Version 22-04/24

User's Manual

AMOZ ELISA Kit

REF **DEIANS058**

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

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

Creative Diagnostics

Address: 45-1 Ramsey Road, Shirley, NY 11967, USA

Tel: 1-631-624-4882 (USA) 44-161-818-6441 (Europe) **Fax: 1-631-938-8221**

Email: info@creative-diagnostics.com **Web: www.creative-diagnostics.com**

PRODUCT INFORMATION

Intended Use

A competitive enzyme immunoassay for screening and quantitative analysis of AMOZ.

General Description

The nitrofurans are a group of synthetic broad-spectrum antibiotics, which have been widely and effectively used for the prevention and treatment of gastrointestinal infections caused by Escherichia coli, Salmonella spp., Mycoplasma spp., Coccidia spp., coliforms and other protozoa in animal production and aquaculture. Moreover, nitrofurans have been employed as growth promoters in livestock. The four major nitrofurans are furazolidone, furaltadone, nitrofurantoin and nitrofurazone. They have been banned in the EU for use as veterinary drugs, due to their toxic an suspected carcinogenic and mutagenic properties (Commission Regulation 1442/95). In 2003 a definitive MRPL(Minimum Required Performance Limit) was set at 1 ng/g (ppb) in the EU for all four of the above mentioned nitrofurans in poultry and aquaculture products (Commission Decision 2003/181/EC). Various studies have demonstrated that the nitrofuran parent molecules are rapidly metabolised by animals and that their in vivo stability is not longer than a few hours. As a result, persistent protein-bound residues are formed. Unlike the parent molecules, these protein-bound metabolites are stable and persistent in the body. It is possible to free these residues from proteins by acid hydrolysis. Testing for the presence of nitrofurans is thus equivalent to testing for the presence of a part of the parent molecule, i.e. the free residue. Nitrofuran residues are found after administration of furaltadone (3-amino-5-morpholinomethyl-2-oxazolidinone = AMOZ), furazolidone (3-amino-2-oxazolidinone = AOZ), nitrofurantoin (1-aminohydantoin = AHD) and nitrofurazone (semicarbazide = SEM).

Principles of Testing

The microtiter plate based AMOZ ELISA consists of one precoated plate(12 strips,8 wells each). Horseradish peroxidase (-HRP) labeled AMOZ and standard solution or sample are added to the wells. Free AMOZ from the samples or standards and AMOZ-HRP conjugate compete for the specific antibody binding sites (competitive enzyme immunoassay). After an incubation step of 30 minutes, the non-bound reagents are removed in a washing step. The amount of bound AMOZ-HRP conjugate is visualized by the addition of a substrate/chromogen solution (tetramethylbenzidine, TMB). Bound AMOZ-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 AMOZ concentration in the sample.

Reagents And Materials Provided

1. One sealed (96-wells) microtiter plate (12 strips, 8 wells each), coated antibody. Ready-to-use.
2. Position of the reagents in the kit. For preparation of the reagents see Reagent Preparation.
3. Sample dilution buffer (40 ml, ready-to-use)
4. Rinsing buffer (30 ml, 20× concentrated)
5. Substrate solution (12 ml, ready-to-use)

6. Stop solution (15 ml, ready-to-use)
7. Conjugate solution (100 µl, 100 times concentrated)
8. Dilution buffer (15 ml, ready-to-use)
9. AMOZ-stock solution (1 ml 100 ng/ml for spiking samples)
10. Zero Standard (2ml, ready-to-use)
11. Standard solution 1 (1 ml, ready-to-use) 0.0625 ng/ml AMOZ-NP
12. Standard solution 2 (1 ml, ready-to-use) 0.125 ng/ml AMOZ-NP
13. Standard solution 3 (1 ml, ready-to-use) 0.25 ng/ml AMOZ-NP
14. Standard solution 4 (1 ml, ready-to-use) 0.50 ng/ml AMOZ-NP
15. Standard solution 5 (1 ml, ready-to-use) 1.00 ng/ml AMOZ-NP
16. Standard solution 6 (1 ml, ready-to-use) 2.00 ng/ml AMOZ-NP

Materials Required But Not Supplied

1. 4 ml glass tubes
2. 15 ml tubes with screw cap
3. Scales and weighing vessels
4. Gloves
5. Fume hood
6. Homogeniser (vortex, mixer)
7. Centrifuge (2000 x g)
8. Automated microtiter plate washer or 8-channel micropipette 100 – 300 µl
9. Microtiter plate shaker
10. Microtiter plate reader with 450 nm filter
11. Glass test tubes (10 – 15 ml)
12. Micropipettes, 100 – 1000 µl
13. Multipipette with 2.5 ml combitips
14. Aluminium foil or parafilm
15. Distilled water (bidest)
16. Ethyl acetate
17. Dimethyl-sulfoxide (DMSO)
18. n-Hexane
19. 1 M HCl
20. 2-nitrobenzaldehyde
21. 0.25 M K_2HPO_4 (43.5 gram/L distilled water)
22. 1 M NaOH (40 gram/L distilled water)

Storage

Store the kit at +2°C to +8°C in a dark place. For repeated use store kit components as specified under Reagent Preparation.

