Technical Manual

DZP (Diazepam) ELISA Kit

- Catalogue Code: FSES0022
- Competitive ELISA Kit
- Research Use Only

Contents

1. Key features and Sample Types

Sensitivity:

0.3 ppb (ng/mL)

Assay Procedure:

25°C, 30 min~30 min~15 min

Detection Limit:

Urine - 5 ppb; Muscle - 5 ppb; Formula feed - 50 ppb; Other feed - 100 ppb.

Cross Reactivity:

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

Sample Recovery rate:

Muscle - 90%±20%; Urine - 85%±20%; Feed - 80%±20%;

Storage:

2-8°C for 6 months.

Expiry:

See Kit Label

2. Storage

Store the kit at 2~8°C. Do not freeze any test kit components.
Return any unused microwells to their original foil bag and reseal them together with the desiccant provided and further store at 2 - 8°C.

3. Test Principle

This kit uses a Competitive-ELISA method. It can detect Diazepam (DZP) in samples, such as muscle, feed. This kit is composed of ELISA Microtiter plate, HRP conjugate, antibody working solution, standard and other supplementary reagents. The microtiter plate in this kit has been pre-coated with coupled antigen. During the reaction, DZP in the samples or standard competes with coupled antigen on the solid phase supporter for sites of anti-DZP antibody. Then Horseradish Peroxidase (HRP) conjugate is added to each microtiter plate well, and substrate reagent is added for color development. There is a negative correlation between the OD value of samples and the concentration of DZP. The concentration of DZP in the samples can be calculated by comparing the OD of the samples to the standard curve.

4. Kit Contents

Each kit contains reagents for 96 assays including:

No.	Component	96-WellKit
1	ELISA Microtiter plate	96 wells
2	Standards	1mL each (0 ppb, 0.3 ppb, 0.9 ppb, 2.7 ppb, 8.1 ppb, 24.3 ppb)
3	HRP Conjugate	11 mL
4	Antibody Working Solution	5.5 mL
5	Substrate Reagent A	6 mL
6	Substrate Reagent B	6 mL
7	Stop Solution	6 mL
8	20×Concentrated Wash Buffer	40 mL
9	2×Reconstitution Buffer	50 mL
10	Plate Sealer	3 pieces
11	Sealed Bag	1 piece
12	Manual	1 copy

Note: All reagent bottle caps must be tightened to prevent evaporation and microbial pollution.

Additional materials required:

Other materials required but not supplied

- **Instruments:** Microplate reader, Printer, Homogenizer, Vortex mixer, Nitrogen evaporators, Water bath, Centrifuge, Graduated pipette, Balance (sensitivity 0.01 g).
- **Micropipette:** Single channel (20-200 µL, 100-1000 µL), Multichannel (30-300 µL).
- **Reagents:** NaOH, N-hexane.

5. Experimental Preparation

Bring all reagents and samples to room temperature before use.

Open the microplate reader in advance, preheat the instrument, and set the testing parameters.

1. **Sample pre-treatment Notice:**

Experimental apparatus should be clean, and the pipette should be disposable to avoid cross- contamination during the experiment.

2. **Solution preparation**

Please prepare solution according to the number of samples. Don't use up all components in the kit at once!

Solution 1: Reconstitution Buffer

Dilute the **2×Reconstitution Buffer** with deionized water. (2×Reconstitution Buffer: Deionized water=1:1). The reconstitution buffer can be store at 4°C for a month.

Solution 2: Wash Buffer

Dilute the **20× Concentrated Wash Buffer** with deionized water 20× Concentrated Wash Buffer: Deionized water=1:19).

Solution 3: 0.1 M NaOH Solution

Dissolve 4 g of **NaOH** to 1000 mL with deionized water.

3. **Sample pre-treatment procedure**

Targets may be distributed unevenly, resulting in no detection. To avoid this, ensure to take sufficient samples when sampling.

3.1 Pre-treatment of muscle (livestock) sample:

1. Weigh 2±0.05 g of homogenate muscle sample, add 8 mL of **0.1 M NaOH Solution (**Solution 3). Vortex fully for 5 min, centrifuge at 4000 r/min for 10 min at room temperature.
2. Take 1 mL of the supernatant, add 10 mL of **N-hexane**. Vortex fully for 5 min, centrifuge at 4000 r/min for 5 min at room temperature.
3. Take 5 mL of the upper N-hexane phase to glass tube and dry at 50-60°C with nitrogen evaporators or water bath.
4. Take 1 mL of the **Reconstitution Buffer** (Solution 1) to redissolve dry material.
5. Take 50 µL for analysis.

Note: Sample dilution factor: 10, detection limit: 5 ppb

3.2 Pre-treatment of urine (swine)* sample:

(*Data validated in swine urine but pre-treatment can be applied for urine samples of multiple species.)

1. Take 1 mL of clear urine sample into 50 mL centrifuge tube. Add 4 mL of **0.1 M NaOH Solution (**Solution 3). Vortex fully for 2 min.
2. Take 1 mL of the mixture, add 10 mL of **N-hexane**. Vortex fully for 5 min, centrifuge at 4000 r/min for 5 min at room temperature.
3. Take 5 mL of the upper N-hexane phase and to glass tube and dry at 50-60°C with nitrogen evaporators or water bath.

