Furazolidone Metabolite (AOZ) ELISA Kit

Technical Manual

(ELISA)

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1 Principle and Application

This kit adopts the method of indirect competitive enzyme-linked immunoassay (ELISA) to detect Furazolidone Metabolite (AOZ) in the sample such as honey, fish, shrimp, poultry and liver. The kit is composed of Microtiter Plate coated with coupled antigens, HRP conjugate, antibodies, standards and other supporting reagents. During the detection, with adding standards or samples, the AOZ in the samples will compete with the coupled antigens to combine with anti-AOZ antibodies. After adding enzyme conjugates, take coloration with TMB substrates. Absorbance value of the samples is a negative correlation with AOZ content. Lastly, by comparing the obtained absorbance values with the standard curve, we can calculate the AOZ content in the sample.

2 Technique Data

2.1 Kit Sensitivity: 0.05ppb (ng/mL)

2.2 Reactive Mode: 25°C, 45min ~ 15min

2.3 Detection Limits:

Sample	Detection Limits
Tissue, liver, eggs	0.1ppb
Honey, milk, casings	0.1ppb
Milk powder, egg powder, feed	0.1ppb
Delicatessen	0.1ppb

2.4 Cross-reaction Rate:

Drug name	Cross-reaction Rate
Furazolidone Metabolite (AOZ)	100%
Nitrofurantoin Metabolite (AHD)	<0.1%
Furaltadone Metabolite (AMOZ)	<0.1%
Nitrofurazone Metabolite (SEM)	<0.1%

2.5 Sample Recovery Rate:

Sample	Recovery rate
Tissue, liver, eggs	80±25%
Honey, milk, casings	75±15%
Milk powder, egg powder, feed	85±25%
Delicatessen	75±15%

3 Composition of the Kit

Reagent	Specification
Microtiter Plate	8wells× 12strips
Standard: 0ppb, 0.05ppb, 0.15ppb, 0.45ppb, 1.35ppb, 4.05ppb	1×1.0mL
High Standard: 100ppb(red cap)	1×1.0mL
Derivatization Reagent(black cap)	1×10mL
Antibody solution (blue cap)	1×5.5mL
HRP conjugate(red cap)	1×5.5mL
Substrate Reagent A (white cap)	1×6mL
Substrate Reagent B (black cap)	1×6mL
Stop Solution (yellow cap)	1×6mL
Concentrated Wash Buffer (20×)(white cap)	1×40mL
Concentrated Reconstitution Buffer(2×) (yellow cap)	1×50mL
Instruction	1
Adhesive Membrane	1
Sealed bag	1

4 Materials Required but Not Supplied

4.1 Equipment: microplate reader, printer, grinder (for homogenizing solid samples), nitrogen evaporator, vortex mixer (for shake and mix), centrifuge, graduated transfer pipette, and balance with a division value of 0.01 g, constant temperature device, water bath;

4.2 Micropipette: single-channel (20-200μL and 100-1000μL), and multi-channel 300μL;

4.3 Reagents: Ethyl acetate, n-Hexane, Sodium hydroxide, Concentrated hydrochloric acid, Dipotassium hydrogen phosphate trihydrate ($K_2HPO_4 \cdot 3H_2O$), Sodium nitroprusside ($Na_2Fe(CN)_5(NO) \cdot 2H_2O$), Methanol, Acetonitrile, Zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$).

5 Experimental preparation

Restore all reagents and samples to room temperature (adjust to around 25°C) for more than 30 min before use. This is a crucial step to ensure there is no precipitation in the reagents.

5.1 Notice Before Sample Processing:

Please note that the labware must be clean. Use disposable pipette tips to avoid contamination of interference results.

5.2 Solution preparation:

Solution 1: 0.36 M Sodium nitroprusside solution (for milk and milk powder)

Weigh 10.7g of sodium nitroprusside($Na_2Fe(CN)_5(NO)\cdot 2H_2O$), add deionized water, mix until dissolved, and make up to 100mL.

