Importance of E_2 Determination

There is a growing concern about low level contamination of surface waters by hormones. Kolpin et. al 2002, reported that hormones were found in measurable quantities, up to 200 ppt in 11% of 139 streams sampled across the United States.

Estrogens are introduced directly into surface waters (0-200 ppt) through municipal wastewater treatment plant (WWTP) effluent sources, also called sewage treatment works (STW), and from runoff (0-1,400 ppt) from fields were animals graze or where animal wastes are applied. Currently, more than half of the biosolids produced by municipal wastewater treatment systems is applied to land as a soil conditioner or fertilizer and the remaining solids are incinerated or landfilled. These disposal practices provide numerous routes for estrogens reentry into environmental media and ultimately into surface water.

Of the hormones of concern, the most potent natural estrogen is 17-ßEstradiol (E_2). Elevated levels of E_2 in water (> 25 ppt) have been linked to adverse reproductive and developmental effects in fish.

For quantitation of estrogen hormones, instrumental anlaysis such as HPLC, LC-MS, and LC-MS/MS are generally employed. These analytical methods are highly reliable, however, they have several potential drawback including expensive instrumentation, large sample volume, xtensive purification, utilization of large amount of solvents, and need highly technical expertise in operation. Due to these shortcomings, the analysis of large number of samples may be both cost and time prohibitive. Therefore, there is a strong need for rapid, simple, and cost-effective methods for quantitative analysis of these contaminants, such as this ELISA.

Performance Data

Test sensitivity: The detection limit for this assay is 2.7 ppt (pg/mL). If lower detection needed, please contact Abraxis for Technical Bulletin.

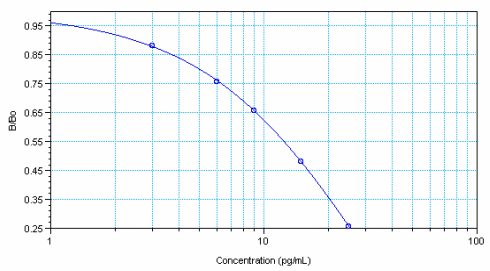
Test reproducibility: Coefficients of variation (CVs) for standards: <13%, for samples: < 20%.

Selectivity: The assay exhibits very good cross-reactivity with E2 and not with other non-related compounds tested.

Cross-reactivities:

E2	100% (per definition)
Estrone	75%
16-Keto E2	27%
Estriol	4.1%
17 alpha EE2	0.36%

The following compounds had cross-reactivity of < 1%: E2- 3 Glucoronide; E2-3 Sulfate; Cholesterol



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R100710

17-ßeta Estradiol ELISA (Microtiter Plate)

Enzyme-Linked Immunosorbent Assay for the Determination of 17-ßeta Estradiol (E_2) in Water Samples

Product No. 580004

1. General Description

The Abraxis E_2 ELISA is an immunoassay for the quantitative and sensitive detection of E_2 in water samples. A pre-sample concentration is not required. If necessary, positive samples can be confirmed by HPLC, or other conventional methods.

2. Safety Instructions

The substrate solution contains tetramethylbenzidine (TMB) and the stop solution contains diluted sulfuric acid. Avoid contact of the TMB and stopping solution with skin and mucous membranes. If these reagents come in contact with the skin, wash with water.

3. Storage and Stability

The E_2 ELISA should be stored in the refrigerator (4–8°C). Solutions should be allowed to reach room temperature (20-25°C) before use. Reagents may be used until the expiration date on the box. Consult state, local and federal regulations for proper disposal of all reagents.

4. Test Principle

The test is a direct competitive ELISA that allows the detection of E_2. It is based on the recognition of E_2 by specific antibodies. E_2, when present in a sample, and a E_2-HRP analogue compete for the binding sites of rabbit anti-E_2 antibodies in solution. The E_2 antibodies are then bound by a second antibody (goat anti-rabbit) immobilized in the plate. After a washing step and addition of the substrate solution, a color signal is generated. The intensity of the blue color is inversely proportional to the concentration of the E_2 present in the sample. The color reaction is stopped after a specified time and the color is evaluated using an ELISA reader. The concentrations of the samples are determined by interpolation using the standard curve constructed with each run.

