Ecologiena®

Ethynylestradiol(EE2) ELISA KIT (Microplate)

User's Guide

Japan EnviroChemicals, Ltd.

Ethynylestradiol ELISA KIT (Microplate)
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LIMITED WARRANTY

Japan EnviroChemicals, Ltd. (the Company, hereunder) warrants its products. (the Product, hereunder) to be manufactured in accordance with its specifications and free from defects in material. This warranty is expressly limited to the refund of the price of any defective Product or the replacement of any defective Product with new Product. This warranty applies only when the Buyer gives written notice to the Company within thirty (30) days after the receipt of the Product by the Buyer. In addition, this warranty applies under conditions of normal use, but does not apply to defects that result from intentional damage, negligence or unreasonable use.

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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

- ✧ EE2 monoclonal antibody binds exclusively with EE2 and does not show cross-reaction with other hormones or chemicals of similar structures. A monoclonal antibody is uniform in quality, generating very little lot-to-lot variation.
- ✧ The quantitative analysis ranges from 0.05 g/L to 3 g/L (ppb), sensitive enough to detect EE2 in field or biological specimens such as river water, wastewater, or blood, etc.
- ✧ With ease of handling, the total time for measurement is only 2.5 hours.
- ✧ The ELISA measurement is highly reproducible; the coefficient of variation (CV) is generally under 10%.
- ✧ The kit, a 96-well microplate format, enables simultaneous measurement of multiple samples at more reasonable cost.

Measuring Principle
(Competitive ELISA)

1. Competitive Reaction

The test is based on the recognition of EE2 by specific monoclonal antibodies. EE2 present in the sample and a EE2-enzyme conjugate (i.e. EE2 labeled with a coloring enzyme) 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 EE2 concentration is higher relative to the enzyme conjugate, the EE2 will predominantly bind the antibody and vice versa.

2. Chromogenic Reaction

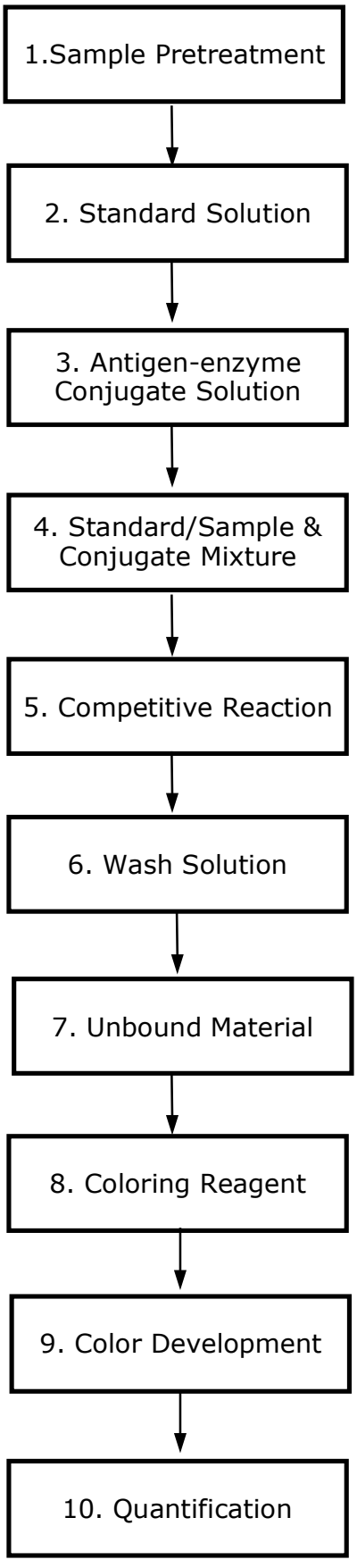
Unbound EE2 and excess EE2-enzyme conjugates are washed out. The presence of EE2 is detected by adding a chromogenic substrate. The enzyme-labeled EE2 bound to the EE2 antibody in the plate, catalyzes the conversion of the substrate to a colored product. After an incubation period, the reaction is stopped by the addition of a diluted acid. The higher the EE2 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 EE2 standards, is determined from the absorbance at 450nm. The EE2 concentration in each sample is accurately calculated by interpolation using the absorbance intensity obtained from the standard curve.

Flowchart for EE2 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.

Dilute the EE2 Standard Concentrate (#2) 10 fold with 10% methanol to prepare 10_g/L EE2 diluted stock solution. Then, further dilute this solution to prepare designated concentration of EE2 standards, from 0.05_g/L to 3_g/L.

Reconstitute Antigen-enzyme Conjugate powder (#3) with 7mL out of 8mL Buffer Solution (#4, white cap).

Mix 100_L of EE2 standard (or sample) and 100_L of conjugate solution in each well of uncoated microplate (#9).

Dispense 100_L 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 distilled water to prepare a wash solution.

