

RIDASCREEN[®] Nitrofuran (AOZ)

Enzymimmunoassay zur quantitativen Bestimmung von
AOZ

Enzyme immunoassay for the quantitative analysis of
AOZ

Art. No.: R3701

研 究 用 試 薬

In vitro Test

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Storage at 2 - 8 °C

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R-Biopharm AG

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Enzyme immunoassay for the quantitative analysis of
AOZ

Brief information

RIDASCREEN[®] Nitrofuran (AOZ) (Art. No.: R3701)

Competitive enzyme immunoassay for the quantitative analysis of AOZ in shrimps, meat (chicken, pig, beef) and milk.

All reagents required for the enzyme immunoassay - including standards - are contained in the test kit.

The test kit is sufficient for 96 determinations (including standards).

A microtiter plate spectrophotometer is required for quantification.

Sample preparation:	homogenization (milk samples: precipitation), derivation, extraction, centrifugation, concentration, and degreasing
Time requirement:	sample preparation (for 10 samples) 1. day approx. 2 h over night incubation approx. 16 h 2. day approx. 2 h test implementation (regardless of the number of samples) 1.5 h
Detection limit:	approx. 100 ppt
Recovery rate:	in shrimp approx. 90 - 100 % in meat and milk approx. 80 - 90 %
Cross-reactions:	AMOZ < 0.01 % AHD < 0.01 % SEM < 0.01 %
Note:	For the determination of AMOZ we refer to the RIDASCREEN [®] Nitrofuran (AMOZ) test with Art. No. R3711

1. Intended use

RIDASCREEN[®] Nitrofurantoin (AOZ) is a competitive enzyme immunoassay for the quantitative analysis of AOZ in shrimps, meat (chicken, pig, beef) and milk.

2. General

Nitrofurans are synthetic broad-spectrum antibiotics, which are frequently employed in animal production for its excellent antibacterial and pharmacokinetic properties. They had been also used as growth promoters in the pig production, the poultry and fish sector. In long term studies with experimental animals the parent drugs and their metabolites showed carcinogenic and mutagenic characteristics. This has led to a prohibition of nitrofurans for the treatment of animals used for food production. The nitrofurantoin, nitrofurazone and nitrofurazone were banned from use in food animal production in the EU in 1993, and the use of furazolidone was prohibited in 1995.

The analysis of residues of nitrofurantoin drugs needs to be based on the detection of the tissue bound metabolites of the nitrofurantoin parent drugs. Since the parent drugs are very rapidly metabolized, they are not detectable shortly after treatment. The tissue bound nitrofurantoin metabolites are detectable for a long time after administration and therefore they are used for the detection of the abuse of nitrofurantoin. Nitrofurantoin metabolites are found after administration of Furazolidone (metabolite: 3-amino-2-oxazolidinone = AOZ), Furaltadone (metabolite: 3-amino-5-morpholinomethyl-2-oxazolidinone = AMOZ), Nitrofurantoin (metabolite: 1-aminohydantoin = AHD) and Nitrofurazone (metabolite: semicarbazide = SEM).

AOZ-residues are determined most commonly by LC-UV, LC-MS, or LC-MS/MS techniques. Enzyme immunoassays, compared with chromatographic methods, show considerable advantages regarding sensitivity, detection limit, technical equipment and time requirement.

Using the RIDASCREEN[®] Nitrofurantoin (AOZ) test, it is possible to detect AOZ in shrimps, meat (chicken, pig, beef) and milk with little preparation of the sample.

3. Test principle

The basis of the test is the antigen-antibody reaction. The microtiter wells are coated with capture antibodies directed against anti-AOZ antibodies. AOZ standards or sample solution, AOZ enzyme conjugate and anti-AOZ antibodies are added. Free AOZ and AOZ enzyme conjugate compete for the AOZ antibody binding sites (competitive enzyme immunoassay). At the same time, the anti-AOZ antibodies are also bound by the immobilized capture anti-bodies. Any unbound enzyme conjugate is then removed in a washing step. Substrate/chromogen is added to the wells and incubated. Bound enzyme conjugate converts the chromogen into a blue product. The addition of the stop solution leads to a color change from blue to yellow. The measurement is made photometrically at 450 nm (optional reference wavelength ≥ 600 nm). The absorption is inversely proportional to the AOZ concentration in the sample.