Solution 2: 1.04M Zinc sulfate solution (for milk and milk powder)

Weigh 29.8g of zinc sulfate heptahydrate($ZnSO_4\cdot 7H_2O$), add deionized water, mix until dissolved, and make up to 100mL.

Solution 3: 0.1M Dipotassium hydrogen phosphate solution

Weigh 11.4g of Dipotassium hydrogen phosphate trihydrate ($K_2HPO_4\cdot 3H_2O$), add deionized water, mix until dissolved, and make up to 500 mL.

Solution 4: 1M hydrochloric acid solution

Take 8.6 mL concentrated hydrochloric acid, add deionized water, mix until dissolved, and make up to 100 mL

Solution 5: 1M sodium hydroxide solution

Weigh 4g of sodium hydroxide, add deionized water, mix until dissolved, and make up to 100 mL

Solution 6: Reconstitution Buffer

Dilute the Concentrated Reconstitution Buffer (2×) 2 times with deionized water (Concentrated Reconstitution Buffer (2×): deionized water=1:1) . It can be stored at 4 °C for one month.

Solution 7: Working Wash Buffer

Dilute the concentrated wash buffer (20×) by a factor of 20,

(Concentrated wash buffer/Deionized water= 1: 19)

5.3 Sample pretreatment steps:

5.3.1 Milk treatment.

1) Take 5mL of milk in a centrifuge tube, add 250μL of 0.36M sodium nitroprusside solution (Solution 1), shake for 30 seconds, add 250μL of 1.04M zinc sulfate solution (Solution 2), shake for another 30 seconds, centrifuge at 4000 rpm for 10 minutes at room temperature.

2) Take 1.1 mL of the supernatant, add 4 mL of deionized water, 0.5 mL of 1M hydrochloric acid solution (Solution 4), and 100 μL of derivatization reagent, shake for 5 minutes.

3) Incubate overnight at 37°C (approximately 16 hours) or at 50°C (incubation for more than 50°C may affect the separation effect) in a water bath for 3 hours.

4) Add 5mL of 0.1M Dipotassium hydrogen phosphate solution (Solution 3), 0.4mL of 1M sodium hydroxide solution (Solution 5), and 5 mL of ethyl acetate separately, shake for 5 minutes.

5) Centrifuge at 4000 rpm at room temperature for 10 minutes.

6）Transfer 2.5 mL of the upper layer liquid to another centrifuge tube, and evaporate it to dryness under nitrogen or air at 50°C-60°C.

7) Dissolve the residue in 1 mL n-hexane, add 1 mL of reconstitution buffer (Solution 6), shake thoroughly for 30 seconds; centrifuge at 4000 rpm for 10 minutes at room temperature.

8) Remove the upper layer and take 50μL of the lower layer liquid for analysis.

Dilution times of the sample:2 Detection limits: 0.1ppb

5.3.2 Milk powder and egg powder treatment.

1) Weigh 1g ± 0.05g of homogeneous sample into a centrifuge tube, add 4mL of deionized water, 0.5 mL of 1M hydrochloric acid solution (Solution 4), and 100μL of derivatization reagent. Shake for 5 minutes.

2) Incubate overnight at 37°C (approximately 16 hours) or at 50°C (incubation for more than 50°C may affect the separation effect) in a water bath for 3 hours.

3) Add 250μL of 0.36M sodium nitroprusside solution (Solution 1), shake for 30 seconds, add 250μL of 1.04M zinc sulfate solution (Solution 2), shake for another 30 seconds, and centrifuge at 4000 rpm for 10 minutes at 15°C.

4) Transfer all of the supernatant to another centrifuge tube, add 5mL of 0.1M Dipotassium hydrogen phosphate solution (Solution 3), 0.4mL of 1M sodium hydroxide solution (Solution 5), and 5 mL of ethyl acetate separately, shake for 5 minutes.

