

DIMETRIDAZOLE ELISA

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

EUROPROXIMA DIMETRIDAZOLE ELISA

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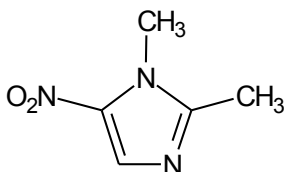
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BRIEF INFORMATION

The dimetridazole ELISA is a competitive enzyme immunoassay for measurement of the concentration of dimetridazole. With this ELISA-kit 96 analyses can be performed. Samples and standards are measured in duplicate which means that a total of 40 samples can be analysed.

The ELISA kit contains all reagents to perform the test. However. Reagents for sample preparation are not included in the kit.

1. INTRODUCTION



Chemical structure of dimetridazole

Dimetridazole (DMZ) is a 5-nitroimidazole drug traditionally used for the prevention and treatment of histomoniasis in turkeys, trichomoniasis in pigeons, genital trichomoniasis in cattle and the prevention and treatment of haemorrhagic enteritis in pigs. DMZ is listed in Annex IV of the Council Regulation 2377/90/EC, and is thus banned from the use in food-producing animals. Poisoning is manifested by infertility, and hepatic and renal insufficiency.

2. PRINCIPLE OF THE DIMETRIDAZOLE ELISA

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

After an incubation step of 1 hour the non-bound reagents are removed in a washing step. The amount of bound dimetridazole-HRP conjugate is visualized by the addition of a substrate/chromogen solution (H₂O₂/TMB). Bound dimetridazole-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 dimetridazole concentration in the sample.

3. SPECIFICITY AND SENSITIVITY

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

Cross-reactivity:	dimetridazole	100%
	metronidazole	7.4%
	hydroxydimetridazole	5.3%
	ronidazole	8.5%
	ipronidazole	7.4%
	nicarbazin	0.1%
	halofuginone	< 0.02%
	diclazuril	< 0.02%
	robenidine	< 0.02%
	hydroxymetronidazole	0.1%
	hydroxyipronidazole	0.5%

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) is determined under optimal conditions. Cut-off criteria need critical consideration.

Matrix	Procedure	LOD ppb
Shrimps	8.1	0.8
Tissue	8.2	0.3
Milk	8.3	0.3
Egg	8.2	0.3
Serum	8.4	0.3

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 (2°C to 8°C) 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:

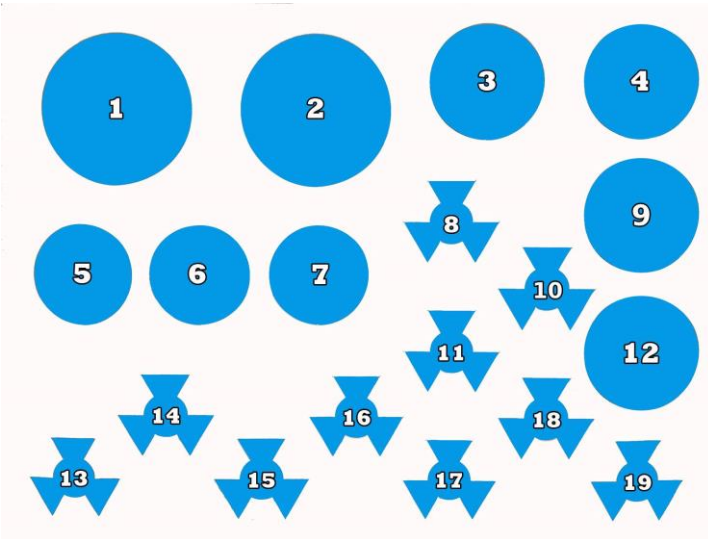
- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells ($E_{450nm} < 0.8$).

5. KIT CONTENTS

Manual

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

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



1. **Dilution buffer** (20 ml, 10x concentrated)
2. **Rinsing buffer** (30 ml, 20x concentrated)
3. **Substrate solution** (12 ml, Ready-to-use)
4. **Stop solution** (15 ml, Ready-to-use)
5. not in use
6. not in use
7. not in use
8. **Conjugate** (100x concentrated)
9. not in use
10. not in use
11. not in use
12. not in use
13. **Zero Standard solution** (2ml, Ready-to-use)
14. **Standard solution 1** (1ml, Ready-to-use) **0.313 ng/ml**
15. **Standard solution 2** (1ml, Ready-to-use) **0.625 ng/ml**
16. **Standard solution 3** (1ml, Ready-to-use) **1.25 ng/ml**
17. **Standard solution 4** (1ml, Ready-to-use) **2.5 ng/ml**
18. **Standard solution 5** (1ml, Ready-to-use) **5 ng/ml**
19. **Standard solution 6** (1ml, Ready-to-use) **10 ng/ml**

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- Scales and weighing vessels
- Gloves
- Fume hood
- Homogeniser (vortex, mixer)
- Centrifuge (2000 x g)
- Automated microtiter plate washer or 8-channel micropipette 100 – 300 µl
- Microtiter plate shaker
- Microtiter plate reader with 450 nm filter
- Micropipettes, 100 – 1000 µl
- Multipipette with 2.5 ml combitips
- Methanol 100%
- Acetonitrile
- n-hexane
- 4 ml glass tubes
- 15 ml tubes with screw cap (polypropylene)

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 +4°C.
- 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.

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8. SAMPLE PREPARATIONS

8.1 Shrimps

- Weigh 2 g of homogenised sample into a 15 ml tube
- Add 8 ml of acetonitrile and vortex vigorously
- Place the tubes in an ultrasonic bath for 5 minutes
- Centrifuge for 10 minutes at 2000 x g
- Pipette 4 ml of the supernatant into a tube
- Evaporate to dryness under a mild stream of nitrogen at 50°C
- Dissolve the residue in 100 µl methanol, vortex, add 0.9 ml dilution buffer, vortex
- An aliquot of 50 µl is used in the ELISA test.

8.2 Chicken muscle and egg

- Weigh 2 g of homogenised egg or minced muscle into a 50 ml tube.
- Add 8 ml of acetonitrile and immediately vortex for 1 minute.
- Place the tubes in an ultrasonic bath for 5 minutes.
- Centrifuge at 2000 x g for 10 minutes at room temperature.
- Transfer the supernatant into a 10 ml tube and evaporate to dryness under a mild stream of nitrogen at 50°C.
- Add 1 ml of n-hexane and mix well. Add 1 ml of methanol/H₂O (3:1) and vortex for 10 seconds.
- Leave in a water bath at 40°C for 5 minutes.
- Centrifuge at 2000 x g for 5 minutes at room temperature.
- Delicately remove the hexane layer and any slight emulsions that appear at the interface. After elimination, approximately 1 ml must be present in the tube.
- Evaporate to dryness.
- Reconstitute the samples in 100 µl of 100% methanol, then in 1.9 ml of dilution buffer. Mix well. Store them at 2-8°C for at least one hour before use in the ELISA.
- An aliquot of 50 µl is used in the ELISA test.

8.3 Milk

- Neutralize the pH of the milk if necessary. The pH must be 7 +/- 0.5
- Dilute the milk sample 2 times in dilution buffer. 100 µl milk + 100 µl dilution buffer
- Mix the diluted milk
- An aliquot of 50 µl is used in the ELISA test.

8.4 Serum

- Centrifuge the serum if it is turbid, 15 minutes at 2000 x g.
- Dilute the serum 4 times in dilution buffer. 100 µl serum + 300 µl dilution buffer
- Mix the diluted serum
- An aliquot of 50 µl is used in the ELISA test.

9. PREPARATION 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 +2°C to +8°C. Prepare reagents fresh before use.

Microtiter plate

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

Rinsing buffer

The rinsing buffer is 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 solution

The substrate solution (ready-to-use) precipitates 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.

Dilution buffer

The sample dilution buffer is 10 times concentrated. Before dilution (10 ml buffer + 90 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.