4. Reagents provided

Each kit contains sufficient materials for 96 measurements (including standard analyses). Each test kit contains:

- 1 x Microtiter plate with 96 wells (12 strips with 8 removable wells each)
coated with capture antibodies against anti-AOZ-antibodies
- 6 x AOZ standards, 1.3 ml each
0 ppt (zero standard), 50 ppt, 150 ppt, 450 ppt, 1350 ppt, 4050 ppt
AOZ in aqueous solution
- 1 x Conjugate (6 ml)red cap
peroxidase conjugated AOZ
- 1 x Anti-AOZ antibody (6 ml) black cap
- 1 x Substrate/Chromogen (10 ml) blue cap
stained red, contains tetramethylbenzidine
- 1 x Stop solution (14 ml)yellow cap
contains 1 N sulfuric acid
- 1 x Sample- and washing buffer (salt)
for preparation of a 10 mM Phosphate Buffer, pH 7.4
contains 0.05 % Tween 20

5. Materials required but not provided

5.1. Equipment:

- Microtiter plate spectrophotometer (450 nm)
- Centrifuge
- Shaker (Vortex)
- Mixer (Stomacher, Ultraturrax)
- Rotary evaporator
- Magnetic stirrer
- Pasteur pipettes
- Graduated pipettes
- 50 µl-, 100 µl-, 1000 µl- micropipettes, adjustable

5.2. Reagents:

- 2-Nitrobenzaldehyd (10 mM in Dimethylsulfoxid)
- 0.1 M K_2HPO_4
- Ethyl acetate
- n-hexane (or n-heptane)

for milk samples:

- Carrez I: 0.36 M Potassium ferrocyanide (II) x 3 H₂O
- Carrez II: 1.04 M Zinc sulfate x 7 H₂O

6. Warnings and precautions for the users

The standards contain AOZ, particular care should be taken.

The stop solution contains 1 N sulfuric acid. Avoid contact of the reagent with the skin.

Do not use RIDASCREEN® Nitrofurantoin (AOZ) kit past the expiration date on the kit label. Dilution or adulteration of these reagents may result in loss of sensitivity.

Do not interchange individual reagents between kits of different lot numbers.

7. Storage instructions

Store the kit at 2 - 8 °C (36 - 46 °F). DO NOT FREEZE.

Return any unused microwells to their original foil bag and reseal them together with the desiccant provided.

The substrate/chromogen solution is light sensitive, therefore, avoid exposure to direct light.

8. Indication of deterioration of reagents

Any bluish coloration of the red substrate/chromogen solution is indicative of deterioration and the reagent should be discarded.

A value of less than 0.6 absorbance units ($A_{450\text{ nm}} < 0.6$) for the zero standard may indicate deterioration of reagents.

9. Preparation of Samples

The samples should be stored in a cool place, protected against light.

9.1. Shrimps and meat samples:

- homogenize the sample, use stomacher or mixer
- continue as described in 9.3.

9.2. Milk samples:

- transfer 5 ml of milk into a centrifugal glass vial
- add 250 µl Carrez I and 250 µl Carrez II (see 5.2.)
- mix thoroughly (Vortex) and centrifuge: 10 min / 3000 g / 4 – 12 °C (39 – 54 °F)
if a refrigerated centrifuge is not available, chill sample to approx. 8 °C (46 °F)
prior to centrifugation
- continue as described in 9.3.

9.3. Derivation:

- mix 1 g of the homogenized sample (shrimps / meat) or respectively 1.1 ml of the supernatant (corresponding to 1 ml milk) with 4 ml aqua dist., 0.5 ml 1 M HCl and 100 µl 10 mM 2-Nitrobenzoic aldehyde (in DMSO) by shaking properly
- incubate at 37 °C over night (approx. 16 h)
- add 5 ml 0.1 M K₂HPO₄, 0.4 ml 1 M NaOH and 5 ml ethyl acetate, shake vigorously for 30 sec.
- centrifuge: 10 min / 3000 g / at room temperature (20 - 25 °C / 68 - 77 °F)
- transfer 2.5 ml of the ethyl acetate layer into a new vessel and reduce to dryness
- dissolve the residue in 1 ml n-hexane (or n-heptane) and mix properly with 1 ml sample buffer
- centrifuge: 10 min / 3000 g / at room temperature (20 - 25 °C / 68 - 77 °F)
- use 50 µl of the lower, aqueous phase per well in the assay

10. Test implementation

10.1. Preliminary comments

1. Bring all reagents to room temperature (20 - 25 °C / 68 - 77 °F) before use.
2. Return all reagents to 2 - 8 °C (36 - 46 °F) immediately after use.
3. Do not allow microwells to dry between working steps.
4. Reproducibility in any EIA is largely dependent upon the consistency with which the microwells are washed. Carefully follow the recommended washing sequence as outlined in the EIA test procedure.
5. Avoid direct sunlight during all incubations. Covering the microtiter plates is recommended.

10.2. Preparation of the sample- and washing buffer

The kit contains a packet with buffer salt. One pouch is dissolved in 1 liter of dist. water. The prepared sample- and washing buffer expires after 4 weeks at 2 - 8 °C (36 - 46 °F).