5) Centrifuge at 4000 rpm at room temperature for 10 minutes.

6）Transfer 2.5 mL of the upper layer liquid to another centrifuge tube, and evaporate it to dryness under nitrogen or air at 50°C-60°C.

7) Dissolve the residue in 1 mL n-hexane, add 1 mL of reconstitution buffer (Solution 6), shake thoroughly for 30 seconds; centrifuge at 4000 rpm for 10 minutes at room temperature.

8) Remove the upper layer and take 50μL of the lower layer liquid for analysis.

Dilution times of the sample:2 Detection limits: 0.1ppb

5.3.3 Honey, tissues, casings, liver, feed, and eggs treatment.

1) Weigh 1g ± 0.05g of homogeneous sample into a centrifuge tube, add 4mL deionized water, 0.5 mL 1M hydrochloric acid solution (Solution 4), and 100μL derivatization reagent. Shake for 5 minutes.

2) Incubate overnight at 37°C (approximately 16 hours) or at 50°C (incubation for more than 50°C may affect the separation effect) in a water bath for 3 hours.

3) Add 5mL of 0.1M Dipotassium hydrogen phosphate solution (Solution 3), 0.4mL of 1M sodium hydroxide solution (Solution 5), and 5 mL of ethyl acetate separately, shake for 5 minutes.

4) Centrifuge at 4000 rpm at room temperature for 10 minutes.

5）Transfer 2.5 mL of the upper layer liquid to another centrifuge tube, and evaporate it to dryness under nitrogen or air at 50°C-60°C.

6) Dissolve the residue in 1 mL n-hexane, add 1 mL of reconstitution buffer (Solution 6), shake thoroughly for 30 seconds; centrifuge at 4000 rpm for 10 minutes at room temperature.

7) Remove the upper layer and take 50µL of the lower layer liquid for analysis.

Dilution times of the sample:2 Detection limits: 0.1ppb

5.3.4 Delicatessen (cooked food) treatment.

1) Weigh 1g ± 0.05g of homogeneous sample into a 50mL centrifuge tube, add 4.5mL of methanol and 0.5mL of deionized water, shake for 2 minutes, centrifuge at 4000 rpm for 5 minutes at room temperature, and remove all the liquid.

2) Add 5mL of acetonitrile and 5mL of n-hexane, shake for 2 minutes at room temperature and 4000 rpm, centrifuge for 5 minutes, and remove all the liquid.

3) Add 4mL of deionized water, 0.5mL of 1M hydrochloric acid solution (Solution 4), and 100µL of derivatization reagent to the remaining precipitate, shake for 5 minutes.

Note: In the addition recovery experiment for cooked food, please introduce high standards at this step.

4) Incubate overnight at 37°C (approximately 16 hours) or at 50°C (incubation for more than 50°C may affect the separation effect) in a water bath for 3 hours.

5) Add 5mL of 0.1M Dipotassium hydrogen phosphate solution (Solution 3), 0.4mL of 1M sodium hydroxide solution (Solution 5), and 5 mL of ethyl acetate separately, shake for 5 minutes.

6) Centrifuge at 4000 rpm at room temperature for 10 minutes.

7）Transfer 2.5 mL of the upper layer liquid to another centrifuge tube, and evaporate it to dryness under nitrogen or air at 50°C-60°C.

8) Dissolve the residue in 1 mL n-hexane, add 1 mL of reconstitution buffer (Solution 6), shake thoroughly for 30 seconds; centrifuge at 4000 rpm for 10 minutes at room temperature.

9) Remove the upper layer and take 50µL of the lower layer liquid for analysis.

Dilution times of the sample:2 Detection limits: 0.1ppb

6 ELISA procedure

Place all reagents and samples to room temperature (adjust to around 25°C) for 30min. Gently shake the reagent bottles before use.

Take out the frame of the microplate along with the required number of wells. Then place the unused microplate wells into the sealed bag with the desiccant provided. Store the remaining kit in the refrigerator at 2-8°C.

