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BRAXIS

Ecologiena®

new AP ELISA KIT
(Microplate)
User's Guide

Alkylphenol (AP) 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

- ✧ The monoclonal antibody binds exclusively with Alkylphenol(AP) 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 5μg/L and 500μg/L. A simple concentration protocol based on solid phase extraction is available to determine much lower concentration.
- ✧ The ELISA measurement is highly reproducible; the coefficient of variation (CV) is mostly under 10%.
- ✧ 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 AP by specific monoclonal antibodies. AP present in the sample and a AP-enzyme conjugate (i.e. AP 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 AP concentration is higher relative to the enzyme conjugate, the AP will predominantly bind the antibody and vice versa.

2. Chromogenic Reaction

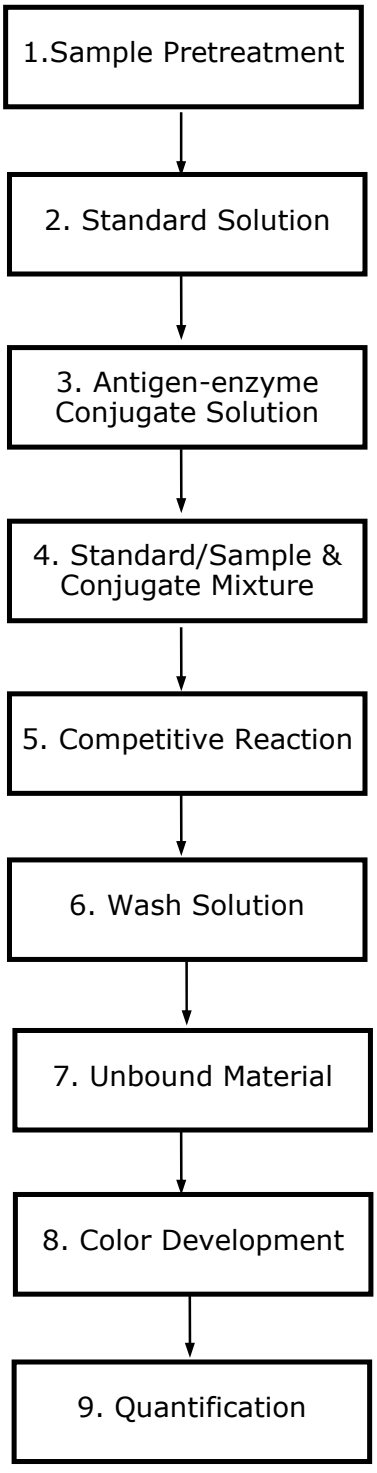
Unbound AP and excess AP-enzyme conjugates are washed out. The presence of AP is detected by adding a chromogenic substrate. The enzyme-labeled AP bound to the AP antibody in the plate, catalyzes the conversion of the substrate to a colored product. After an incubation period, the reaction is stopped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

Flowchart for AP 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 1% (v/v) DMSO and 10% (v/v) MeOH. Solid phase extraction may be necessary for some samples.

Dilute the AP Standard Concentrates (#2) 10 fold with methanol solution (Methanol/distilled water=0.8/8.2) to prepare designated concentrations of AP standards, from 5µg/L to 500µg/L.

Dispense methanol solution first then add Standard Concentrates.

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

Mix 100µL of conjugate solution and 100µL of AP 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 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.

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, a black cap) to terminate reaction.

Measure the absorbance at 450nm for each standard solution and generate a standard curve. Calculate the quantity of AP 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	AP Standard 0µg/L (10%DMSO 20%MeOH)	1.5mL each	1 Vial each	2-8°C
	AP Standard 50µg/L (10%DMSO 20%MeOH)			
	AP Standard 200µg/L (10%DMSO 20%MeOH)			
	AP Standard 1000µg/L (10%DMSO 20%MeOH)			
	AP Standard 5000µg/L (10%DMSO 20%MeOH)			
3	Antigen-enzyme Conjugate powder		2 Vials	2-8°C
4	Buffer Solution ***– white cap -***	7mL	2 Vials	2-8°C
5	Wash Solution (6-fold concentration)	50mL	1 Vial	2-8°C
6	Color Solution ***- brown vessel -***	15mL	1 Vial	2-8°C
7	Stop Solution ***– black cap -***	15mL	1 Vial	2-8°C
8	Uncoated Microplate	96 Wells	1 Plate	---
9	Plate Cover	---	1	---
10	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 AP 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)
9. DMSO (HPLC grade)

Essential - When Sample Concentration through SPE is Required.

1-9. the same as above

10. Solid phase extraction cartridge (e.g. NEXUS SPE Cartridge Producer: VARIAN PART#:1210-3102 ABS ELUT-NEXUS,200MG 6ML,30/PK)
11. Acetone (HPLC grade)
12. Dichloromethane (analytical reagent)

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.