10.3. Antibody-coated microtiter strips

The foil bag is cut open along the transverse side beyond the zip. The required wells are extracted together with the frame. Those wells not required are kept together with the drying agent, well sealed in the foil bag, and should continuously be stored at 2 - 8 °C (36 - 46 °F).

10.4. Test procedure

1. Insert a sufficient number of wells into the microwell holder for all standards and samples to be run in duplicate. Record standard and sample positions.
2. Add 50 µl of each standard solution or prepared sample to separate duplicate wells. Use a new pipette tip for each standard or sample.
3. Add 50 µl of the enzyme conjugate (red cap) to the bottom of each well.
4. Add 50 µl of the antibody (black cap) to each well, mix gently by rocking the plate manually and incubate for 1 h at room temperature (20 - 25 °C / 68 - 77 °F).
5. Pour the liquid out of the wells and tap the microwell holder upside down vigorously (three times in a row) against absorbent paper to ensure complete removal of liquid from the wells. Fill all the wells with 250 µl washing buffer and pour out the liquid again. Repeat two more times.
6. Add 100 µl of substrate/chromogen (blue cap) to each well. Mix gently by rocking the plate manually and incubate for 15 min at room temperature (20 - 25 °C / 68 - 77 °F) in the dark.
7. Add 100 µl of the stop solution to each well. Mix gently by rocking the plate manually and measure the absorbance at 450 nm against an air blank. Read within 60 minutes after addition of stop solution.

11. Results

The mean values of the absorbance values obtained for the standards and the samples are divided by the absorbance value of the first standard (zero standard) and multiplied by 100. The zero standard is thus made equal to 100 % and the absorbance values are quoted in percentages.

$$\frac{\text{absorbance standard (or sample)}}{\text{absorbance zero standard}} \times 100 = \% \text{ absorbance}$$

The values calculated for the standards are entered in a system of coordinates on semilogarithmic graph paper against the AOZ concentration in [ng/kg]. The calibration curve should be virtually linear in the 150 - 1350 ng/kg (ppt) range. The

AOZ concentration in ng/kg (ppt) corresponding to the absorbance of each sample can be read from the calibration curve.

In order to obtain the AOZ concentration in ng/kg actually contained in a sample, the concentration read from the calibration curve must be further multiplied by the corresponding dilution factor. When working in accordance with the regulation stated, **the dilution factor is 2.**

Please note:

For evaluation of the ELISA kits special software for the RIDASCREEN® tests has been developed by R-Biopharm. The RIDA® SOFT Win can be ordered from your local distributor.

12. Sensitivity

The mean lower detection limit of the RIDASCREEN® Nitrofurantoin (AOZ) test is about 50 ng/kg. According to the test preparation record, the detection limit is 100 ng/kg (ppt).