Rinse each microplate well with approximately 300_L 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.

Mix Chromogen Solution (#6) and Substrate Solution (#7) at a ratio of 1:100 to prepare a coloring reagent.

Dispense 100_L of the coloring reagent into each well and incubate it for 30 minutes at room temperature (18-25°C). Then, add 100_L of Stop Solution (#8, a black cap) to terminate reaction.

Measure the absorbance at 450nm for each standard solution and generate a standard curve. Calculate the quantity of EE2 in a sample from the 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	EE2 Standard Concentrate (100_g/L 10%MeOH)	4mL	1 Vial	2-8°C
3	Antigen-enzyme Conjugate	7mL	2 Vials	2-8°C
4	Buffer Solution ***– white cap -***	8mL	2 Vials	2-8°C
5	Wash Solution (6-fold concentration)	50mL	1 Vial	2-8°C
6	Chromogen Solution	250_L	1 Vial	2-8°C
7	Substrate Solution ***– red marker -***	15mL	1 Vial	2-8°C
8	Stop Solution ***– black cap -***	15mL	1 Vial	2-8°C
9	Uncoated Microplate	96 Wells	1 Plate	---
10	Plate Cover	---	1	---
11	Instruction Booklet	---	1	---

Other Essential Reagents/Materials

Essential - When Sample Concentration is NOT Required.

1. Disposable test tubes (e.g. IWAKI, item No. 9831-1207)
   *Be sure to use disposable tubes to avoid EE2 adsorption.
2. Glass fiber filters (e.g. ADVANTEC Co., item No. 36481047 _47mm) and filtering equipment
3. Micropipettes (20_L - 200_L and 100_L -1000_L, e.g. Gilson Pipetman P-200, P-1000) and tips (e.g. ICN Superpack 96NS)
4. Multichannel pipettes (50_L - 300_L e.g. LabSystems Finnpipette Digital 8-channel Pipettor) and tips (e.g. ICN Superpack 96NS)
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)

Essential - When Sample Concentration through SPE is Required.

1-8. the same as above

9. Solid phase extraction cartridge (e.g. J.T. Baker SPE Column C18, cat # 562-20014; Bond Elut C18 Octadecyl, cat # 5010-11024)
10. Dichloromethane, Hexane (HPLC grade)

IMPORTANT

- Comparative tests should be performed 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 (1_m pore diameter). If there remains sediment on the filter, pour MeOH to extract the analyte from the solid and add the eluant to the filtrate.
Make sure the amount of MeOH dose not exceed 1% of the total volume of the filtrate. i.e. For 1L of filtered sample, the amount of MeOH 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. 500mg) preconditioned with methanol (ex. 5ml) and distilled water (ex. 10ml).
2) Wash the cartridge with distilled water (ex. 5ml). Keep suctioning for about a minute to dry the cartridge. Then, wash the cartridge with hexane (ex. 5ml).
3) Elute the analyte with dichloromethane (ex. 5ml). Then, evaporate the solvent with nitrogen gas.
4) Add 100% methanol to the residue and stir the mixture with a vortex. Terminate the mixing and add distilled water to adjust the content at 10% methanol (v/v).
   If there remains residue, add 100% DMSO and MeOH to adjust the ratio at 1%DMSO and 10%MeOH solution. Then, the solvent composition in each standard solution must be prepared as 1%DMSO and 10%MeOH.

The sample pretreatment protocol is under constant review. Please refer to our web site for the latest information (http://www.jechem.co.jp/eco/index-e.html).

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. Standard Solution

1) 10-fold Diluted Stock

!! IMPORTANT !!

First, dilute the 100_g/L EE2 concentrate solution (#2, 10% v/v methanol) with 9 times of its volume of 10% v/v methanol to prepare the 10_g/L EE2 diluted stock solution.

2) EE2 Standard Preparation

Dilute the 10_g/L solution, prepared above, in 10% methanol to obtain EE2 from 0.05_g/L to 3_g/L, which represents the dynamic range of this kit. The following is an example.

Standard solution	(_g/L)	3.0	1.0	0.3	0.1	0.05	0
10_g/L EE2 concentrate	(_L)	300	100	30	20	20	0
10% Methanol	(_L)	700	900	970	1980	3980	1000
Total	(_L)	1000	1000	1000	2000	4000	1000

※If you miss 10-fold dilution step to prepare standard solutions directly from the 100 _g/L EE2 concentrate (#2), the resulting concentration is 10 times as high as that of originally anticipated and the absorbance reading is lowered around 0.2-0.5. Be sure to dilute the concentrate 10 fold as the first step.

- Prepare the standard EE2 solution just before the test. Standard solutions, once diluted from the concentrate, are NOT reusable at a later date. Prepare new standard solution for every test session.
- Disposable glass tubes are recommended for dilution to minimize adsorption and contamination.
- In order to minimize adsorption on the wall, be sure to dispense 100_g/L EE2 concentrate (#2) first in a tube and then add 10% methanol to prepare 10_g/L solution.
- Dilute directly from 10_g/L EE2 stock solution to prepare the designated concentration of EE2 (0.05, 0.1, 0.3, 1.0, 3.0_g/L) to minimize adsorption on the walls of the tube.
- Be sure the standard concentrate is tightly capped after use and store it in a refrigerator. The standard solution must also be sealed or capped tightly to avoid methanol evaporation.
- Keep the methanol concentration of standard solutions at 10%. Higher methanol content in the sample may damage antibody and lower content may result in inaccurate readings.
- Mix by filling the tip and expelling the contents with a pipette. Do not stir vigorously, with a Vortex mixer for example to prevent its non-specific adsorption onto the test tube surface.
- Do not discharge any untreated waste liquid. For example, soak cloth or paper in fluid for incineration. Dispose according to local, state or federal regulations.

3. Antigen-enzyme Conjugate Solution

Reconstitute a bottle of antigen-enzyme conjugate powder (#3) with 7mL out of 8 mL buffer solution (#4, white cap) 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.

4. Mixture of Standard/Sample and Conjugate Solution

Transfer 100_L of EE2 standard, prepared in Section 2-2), or 100_L of sample, prepared as 10 % (v/v) methanol solution, and then transfer 100_L of conjugate solution into each well of the uncoated microplate (#9) and mix by filling the tip and expelling the contents with a pipette.

- Dispense standard solution first, and then add conjugate solution 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 solution as a blank.

5. Competitive Reaction

Dispense 100_L aliquots of the mixture, prepared in the above Section 4, into each coated well of the microplate (#1). Tap the plate lightly 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 the 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 the 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 (18-30°C) is recommended.
- Secure the constant reaction time for each well, particularly to measure multiple samples.

6. Wash Solution

Dilute Wash Solution (6-fold concentration) (#5) in 5 times of its volume of distilled water to prepare a wash solution, e.g. 20mL of concentrate and 100mL of distilled 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 120 mL for a whole plate.
- The wash solution must be stored at 2-8°C; it will be stable approximately for a month after preparation.

7. Unbound Material

Rinse each microplate well with approximately 300_L 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.

8. Coloring Reagent

Mix Chromogen Solution (#6) and Substrate Solution (#7, a red mark on a white cap) at a ratio of 1:100 to prepare the coloring reagent, e.g. add 120_L of Chromogen Solution (#6) to 12 mL of Substrate Solution (#7) while stirring gently with a pipette tip.

- Prepare the coloring reagent within 15 minutes before the reaction.
- Dispense Substrate Solution first and then add Chromogen Solution.
- Prepare the mixture to the minimum necessary. 1mL of mixture is enough for 8 wells; approximately 12 mL is necessary for the whole plate. Screw the caps tightly and keep them in a refrigerator.
- The solution cannot be stored even under refrigeration.

9. Color Development

Dispense 100_L of the coloring reagent mixture, prepared in Section 8, into each microplate well and incubate the microplate for 30 minutes at room temperature (18-25°C). Then, add 100_L of Stop Solution (#8, a black cap) to terminate the reaction.

- A temperature-controlled bath (18-30°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.

10. Quantification

Measure the absorbance at 450nm for each standard solution and generate a standard curve. Calculate the quantity of EE2 in a sample from the absorbance reading and interpolation from the standard curve.

- 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 0.05_g/L and 3_g/L. Samples of concentration beyond 5_g/L must be diluted with 10% methanol and re-tested. If the concentration of EE2 in a sample is completely unknown,

more than one dilution of each pretreated sample is recommended to be included in the assay.

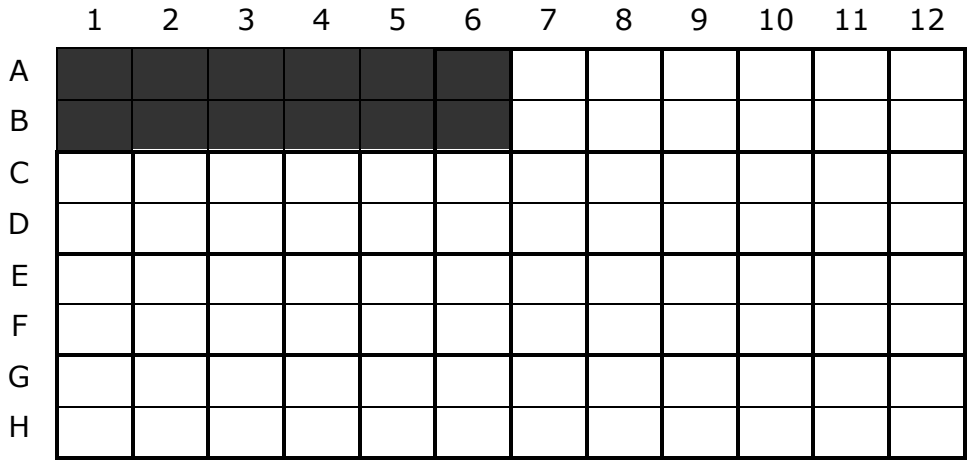
APPENDIX

1. Plate Layout

EE2 MoAb-Coated Microplate has 96 wells breakable into 8 x 12 strips.

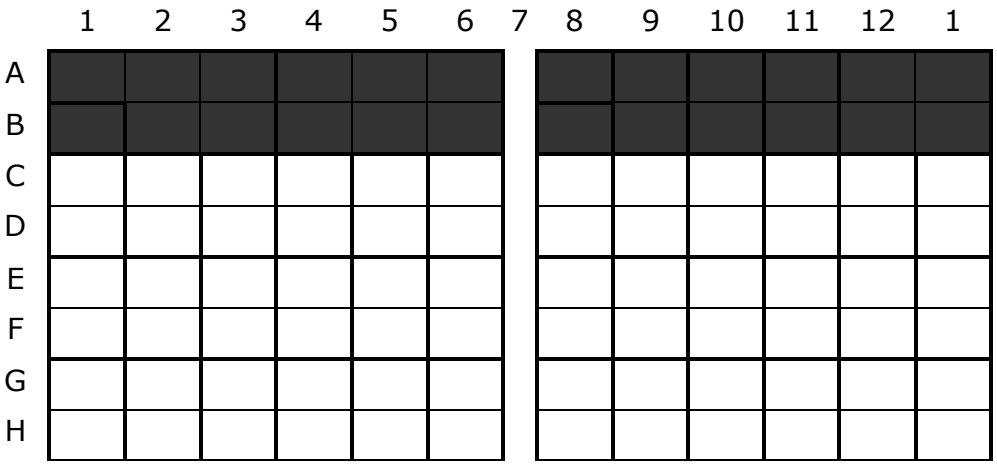
Example 1) Full Plate Format

Six different concentrations of EE2 standards (0, 0.05, 0.1, 0.3, 1.0, 3.0_g/L) are assayed in duplicates. The standards take up 12 wells, leaving the rest of 84 wells for samples. With duplicate measurement, the whole plate can take 42 samples altogether.

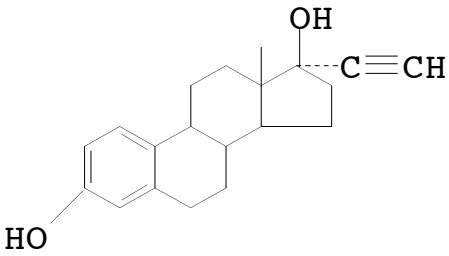


Example 2) Partial Plate Format

Six different concentrations of EE2 standards are assayed in duplicates. The plate is split into two for independent assays. Half a plate can take up to 18 samples with duplicate measurement.



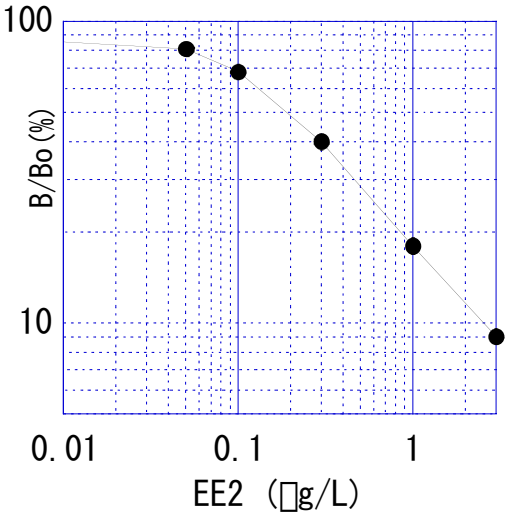
2. Chemical Structure of EE2 Standard



3. Cross-reactivity Pattern

Compound	Reactivity (%)
Ethynylestradiol(EE2)	100.0
Estrone(E1)	<0.2
2-methoxy E1	<0.2
17β-Estradiol(E2)	<0.2
16-keto-E2	<0.2
E2-17-glucuronide	<0.2
E2-3-glucuronide	<0.2
E2-3-sulfate-17-glucuronide	<0.2
Estriol(E3)	<0.2
16-epi-E3	<0.2
E3-16-glucuronide	<0.2

4. Ethynylestradiol Standard Curve



Samples containing Ethynylestradiol (EE2) within the dynamic range (0.05_g/L- 3_g/L) can be directly applied to assay after filtration.

Samples with Ethynylestradiol 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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