Importance of Phthalates Determination

Phthalates are a group of compounds, which are used to increase flexibility and durability in plastics, such as PVC (polyvinyl chloride), and also as solvents for other compounds. These functional qualities have made Phthalates useful for a wide variety of purposes and resulted in their inclusion in products ranging from building materials to personal care products and pharmaceuticals.

Exposure to Phthalates occurs through consumption of foods and drinks prepared or stored in Phthalate containing plastic containers or packaging, the use of Phthalate containing personal care products or products stored in Phthalate containing plastic packaging, from Phthalate containing pharmaceuticals, or from breathing contaminated dust. Concern regarding possible adverse effects from exposure to Phthalates, including endocrine disruption (linked to obesity and insulin resistance), allergies and asthma in children, low birth weight and premature delivery, Attention Deficit Hyperactivity Disorder (ADHD), and cancer, have led to regulations and/or bans on the use of certain Phthalates in various products in a number of countries. In the United States, amounts greater than 0.1% of the Phthalates Di(2-ethylhexyl) Phthalate (DEHP), Dibutyl Phthalate (DBP), and Benzyl Butyl Phthalate (BBP) have been banned in all children's toys and also in care items used to facilitate sleeping, feeding, or teething for children up to 3 years old. These care items include sleepwear, bottles, cups, utensils, bibs, pacifiers, and teething items. An interim ban has also been established for Diisononyl Phthalate (DINP), Diisodecyl Phthalate (DIDP), and Di-n-octyl Phalate (DnOP) in levels greater than 0.1% in any children's toys which can be placed in a child's mouth as well as in care items used to facilitate sleeping, feeding, or teething for children up to 3 years old. The Environmental Protection Agency (EPA) has also placed regulatory limits on the amount of DEHP, the most commonly used Phthalate, in drinking water. The maximum contaminant level (MCL) for DEHP in drinking water is 6 ppb.

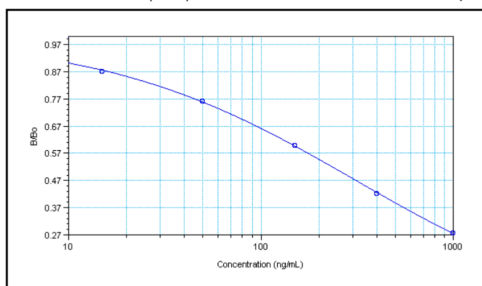
The ABRAXIS® Phthalates ELISA allows for the analysis of 42 samples in duplicate determination. Only a few milliliters of sample are required. The test can be performed in approximately 1 hour.

Performance Data

Test sensitivity: The ABRAXIS® Phthalates ELISA can detect Phthalates in water samples at two different levels of sensitivity. The general screening level provides a limit of detection of approximately 30 ppb in water samples. For the analysis of samples in the EPA regulatory range (6 ppb), samples can be concentrated prior to analysis, providing a limit of detection of approximately 5 ppb in concentrated water samples. See section D, Sample Preparation, for sample preparation procedures for the analysis of concentrated and non-concentrated water samples.

The concentration of residue necessary to cause 50% inhibition (50% B/B_0) is approximately 525.0 ppb in non-concentrated water samples and 87.5 ppb in concentrated water samples. Determinations closer to the middle of the calibration range of the test yield the most accurate results.

Test reproducibility: Coefficients of variation (CVs) for standards: <10%; CVs for samples: <15%.



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ABRAXIS® Phthalates ELISA Microtiter Plate

Enzyme-Linked Immunosorbent Assay for the Determination of Phthalates in Water

Product No. 530050

1. General Description

The ABRAXIS® Phthalates ELISA is an immunoassay for the detection of Phthalates in surface water. This test is suitable for the quantitative and/or qualitative detection of Phthalates in contaminated samples. Positive samples should be confirmed by HPLC, GC/MS, or other conventional methods.

