***Ecologiena*®**

APE ELISA KIT

(Microplate)

User's Guide

Japan EnviroChemicals, Ltd.

APE 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

- ✧ Alkylphenol Ethoxylate (APE) monoclonal antibody binds exclusively with APE 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 0.02mg/L to 1mg/L (ppm).
- ✧ A simple filtration through glass filter is generally sufficient as a pretreatment before measurement. Solid phase extraction may be necessary if sample is required for concentration and/or clean-up.
- ✧ 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 APE by specific monoclonal antibodies. APE present in the sample and an APE-enzyme conjugate (i.e. APE 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 APE concentration is higher relative to the enzyme conjugate, the APE will predominantly bind the antibody and vice versa.

2. Chromogenic Reaction

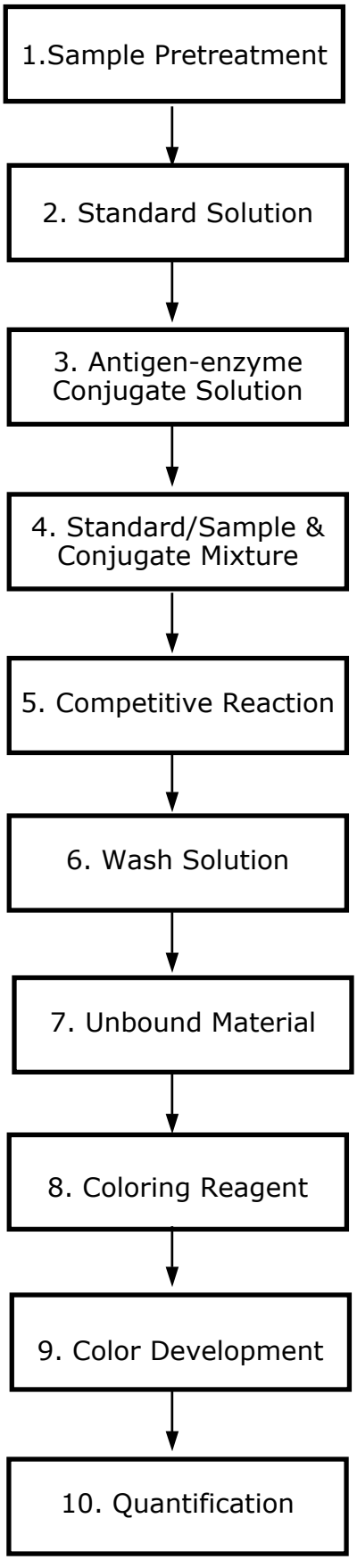
Unbound APE and excess APE-enzyme conjugates are washed out. The presence of APE is detected by adding a chromogenic substrate. The enzyme-labeled APE bound to the APE antibody in the plate, catalyzes the conversion of the substrate to a colored product. After an incubation period, the reaction is stopped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

Flowchart for APE 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 APE Standard Concentrate (#2) to prepare designated concentration of APE standards, from 0.02mg/L to 1mg/L.

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

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

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 distilled water to prepare a wash solution.

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.

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

Dispense 100µL of the coloring reagent into each well and incubate it for 30 minutes at room temperature (18-25°C). Then, add 100µL 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 APE 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	APE Standard Concentrate (NP10EO, 4mg/L, 20%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	250µL	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. Glass disposable test tubes (e.g. IWAKI, item No. 9831-1207)
   *Be sure to use disposable tubes to avoid APE 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 (e.g. ICN Superpack 96NS)
4. Multichannel pipettes (50µL - 300µL 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. Bakerbond spe™ Octadecyl, Disposable Extraction Columns Code. No. 562-20014)

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).

Test Protocol (continued)

- 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

Clear Sample: Add 100% MeOH to be 10% (v/v) methanol solution. 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.

Turbid Sample: Filter the sample through a glass fiber filter (To save time, suctioning with a vacuum pump is recommended). Wash the residue, if any, with 100% MeOH (Make sure the amount of MeOH dose not exceed 1% of the total volume of the filtrate). Adjust MeOH content of the filtrate to be 10% (v/v). Confirm the pH of the solution as described in "Clear Sample."