5. Limitations of the E_2 ELISA, Possible Test Interference

Numerous organic and inorganic compounds commonly found in water samples have been tested and found not to interfere with this test. However, due to the high variability of compounds that might be found in water samples, test interferences caused by matrix effects can't be completely excluded. The presence of the following substances up to 10,000 ppm were found to have no significant effect on the E_2 Assay results: aluminum oxide, calcium chloride, calcium sulfate, manganese sulfate, magnesium sulfate, magnesium chloride, phosphate, sodium thiosulfate, sodium nitrate. Copper Chloride up to 1,000 ppm. ferric sulfate, zinc sulfate up to 100 ppm. Humic acid up to 10 ppm. Sodium Chloride up to 100,000 ppm.

The assay can tolerate up to 20% methanol and 10% acetonitrile.

Mistakes in handling the test can also cause errors. Possible sources for such errors can be:
Inadequate storage conditions of the test kit, wrong pipetting sequence or inaccurate volumes of the reagents, too long or too short incubation times during the immune and/or substrate reaction, and extreme temperatures during the test performance (lower than 10°C or higher than 30°C). The assay procedure should be performed away from direct sun light.

As with any analytical technique (GC, HPLC, etc…..) positive results requiring some action should be confirmed by an alternative method.

A. Materials Provided

1. Microtiter plate coated with a second antibody (goat anti rabbit).
2. Standards (6): 0, 3, 6, 9, 15, 25 pg/mL
3. Antibody solution (rabbit anti-E_2), 6 mL
4. E_2-HRP, 6 mL
5. Diluent/zero, 25 mL. Use to dilute samples with concentration above 25 ppt
6. Wash Solution 5X Concentrate, 100 mL
7. Color Solution (TMB), 16 mL
8. Stop Solution, 12 mL

B. Test Preparation

Micro-pipetting equipment and pipette tips for pipetting the standards and the samples are necessary. We recommend using a multi-channel pipette or a stepping pipette for adding the antibody, enzyme conjugate, substrate solution, and the stop solution in order to equalize the incubations periods of the standard solutions and the samples on the entire microtiter plate. Please only use the reagents and standards from one package lot in one test, as they have been adjusted in combination. Read and understand the instructions and precautions given in this insert before proceeding.

1. Adjust the microtiter plate and the reagents to room temperature before use.
2. Remove the number of microtiter plate strips required from the foil bag. The remaining strips are stored in the foil bag and zip-locked closed. Store the remaining kit in the refrigerator (4-8°C).
3. The standard, control, antibody solution, enzyme conjugate, substrate and stop solutions are ready to use and do not require any further dilutions.
4. The wash solution is a 5X concentrated solution and needs to be diluted with deionized water. In a 1L container dilute the 5X solution 1:5 (i.e. 100 mL of the 5X wash solution plus 400 mL of deionized water). The diluted solution is used to wash the microtiter wells.
5. The stop solution has to be handled with care as it contains diluted H_2SO_4.