2. Safety Instructions

The standard solutions in this test kit contain small amounts of Phthalates. In addition, the substrate solution contains tetramethylbenzidine and the stop solution contains diluted sulfuric acid. Avoid contact of these solutions with skin and mucous membranes. If these reagents come in contact with skin, wash thoroughly with water.

3. Storage and Stability

The ABRAXIS® Phthalates ELISA Kit should to be stored in the refrigerator (2-8°C). The solutions must be allowed to reach room temperature (20-25°C) before use. Reagents may be used until the last day of the month as indicated by the expiration date on the box. The conjugate is supplied in lyophilized form (3 vials). Before each assay, the required volume of lyophilized conjugate must be reconstituted (see Test Preparation, section C). Reconstitute only the amount needed for the samples to be run, as the reconstituted solution will only remain viable for one week (store frozen).

4. Test Principle

The test is a direct competitive ELISA based on the recognition of Phthalates by specific antibodies. Phthalates, when present in a sample and a Phthalates-enzyme conjugate compete for the binding sites of anti-Phthalates antibodies in solution. The Phthalates antibodies are then bound by a second antibody (goat anti-rabbit) immobilized on the microtiter plate. After a washing step and addition of the substrate solution, a color signal is produced. The intensity of the blue color is inversely proportional to the concentration of Phthalates present in the sample. The color reaction is stopped after a specified time and the color is evaluated using a microplate ELISA photometer. The concentrations of the samples are determined by interpolation using the standard curve constructed with each run.

5. Limitations of the ABRAXIS® Phthalates ELISA, Possible Test Interference

Numerous organic and inorganic compounds commonly found in samples have been tested and found not to interfere with this test. However, due to the high variability of compounds that might be found in samples, test interferences caused by matrix effects cannot be completely excluded. Mistakes in handling the test can also cause errors. Possible sources for such errors can include:

Inadequate storage conditions of the test kit, incorrect pipetting sequence or inaccurate volumes of the reagents, too long or too short incubation times, exposure to direct or indirect sunlight during the substrate reaction, and incorrect temperatures during the immune and/or substrate reactions (lower than 10ºC or higher than 30°C).

Extreme care must be taken to avoid contamination of samples. Laboratory reagents, equipment, and supplies (both plastic and glass) often contain Phthalates or are contaminated with Phthalates during the manufacturing process. All materials (such as vials, caps, pipette tips, solvents, etc.) used for sampling, storage, and analysis should be evaluated for the presence of Phthalates prior to use in order to ensure that they will not contaminate samples and produce inaccurate results.

The ABRAXIS® Phthalates ELISA kit provides screening results. As with any analytical technique (GC, HPLC, etc.), positive samples requiring regulatory action should be confirmed by an alternative method.

6. Working Instructions

A. Materials Provided

1. Microtiter plate coated with a second antibody (goat anti-rabbit)
2. Phthalates Standards (6): 0, 15, 50, 150, 400, and 1000 ng/mL (ppb), 1 mL each
3. Phthalates-HRP Conjugate, 3 vials (lyophilized), must be reconstituted before use, see Test Preparation (Section C)
4. Conjugate Diluent, 12 mL
5. Anti-Phthalate Antibody, 6 mL
6. ABRAXIS® Wash Buffer (5X) Concentrate, 100 mL, must be diluted before use, see Test Preparation (Section C)
7. Substrate (Color) Solution (TMB), 16 mL
8. Stop Solution, 12 mL

B. Additional Materials (not included with the test kit)

1. Micro-pipettes with disposable plastic tips (Phthalate-free, 20-200 µL)
2. Multi-channel pipette or stepper pipette (50-250 µL) with disposable plastic tips (Phthalate-free)
3. Methanol (Phthalate-free)
4. Deionized or distilled water
5. Glass graduated cylinder
6. Glass container with 500 mL capacity (for 1X diluted Wash Buffer, see Test Preparation, Section C)
7. Aluminum foil
8. Timer
9. Paper towels or equivalent absorbent material
10. Hexane
11. Glass vials with Teflon-lined caps (Phthalate-free, 60 mL and 4 mL)
12. Heat block or hot plate
13. Fume hood
14. Microtiter plate reader (wave length 450 nm)