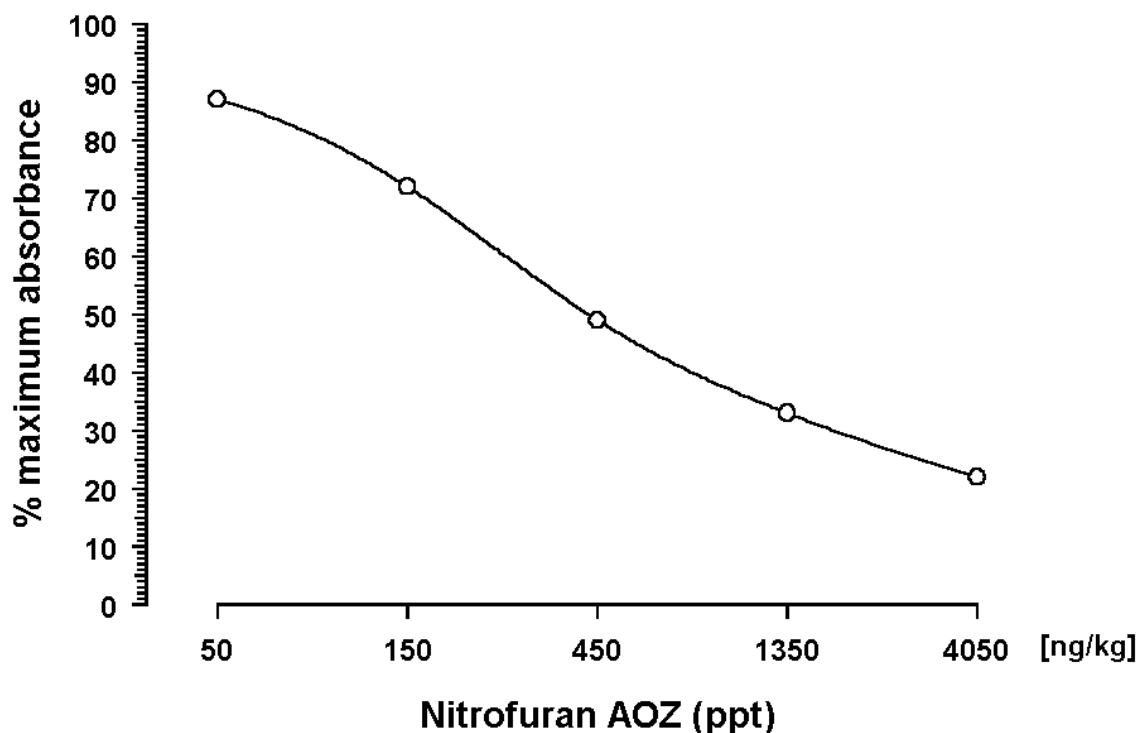


Fig. 1: Calibration curve of a RIDASCREEN® Nitrofurantoin (AOZ) kit

13. Specificity

The specificity of the RIDASCREEN® Nitrofuran (AOZ) test was established by analyzing the cross-reactivity to corresponding nitrofuran-metabolites:

Cross-reactions:	AMOZ.....	< 0.01 %
	AHD	< 0.01 %
	SEM	< 0.01 %

14. Reproducibility

The precision within a series was determined from the results of three different experiments. Fig. 2 shows the interassay precision profile of RIDASCREEN® Nitrofuran (AOZ). The coefficients of variation (% CV) for the absorbance values of the standards are entered against the corresponding AOZ concentrations. The coefficients of variation are so low with respect to the whole range of the figure that a high reproducibility of the results is ensured.

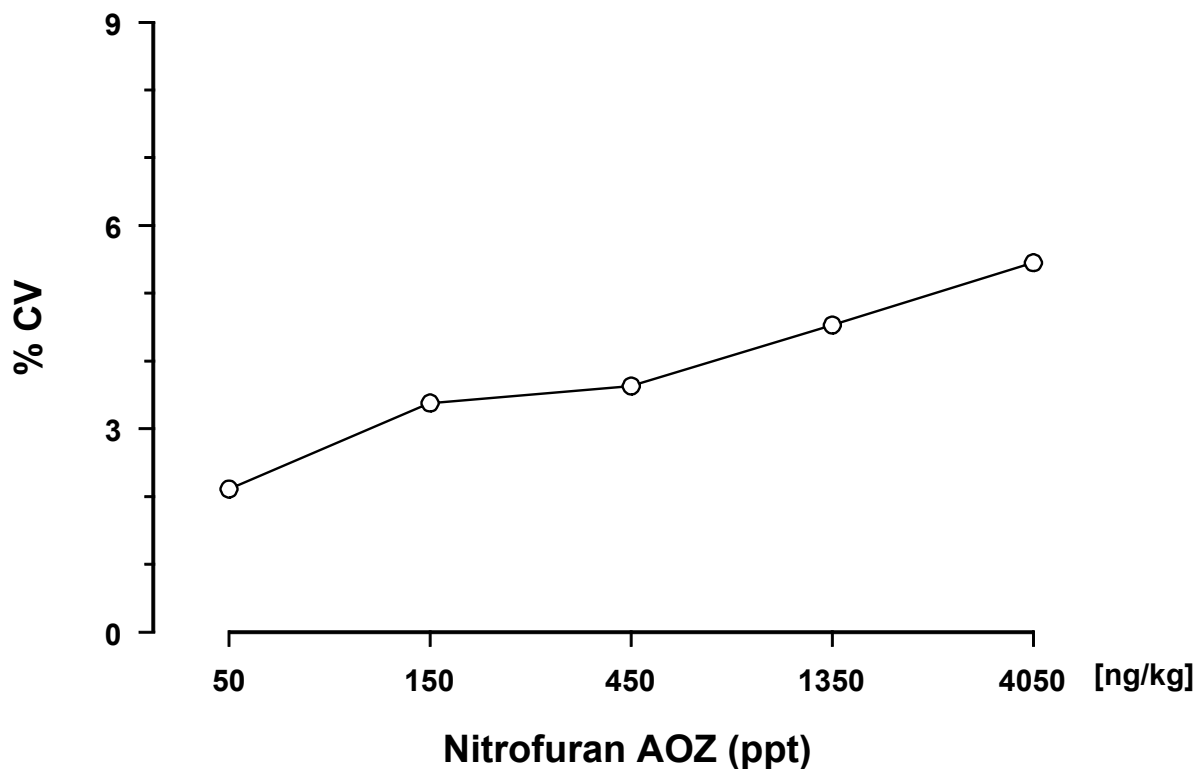


Abb. 2: Interassay precision profile of RIDASCREEN® Nitrofuran (AOZ)

15. Recovery rate

The recovery rate in spiked shrimps is approx. 90 - 100 %. For meat (chicken, pig, beef) and milk the recovery rate is approx. 80 - 90 %.

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Appendix

Literature

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