Diethylstilbestrol (DES) ELISA Quantitation Kit

Manual

Catalog number: 40-784-140007

Immunoperoxidase Assay for Determination of Diethylstilbestrol (DES) in the Feed, Urine, Liver, Meat, Shrimp and Fish.
This kit is for research use only, and is not for use in diagnostic procedures.

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1. **PRINCIPLE OF THE TEST**

This test kit is based on the competitive enzyme immunoassay for the detection of Diethylstilbestrol (DES) in the feed, urine, liver, meat, shrimp and fish. The conjugate antigen is pre-coated on the micro-well stripes. The Diethylstilbestrol in the testing sample competes with the conjugate antigen pre-coated on the micro-well stripes, to interact with the antibodies against Diethylstilbestrol. After the addition of the enzyme conjugate, the TMB substrate is added for coloration. The optical density (OD) value of the testing sample has a negative correlation with the content of Diethylstilbestrol in the sample. This value is compared to the standard curve and the content of the corresponding Diethylstilbestrol is subsequently obtained.

2. **TECHNICAL SPECIFICATIONS**

**Sensitivity**

0.1ppb

**Detection Limit**

Shrimp, Fish ------------------------------0.2 ppb  
Meat, Liver-------------------------------2 ppb  
Urine--------------------------------------0.6 ppb  
Feed--------------------------------------20 ppb

**Recovery Rate**

Urine-----------------------------70±10%  
Feed------------------------------- 90±10%  
Tissue------------------------------- 85±10%

**Cross-Reaction Rate:**

DES ----------------------------100%  
Dienestrol------------------------38.5%  
Hexestrol-------------------------8.5%  
Ethinylestradiol--------------------< 0.1%  
Estriol---------------------------< 0.1%

3. **COMPONENTS**

1. Micro-well strips: 12 strips with 8 removable wells each  
2. 6× standard solution (1ml each): 0ppb, 0.1ppb, 0.3ppb, 0.9ppb, 2.7ppb and 8.1ppb  
3. Enzyme conjugate (12ml) ..............................red cap  
4. Antibody working solution (7ml) ......................blue cap  
5. Substrate A solution (7ml) ............................white cap  
6. Substrate B solution (7ml) ............................ black cap  
7. Stop solution (7ml) .................................yellow cap  
8. 20× concentrated washing buffer (40ml)..............white cap  
9. 2× concentrated re-dissolving solution (50ml)....... transparent cap
4. MATERIALS REQUIRED BUT NOT PROVIDED

1. Equipments
   - Microplate reader
   - Printer
   - Mixer or stomacher
   - Nitrogen-drying device
   - Oscillator
   - Centrifuge
   - Measuring pipets
   - Balance with a reciprocal sensibility of 0.01g

2. Micropipettors
   - Single-channel 20 to 200µl and 100 to 1000µl
   - Multi-channel 250µl

3. Reagents
   - NaOH
   - Acetonitrile (CH₃CN)
   - Acetone
   - Deionized water
   - H₃PO₄ (85%)
   - CHCl₃

5. SAMPLE PRE-TREATMENT

Instructions

The following points must be dealt with before the pre-treatment of any kind of sample:

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. 6M H₃PO₄: dissolve 100 ml H₃PO₄ in 150 ml of the deionized water, mix properly.

2. 1M NaOH: dissolve 4 g NaOH in deionized water to 100 ml.

3. 2M NaOH: dissolve 8 g NaOH in deionized water to 100 ml.

4. Acetonitrile-Acetone: mix 80 ml Acetonitrile and 20 ml Acetone evenly.
5. The 5× concentrated redissolving solution is mixed with deionized water at 1:5 (1 ml concentrated redissolving solution + 4 ml deionized water). The resulting solution will be used for the treated sample redissolving.

5.1 Feed

1. Weight 2±0.05g of the homogenized sample, add 8 ml Acetonitrile, shake properly for 510 min, centrifuge at above 3000 r/min at 15°C for 10 min.

