Ecologiena®

APE ELISA KIT

(Microplate)

User's Guide

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

APE ELISA KIT (Microplate)
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LIMITED WARRANTY

Tokiwa Chemical Industries CO.,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 Alkylphenoxy polyethoxy acetic acid (APnEC), 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 :HRP) 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

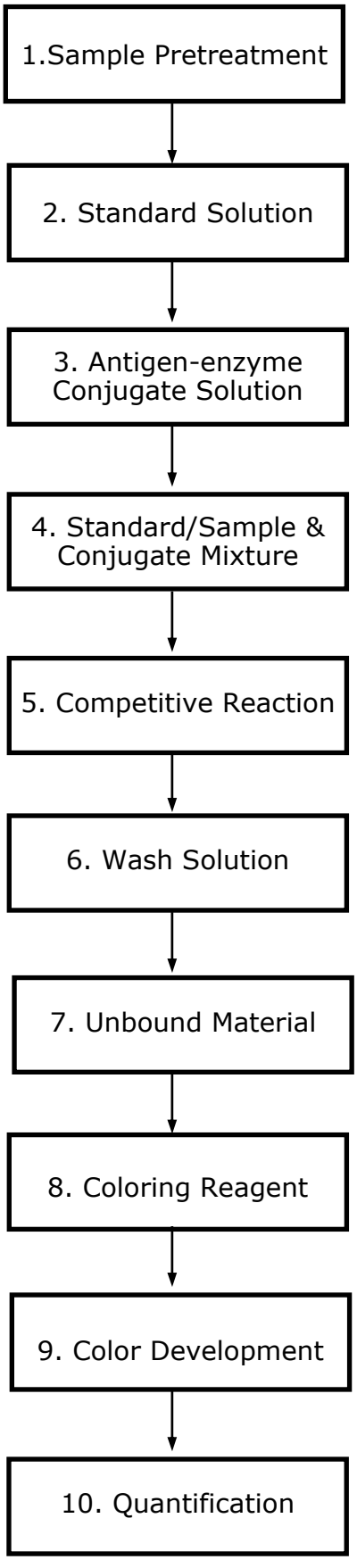
Unbound APE and excess APE-enzyme conjugate are washed out. The presence of APE is detected by adding a chromogenic substrate:TMB. The enzyme-labeled APE bound to the APE antibody in the plate, catalyzes the conversion of the substrate to a colored product. After an incubation period, the reaction is 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).

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

**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.

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) 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% Methanol)	4mL	1 Vial	2-8°C
3	Antigen-enzyme Conjugate powder		2 Vials	2-8°C
4	Buffer Solution	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	15mL	1 Vial	2-8°C
9	Uncoated Microplate	96 Wells	1 Plate	---
10	Adhesive 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. ASAHI TECHNO GLASS, 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
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. 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; Sep-Pak PS-2 N20131, Waters)

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

Clear Sample:	Add 100% methanol 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, suction with a vacuum pump is recommended). Wash the residue, if any, with 100% methanol (Make sure the amount of methanol does not exceed 1% of the total volume of the filtrate). Adjust methanol 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 pure water through a solid phase extraction column.
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 pure water and then dry it with vacuuming or nitrogen gas flow.
5) Elute the analyte with 10 ml of 100% methanol.
6) Dilute the eluant with pure water to prepare 10% methanol solution. If concentration is necessary, evaporate the eluant and dissolve the residue in 10% methanol.

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

2. Standard Solution

Dilute the 4mg/L APE concentrate (#2) with pure 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	0.1	0.02	0
100% methanol	(µL)	50	95	396	100
Pure Water	(µL)	700	880	3584	900
4mg/L APE concentrate	(µL)	250	25	20	0
Total	(µL)	1000	1000	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 100% methanol first in a tube and then add pure water and 4mg/L APE concentrate

(#2) to prepare each solution.

- 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.
- Use 10% methanol as a blank.

3. Antigen-enzyme Conjugate Solution

Reconstitute a bottle of antigen-enzyme conjugate powder (#3) with 7mL out of 8 mL 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.

4. Mixture of Standard / Sample and Conjugate Solution

Transfer 100µL of conjugate solution first, and then transfer 100µL of APE standard, prepared in Section 2, or 100µL of sample, prepared as 10 % (v/v) methanol solution into each well of the uncoated microplate (#9) 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.