Test Protocol (continued)

- 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), and add DMSO and methanol to the filtrate to be at a final methanol concentration of 1% (v/v) DMSO and 10% (v/v) MeOH.
If a sample concentration and clean-up are necessary, follow the following solid phase extraction.
[Example]

1) Pour the filtrate, prepared above, at 10mL/min through a NEXUS cartridge preconditioned with Dichloromethane (ex. 10ml), methanol (ex. 5ml) and distilled water (ex. 5ml).
2) Wash the NEXUS cartridge with distilled water (ex. 5ml) and 50% methanol solution (ex. 5ml), then dry the column with vacuuming for more than 45 minutes.
3) Elute the analyte with dichloromethane (ex. 6ml). Then, evaporate the solvent with nitrogen gas under 30 degree Celsius or lower temperature.
4) Dissolve the residue in DMSO and methanol solution to be a final concentration of 1% DMSO and 10% methanol.

2. Standard Solution

!! IMPORTANT !!

1) Prepare methanol solution (methanol/distilled water=0.8ml/8.2ml) for diluting the Standard Concentrates (#2, 10% v/v DMSO, 20% v/v methanol).

2) Dilute the each AP Standard Concentrates (#2) with 9 times of its volume of the methanol solution above to prepare designated concentration of AP solutions (1% v/v DMSO, 10% v/v methanol).

Dispense methanol solution first then add Standard Concentrates(#2)

- Be sure to dispense methanol solution first in a tube and then add each AP Standard Concentrates (#2) to prepare designated concentration of AP solutions, in order to minimize adsorption on the walls of the tube.
- Disposable glass tubes are recommended for dilution to minimize adsorption and contamination.
- If you miss 10-fold dilution, 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 AP standard solutions just before the test. Standard solution, once diluted from the concentrate, is NOT reusable at a later date. Prepare new standard solution for every test session.
- Mix by filling the tip and expelling the contents with a pipette. Should not stir vigorously, with a Vortex mixer for example to prevent its foaming and adsorption onto the test tube surface.
- Be sure the Standard Concentrates are 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.
- 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 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. Conjugate Solution and Mixture of Standard/Sample

Transfer 100µL of conjugate solution, and then transfer 100µL of AP standards, prepared in Section 2, or 100µL of sample, prepared as 1% (v/v) DMSO and 10% (v/v) MeOH 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 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 1% DMSO and 10% methanol solution 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 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.

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. Color Development

Dispense 100µL of the color solution (#6, 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, a black cap) 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.

9. Quantification

Read 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 5µg/L and 500µg/L. Samples of concentration beyond 500µg/L must be diluted with 10% methanol + 1% DMSO and re-tested. If the concentration of AP 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 concentration of AP 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 : NP concentration
Y-axis : Optical Density(OD) or Inhibition Rate(B/B0%)

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

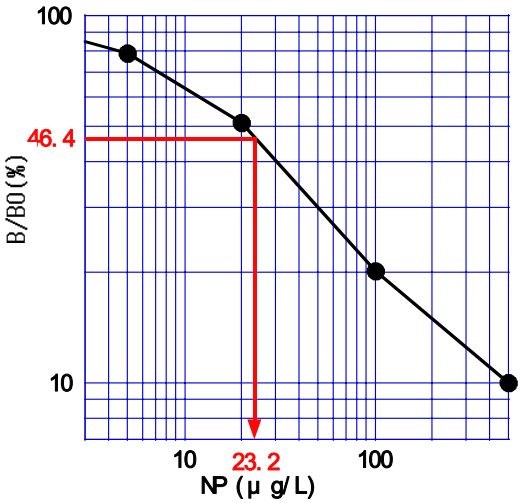
(Example)

Standard OD or B/B0%

NP(ug/L)	OD	B/B0%
0	1.247	100
5	0.985	79.0
20	0.636	51.0
100	0.251	20.1
500	0.125	10.0
Sample	0.579	46.4