4. Take 1 mL of the **Reconstitution Buffer** (Solution 1) to redissolve dry material.
5. Take 50 µL for analysis.

Note: Sample dilution factor: 10, detection limit: 5 ppb.

3.3 Pre-treatment of feed sample:

1. Weigh 1±0.05 g of homogenate feed sample, add 1 mL of deionized water and 3 mL of **0.1 M NaOH Solution (**Solution 3). Vortex fully for 2 min.
2. Add 10 mL of **N-hexane**. Vortex fully for 10 min, centrifuge at 4000 r/min for 10 min at room temperature.
3. Take 1 mL of the upper N-hexane phase and to glass tube and dry at 50-60℃ with nitrogen evaporators or water bath.
4. Take 1 mL of the **Reconstitution Buffer** (Solution 1) to redissolve dry material. Then dilute it with the following ratio.
5. **For formula feed sample:** Dilute the sample solution [Step (3)] with **Reconstitution Buffer** (Solution 1) for 10 times (sample solution: Reconstitution buffer = 1:9).

   Note: Sample dilution factor: 100, detection limit: 50 ppb

   For other feed sample: Dilute the sample solution [Step (3)] with **Reconstitution Buffer** (Solution 1) for 20 times (sample solution: Reconstitution buffer = 1:19).

   Note: Sample dilution factor: 200, detection limit: 100 ppb

6. Assay Procedure

Bring all reagents and samples to room temperature before use. All the reagents should be mixed thoroughly by gently swirling before pipetting. Avoid foaming. The unused ELISA Microtiter plate should be sealed as soon as possible and stored at 2~8°C.

1. **Number:** number the sample and standard in order (multiple well), and keep a record of standard wells and sample wells. **Standard and Samples must be tested in duplicate.**
2. **Add Sample:** add 50 µL of **Standard or Sample** per well, then add 50 µL **Antibody Working Solution**, cover the plate with plate sealer. Vortex for 5s gently to mix thoroughly. Incubate at 25°C for 30 min away from direct sunlight.
3. **Wash:** uncover the sealer carefully, remove the liquid in each well. Immediately add 300 µL of **Wash Buffer (**Solution 2) to each well and wash. Repeat wash procedure for 5 times, 30s intervals/time. Invert the plate and pat it against absorbent paper (If bubbles exist in the wells, clean tips can be used to prick them).
4. **HRP Conjugate:** add 100 µL of **HRP Conjugate** to each well. Incubate at 25°C for 30 min away from direct sunlight
5. **Wash:** Repeat Step 3.
6. **Colour Development:** add 50 µL of **Substrate Reagent A** to each well, and then add 50 µL of **Substrate Reagent B**. Gently vortex for 5s to mix thoroughly. Incubate at 25°C for 15 min away from direct sunlight.
7. **Stop Reaction:** add 50 µL of **Stop Solution** to each well, gently vortex for 5s.
8. **OD Measurement:** determine the optical density (OD value) of each well at 450 nm (reference wavelength 630 nm) with a microplate reader. This step should be finished in 10 min after stop reaction.

7. Data Analysis

1. **Absorbance% = $A/A_0 \times 100\%$**
   A: Average absorbance of standard solution or sample
   A_0: Average absorbance of 0 ppb Standard solution

2. **Drawing and calculation of standard curve**
   Create a standard curve by plotting the absorbance percentage of each standard on the y-axis against the log concentration on the x-axis to draw a semi-logarithmic plot. Add the average absorbance value of sample to standard curve to get corresponding concentration. **If samples have been diluted, the concentration calculated from the standard curve must be multiplied by the dilution factor.**

   For this kit, it is more convenient to use professional analysis form for accurate and fast analysis on many samples.

8. Notes

1. The overall OD value will be lower when reagents have not been brought to room temperature before use or room temperature is below 25°C.
2. If the wells turn dry during the washing procedure, it will lead to bad linear standard curve and poor repeatability. Operate the next step immediately after wash.
3. Mix thoroughly and wash the plate completely. The consistency of wash procedure can strongly affect the reproducibility of this ELISA kit.
4. ELISA Microplate should be covered by plate sealer. Avoid the kit to strong light.
5. **Each reagent is optimized for use in the FSES0022. Do not substitute reagents from any other manufacturer into the test kit. Do not combine reagents from other FSES0022 with different lot numbers.**
6. Substrate Reagent should be abandoned if it turns blue colour. When OD value of standard (concentration: 0) < 0.5 unit (A450nm < 0.5), it indicates the reagents are deteriorated.
7. Stop solution is caustic, avoid contact with skin and eyes.
8. As the OD values of the standard curve may vary according to the conditions of the actual assay performance (e.g. operator, pipetting technique, washing technique or temperature effects), the operator should establish a standard curve for each test.
9. Even the same operator might get different results in two separate experiments. In order to get reproducible results, the operation of every step in the assay should be controlled.
10. If the samples are not indicated in the manual, a preliminary experiment to determine the validity of the kit is necessary.
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, etc. can be used for quantitative confirmation.

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