2. Take 2 ml of the supernatant into a new vessel, blow to dry with 60°C nitrogen.

3. Add 0.5 ml CHCl₃, vortex for 20 s, add 2ml 1 M NaOH, vortex for 30 s, centrifuge at above 3000 r/min for 5 min.

4. Take 1 ml the clear supernatant, add 10 ul 6 M H₃PO₄, vortex for 5s.

5. Dilution: Compound feed----------take 50 ul sample, add 950ul diluted redissolving solution Concentrated / Premixed feed----take 25 ul sample, add 975 ul diluted redissolving solution.

6. Take 50 ul for analysis.

Fold of dilution of the sample:

<table>
<thead>
<tr>
<th>Compound Feed</th>
<th>Concentrated / Premixed Feed</th>
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<tbody>
<tr>
<td>100</td>
<td>200</td>
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</table>

5.2 Meat, Liver, Shrimp, Fish

1. Weight 2±0.05g of the homogenized sample, add 6 ml Acetonitrile- Acetone, shake for 10 min, and centrifuge at above 3000 r/min at 15°C for 10 min.

2. Transfer 3 ml of the supernatant into a new vessel, blow to dry with 60°C nitrogen /air. Add 0.5 ml CHCl₃, vortex for 20 s, add 2ml 1 M NaOH, vortex for 30 s, centrifuge at above 3000 r/min for 5 min.

3. Take 1 ml the clear supernatant, add 200ul 6 M H₃PO₄, vortex for 5s.

4. Add 3 ml Acetonitrile(CH₃CN) for extraction, shake properly for 10 min, centrifuge at above 3000 r/min at room temperature(20-25°C) for 10 min, take the upper layer, blow to dry with 60°C nitrogen/air.

5. Dissolve dry residues in 1 ml of the diluted redissolving solution.

6. Dilution: shrimp and finish-----directly take 50 ul water phase for detection
Meat and liver------ take 50ul water phase, add 450ul of the diluted redissolving solution and shake properly.

Fold of dilution of the sample:

<table>
<thead>
<tr>
<th>Shrimp and Fish</th>
<th>Meat and Liver</th>
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<tbody>
<tr>
<td>2</td>
<td>20</td>
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5.3 Urine

1. Take 2 ml urine into centrifuge tube, centrifuge at above 3000r/min at room temperature (20-25°C) for 10 min and stop when it is clear.

2. Transfer 1 ml clear urine into centrifuge tube, add 1M NaOH, shake vigorously for 5 min.
3. Add 100 ul 6 M H₃PO₄, vortex for 30 s.

4. Add 8 ml CHCl₃ for extraction; shake properly for 10 min, centrifuge at above 3000 r/min at 15˚C for 10min.

5. Remove the upper layer (water phase), take 4 ml of the lower layer, blow to dry with 60˚C nitrogen/air.

6. Dissolve dry residues in 3 ml of the diluted redissolving solution, take 50 ul for analysis.

**Fold of dilution of the sample: 6**

### 6. ASSAY PROCEDURE

#### 6.1 Instructions

1. Bring all reagents and micro-well strips to the room temperature (20 to 25˚C).

2. Return all reagents to 2 to 8˚C 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 micro-plate 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 to 25˚C) for at least 30min. 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 micro-plate, stored at 2 to 8˚C, not frozen.

3. Solution preparation: dilute the 20× concentrated washing buffer with the distilled or deionized water to a final volume of 800ml (or just to the required volume) for use.

4. Ordering: give different serial numbers to the different micro-wells each corresponding to a testing sample or a standard preparation; each testing sample or standard preparation should have two duplicate micro-wells; record their locations.

5. Add the testing sample and the standard preparation into each well, 50 ul each; add 50µl of the antibody working solution into each well. Seal the micro-plate with the cover membrane, and incubate at 25˚C for 30 min.

6. Pour the liquid, wash the micro-plate with the washing buffer at 250µl/well for four to five times. Each time soak the well with the washing buffer for 10S, flap to dry with absorbent paper (if there are the bubbles after flapping, cut them with the clean tips).

7. Add 100 ul enzyme conjugate into every well, seal the micro-plate with the cover membrane, react at 37˚C for 30 min and continue as step 6.

8. Coloration: add 50µl of the substrate solution and then 50µl of the B solution into each well. Vortex gently to mix evenly, seal the micro-plate with the cover membrane and incubate at 37˚C for 15 min at dark for coloration.
9. Determination: add 50µl of the stop solution into each well. Vortex gently to mix evenly. Set the wavelength of the micro-plate reader at 450nm to determine the OD value (we recommend to read the OD value at the dual-wavelength 450/630nm within 5m.