Sample with Low APE: Concentrate the sample with solid phase extraction as follows.

1) Pour 5 ml methanol followed by 5 ml distilled water through a solid phase extraction column such as Sep-Pak PS-2.
2) 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.
3) Pressurize or vacuum the column to adjust a flow rate at 10-20ml/minute.
4) Wash the column with 10 ml distilled water and then dry it with vacuuming or nitrogen gas.
5) Elute the analyte with 10 ml of 100% MeOH.
6) Dilute the eluant with distilled water to prepare 10% MeOH solution. If concentration is necessary, evaporate the eluant and dissolve the residue in 10% MeOH.

This sample pretreatment method is also applicable to Linear Alkylbenzene Sulfonate (LAS) and AE (AlkylEthoxylate) ELISA kits. 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).

2. Standard Solution

Dilute the 4mg/L APE concentrate (#2) with distilled water and methanol to obtain APE standards from 0.02mg/L to 1mg/L, which represents the dynamic range of this kit. The following is an example.

Standard solution	(mg/L)	1	0.4	0.1	0.05	0.02	0
4mg/L APE concentrate	(µL)	250	100	25	25	20	0
100% methanol	(µL)	50	80	95	195	396	100
Distilled Water	(µL)	700	820	880	1780	3584	900
Total	(µL)	1000	1000	1000	2000	4000	1000

- Prepare the standard APE 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 APE adsorption on the walls of the tube, be sure to dispense APE concentrate (#2) first in a tube and then add 10% methanol and then distilled water.
- 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 foaming and non-specific adsorption onto the glass surface.
- 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.
- 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 100µL of APE standard, prepared in Section 2, or 100µL of sample, prepared as 10 % (v/v) methanol solution, and then transfer 100µL 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 or sample first, 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 as a blank.

5. Competitive Reaction

Dispense 100µL 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 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.

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 120µL 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 100µL 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 100µL 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 APE 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 0.02mg/L and 1mg/L. Samples of concentration beyond 1mg/L must be diluted with 10% methanol and re-tested. If the concentration of APE in a sample is completely unknown, more than one dilution of each pretreated sample is recommended to be included in the assay.

