Ecologiena®

Estrogen(E1/E2/E3) ELISA KIT

(Microplate)

User's Guide

TOKIWA CHEMICAL INDUSTRIES CO.,LTD.
常盤化学工業株式会社

Estrogen (E1/E2/E3) ELISA KIT (Microplate)
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LIMITED WARRANTY

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The design of the Product is under constant review and every effort is made to keep this guide up to date, the Company reserves the right to change specifications and equipment at any time without prior notice.

Kit Feature

- ✧ ES(Estrogen) monoclonal antibody binds exclusively with E1, E2, and E3 and does not show cross-reaction with other chemicals of similar structures. A monoclonal antibody is uniform in quality, generating very little lot-to-lot variation.
- ✧ The quantitative analysis ranges from 0.05μg/L to 3μg/L (ppb).
- ✧ The ELISA measurement is highly reproducible; the coefficient of variation (CV) is mostly under 10%.
- ✧ The assay requires less amount of harmful solvent than instrument analyses.
- ✧ With ease of handling, the total time for measurement is only 2.5 hours.
- ✧ The kit, a 96-well microplate format, enables simultaneous measurement of multiple samples at more reasonable cost.

Measuring Principle

1. Competitive Reaction

The test is based on the recognition of ES(Estrogen) by specific monoclonal antibodies. ES present in the sample and an E2-enzyme conjugate (i.e. E2 labeled with a coloring enzyme:HPR) are premixed and added into each well of a microplate, and allowed to compete for limited number of binding sites of specific antibodies immobilized on the surface of the wells. When the ES concentration is higher relative to the enzyme conjugate, the ES will predominantly bind the antibody and vice versa.

2. Chromogenic Reaction

Unbound ES and excess E2-enzyme conjugates are washed out. The presence of ES is detected by adding a chromogenic substrate:TMB. The enzyme-labeled E2 bound to the ES antibody in the plate, catalyzes the conversion of the substrate to a colored product. After an incubation period, the reaction is stopped by the addition of a diluted acid. The higher the ES concentration in a sample, for example, leads to less antigen-enzyme conjugate bound to the antibody binding sites in a microplate well, generating a lighter color, i.e. lower absorbance.

3. Quantitative Analysis

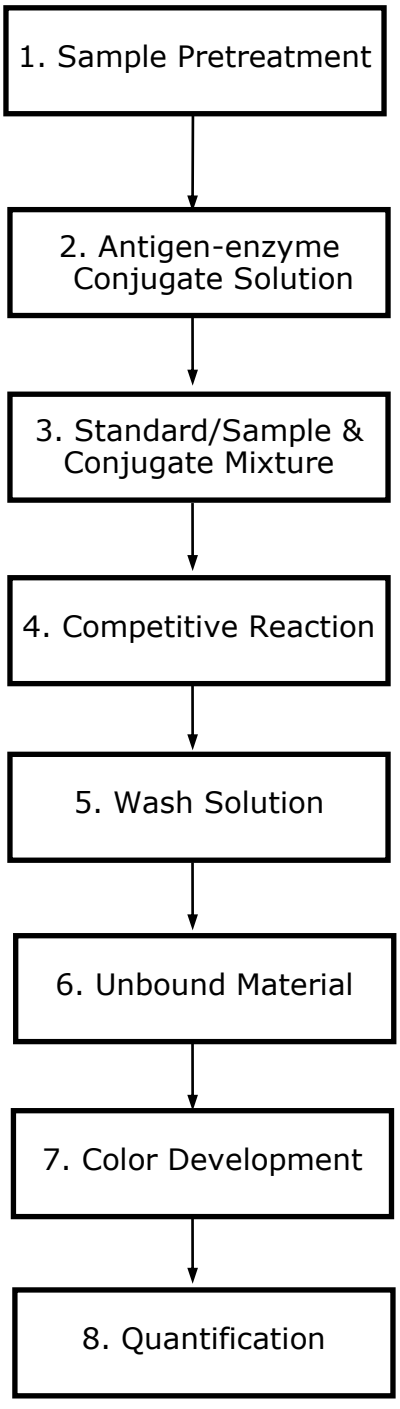
The standard curve, a dose-response curve obtained from known concentrations of E2 standards, is determined from the absorbance at 450nm. The ES concentration in each sample is accurately calculated by interpolation using the absorbance intensity obtained from the standard curve.