C. Test Preparation

Micro-pipetting equipment and pipette tips for pipetting the standards and samples are necessary. A multi-channel pipette or a stepping pipette is recommended for adding the conjugate, antibody, substrate, and stop solutions in order to equalize the incubations periods of the solutions on the entire microtiter plate. Please use only the reagents and standards from one package lot in one test, as they have been adjusted in combination.

1. Allow the microtiter plate, reagents, and samples to reach room temperature before beginning the test.
2. Remove the number of microtiter plate strips required from the foil bag. The remaining strips must be stored in the foil bag with desiccant and zip-locked closed. Store the remaining kit in the refrigerator (2-8°C).
3. The standard solutions, antibody, color (substrate), and stop solutions are ready to use and do not require any further dilutions.
4. The conjugate provided is lyophilized (3 vials). Before each assay, calculate the volume required (when reconstituted, each vial will provide enough solution for approximately 40 wells). Reconstitute only the amount necessary for the samples to be analyzed. Once reconstituted, the solution will only remain viable for one week (store frozen). If additional samples are to be analyzed greater than one week after reconstitution, a new vial of conjugate will need to be prepared. To reconstitute, add 2.0 mL of Conjugate Diluent to each vial of conjugate required and vortex thoroughly. If using multiple vials of reconstituted conjugate solution in one assay, reconstitute each vial as described above then combine the reconstituted conjugate solutions in an amber glass vial. Vortex thoroughly before use.
5. Sample diluent, for the dilution of samples which exceed the calibration range of the assay, can be prepared by combining equal parts methanol and deionized or distilled water. For example, add 50 mL of methanol and 50 mL of deionized or distilled water to a Phthalate-free glass container and mix thoroughly.
6. Dilute the wash buffer concentrate at a ratio of 1:5 with deionized or distilled water. If using the entire bottle (100 mL), add to 400 mL of deionized or distilled water.
7. The stop solution must be handled with care as it contains diluted sulfuric acid.

D. Sample Preparation

Samples should be collected in Phthalate-free glass sample containers and tested within 1 week (store refrigerated). If samples must be held for longer periods (greater than 1 week), store frozen.

Water Samples (30 ppb limit of detection)

Prior to analysis, water samples should be combined with methanol at a 1:1 ratio. For example, add 2 mL of water sample to a Phthalate-free glass vial, then add 2 mL of methanol, cap tightly and mix thoroughly.

The Phthalates concentration contained in water samples is determined by multiplying the ELISA result by the dilution factor of 2. Highly contaminated samples (those outside of the calibration range of the assay) must be diluted further in 1:1 methanol/deionized water and re-analyzed.

Concentrated Water Samples (5 ppb limit of detection)

1. Prepare 60 mL glass vial as follows:
   1a. Add approximately 5 mL of methanol to the vial. Cap tightly and vortex for 30 seconds.
   Note: Methanol should contact all inside surfaces of the vial during the methanol rinse to ensure the removal of any Phthalates which may have contaminated the vial during manufacturing and could produce inaccurate results if not removed prior to sample concentration.
   Decant methanol into an appropriate waste container.
   1b. Add approximately 5 mL of sample to the vial. Cap tightly and manually shake for 30 seconds. Decant into an appropriate waste container.
   1c. Add a second (approximately 5 mL) aliquot of sample to the vial. Cap tightly and manually shake for 30 seconds. Decant into an appropriate waste container.
2. Add 60 mL of sample to the 60 mL glass vial prepared above in step 1.
3. Add 1 mL of hexane. Vortex for 2 minutes. Allow to separate for approximately 15 minutes.
4. