**DIMETRIDAZOLE ELISA**

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

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## 12. LITERATURE


1. 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. LAST MUTATIONS

Chapter 6: “equipment and materials required but not provided” is added.
Sample treatment for shrimps is added.


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7. Pipette 100 µl substrate solution into each well. Incubate 30 min. at room temperature in the dark (approximately 20ºC - 25ºC).

8. Add 100 µl stop solution to each well.

9. Read the absorbance values immediately at 450 nm.

## 11. INTERPRETATION OF RESULTS

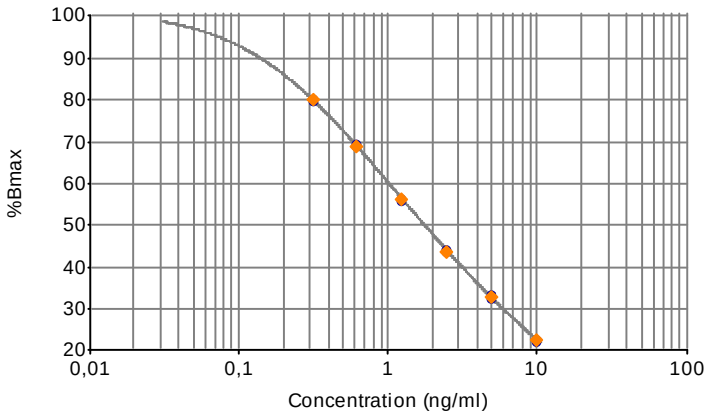
Subtract the mean optical density (O.D.) of the wells H1 and H2 (Blank) from the individual O.D. of the wells containing the standards and the samples.

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% = % 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.

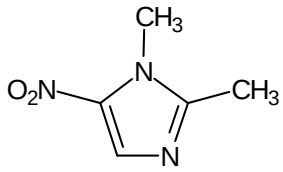





## BRIEF INFORMATION

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 [1]. 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 ($H_2O_2$/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 limit of detection (LOD) is calculated as: Xn + 3SD and is determined under optimal conditions.

| 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 |

## 4. HANDLING AND STORAGE

- Store the kit at +2ºC to +8ºC in a dark place. For repeated use store kit components as specified under chapter 9.
- After the expiry date (see kit label) has passed, quality claims are not accepted.
- Before opening the sealed plate, the plate should be at ambient temperature in order to avoid condensation in the ELISA.
- Dilute the kit components immediately before use, but after the components are at ambient temperature.
- The substrate chromogen solution can be stored in a refrigerator (+2ºC to + 8ºC) until the expiry date stated on the label.
- Any direct action of light on the substrate chromogen solution should be avoided.

Degeneration of the reagents may have occurred when the following phenomena are observed:
- A blue colouring of the substrate solution before transferring it into the wells.
- A weak or absent colour reaction of the zero standard (Bmax, E450nm < 0.8).



## 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 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 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 (Sample preparation) and prepare reagents according to Chapter 9 (Preparation of reagents).

2. Pipette 100 µl of zero standard in duplicate (well H1, H2).
   Pipette 50 µl of zero standard in duplicate (well A1, A2).
   Pipette 50 µl 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 µl of each sample solution in duplicate into the remaining wells of the microtiter plate (40 samples; 80 wells).

3. Add 50 µl 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 (2ºC - 8ºC).

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

## 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 +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 is delivered 20 times concentrated. Prepare dilutions freshly before use. Per strip 40 ml of diluted rinsing buffer is used (2 ml concentrated rinsing buffer + 38 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 (10x concentrated)
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. The 10 times diluted buffer can be stored in a refrigerator (+2ºC to +8ºC) until the expiry date stated on the kit label.

Conjugate solution (100x concentrated)
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.

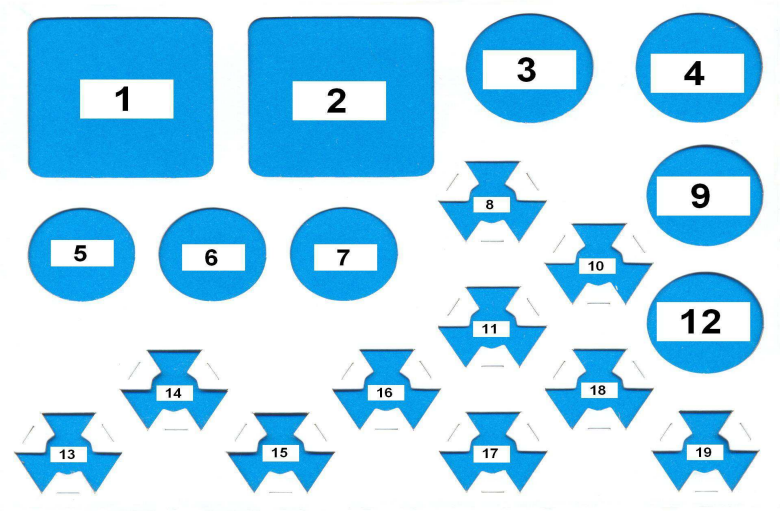


## 5. KIT CONTENTS

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 8.




1. **Dilution buffer** (20 ml, 10x concentrated)
2. **Wash Buffer** (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** (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
- 4 ml glass tubes
- 15 ml tubes with screw cap (Greiner, polypropylene)

## 7. PRECAUTIONS

- Dimetridazole is a toxic compound. Avoid contact with mouth and skin. Be aware that the dimetridazole is not inhaled.
- The stop reagent contains 0.5 M sulfuric acid. Do not allow the reagent to get into contact with the skin.
- 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.
- TMB is toxic by inhalation, in contact with skin and if swallowed; observe care when handling the substrate.
- Do not use components past expiry date and do not intermix components from different serial 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, which crystallises at 4ºC.
- Optimal results will be obtained by strict adherence to this protocol. Careful pipetting and washing throughout this procedure are necessary to maintain precision and accuracy.



## 8. SAMPLE TREATMENT

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/$H_2O$ (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.
- As samples must be well cooled before use in the ELISA (this is essential), they should be kept on ice during preparation of the sample.
- An aliquot of 50 µl is used in the ELISA test.

8.3 Milk

Note :

The pH of the sample is a potential pitfall. Sour milk disturbs the ELISA, neutralization of the pH is essential.

- 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.