7. RESULTS

There are two methods to judge the results; the first one is used for the rough judgment, while the second for the quantitative determination. Note that the OD value of the testing sample has a negative correlation with the content of Diethylstilbestrol (DES).

7.1 Qualitative Determination

The concentration range (ng/ml) can be obtained form the comparison the average OD value of the testing sample with that of the standard preparation. Assuming that the OD value of the sample I is 0.310, and that of the sample II is 0.820, the OD value of standard preparations is: 1.510 for 0 ppb, 1.320 for 0.1 ppb, 1.03 for 0.3 ppb, 0.660 for 0.9 ppb, 0.389 for 2.7 ppb, 0.198 for 8.1 ppb, accordingly the concentration range of the sample I is 0.8 to 1.6 ppb, and that of the sample II is 0.2 to 0.4 ppb. (multiplied by the corresponding dilution fold)

7.2 Quantitative Determination

The mean values of the absorbance values is equivalent to the percentage of the average OD value (B) of the testing sample and the standard preparation divided by the OD value (B0) of the first standard preparation (0 standard) and subsequently multiplied by 100%, that is,

\[
\text{Percentage of Absorbance Value} = \left( \frac{B}{B_0} \right) \times 100\%
\]

\[
B = \text{the average (double wells) OD value of the testing sample or the standard preparation}
\]

\[
B_0 = \text{the average OD value of the 0ng/ml standard preparation}
\]

Draw the standard curve with the absorption percentages of the standard preparations and the semi logarithm values of the Diethylstilbestrol (DES) standard preparations (ng/ml) as Y- and X-axis, respectively. Read the corresponding concentration of the testing 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 the actual concentration of Diethylstilbestrol (DES) in the testing 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. Bring all reagents and micro-well strips to the room temperature (20 - 25°C).

2. Return all reagents to 2 to 8°C 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.

5. The room temperature below 20°C or the temperature of the reagents and the testing samples being not returned to the room temperature (20 to 25°C) will lead to a lower standard OD value.
6. 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.

7. Mix evenly; otherwise there will be the undesirable reproducibility.

8. The stop solution is the 2M sulfuric acid solution, being avoided contacting with the skin.

9. 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;

10. Put the unused microplate into an auto-sealing bag to re-seal it. The standard substance and the colorless color former is light sensitive, and thus they cannot be directly exposed to the light;

11. Discard the coloration solution with any color that indicates the degeneration of this solution. The detecting value of the 0 standard preparation of less than 0.5 indicates its degeneration.

9. STORAGE AND EXPIRATION DATE

Storage: Store at 2-8°C, not frozen.
Expiry Date: 6 Months; Date of production is on the box.

Troubleshooting

The following are some common problems encountered with the use of ELISA kits, and some of the causes of these problems.

1. Problem: Low absorbance
   □ Incorrect dilutions or pipetting errors.
   □ Improper incubation times
   □ Improper mixing of the TMB substrate. Each component is mixed in equal parts.
   □ Wrong filter on microtiter reader. Wavelength should be 450 nm for TMB, 490 nm for OPD, or 405 nm for ABTS.
   □ Kit materials or reagents are contaminated or expired.
   □ Incorrect reagents used.

2. Problem: High Absorbance
   □ Cross contamination from other samples or positive control.
   □ Incorrect dilutions or pipetting errors.
   □ Improper washing.
   □ Wrong filter on microtiter reader.
   □ Contaminated buffers or enzyme substrate.
   □ Improper incubation times.
   □ Kit materials or reagents are contaminated or expired.

3. Problem: Poor Duplicates
   □ Poor mixing of specimens.
   □ Incorrect dilutions or pipetting errors.
4. **Problem: All wells are positive**
   - Technical error.
   - Inconsistency in following ELISA protocol.
   - Inefficient washing.

5. **Problem: All wells are negative**
   - Procedure not followed correctly.
   - Contaminated buffers or enzyme substrate.
   - Contaminated conjugate.
   - Kit materials or reagents are contaminated or expired.