Note

The ES antibody binds with E1, E2, and E3. The total concentration of estrogens is reduced to the amount of E2, or 17β-Estradiol in the final quantitative analysis.

Flowchart for ES Measurement

<Please follow the steps described in Test Protocol (PP6-8)>



Take out the kit from the refrigerator for approximately half an hour before the assay and allow it to reach room temperature: 18-25°C. Filter the samples and add methanol to obtain a final methanol concentration of 10% (v/v). Solid phase extraction may be necessary for some samples.

Reconstitute Antigen-enzyme Conjugate powder (#3) with Buffer Solution (#4).

Mix 100µL of conjugate solution and 100µL of E2 standard (or sample) in each well of uncoated microplate (#8).

- *Dispense conjugate solution first then add standard solution or sample.*

Dispense 100µL aliquots of the above mixture into each coated well of a microplate (#1). Incubate it for 60 minutes at room temperature (18-25°C).

Dilute Wash Solution (6-fold concentration) (#5) in 5 times of its volume of pure water to prepare a wash solution.

Tap off the reaction liquid and rinse each microplate well with approximately 300µL of the wash solution and repeat the step twice more. Firmly tap out the plate to remove solution from the microplate. Blotting the plate against a paper towel is helpful.

Dispense 100µL of the color solution (#6, a brown vessel) into each well and incubate it for 30 minutes at room temperature (18-25°C). Then, add 100µL of Stop Solution (#7) to terminate reaction.

Measure the absorbance at 450nm for each standard solution and generate a standard curve. Calculate the quantity of ES in a sample from an absorbance reading and interpolation from the standard curve.

Kit Content

#	Contents	Volume	Quantity	Storage
1	MoAb-Coated Microplate	96 Wells	1 Plate	2-8°C
2	E2 Standard 0µg/L (10% Methanol)	1.5mL each	1 Vial each	2-8°C
	E2 Standard 0.05µg/L (10% Methanol)			
	E2 Standard 0.15µg/L (10% Methanol)			
	E2 Standard 0.5µg/L (10% Methanol)			
	E2 Standard 3.0µg/L (10% Methanol)			
3	Antigen-enzyme Conjugate powder		2 Vials	2-8°C
4	Buffer Solution	7mL	2 Vials	2-8°C
5	Wash Solution (6-fold concentration)	50mL	1 Vial	2-8°C
6	Color Solution (TMB) ***- brown vessel -***	15mL	1 Vial	2-8°C
7	Stop Solution (Citric acid)	15mL	1 Vial	2-8°C
8	Uncoated Microplate	96 Wells	1 Plate	---
9	Adhesive Plate Cover	---	1	---
10	Instruction Booklet	---	1	---

Other Essential Reagents/Materials

Essential - When Sample Concentration is NOT Required.

1. Disposable test tubes (e.g. ASAHI TECHNO GLASS, item No. 9831-1207)
   *Be sure to use disposable tubes to avoid ES adsorption.
2. Glass fiber filters (e.g. ADVANTEC Co., item No. 36481047 Φ47mm) and filtering equipment
3. Micropipettes (20µL - 200µL and 100µL - 1000µL, e.g. Gilson Pipetman P-200, P-1000) and tips
4. Multichannel pipettes (50µL - 300µL e.g. Finnpipette Digital 8-channel Pipettor) and tips
5. Microplate reader (450nm wavelength) (e.g. TECAN Sunrise Remote)
6. Stop watch
7. Strip ejector (e.g. COSTAR, No.2578)
8. Methanol (HPLC grade)
9. Pure water

Essential - When Sample Concentration through SPE is Required.

1-9. the same as above

10. Solid phase extraction cartridge (e.g. J.T. Baker SPE Column C18, cat # 562-20014; Bond Elut C18 Octadecyl, cat # 5010-11024)
11. Solid phase extraction cartridge (e.g. Waters Corporation, Sep-Pak Plus NH2 Cartridges CodeNo.020535)
12. Dichloromethane, n-Hexane

IMPORTANT

- Comparative tests should be needed if an alternate supplier is used for specified reagents or materials.

Test Protocol

IMPORTANT

- For research use only, not for human use.
- Take out all the kit contents from the refrigerator and let them reach room temperature (18-25°C) for approximately 30 minutes prior to the assay.
- Do not mix reagents from different kits.
- Store reagents under refrigeration (2-8°C)
- Do not use expired kits.
- Dispose of kit components in accordance with applicable regulations after use.
- Duplicate measurement is recommended for more accurate determination.

CAUTION

Wear appropriate protective clothing, gloves and eyewear to avoid any accidental contacts.

1. Sample Pretreatment

Filter raw water samples through the specified glass fiber filter (1µm pore diameter).
If there remains sediment on the filter, pour methanol to extract the analyte from the solid and add the eluant to the filtrate.
Make sure the amount of methanol does not exceed 1% of the total volume of the filtrate. i.e. For 1L of filtered sample, the amount of methanol should be less than 10mL.
Confirm the pH of the filtrate is between 5 and 8. If pH is out of this range, add acid or base to adjust pH.
If a sample concentration and clean-up are necessary, follow the solid phase extraction.

[Example]

1) Pour the filtrate, prepared above, through a C18 cartridge (ex. 500ml) preconditioned with methanol (ex. 5ml) and pure water (ex. 10ml).
2) Wash the C18 cartridge with pure water (ex. 5ml) and dry the column with vacuuming for about a minute. Wash the cartridge with hexane (ex. 5ml).
3) Elute the analyte with dichloromethane (ex. 5ml). Then, evaporate the solvent with nitrogen gas.
4) Dissolve the dried material in methanol (ex. 1ml), then pour it through an aminopropyl cartridge, which has been preconditioned with methanol (ex. 5ml). Keep the filtrate of the loaded sample in a tube.
5) Elute the remaining estrone with methanol (ex. 5ml) and receive the eluate into a tube, which contains the previously obtained filtrate. Then, evaporate the solvent with nitrogen gas.
6) Add 100% methanol to the residue and stir the mixture with a vortex. Terminate the mixing and pour pure water to adjust the content at 10% methanol (v/v).

IMPORTANT

- Dichloromethane is a possible carcinogen, classified as Group B in NTP and as Group 2B in IARC. Follow the applicable regulation when you use it.
- Keep the methanol concentration to be 10%. Higher methanol content may result in inaccurate readings.
- Use a new cartridge for each filtrate.

2. Antigen-enzyme Conjugate Solution

Reconstitute a bottle of antigen-enzyme conjugate powder (#3) with each buffer solution (#4) to prepare antigen-enzyme conjugate solution.

- Store the conjugate solution at 2-8°C; it will be stable for approximately 2 weeks. 7mL is sufficient for approximately 50 wells.
- Mix by filling the tip and expelling the contents with a pipette. Be sure not to generate bubbles when you transfer liquid.
- Mix a pair of reconstituted solutions when you use them altogether.

3. Mixture of Standard/Sample and Conjugate Solution

Transfer 100µL of conjugate solution and then transfer 100µL of E2 standard or 100µL of sample, prepared as 10 % (v/v) methanol solution, into each well of the uncoated microplate(#8) and mix by filling the tip and expelling the contents with a pipette.

- Dispense conjugate solution first, then add standard solution or sample to avoid non-specific adsorption on the inner surface of the well.
- Mix by filling the tip and expelling the contents with a pipette. Be sure not to generate bubbles when you transfer liquid.
- Use 10% methanol as a blank.

4. Competitive Reaction

Dispense 100µL aliquots of the mixture, prepared in the above Section 3, into each coated well of the microplate (#1). Tap the plate to make the liquid level horizontal. Incubate the microplate for 60 minutes at room temperature (18-25°C).

- Split the microplate, with a strip ejector for example, to use necessary number of wells. This microplate is breakable into 12 strips, each of which consists of 8 wells. Place back the unused plate strips in the packet, seal and store them at 2-8°C.
- Be sure not to generate bubbles when you transfer liquid to avoid erroneous reading. To remove them, tap a plate lightly.
- Cover a microplate with film, to avoid contamination and evaporation.
- Do not move or shake a microplate during the reaction.
- A temperature-controlled bath (18-25°C) is recommended.
- Secure the constant reaction time for each well, particularly to measure multiple samples.

5. Wash Solution

Dilute Wash Solution (6-fold concentration) (#5) in 5 times of its volume of pure water to prepare a wash solution, e.g. 20mL of concentrate and 100mL of pure water.

- Prepare the necessary amount of solution if you plan to run assays on different days with a split plate. The rule of thumb is 1.2mL of wash solution is required per well, i.e. approximately 120mL for a whole plate.
- The wash solution must be stored at 2-8°C; it will be stable approximately for a month after preparation.