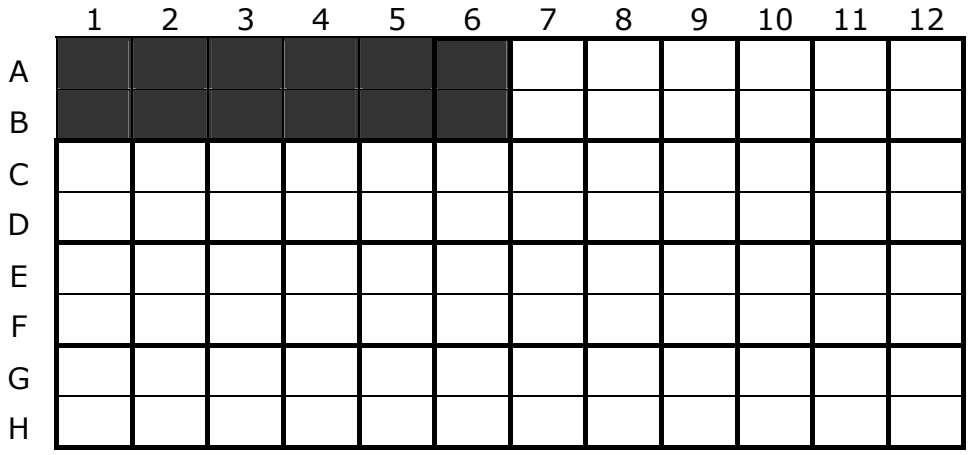
APPENDIX

1. Plate Layout

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

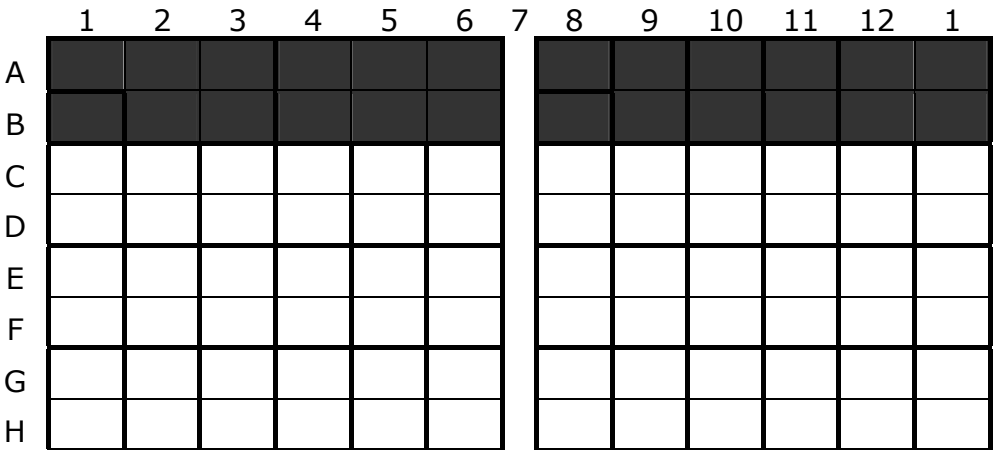
Example 1) Full Plate Format

Six Seven different concentrations of APE standards (0, 0.02, 0.05, 0.1, 0.4, 1mg/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.

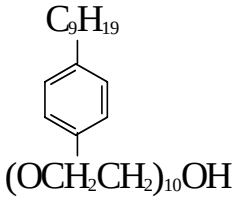


Example 2) Partial Plate Format

Six different concentrations of APE 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 Alkylphenol Ethoxylate (APE)



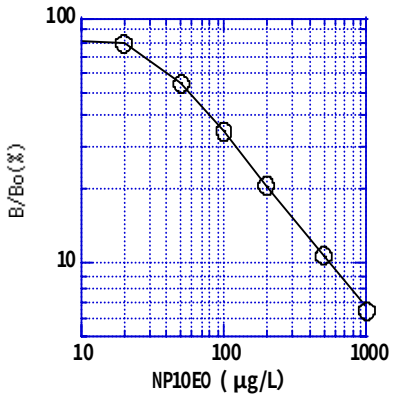
Average length of EO chain = 10

3. Cross-reactivity Pattern

Compound		% reactivity
Nonionic surfactants		
NPE(Nonylphenol ethoxylate)		
NP10EO	(Ave. EO chain length 10)	100
NP7.5EO	(Ave. EO chain length 7.5)	100
NP5EO	(Ave. EO chain length 5)	80
NP2EO	(Ave. EO chain length 2)	40
NP1EO	(Nonylphenol)	20
OPE(Octylphenol ethoxylate)		
OP10EO	(Ave. EO chain length 10)	230
AE (Alkyl ethoxylate)		<0.2
Anionic surfactants		
LAS	(Linear alkylbenzene sulfonate)	<0.2
SOAP	(Sodium laurate)	<0.2
SDS	(Sodium dodecyl sulfate)	<0.2
AES	(Alkylether sulfate)	<0.2
Phenol		<0.2
PEG	(Polyethylene glycol)	<0.2

The monoclonal antibody has a high specificity to APE with various polyethoxylic chain length (n=1-22) and doesn't cross-react with other surfactants or compounds of similar structure.

4. APE Standard Curve



This test kit has a wide detection range between 0.02mg/ and 1mg/L.
Samples within this range can be directly applied to assay only after filtration.

Samples outside the upper limit must be diluted with 10% methanol. Samples with APE 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.

Ver 0311

Japan EnviroChemicals, Ltd.

Tokyo Office: Seavans North 9F, 2-1 Shibaura, 1-chome, Minato-ku, Tokyo 105-0023
TEL +81-3-5444-9891 FAX +81-3-5444-9860
E-mail eco@jechem.co.jp

URL: http://www.jechem.co.jp/eco/index-e.html