

Ecologiena[®]

**Alkylethoxylate (AE)
ELISA KIT
(Microplate)
User's Guide**

Japan EnviroChemicals, Ltd.

Alkylethoxylate (AE) 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.

THE COMPANY MAKES NO WARRANTIES, EITHER EXPRESS OR IMPLIED, EXCEPT AS PROVIDED HEREIN, INCLUDING WITHOUT LIMITATION THEREOF, WARRANTIES AS TO MARKETABILITY, MERCHANTABILITY, FITNESS FOR A PARTICULAR PURPOSE OR USE, OR NON-INFRINGEMENT OF ANY INTELLECTUAL PROPERTY RIGHTS. IN NO EVENT SHALL THE COMPANY BE LIABLE FOR ANY INDIRECT, INCIDENTAL, OR CONSEQUENTIAL DAMAGES OF ANY NATURE, OR LOSSES OR EXPENSES RESULTING FROM ANY DEFECTIVE PRODUCT OR THE USE OF ANY PRODUCT.

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 C10-12 AEs regardless of ethylene oxide (EO) chain length (n=1-25) 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 and 1.0mg/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%.
- ✧ 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 AE by specific monoclonal antibodies. AE present in the sample and an AE-enzyme conjugate (i.e. AE 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 AE concentration is higher relative to the enzyme conjugate, the AE will predominantly bind the antibody and vice versa.

2. Chromogenic Reaction

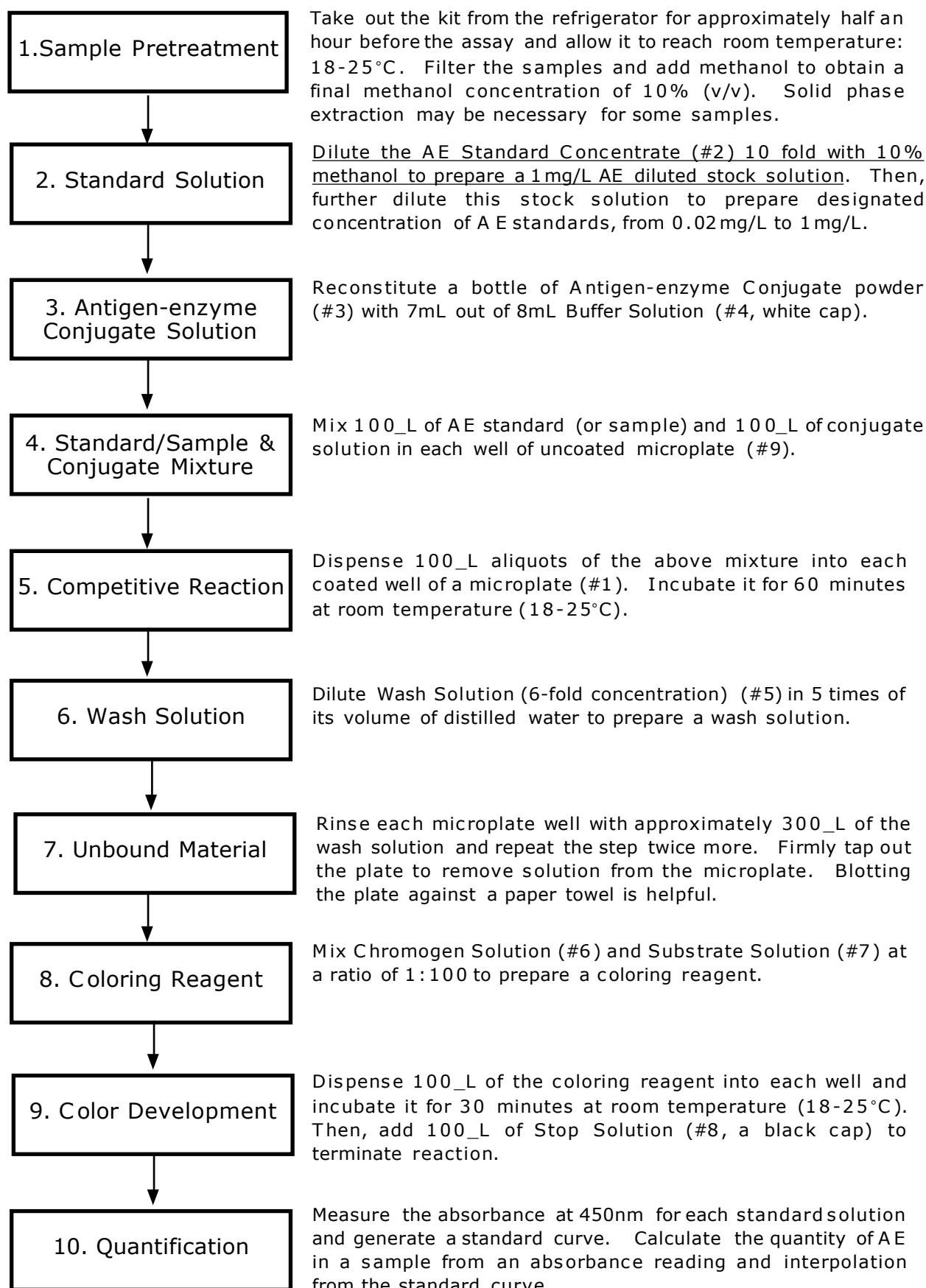
Unbound AE and excess AE-enzyme conjugates are washed out. The presence of AE is detected by adding a chromogenic substrate. The enzyme-labeled AE bound to the AE antibody in the plate, catalyzes the conversion of the substrate to a colored product. After an incubation period, the reaction is stopped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

Flowchart for AE Measurement

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



Kit Content

#	Contents	Volume	Quantity	Storage
1	MoAb-Coated Microplate	96 Wells	1 Plate	2-8°C
2	AE Standard Concentrate (C ₁₂ EO ₇ 10mg/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_μL	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 AE adsorption.
2. Glass fiber filters (e.g. ADVANTEC Co., item No. 36481047_47mm) and filtering equipment
3. Micropipettes (20_μL - 200_μL and 100_μL - 1000_μL, e.g. Gilson Pipetman P-200, P-1000) and tips (e.g. ICN Superpack 96NS)
4. Multichannel pipettes (50_μL - 300_μL 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. Sep-Pak PS-2 N20131 , Waters)

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.

Test Protocol (continued)

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 AE: 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 Alkylphenol Ethoxylate (APE) 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

- 1) 10-fold Diluted Stock

!! IMPORTANT !!

First, dilute the 10mg/L AE concentrate solution (#2, 10% v/v methanol) with 9 times of its volume of 10% v/v methanol to prepare the 1mg/L AE solution

- 2) AE Standard Preparation

Dilute the 1mg/L solution, prepared above, in 10% methanol to obtain AE 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
1mg/L AE concentrate	(_L)	1000	400	100	50	20	0
10% methanol	(_L)	0	600	900	950	980	1000

Total	(_L)	1000	1000	1000	1000	1000	1000
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※If you miss 10-fold dilution step to prepare standard solutions directly from the 10mg/L AE 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 AE solution 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.
- Disposable glass tubes are recommended for dilution to minimize adsorption and contamination.
- Be sure to dispense 10mg/L AE concentrate (#2) first in a tube and then add 10% methanol to prepare 1mg /L solution, in order to minimize dsorption on the walls of the tube.
- Dilute directly from 1mg /L AE stock solution to prepare the designated concentration of AE (0.02, 0.05, 0.1, 0.4mg/L) to minimize adsorption on the wall.
- 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 test tube 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.
- 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 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_μL of AE standard, prepared in Section 2-2), or 100_μL of sample, prepared as 10 % (v/v) methanol solution, and then transfer 100_μL 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 100_μL 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 (18-30°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_μL 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_μL 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_μL 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_μL 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 AE 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 AE 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

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

Example 1) Full Plate Format

Six different concentrations of AE 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.

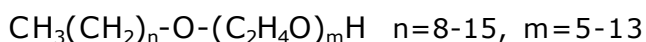
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Example 2) Partial Plate Format

Six different concentrations of AE 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.

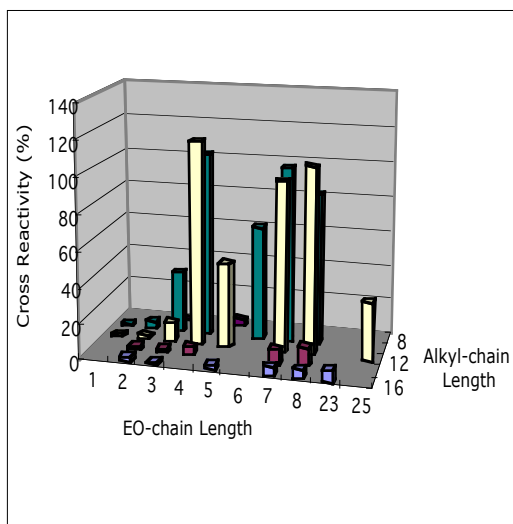
	1	2	3	4	5	6	7	8	9	10	11	12	1
A													
B													
C													
D													
E													
F													
G													
H													

2. Chemical Structure of Alkylethoxylate (AE)



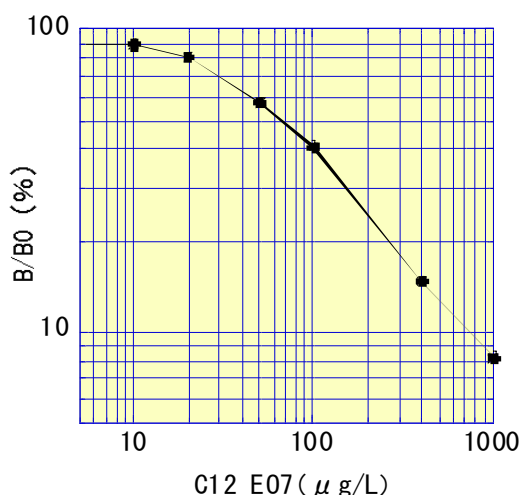
3. Cross-reactivity Pattern

The monoclonal antibody has a high specificity to C10-12 AEs with various EO chain length (n=1-25).



Compound	% reactivity
Nonionic surfactants	
AE (C12EO7)	100
Nonylphenol ethoxylate (NP10EO) (Ave. EO = 7.5)	<1
Polyethylene glycol (PEG) (Ave. EO = 2)	<1
Tween-20 (Ave. EO = 2, -COOH)	3
Anionic surfactants	
Linear alkylbenzene sulfonate (LAS)	<1
Sodium laurate	10
Sodium dodecyl sulfate	16
Alkylether sulfate	31
Cationic surfactants	
Hexadecyl trimethyl ammonium chloride	<1

4. AE Standard Curve



Samples containing AE within the dynamic range (0.02mg/L- 1.0mg/L) can be directly applied to assay after filtration. Samples with AE 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 10% methanol. Samples with LAS content below the range must be concentrated with solid phase extraction prior to the ensuing session.

Coefficient of variation (CV) is generally

Ver.0311

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