Diazepam ELISA Test Kit

1. Principle

This test kit is based on the competitive enzyme immunoassay for the detection of Diazepam in the feed, urine, liver and meat. The coupling antigen is pre-coated on the micro-well stripes. The Diazepam in the sample and the coupling antigen pre-coated on the micro-well stripes compete for the anti-Diazepam antibodies. After the addition of the enzyme conjugate, the TMB substrate is added for coloration. The optical density (OD) value of the sample has a negative correlation with the Diazepam in it. This value is compared to the standard curve and the Diazepam concentration is subsequently obtained.

2. Technical specifications

Sensitivity: 0.1 ppb

Detection limit

Tissue.....1 ppb
Urine .....1 ppb
Feed.....10 ppb

Recovery rate

Urine ..... 80 ±10%
Feed..... 75 ±10%
Tissue(meat, liver) ..... 85 ±10%

Cross-reactions rate

Diazepam..... 100%
Nitrazepam..... 7.6%
Oxazepam..... 8.8%

3. Components

1） Micro-well strips: 12 strips with 8 removable wells each
2） 6× standard solution (1 mL each): 0 ppb, 0.1 ppb, 0.3 ppb, 0.9 ppb, 2.7 ppb, 8.1 ppb
3） Enzyme conjugate (12 mL) .....red cap
4） Antibody working solution (7 mL).....blue cap
5） Substrate A solution (7 mL)..... white cap
6） Substrate B solution (7 mL)..... black cap
7） Stop solution (7 mL) .....yellow cap
8） 20× concentrated washing buffer (40 mL) ..... white cap
9） 2× concentrated redissolving solution (50 mL) .....transparent cap

4. Materials required but not provided

1) **Equipments:** microplate reader, printer, homogenizer, nitrogen-drying device, votex, centrifuge, measuring pipets, balance (a sensibility reciprocal of 0.01 g).
2) **Micropipettors:** single-channel 20 to 100 µL and 200 to 1000 µL, and multi-channel 250 µL.
3) **Reagents:** N-hexane, NaOH,Dichloromethane(CH_2Cl_2), CH_3CN.

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5. Sample pre-treatment

Instructions (The following points must be dealt with before the pre-treatment)

1) Only the disposable tips can be used for the experiments and the tips must be changed when used for absorbing different reagents;
2) Before the experiment, each experimental utensil must be clean and should be re-cleaned if necessary, in order to avoid the contamination that interferes with the experimental results.

Solution preparation before sample pre-treatment:

1) 0.1M NaOH:dissolve 0.1g NaOH in 100ml deionized water
2) 1 M NaOH: dissolve 4 g NaOH in 100 mL deionized water.
3) 2M NaOH: dissolve 8 g NaOH in 100 mL deionized water.
4) The 2×concentrated redissolving solution is mixed with deionized water at 1:1 (1 mL concentrated redissolving solution + 1 mL deionized water), used for the treated sample redissolving.

3) N-hexane-CH_2Cl_2 solution: $V_{N\text{-}hexane}$: V_{CH2Cl2} = 5:3.

5.1 Animal tissues (meat ,liver)

1. Take the sample, homogenize at 10000 r/min for 1 min,
2. Weigh 2 ± 0.05 g of the homogenized sample, put into 50 mL centrifugal tube, add 5 mL CH_3CN,mix up;add 1 mL 2 M NaOH, shake properly for 5min, centrifuge at above 4000 r/min at 10 ℃for 10 min,
3. Take 3 mL supernatant(upper layer) into a new centrifugal tube, add 200 µL 2 M NaOH, 6 mL N-hexane-CH_2Cl_2 solution, shake for 5 min, and centrifuge at above 4000 r/min at 20-25 ℃for 5 min,
4. Static for 5-10 min, transfer all supernatant into a new centrifugal tube, blow to dry with nitrogen,
5. Dilute residues in 1 mL of the diluted redissolving solution,mix for 30s
6. Take sample solution, dilute at 1:9 (50 µL sample solution + 450 µL diluted redissolving solution), mix for 30s
7. Take 50 µL for further analysis.

Fold of dilution of the sample: 10 Detection limit: 1 ppb

5.2 Urine

1. Put 1 mL clear sample into 50 mL centrifuge tube, add 4 mL 0.1 M NaOH, shake properly for 2-5 min,
2. Transfer 1 mL liquid into another centerifugal tube, add 10 mL N-hexane, shake for 5 min, and centrifuge at above 4000 r/min at 20-25 ℃for 5 min,
3. Transfer 5 mL supernatant into a new centrifugal tube, blow to dry with nitrogen,
4. Dilute residues with 1 mL of the diluted redissolving solution,
5. Take 50 µL for further analysis.

Fold of dilution of the sample: 10 Detection limit: 1ppb

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5.3 Feed

1. Put 1.0 ± 0.05 g feed into 50 mL centrifugal tube, add 6 mL deionized water and 1 mL 1 M NaOH, vortex for 1 min, add 6 mL N-hexane-CH_2Cl_2 solution(5:3), shake properly for 5 min, centrifuge at above 4000 r/min at room temperature for 5 min,
2. Take 3 mL supernatant(upper layer), blow to dry with nitrogen at 50 ℃,
3. Dilute residues with 1 mL of the diluted redissolving solution,shake for 30s
4. Dilution: at 1:49(10 µL sample + 490 µL the diluted redissolving solution). shake for 30s
5. Take 50 µL for further analysis

Fold of dilution of the sample: 100 ***Detection limit: 10 ppb***

Notice: We recommend the standard 3 as cut off because of some interference.