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

Any direct action of light on the 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 (E450nm < 0.8).

Specimen Collection And Preparation

Each matrix requires its own specific derivatisation step followed by a general extraction procedure.

For optimal recovery of the derivatisation procedure, each laboratory has to run its own in-house validation. For individual nitrofurans and matrices we advise the optimal concentration of the derivative reagent as well as the optimal derivatisation time as validated in our laboratory and stated in the manual. However, these figures are guidelines and have to be tested for optimal results in each individual lab.

Derivatisation

1. Milk

Centrifuge cold milk samples for 10 minutes at 2000 x g at 4°C.

Remove the upper fat layer using a spatula. Use 1 ml defat sample for derivatisation.

Derivation

Mix gently 1 ml of the homogenized defatted milk sample with 4 ml double distilled water, 0.5 ml of 1 M HCL and 250 µl derivative reagent* in a 15 ml centrifuge tube.

Mix carefully head over head for 1 minute (when shaking is too rigorous, the extract becomes jelly).

Incubate at 37°C 1 hour.

2. Honey

Mix 1 gram of the honey sample with 4 ml double distilled water, 0.5 ml of 1 M HCL and 200 µl derivative reagent* in a 15 ml centrifuge tube.

Mix head over head until the honey is completely dissolved approximately 5 minutes.

Incubate at 37°C 1 hour.

3. Egg

Mix gently 1 gram of the homogenized whole egg sample with 4 ml double distilled water, 0.5 ml of 1 M HCL and 200 µl derivative reagent* in a 15 ml centrifuge tube.

Mix carefully head over head for 1 minute (when shaking is too rigorous, the extract becomes jelly).

Incubate at 37°C 1 hour

4. Egg powder

Weigh 1 gram of egg powder in a tube, add 5 ml of water, mix until a homogeneous solution.

Mix 1 ml of the homogenized sample with 4 ml double distilled water, 0.5 ml of 1 M HCl, and 200 µl derivative reagent*.

Mix carefully head over head for 1 minute (when shaking is too rigorous, the extract becomes jelly).

Incubate at 37°C 1 hour

5. Shrimps

Mix 1 gram of the homogenized shrimps sample with 4 ml double distilled water, 0.5 ml of 1 M HCL and 150 µl derivative reagent* in a 15 ml centrifuge tube.

Mix head over head for 5 minutes.

Incubate at 37°C for 1 hour, or 37°C for 3 hours or 37°C overnight.

6. Tissue/fish

Mix 1 gram of the homogenized sample with 4 ml double distilled water, 0.5 ml of 1 M HCL and 250 µl derivative reagent* in a 15 ml centrifuge tube.

Mix head over head for 5 minutes.

Incubate at 37°C for 1 hour, or 37°C for 3 hours or 37°C overnight.

7. Liver

Mix 1 gram of the homogenized sample with 4 ml double distilled water, 0.5 ml of 1 M HCL and 50 µl derivative reagent* in a 15 ml centrifuge tube.

Mix head over head for 5 minutes.

Incubate at 37°C 1 hour

8. Urine

Centrifuge the urine samples 5 minutes at 2000 x g.

Mix 1 ml of the clear urine sample with 4 ml double distilled water, 0.5 ml of 1 M HCL and 200 µl derivative reagent* in a 15 ml centrifuge tube.

Mix head over head for 5 minutes.

Incubate at 37°C 1 hour

*** Derivative reagent**

10 mM 2-nitrobenzaldehyde in Dimethylsolfoxid (DMSO) This solution has to be prepared directly before use. i.e. dissolve 15.2 mg 2-nitrobenzaldehyde in 10 ml DMSO.

Note: 2-nitrobenzaldehyde is sensitive to oxidizing agents. Therefore, keep away from open air or other incompatible substances. Keep container tightly closed when not in use. Do not use material that has been expired.

9. Extraction procedure

a. Add to the derivatized sample 2.5 ml 0.25 M K_2HPO_4 , 0.4 ml of 1 M NaOH and 5 ml ethyl acetate.

b. Mix carefully head over head (when shaking too rigorous, the extract becomes jelly), for 1 minute.

c. Centrifuge for 10 minute at 2000 x g.

d. Transfer 2.5 ml of the ethyl acetate layer (upper layer) into a 4 ml glass tube.

e. Evaporate to dryness at 50°C under a mild stream of nitrogen.

f. Dissolve the residue in 1 ml n-hexane. Add 1 ml sample dilution buffer.

g. Vortex for 1 minute.

h. Centrifuge for 10 minute at 2000 x g.

i. Remove the upper, Hexane layer.

j. If not separate to two phases, heat the above sample for about 3 minutes (80°C~100°C)

k. Centrifuge for 10 minute at 2000 x g.

l. Use 50 µl of the aqueous phase in the ELISA.

Plate Preparation

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.

Reagent Preparation

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 freshly before use

1. Rinsing buffer

The rinsing buffer is delivered 20 times concentrated. Prepare dilutions freshly before use. For each strip 40 ml of diluted rinsing buffer is used (2 ml concentrated rinsing buffer + 38 ml distilled water).