C. Assay Procedure

1. Add 50 µL of the standard solutions, control or samples into the wells of the test strips according to the working scheme given. We recommend using duplicates or triplicates.
2. Add 50 µL of E_2 antibody solution successively to each well using a multi-channel or a stepping pipette. Cover wells with parafilm or tape to prevent contamination and evaporation. Thoroughly mix the contents of the wells by moving the strip holder in a rapid circular motion on the benchtop for a full 20-30 seconds. Be careful not to spill the contents. Incubate at ambient temperature for 30 minutes.
3. Add 50 µL of E_2 HRP conjugate solution to the individual wells successively using a multi- channel pipette or a stepping pipette. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a rapid circular motion on the bench top for about 30 seconds. Be careful not to spill contents. Incubate the strips for ninety (90) minutes at room temperature.
4. After incubation, remove the covering and vigorously shake the contents of the wells into a sink. Wash the strips four times using the 1X washing buffer solution. Please use at least a volume of 250 µL of washing buffer for each well and each washing step. Remaining buffer in the wells should be removed by patting the plate dry on a stack of paper towels.
5. Add 150 µL of substrate/color solution to the wells using a multi- channel pipette or a stepping pipette. The strips are incubated for 20 minutes at room temperature. Protect the strips from sunlight.
6. Add 100 µL of stop solution to the wells in the same sequence as for the substrate/color solution using a multi- channel pipette or a stepping pipette.
7. Read the absorbance at 450 nm using a microplate ELISA photometer within 15 minutes after stopping the reaction.

D. Evaluation

The evaluation of the ELISA can be performed using commercial ELISA evaluation programs (4-parameters, Logit/Log or alternatively point to point). For a manual evaluation, calculate the mean absorbance value for each of the standards. Calculate the $\%B/B_0$ for each standard by dividing the mean absorbance value for each standard by the Zero Standard (Standard 0) mean absorbance. Construct a standard curve by plotting the $\%B/B_0$ for each standard on a vertical linear (y) axis versus the corresponding E_2 concentration on horizontal logarithmic (x) axis on graph paper. $\%B/B_0$ for controls and samples will then yield levels in ppt of E_2 by interpolation using the standard curve.

The concentrations of the samples are determined using the constructed standard curve (do not use a previously stored curve). Samples showing a lower concentration than 3 ppt of E_2 should be reported as < 3 ppt. Samples showing a higher concentration than standard 6 (25 ppt) must be diluted to obtain more accurate results.

E. Additional Materials (not delivered with the test kit)

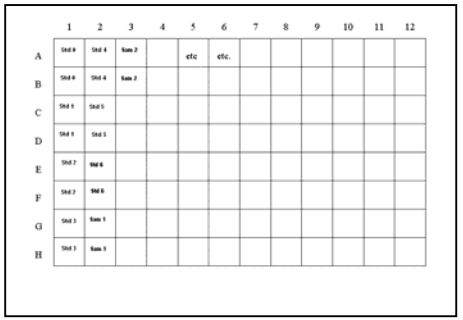
1. Micro-pipettes with disposable plastic tips (50-250 µL)
2. Multi-channel pipette (50-250 µL) or stepper pipette with plastic tips (50-250 µL)
3. Reagent reservoir for multichannel pipettes
4. Microtiter plate washer (optional)
5. Microtiter plate reader (wave length 450 nm)
6. Shaker for microtiter plates (optional)

F. Working Scheme

The microtiter plate consists of 12 strips of 8, which can be used individually. The standards must be run with each test. Never use the values of standards which have been determined in a test performed previously.

Std 0-Std 6: Standards

Sam1, Sam2, Sam3, etc.: Samples



Recovery

Three (3) groundwater samples, were spiked with various levels of E_2 and then assayed using the Abraxis E_2 Assay. The following results were obtained:

Amount of E2 Added (ppt)	Recovery Mean (ppt)	Recovery SD (ppt)	Recovery (%)
5	4.53	1.07	82
10	9.35	1.17	91
20	20.4	1.16	101
Average			95

Precision

The following results were obtained:

Control	1	2	3
Replicates	5	5	5
Days	5	5	5
n	25	25	25
Mean (ppt)	6.14	11.03	21.58
% CV (within assay)	5.0	3.3	3.2
% CV (between assay)		8.1	4.6
3.7			

Sensitivity

The Abraxis E2 ELISA has an estimated minimum detectable concentration, based on 90% B/Bo of 2.7 ppt.

G. References

(1) Kolpin, D.W., E.T. Furlong, M.T. Meyer, E.M. Thurman, S.D. Zaugg, L.B. Barber, and H.T. Buxton. 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: A national reconnaissance. *Environmental Science & Technology* 36, 1202-1211.