen at 50°C.
- Dissolve the residue in 0.75 ml of sample dilution buffer (see chapter 9).
- Vortex vigorously.
- Use 50 µl of the sample in the ELISA.

9. PREPARATION AND HANDLING OF REAGENTS

Before beginning the test, the reagents should be brought up to ambient temperature. Any reagents not used should be put back into storage immediately at +2ºC to +8ºC. Prepare reagents fresh before use.

Microtiter plate
Return unused strips into the resealable bag with desiccant and store at 2ºC to 8ºC for use in subsequent assays. Retain also the strip holder.

Rinsing buffer
The rinsing buffer, delivered 20 times concentrated. Prepare dilutions freshly before use. Per strip 20 ml of diluted rinsing buffer is used (1 ml concentrated rinsing buffer + 19 ml distilled water).

Substrate/chromogen solution
The substrate/chromogen solution (ready-to-use) tends to precipitate at 4°C. Take care that this vial is at room temperature (keep in the dark) and mix the content before pipetting into the wells.

Conjugate solution
Reconstitute the vial of lyophilised conjugate (Ivermectin-HRP) with 4 ml of dilution buffer, mix thoroughly and keep in the dark until use.

Antibody solution
Reconstitute the vial of lyophilised antibody (rabbit anti-ivermectin antibodies with 4 ml of dilution buffer, mix thoroughly and keep in the dark until use.

Dilution buffer
The dilution buffer is 4 times concentrated. Before dilution (20 ml buffer + 60 ml distilled water) the concentrated buffer should be at room temperature and thoroughly mixed. Concentrated buffer can show precipitates of the contents. Mix well before dilution with distilled water. The 4 times diluted buffer can be stored in a

refrigerator (2°C to 8°C) until the expiry date stated on the kit label.

Sample dilution buffer
Add 1 ml 100% methanol to 9 ml dilution buffer.

0.5 M NaCl
Dissolve 2.92 gram NaCl in 100 ml distilled water.

0.25 M NaCl
Dissolve 0.146 gram NaCl in 10 ml distilled water.

10. ASSAY PROCEDURE

Rinsing protocol

In ELISAs, between each immunological incubation step, unbound components have to be removed efficiently. This is achieved by appropriate rinsing. It should be clear that each rinsing procedure must be carried out with care to guarantee good inter- and intra-assay results.
Basically, manual rinsing or rinsing with automatic plate wash equipment can be performed as follows:

Manual rinsing

1. Empty the contents of each well by turning the microtiter plate upside down and remove residual liquid by striking the plate against a paper towel.
2. Fill all the wells to the rims (300 µl) with rinsing solution.
3. This rinsing cycle (1 and 2) should be carried out 3 times.
4. Turn the plate upside down and empty the wells by a firm short vertical movement.
5. Place the inverted plate on absorbent paper towels and tap the plate firmly to remove residual washing solution from the wells.
6. Take care that none of the wells dry out before the next reagent is dispensed.

Rinsing with automatic microtiter plate washing equipment

When using automatic plate wash equipment, check that all wells can be aspirated completely, that the rinsing solution is nicely dispensed reaching the rim of each well during each rinsing cycle. The washer should be programmed to execute three rinsing cycles.

Assay protocol

1. Prepare samples according to Chapter 8 and prepare reagents according to Chapter 9.

2. Pipette 100 µl of the zero standard in duplicate (wells H_1, H_2: blank).
   Pipette 50 µl of the zero standard in duplicate (wells A_1, A_2: zero standard).
   Pipette 50 µl of each of the standard solutions in duplicate (wells B_1, B_2 to G_1, G_2 i.e.).