2. Dilution buffers

This ELISA contains two dilution buffers, both ready to use provided. The dilution buffer for the conjugate (Dilution buffer) and sample dilution buffer (Sample dilution buffer) for dissolving of the samples.

3. Conjugate solution (100 µl)

The conjugate (AMOZ-HRP) is delivered 100× concentrated. Spin down the conjugate in the vial by a short centrifugation step (1 minute, 1000 x g). Add 10 µl of the concentrated conjugate solution to 1 ml of dilution buffer. Per 2×8 wells 800 µl of diluted conjugate is required. Store unused concentrated conjugate at +2°C to +8°C.

4. 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 when used (keep in the dark) and mix the content before pipetting into the wells.

Assay Procedure

Rinsing protocol

In ELISA's, 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 washing solution in 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.

Protocol

1. Prepare samples according to **Specimen Collection And Preparation** and prepare reagents according to **Reagent Preparation**.
2. Pipette 100 µl of the zero standard in duplicate (wells H1, H2, blank). Pipette 50 µl of the zero standard in duplicate (wells A1, A2, maximal signal). Pipette 50 µl of each of the standard solutions in duplicate (wells B1,2 to G1,2 i.e. 0.0625, 0.125, 0.25, 0.5, 1.00 and 2.00 ng/ml).
3. Pipette 50 µl of each sample solution in duplicate into the remaining wells of the microtiter plate (40 samples; 80 wells).
4. Pipette 50 µl of conjugate (HRP) to all wells, except H1 and H2.
5. Seal the microtiter plate and shake the plate for a few seconds on a microtiter plate shaker.
6. Incubate for 30 minutes in the dark at room temperature (20°C to 25°C).
7. Discard the solution from the microtiter plate and wash 3 times with rinsing buffer.
8. Pipette 100 µl of substrate solution into each well.
9. Incubate 15 minutes at room temperature (20°C to 25°C).
10. Add 100 µl of stop solution to each well.
11. Read the absorbance values immediately at 450 nm.

Calculation

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.

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

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:

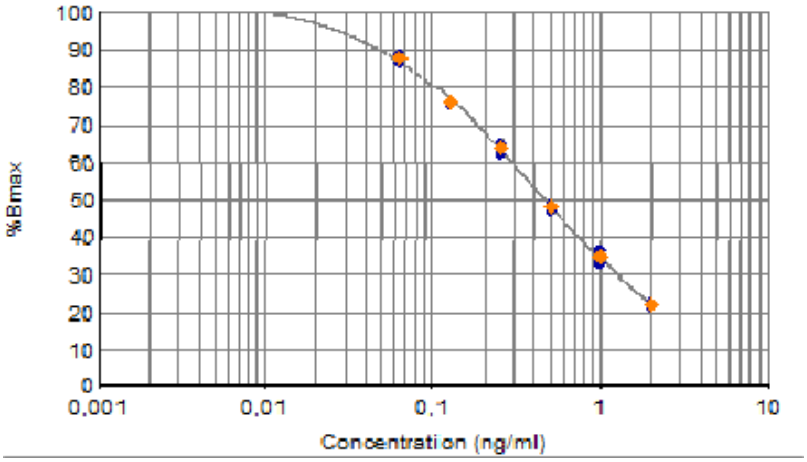
The value of absorption (logit) calculation of the standards are plotted on Y-axis versus the analyte equivalent concentration on a logarithmic X-axis.

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

All matrices have the same calculation factor.

The AMOZ equivalents read from the calibration curve have to be multiplied by 2.

Typical Standard Curve



Sensitivity

The LOD is determined under optimal conditions. Cut-off values need critical consideration.

Matrix	Procedure	LOD ppb
Milk	8.1	0.1
Honey	8.2	0.1
Egg	8.3	0.1
Egg powder	8.4	0.1
Shrimps	8.5	0.1
Tissue/fish	8.6	0.1
Liver	8.7	0.1
Urine	8.8	0.1

Specificity

The AMOZ ELISA utilizes antibodies raised in mouse against protein conjugated AM0Z. The reactivity pattern of the antibody is:

Cross- reactivity:	AMOZ	100%
	AOZ	< 0.01%
	AHD	< 0.01%
	SEM	< 0.01%

Precautions

1. Nitrofurans are toxic compounds. Avoid contact with mouth and skin. Be aware that nitrofurans are not inhaled.
2. The stop reagent contains 0.5 M sulfuric acid. Do not allow the reagent to get into contact with the skin.
3. Avoid contact of all biological materials with skin and mucous membranes.
4. Do not pipette by mouth.
5. Do not eat, drink, smoke, store or prepare foods, or apply cosmetics within the designated work area.
6. Use the derivative reagent in a fume hood to avoid breathing vapor.
7. TMB is toxic by inhalation, in contact with skin and if swallowed; take care when handling the substrate.
8. Do not use components past expiration date and do not use components from different lots.
9. Each well is ultimately used as an optical cuvette. Therefore, do not touch the under surface of the wells, prevent damage and dirt.
10. All components should be completely dissolved before use. Take special attention to the substrate, which crystallises at +4°C
11. 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.