6. Unbound Material

Rinse each microplate well with 300µL of the wash solution and repeat the step twice more. Then, firmly tap out the plate to remove solution from the microplate. Blotting the plate against a paper towel, a clean cloth or a lint-free towel is helpful.

- Be sure to remove any remaining solution, which may cause a measurement error.
- Be sure the bottom of the plate is free from any fingerprints or dirt.
  Otherwise absorbance readings will be significantly altered.
- Do not discharge any untreated waste liquid. For example, soak cloth or paper in fluid for incineration.

7. Color Development

Dispense 100µL of the color solution (#6, a brown vessel) into each microplate well and incubate the microplate for 30 minutes at room temperature (18-25°C). Then, add 100µL of Stop Solution (#7) to terminate the reaction.

- A temperature-controlled bath (18-25°C) is recommended.
- Secure the constant reaction time for each well, particularly to measure multiple samples.
- Each well colored with a blue color from the coloring reagent will turn yellow once the stop solution is added.

8. Quantification

Measure the absorbance at 450nm for each standard solution and samples with a plate reader.

- Measure the absorbance within 15 minutes after the reaction is stopped.
- Prepare a standard curve based on at least duplicate standards for every assay.
- Be sure the bottom of the plate is free from any fingerprints or dirt.
  Otherwise absorbance readings will be significantly altered.

The assay must be performed within the range between 0.05µg/L and 3µg/L. Samples of concentration beyond 3µg/L must be diluted with 10% methanol and re-tested. If the concentration of ES in a sample is completely unknown, more than one dilution of each pretreated sample is recommended to be included i

Several options are available for the calculation of the ES concentration in samples.

(1)Computer aided Calculation

Calculate using microplate analysis software.
A 4-parameter logistic fitting software is recommended, for example " Delta Soft " from BioMetallics, Inc., Princeton, NJ (http://www.microplate.com).

(2)Graph Paper (Section Paper) aided Fitting

Calculate using Log-Log (or Log-Linear) Graph Paper (Section Paper) Fitting.
X-axis : E2 concentration
Y-axis : Optical Density(OD) or Inhibition Rate(B/B0%)

Inhibition Rate(B/Bo%) = (Sample or standard OD)/(OD at E2 standard=0)

(Example)

Standard OD or B/B0%

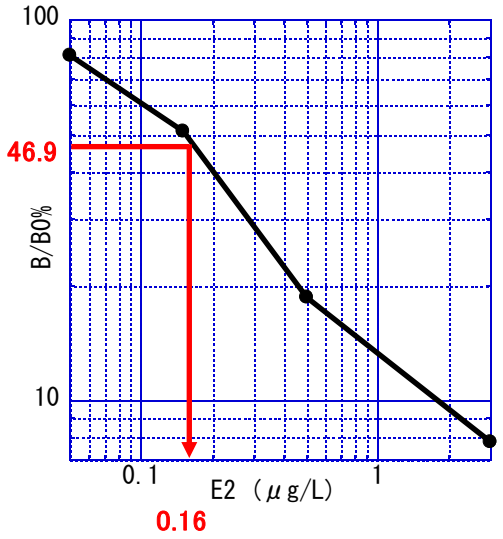
E2(µg/L)	OD	B/B0%
0	1.264	100
0.05	1.019	80.6
0.15	0.646	51.1
0.50	0.236	18.7
3.00	0.099	7.8

Example:

E2(µg/L)	OD	B/B0%
0.16	0.593	46.9

Log-Log Graph Paper Calculation

E2=0.16(µg/L) from B/B0%=46.9%



APPENDIX
1. Plate Layout

ES MoAb-Coated Microplate has 96 wells breakable into 8 x 12 strips.

Example 1) Full Plate Format

Five different concentrations of E2 standards (0, 0.05, 0.15, 0.5, 3μg/L) are assayed in duplicates. The standards take up 10 wells, leaving the rest of 86 wells for samples. With duplicate measurement, the whole plate can take 43 samples altogether.

