Diazepam (DZP) 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 Diazepam (DZP) in the sample such as Tissue (beef, chicken, pork, etc.), feed. 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 DZP in the samples will compete with the coupled antigens to combine with anti-DZP antibodies. After adding HRP conjugate, take coloration with TMB substrates. Absorbance value of the samples is a negative correlation with DZP content. Lastly, by comparing the obtained absorbance values with the standard curve, we can calculate the DZP content in the sample.

2 Technique Data

2.1 Kit Sensitivity: 0.3ppb (ng/mL)

2.2 Reactive Mode: 25°C, 30min ~ 30min ~ 15min

2.3 Detection Limits:

Sample	Detection Limits
Tissue (beef, chicken, pork, etc.)	3ppb
Urine	3ppb
Compound feed	30ppb
Concentrate/Premix	60ppb

2.4 Cross-reaction Rate:

Diazepam 100%
Nitrazepam ..... <10%
Oxazepam ..... <10%

2.5 Sample Recovery Rate:

Sample	Recovery rate
Tissue	90±20%
Urine	85±20%
Compound feed	80±20%
Concentrate/Premix	75±20%

3 Composition of the Kit

Reagent	Specification
Microtiter Plate	8wells× 12strips
Standard: 0ppb, 0.3ppb, 0.9ppb, 2.7ppb, 8.1ppb, 24.3ppb	1.0mL each
High Standard (red cap)	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(25°C);

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

4.3 Reagents: n-Hexane, sodium hydrate.

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: Reconstitution Buffer

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

Solution 2: Working Wash Buffer

Dilute the concentrated wash buffer (20×) by a factor of 20 (Concentrated wash buffer/Deionized water= 1: 19).

Solution 3: 0.1 M NaOH Solution

Dissolve 4g of sodium hydroxide in deionized water to a final volume of 1000mL.

5.3 Sample pretreatment steps:

5.3.1 Tissue treatment.

1) Weigh 2g ± 0.05g of homogeneous sample into a 50mL centrifuge tube. Add 10mL of 0.1 M NaOH Solution (Solution 3), shake for 5 minutes, and centrifuge at 4000rpm for 10 minutes at room temperature.

2) Take 1mL of the supernatant, add 10mL of n-Hexane, shake for 5 minutes, and centrifuge at 4000rpm for 5 minutes at room temperature.

3) Take 5 mL of the upper liquid layer and evaporate it to dryness under nitrogen or air at 50°C-60°C.

4）Dissolve the dried residue in 1mL of Reconstitution Buffer (Solution 1) and take 50μL for analysis.

Dilution times of the sample:10 Detection limits: 3ppb

5.3.2 Urine treatment.

1) Transfer 1 mL of clear urine sample to a 50 mL centrifuge tube, add 4 mL of 0.1 M NaOH Solution (Solution 3), and shake for 2 minutes.

2) Take 1 mL of the mixture, add 10 mL of n-hexane, shake for 5 minutes, and centrifuge at room temperature at 4000 r/min for 5 minutes.

3) Take 5 mL of the upper liquid layer and evaporate it to dryness under nitrogen or air at 50°C-60°C.

4) Dissolve the residue in 1 mL of Reconstitution Buffer (Solution 1) and take 50μL for analysis.

Dilution times of the sample:10 Detection limits: 3ppb

5.3.3 Feed treatment.

1) Weigh 1 g ± 0.05 g of homogenized feed sample into a 50 mL centrifuge tube, add 1 mL of deionized water and 3 mL of 0.1 M NaOH Solution (Solution 3), and shake for 2 minutes.

2) Add 10 mL of n-hexane, shake for 10 minutes, and centrifuge at room temperature at 4000 r/min for 10 minutes.

3) Take 1 mL of the upper liquid layer and evaporate it to dryness under nitrogen or air at 50°C-60°C.

4) Dissolve the dried residue in 1mL of Reconstitution Buffer (Solution 1) and then dilute as follows:

—For Compound feed: Take 20 μL of the solution from step 4), add 180 μL of the Reconstitution Buffer (Solution 1), mix well, and then take 50 μL for testing.

Dilution times of the sample:100 Detection limits: 30ppb

—For concentrated feed/premix: Take 10 μL of the solution from step 4), add 190 μL of the Reconstitution Buffer (Solution 1), mix well, and then take 50 μL for testing.

Dilution times of the sample:200 Detection limits: 60ppb

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: Sample Incubation: Add 50μL of standard or sample into each numbered well, then 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 30 min at 25°C in the dark.

Step 3: Washing: Uncover the adhesive membrane carefully, remove the liquid, pipette 350μL of Working Wash Buffer (Solution 2) 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: Enzyme Incubation: Add 100μL of HRP conjugate into each well. Then cover the Microtiter Plate with the adhesive membrane, incubate for 30 min at 25°C in the dark.

Step 5: Washing: Same as step 3.

Step 6: 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 7: 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 8: 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 DZP 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 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.