3. Pipette 50 µl of each sample solution in duplicate into the remaining wells of the microtiter plate.

4. Pipette 25 µl of conjugate solution (ivermectin-HRP) into all wells, except wells H_1 and H_2.

5. Pipette 25 µl of antibody solution into all wells, except wells H_1 and H_2.

6. Seal the microtiter plate and shake the plate for 1 minute on a microtiter plate shaker.

7. Incubate for 1 hour in the dark at 37ºC.

8. Discard the solution from the microtiter plate and wash 3 times with rinsing buffer.

9. Pipette 100 µl of substrate solution into each well.

10. Incubate 30 minutes at 20ºC - 25ºC.

11. Pipette 100 µl of stop solution into each well.

12. Read the absorbance values immediately at 450 nm.

11. INTERPRETATION OF RESULTS

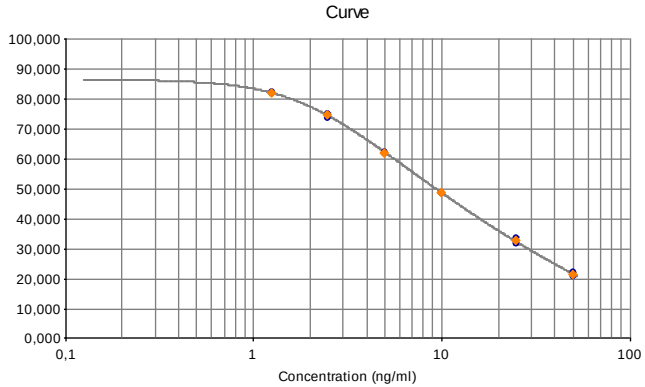
Subtract the mean optical density (O.D.) of the wells H_1 and H_2 (Blank) from the individual O.D. of the wells containing the standards and the samples.

The O.D. values of the six standards and the samples (mean values of the duplicates) are divided by the mean O.D. value of the zero standard/Bmax (wells A_1 and A_2) and multiplied by 100. The zero standard/Bmax is thus equal to 100% (maximal absorbance) and the other O.D. values are quoted in percentages of the maximal absorbance.

$$\frac{\text{O.D. standard (or sample)}}{\text{O.D. zero standard/Bmax}} \times 100 = \text{percentage maximal absorbance}$$

Calibration curve:
The values (% maximal absorbance) calculated for the standards are plotted on the Y-axis versus the analyte equivalent concentration (ng/ml) on a logarithmic X-axis.

Alternative for calibration curve:
The absorbtion value of the standards is plotted on the Y-axis versus the concentration on the X-axis. The Y-axis is in logit the Y-axis is logarithmic.



The amount of ivermectin in the samples is expressed as ivermectin equivalents. The ivermectin equivalents in the samples (ng/ml) corresponding to the % maximal absorbance of each extract can be read from the calibration curve.

8.1 LLE procedure for tissue samples
Method I
The ivermectin equivalents read from the calibration curve have to be multiplied by a factor 4.125 to express the concentration (ng/g) in tissue.

Method II
The ivermectin equivalents read from the calibration curve have to be multiplied by a factor 2.75 to express the concentration (ng/g) in tissue.

8.2 SPE procedure for urine, tissue, milk and serum samples
The ivermectin equivalents read from the calibration curve have to be divided by a factor 2 to express the concentration (ng/g) in the samples.

8.3 TBME procedure for urine and serum (alternative method)
The ivermectin equivalents read from the standard curve have to be divided by a factor 2.

8.4 Ivermectin extraction buffer procedure for liver and corned beef samples
The ivermectin equivalents read from the standard curve have to be multiplied by a factor 5 to express the concentration (ng/g) in liver and corned beef.

12. LITERATURE

Rabel, S.R. *et al*, J. Chromatog. B. 1993, **617**, 79.
Di Netta, J., In Ivermectin and Abamectin, (Springer, New York), 1989, p. 344.
Tway, P.C. *et al*, J. Agric. Food Chem., 1981, **29**, 1059.
Tway, P.C. *et al*, Biomed. Mass Spectrom., 1984, **11**, 172.
Abjean, J.P. *et al*, J. AOAC Int., 1995, **78**, 1141.
Reising, K.S., J. AOAC Int., 1992, **75**, 751.
Reuvers, Th. *et al*, Anal. Chim. Acta, 1993, **275**, 353.
Crooks, S.R.H. *et al*, Analyst, 1998, **123**, 355.
EMEA; The European Agency for the Evaluation of Medicinal Products.
EMEA/MRL/487/98-FINAL, July 1998.

13. ORDERING INFORMATION

For ordering the ivermectin ELISA kit please use cat. code 5141IVER.

14. REVISION HISTORY

The manual is adapted to a new layout of the test kit. Several textual changes are added. The alternative method for milk is removed.

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