	1	2	3	4	5	6	7	8	9	10	11	12
A	**0**	**0**	S04	S04	S12	S12	S20	S20	S28	S28	S36	S36
B	**0.05**	**0.05**	S05	S05	S13	S13	S21	S21	S29	S29	S37	S37
C	**0.15**	**0.15**	S06	S06	S14	S14	S22	S22	S30	S30	S38	S38
D	**0.5**	**0.5**	S07	S07	S15	S15	S23	S23	S31	S31	S39	S39
E	**3.0**	**3.0**	S08	S08	S16	S16	S24	S24	S32	S32	S40	S40
F	S01	S01	S09	S09	S17	S17	S25	S25	S33	S33	S41	S41
G	S02	S02	S10	S10	S18	S18	S26	S26	S34	S34	S42	S42
H	S03	S03	S11	S11	S19	S19	S27	S27	S35	S35	S43	S43

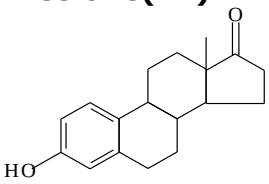
Example 2) Partial Plate Format

Six different concentrations of E2 standards are assayed in duplicates. The plate is split into two for independent assays. Half a plate can take up to 19 samples with duplicate measurement.

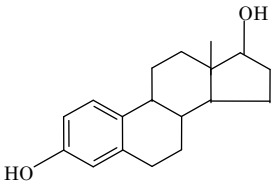
	1	2	3	4	5	6	7	8	9	10	11	12
A	**0**	**0**	S04	S04	S12	S12	**0**	**0**	S04	S04	S12	S12
B	**0.05**	**0.05**	S05	S05	S13	S13	**0.05**	**0.05**	S05	S05	S13	S13
C	**0.15**	**0.15**	S06	S06	S14	S14	**0.15**	**0.15**	S06	S06	S14	S14
D	**0.5**	**0.5**	S07	S07	S15	S15	**0.5**	**0.5**	S07	S07	S15	S15
E	**3.0**	**3.0**	S08	S08	S16	S16	**3.0**	**3.0**	S08	S08	S16	S16
F	S01	S01	S09	S09	S17	S17	S01	S01	S09	S09	S17	S17
G	S02	S02	S10	S10	S18	S18	S02	S02	S10	S10	S18	S18
H	S03	S03	S11	S11	S19	S19	S03	S03	S11	S11	S19	S19

2. Chemical Structure of Estrogens

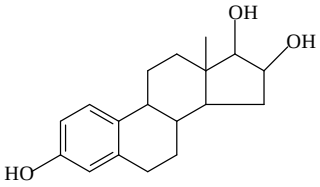
Estrone(E1)



Estradiol(E2)



Estriol(E3)



3. Cross-reactivity Pattern

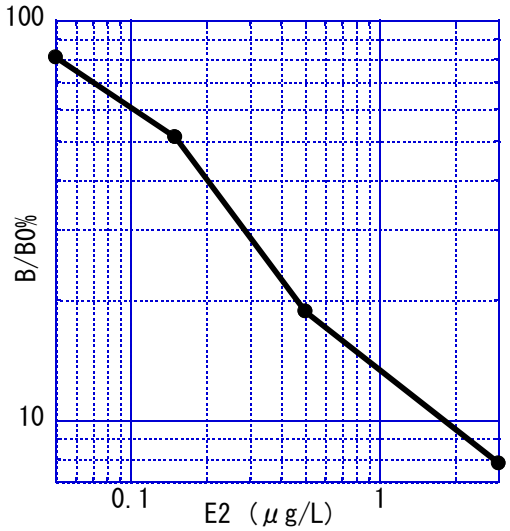
Estrogens

Compound	% Reactivity
Estrone (E1)	87.0
2-methoxy E1	<0.03
E1-3-sulfate	<0.03
17β-Estradiol (E2)	100.0
16-keto E2	118.0
2-methoxy E2	0.2
E2-17-glucronide	5.0
E2-3-glucronide	<0.03
E2-3-sulfate-17-glucronide	0.5
Estriol (E3)	55.0
16-epi-E3	129.0
E3-16-glucronide	48.0
Etynylestradiol (EE2)	0.7

Other Hormones

Compound	% Reactivity
cis-Androsterone	<0.03
trans-Androsterone	<0.03
Cholesterol	<0.03
Dehydroisoandrosterone	<0.03
5α-Dihydrotestosterone	<0.03
Hydrocortisone	<0.03
Pregnenolone	<0.03
Testosterone	<0.03

4. E2 Standard Curve



Samples containing Estrogen within the dynamic range between 0.05μg/L and 3μ/L can be directly applied to assay after filtration.
Estrogen content below the range must be concentrated with solid phase extraction prior to the ensuing session.

Coefficient of variation (CV) is generally under 10% throughout the dynamic range.

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