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 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 120 mL for a whole plate.
- The wash solution must be stored at 2-8°C; it will be stable approximately for

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) 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.
- Several options are available for the calculation of the APE 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 : APE concentration
Y-axis : Optical Density(OD) or Inhibition Rate(B/B0%)

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

Standard OD or B/B0%

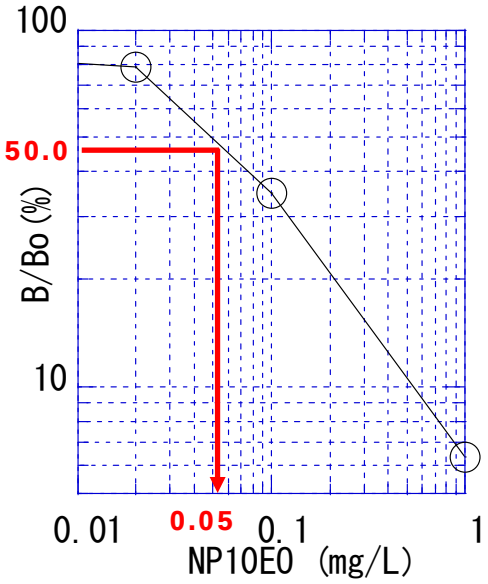
APE (mg/L)	OD	B/B0%
0	1.264	100
0.02	0.995	78.7
0.10	0.440	34.8
1.00	0.081	6.4

Example:

APE (mg/L)	OD	B/B0%
0.050	0.632	50.0

Log-Log Graph Paper Calculation

APE=0.050(mg/L) from B/B0%=52.0%



APPENDIX

1. Plate Layout

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

Example 1) Full Plate Format

Four different concentrations of APE standards (0, 0.02, 0.1, 1mg/L) are assayed in duplicates. The standards take up 8 wells, leaving the rest of 88 wells for samples. With duplicate measurement, the whole plate can take 44 samples altogether.

	1	2	3	4	5	6	7	8	9	10	11	12
A	**0**	**0**	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
B	**0.02**	**0.02**	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
C	**0.1**	**0.1**	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
D	**1.0**	**1.0**	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40
E	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33	S41	S41
F	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34	S42	S42
G	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35	S43	S43
H	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36	S44	S44

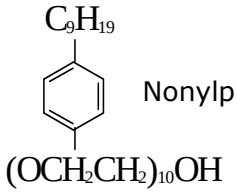
Example 2) Partial Plate Format

Four 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 20 samples with duplicate measurement.

	1	2	3	4	5	6	7	8	9	10	11	12
A	**0**	**0**	S5	S5	S13	S13	**0**	**0**	S5	S5	S13	S13
B	**0.02**	**0.02**	S6	S6	S14	S14	**0.02**	**0.02**	S6	S6	S14	S14
C	**0.1**	**0.1**	S7	S7	S15	S15	**0.1**	**0.1**	S7	S7	S15	S15
D	**1.0**	**1.0**	S8	S8	S16	S16	**1.0**	**1.0**	S8	S8	S16	S16
E	S1	S1	S9	S9	S17	S17	S1	S1	S9	S9	S17	S17
F	S2	S2	S10	S10	S18	S18	S2	S2	S10	S10	S18	S18
G	S3	S3	S11	S11	S19	S19	S3	S3	S11	S11	S19	S19
H	S4	S4	S12	S12	S20	S20	S4	S4	S12	S12	S20	S20

1. Chemical Structure of Alkylphenol Ethoxylate (APE)

Average length of EO chain = 10



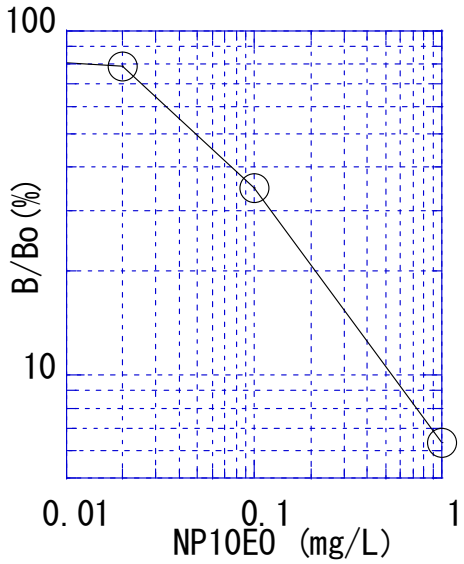
Nonylphenol Ethoxylate

2. Cross-reactivity Pattern

Compound	% reactivity
Nonionic Surfuctants	
Nonylphenol Ethoxylate（NPn EO）	
NPnEO (n = 1)	14
NPnEO (n = 2)	32
NPnEO (n = ca.5)	82
NPnEO (n = ca.10)	100
NPnEO (n = ca.15)	73
NPnEO (n = ca.20)	79
Octylphenol Ethoxylate（OPn EO）	
OPnEO (n = 2)	129
OPnEO (n = ca.5)	244
OPnEO (n = ca.10)	200
OPnEO (n = ca.15)	129
OPnEO (n = ca.20)	92
Nonylphenol Ethoxy Acetic acid (NPnEC)	
NP2EC	267
NP1EC	200
Alcohol Ethoxylate (Alkyl Ethoxylate) (AE)	<0.2
Anionic Surfactants	
Linear Alkylbenzene Sulfonate (LAS)	<0.2
Sodium Dodecyl Sulfate (SDS)	<0.2
Alkylether Sulfate (AES)	<0.2
Sodium Laurate	<0.2
Substances of similar structure	
Phenol	<0.2
Polyethylene Glycol (PEG)	<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.

3. APE Standard Curve



This test kit has a wide detection range between 0.02mg/L 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.

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