Step 1: Number: Number the wells in sequence corresponding to the samples and standard, make 2-well parallel trials for each sample and standard, and record their locations.

Step 2: Incubation: Add 50µL of standard or sample into each numbered well, then add 50µL of HRP conjugate per well. Next, add 50µL of antibody solution per well. Finally, cover the Microtiter Plate with the adhesive membrane, shake gently by hand (or use a microplate shaker) for 5s and incubate for 45 min at 25°C.

Step 3: Washing: Uncover the adhesive membrane carefully, remove the liquid, pipette 350µL of Working Wash Buffer (Solution 7) to every well, let stand for 30 seconds then drain, repeat 5 times. Invert the plate and tap it against a thick absorbent paper (or lint-free cloth), with a soft towel placed underneath. (Bubbles that are not removed after patting dry can be punctured with a clean pipette tip).

Step 4: Color: Add 50µL of Substrate Reagent A to each well, then add 50µL of Substrate Reagent B. Shake gently by hand (or use a microplate shaker) for 5s, and allow to react for 15min at 25°C in the dark. (The reaction can be extended appropriately if the blue colour is too pale.)

Step 5: Stop the reaction: Pipette 50µL of Stop Solution to each well, shake gently by hand (or use a microplate shaker) for 5s. The reaction would be stopped.

Step 6: Calculate: Determine the Optical Density (OD value; absorbance value) at 450nm (Reference wavelength 630nm) with a microplate reader. Finish this step within 10min after stop the reaction.

7 Interpretation of result

7.1 Calculate the percentage of absorbance value

$$\text{Percentage of absorbance value}(\%) = \frac{A}{A0} \times 100\%$$

A—the average OD value of the sample or standard;

A0—the average OD value of the 0ppb standard.

It is used to calculate the percentage absorbance of a standard or sample.

7.2 Draw the standard curve and calculate

Take absorbance percentage(A/A0) of standards as Y-axis and the corresponding log of standards concentration (ppb) as X-axis.

Draw the standard semi-log curves with X-axis and Y-axis.

Take absorbance percentage of samples substitute into standard curve, then can get the corresponding concentration from standard curve. **Last, the resulting concentration values multiplied by the corresponding**

dilution times is the actual concentration of AOZ of samples.

If professional analysis software of the kit is used for calculation, it is more convenient for accurate and rapid analysis of a large number of samples.

8 Attention

8.1 Before test, the reagents and samples should be balanced to room temperature (25°C). If below 25°C, it will lead to all the standard OD value on the low side.

8.2 In the washing process, dry wells may result in non-linear standard curves and undesirable reproducibility. Therefore, proceed to the next step immediately after washing.

8.3 Please mix the contents within the wells uniformly and wash the plate thoroughly. The reproducibility is largely determined by consistency of washing step.

8.4 During the incubation, cover microplates with adhesive membrane to avoid light.

8.5 Do not use kits that are overdue. Do not mix reagents with those from other lots.

8.6 Substrate Reagent A/B is colorless. If not, please discard.

8.7 If absorbance value of 0ppb is below 0.5（A450nm< 0.5), it means that the reagent may be metamorphic.

8.8 Stop solution is corrosives, please avoid contact with skin.

8.9 As the OD values of the standard curve may vary according to the conditions of actual assay performance (e.g. operator, pipetting technique, washing technique or temperature effects), the operator should establish a standard curve for each test.

8.10 For the mentioned sample, fast and efficient extraction methods are included in the kit description. Please consult technical support for the applicability if other sample need to be tested.

8.11 The kit is used for rapid screening of actual samples. If the test result is positive, the instrument method such as HPLC, LC/MS can be used for quantitative confirmation.

9 Storage conditions

The kit shall be stored at 2-8 °C. Avoid freezing.

Shelf Life: 12 months. The date of manufacture is presented in the label of the box.