Conjugate solution

Spin the concentrated conjugate down by a short centrifugation step (1 minute at 1000 x g). For reconstitution, the conjugate is diluted 1 : 100 in dilution buffer, e.g. 10 µl concentrated conjugate + 990 µl dilution buffer. Per 2 x 8 wells 400 µl diluted conjugate is required.

Store unused concentrated conjugate at +2°C to +8°C.

10. ASSAY PROCEDURE

Rinsing protocol

In ELISAs, between each immunological incubation step, unbound components have to be removed efficiently. This is reached 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 μ l) 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 rinsing 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 wash 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 μ l of zero standard in duplicate (well H1, H2).
Pipette 50 μ l of zero standard in duplicate (well A1, A2).
Pipette 50 μ l of each standard dilution in duplicate (well B1,2 to G1,2, i.e. 0.313, 0.625, 1.25, 2.5, 5 and 10 ng/ml).
Pipette 50 μ l of each sample solution in duplicate into the remaining wells of the microtiter plate.
3. Add 50 μ l of conjugate (HRP) to all wells, except wells H1 and H2.
4. Seal the microtiter plate and shake the plate for a few seconds.
5. Incubate for 1 hour. in the dark 4°C (2°C - 8°C).
6. Discard the solution from the microtiter plate and wash 3 times with rinsing buffer.

7. Pipette 100 μ l substrate solution into each well. Incubate 30 min. at room temperature in the dark (approximately 20°C - 25°C).
8. Add 100 μ l stop solution to each well.
9. Read the absorbance values immediately at 450 nm.

11. INTERPRETATION OF RESULTS

Subtract the mean optical density (O.D.) of the wells H1 and H2 (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 A1 and A2) and multiplied by 100. The zero standard/ Bmax is thus made equal to 100% (maximal absorbance) and the other O.D. values are quoted in percentages of the maximal absorbance.

O.D. standard (or sample)

-----x 100 = percentage maximal absorbance

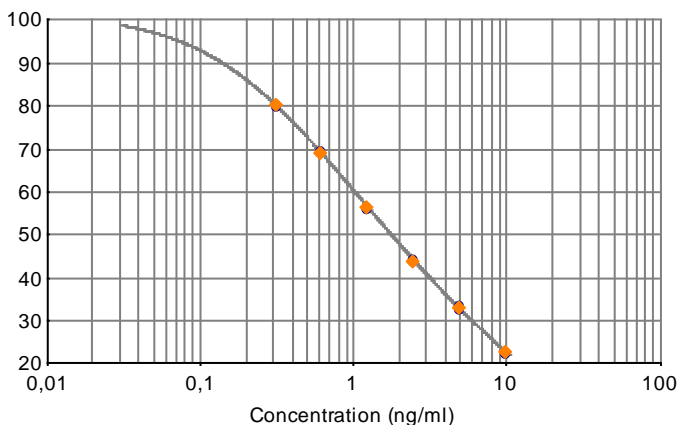
O.D. zero standard/ Bmax

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 value of absorption (logit) calculation of the standards are plotted on Y-axis versus the analyte equivalent concentration on a logarithmic X-axis.



8.1 Shrimp samples

The dimetridazole equivalents calculated from the standard curve has to be multiplied by a factor 1.25.

8.2 Chicken muscle and egg

The dimetridazole equivalents can be read directly from the calibration cuve.

8.3 Milk samples

The dimetridazole equivalents calculated from the standard curve has to be multiplied by a factor 2.

8.4 Serum samples

The dimetridazole equivalents calculated from the standard curve has to be multiplied by a factor 4.

12. LITERATURE

Council Regulation (EEC) No 2377/90 of 26 June 1990 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. Official J. European Union, **L224**, 1-8.

13. ORDERING INFORMATION

For ordering the dimetridazole ELISA kit please use cat. Code 5091DIME.

14. REVISION HISTORY

The manual is adapted to a new layout of the test kit. Several textual changes are added.