Log-Log Graph Paper Calculation

AP=23.2(ug/L) from B/B0%=46.4%



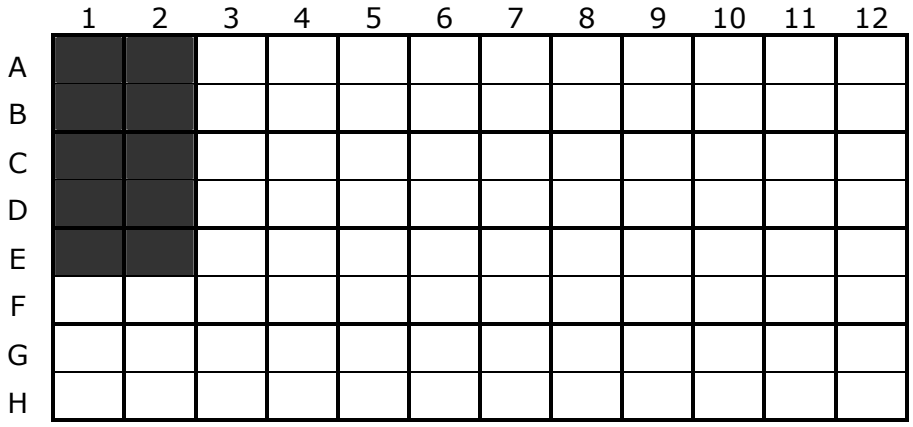
APPENDIX

1. Plate Layout

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

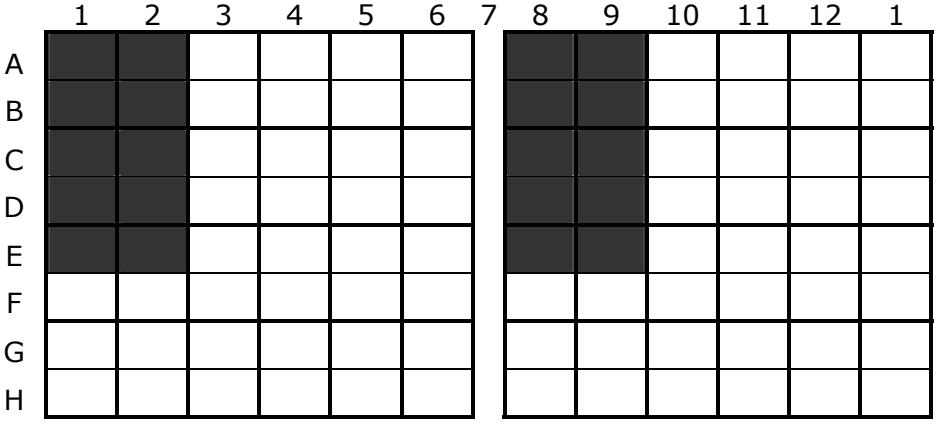
Example 1) Full Plate Format

Five different concentrations of AP standards (0, 5, 20, 100, 500μg/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.

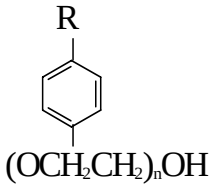


Example 2) Partial Plate Format

Five different concentrations of AP 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.



2. Chemical Structure of Alkylphenol (AP)



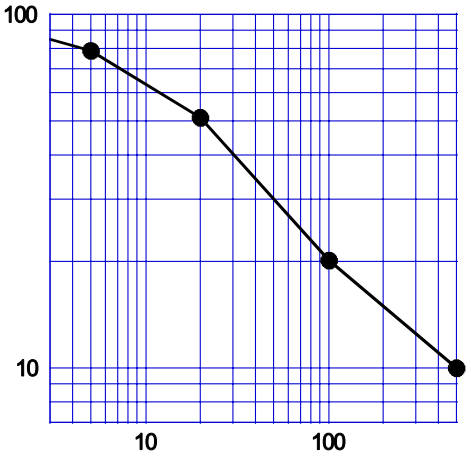
R: C_9H_{19} Nonylphenol (NP)

R: C_8H_{17} Octylphenol (OP)

3. Cross-reactivity Pattern

Compounds	Cross Reactivity (%)
Nonylphenol (NP)	100
Octylphenol (OP)	96
Nonylphenol Ethoxylate (NPnEO)	
NP1EO	1.2
NP2EO	2.1
NPnEO (n≒ 5)	3.2
NPnEO (n≒ 7.5)	4.5
NPnEO (n≒ 10)	4.9
Octylphenol Ethoxylate (OPnEO)	
OPnEO (n≒ 10)	2.9
Nonylphenoxy Acetic Acid (NPnEC)	
NP1EC	0.5
NP2EC	1.5
NP3EC	3.8
Anionic Surfactants	
Linear Alkylbenzene Sulfonates (LAS)	<0.1
Sodium Dodecyl Sulfate (SDS)	<0.1
Alkylether Sulfate (AES)	<0.1
Sodium Laurate (SOAP)	<0.1

4. AP Standard Curve



Samples containing AP within the dynamic range (5μg/L- 500μg/L) can be directly applied to assay after filtration. Samples with AP content below the range must be concentrated with solid phase extraction prior to the ensuing session. Samples outside the upper limit must be diluted with. 1% (v/v) DMSO and 10% (v/v) MeOH.

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

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