Sulfamethoxydiazine (SMD) 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 Sulfamethoxydiazine (SMD) in the sample such as tissue, serum, honey, milk and urine. The kit is composed of Microtiter Plate coated with coupled antigens, HRP enzyme conjugates, antibodies, standards and other supporting reagents. During the detection, with adding standards or samples, the SMD in the samples will compete with the coupled antigens to combine with anti- SMD antibodies. After adding enzyme conjugates, take coloration with TMB substrates. Absorbance value of the samples is a negative correlation with SMD content. Lastly, by comparing the obtained absorbance values with the standard curve, we can calculate the SMD 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 (higher detection limit)	0.05ppb
Tissue (lower detection limit)	0.25ppb
Serum, urine	0.2ppb
Honey	0.05ppb
Milk	1ppb
Eggs	0.1ppb

2.4 Cross-reaction Rate:

Drug name	Cross-reaction Rate
Sulfamethoxydiazine(SMD)	100%
Sulfadiazine(SD/SDZ)	40%
Sulfamerazine(SM1)	25%
Sulfadoxine（SDM'）	35%

2.5 Sample Recovery Rate:

Sample	Recovery rate
Tissue, honey	95±25%
Urine, milk, serum, eggs	85±25%

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
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);

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

4.3 Reagents: Ethyl acetate, n-hexane, acetonitrile, $Na_2HPO_4 \cdot 12H_2O$, NaOH, concentrated hydrochloric acid (36% by mass), $NaH_2PO_4 \cdot 2H_2O$.

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: 0.2M NaOH Solution

Weigh 0.8g of NaOH, dissolve in deionized water, mix thoroughly, and bring the volume up to 100mL.

Solution 2: 0.02M Phosphate Buffer

Weigh 2.58g of $Na_2HPO_4 \cdot 12H_2O$ and 0.44 of $NaH_2PO_4 \cdot 2H_2O$,

And dissolve in deionized water and mix thoroughly. Bring the volume up to 500mL.

Solution 3: 0.5 M hydrochloric acid solution

Dilute 4.3 mL concentrated hydrochloric acid to 100 mL with deionized water, mix thoroughly.

Solution 4: Reconstitution Buffer

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

Solution 5: 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 Tissue (higher detection limit) treatment.

1) Weigh 3g ± 0.05g of homogeneous tissue into a centrifuge

tube. Add 3mL of 0.02M Phosphate Buffer (Solution 2) and mix thoroughly. Then, add 4mL of ethyl acetate and 2mL of acetonitrile, shake vigorously for 10 minutes, and centrifuge at 4000rpm for 10 minutes at room temperature.

2) Transfer 2mL of the upper layer liquid (approximately equivalent to 1g of the sample) and evaporate it to dryness under nitrogen or air at 50°C-60°C.

3) Add 1 mL of n-hexane, then add 1 mL of Reconstitution Buffer (Solution 4), shake for 1 minute, centrifuge at 4000 rpm for 5 minutes at room temperature.

4) Remove the upper layer. Take 50μL of the lower layer for analysis.

Dilution times of the sample:1 Detection limits: 0.05ppb

5.3.2 Tissue (lower detection limit) treatment.

1) Weigh 2g ± 0.05g of homogeneous tissue into a centrifuge tube. Add 8mL of 0.02M Phosphate Buffer (Solution 2) and mix thoroughly. Centrifuge at 4000rpm for 10 minutes at room temperature.

2) Take 50μL of the upper layer for analysis.

Dilution times of the sample:5 Detection limits: 0.25ppb

5.3.3 Serum treatment.

1) Allow the blood sample to stand at room temperature for 30 minutes, then centrifuge at 4000rpm for 10 minutes to separate the serum.

2) Take 1 mL of serum, add 3 mL of 0.02M Phosphate Buffer (Solution 2), and shake for 30 seconds.

3)Take 50μL for analysis.

Dilution times of the sample:4 Detection limits: 0.2ppb

5.3.4 Honey treatment.

1) Weigh 1g ± 0.05g of honey sample into a 50mL centrifuge tube. Add 1mL of 0.5M hydrochloric acid solution (Solution 3) and incubate at 37°C for 30 minutes.

2) Add 2.5mL of 0.2M NaOH Solution (Solution 1), then add 4mL of ethyl acetate, shake for 5 minutes, and centrifuge at 4000rpm for 10 minutes at room temperature.

3) Take 2mL of the upper layer liquid and evaporate under nitrogen or air at 50-60°C. Add 0.5mL of Reconstitution Buffer (Solution 4), and shake for 30 seconds.

4)Take 50μL for analysis.

Dilution times of the sample:1 Detection limits: 0.05ppb

5.3.5 Urine treatment

1) Mix 1 mL of the clarified urine sample with 3 mL of 0.02M Phosphate Buffer (Solution 2) and shake for 30 seconds.

2)Take 50μL for analysis.

Dilution times of the sample:4 Detection limits: 0.2ppb

5.3.6 Milk treatment.

1) Dilute the milk sample 20 times with 0.02M Phosphate Buffer (Solution 2) by adding 20 μL of milk to 380 μL of 0.02M Phosphate Buffer (Solution 2). Shake the mixture for 30 seconds.

2) Take 50μL for analysis.

Dilution times of the sample:20 Detection limits: 1ppb

5.3.7 Egg treatment.

1) Mix egg whites and egg yolks thoroughly.

2) Weigh 2.0±0.05g of the homogenized egg sample (or dissolve 1g of egg powder in 3mL of deionized water, then take 2mL to represent 2g of fresh egg) into a 50mL centrifuge tube. Add 8mL of acetonitrile and shake immediately with a shaker for 10 minutes. Centrifuge at room temperature at 4000 r/min for 5 minutes.

3) Transfer 2mL of the supernatant to a 10mL clean and dry glass test tube, and evaporate to dryness under nitrogen or air at 50-60°C.

4) Add 1 mL of n-hexane and vortex for 30 seconds to dissolve the dried residue. Then add 1 mL of reconstitution buffer (Solution 4), vortex for 1 minute, and centrifuge at room temperature at 4000 r/min for 5 minutes.

5) Remove the upper layer and take 50 μL of the lower 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 into each 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 in the dark.

Step 3: Washing: Uncover the adhesive membrane carefully, discard liquid in the wells, pipette 350μL of Working Wash Buffer (Solution 5) 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 tapping 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 per well. 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 color is too pale.)

Step 5: Stop the reaction: Pipette 50μL of Stop Solution to each well, and shake gently by hand (or use a microplate shaker). 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 SMD 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.