6. ELISA procedures

6.1 Instructions

1. Bring all reagents and micro-well strips to the room temperature (20-25 ℃) before use.
2. Return all reagents to 2-8 ℃ immediately after use.
3. The reproducibility of the ELISA analysis, to a large degree, depends on the consistency of plate washing. The correct operation of plate washing is the key point in the procedures of ELISA.
4. For the incubation at constant temperatures, all the samples and reagents must avoid light exposure, and each microplate should be sealed by the cover membrane.

6.2 Operation procedures

1. Take out the kit from the refrigerated environment. Take out all the necessary reagents from the kit and place at the room temperature (20-25 ℃) for at least 30 min. Note that each liquid reagent must be shaken to mix evenly before use.
2. Take the required micro-well strips and plate frames. Re-sealed the unused microplate, stored at 2-8 ℃, not frozen.
3. Solution preparation: dilute the 20× concentrated washing buffer with the distilled or deionized water to 800 mL (or just to the required volume) for use.
4. Numbering: number the micro-wells according to samples and standard solution; each sample and standard solution should be performed in duplicate; record their positions.
5. Add 50 µL of the sample or standard solution to separate duplicate wells, add 50 µL of the antibody working solution into each well. Seal the microplate with the cover membrane, and incubate at 37 ℃ for 30 min.
6. Pour liquid out of mirowell, add 250 µL/well of washing buffer for 10 sec, repeat four to five times. flap to dry with absorbent paper (if there are the bubbles after flapping, cut them with the clean tips).
7. Add 100 µL enzyme conjugate into every well, seal the microplate with the cover membrane, incubate at 37 ℃ for 30 min, continue as described in 6.
8. Coloration: add 50 µL substrate A solution and 50 µL B solution into each well. Mix gently by shaking the plate manually, seal the microplate with the cover membrane and incubate at 37 ℃

for 15 min at dark for coloration .

9. Determination: add 50 μL stop solution into each well. Mix gently by shaking the plate manually. Set the wavelength of the microplate reader at 450 nm to determine the OD value (Recommend to read the OD value at the dual-wavelength 450/630 nm within 5 min)

7. Result judgment

There are to methods to judge the results; the first one is the rough judgment, while the second is the quantitative determination. Note that the OD value of the sample has a negative correlation with the content of Diazepam.

7.1 Qualitative determination

The concentration range (ng/mL) can be obtained from the comparison the average OD value of the sample with that of the standard solutin. Assuming that the OD value of the sample Ⅰ is 0.211, and that of the sample Ⅱ is 0.785, the OD value of standard solutins is: 2.100 for 0 ppb, 1.580 for 0.1 ppb, 1.010 for 0.3 ppb, 0.580 for 0.9 ppb, 0.308 for 2.7 ppb, 0.120 for 8.1 ppb, accordingly the concentration range of the sample Ⅰ is 2.7 to 8.1 ppb, and that of the sample Ⅱ is 0.3 to 0.9 ppb. (multiplied by the corresponding dilution fold)

7.2 Quantitative determination

The mean values of the absorbance values obtained for the average OD value (B) of the sample and the standard solution divided by the OD value (B0) of the first standard solution (0 standard) and subsequently multiplied by 100%, that is,

$$\text{Percentage of absorbance value} = \frac{B}{B0} \times 100\%$$

B—the average OD value of the sample or the standard solution

B0—the average OD value of the 0ng/mL standard solution

Draw the standard curve with the absorption percentages of the standard solutins and the semilogarithm values of the Diazepam standard solutins (ng/mL) as Y- and X-axis, respectively. Read the corresponding concentration of the sample from the standard curve by incorporating its absorption percentage into the standard curve. The resulting value is subsequently multiplied by the corresponding dilution fold, thus finally obtaining Diazepam concentration in the sample.

Using the professional analyzing software of this kit will be more convenient for the accurate and rapid analysis of a large amount of samples. (Please contact us for this software)

8. Precautions

1. The room temperature below 20 ℃ or the temperature of the reagents and the samples being not returned to the room temperature (20-25 ℃) will lead to a lower standard OD value.
2. Dryness of the microplate in the washing process will be accompanied by the situations including the non-linear standard curves and the undesirable reproducibility; So continue to next step immediately after washing.
3. Mix evenly, otherwise there will be the undesirable reproducibility.

4. The stop solution is the 2 M sulfuric acid solution, avoid contacting with the skin.
5. Do not use the kit exceeding its expiry date. The use of diluted or adulterated reagents from the kits will lead to the changes in the sensitivity and the detecting OD values. Do not exchange the reagents from the kits of different lot numbers to use.
6. Put the unused microplate into an auto-sealing bag to re-seal it. The standard substance and the colourless color former is light sensitive, and thus they cannot be directly exposed to the light.
7. Discard the colouration solution with any color that indicates the degeneration of this solution. The detecting value of the 0 standard solutin of less than 0.5 (A450 nm< 0.5) indicates its degeneration.
8. Colouration time is about 15 min, if the color is light, prolong the time of colouration but don't exceed 30 min.
9. The optimum reaction temperature is 25 ℃, and too high or low temperatures will result in the changes in the detecting sensitivity and OD values.

9. Storage and expiry date

Storage: store at 2-8 ℃, not frozen.

Expiry date: 12 months; date of production is on box.

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Green Earth depends on everyone' efforts

"Build of green Earth needs the cooperation of you and me"

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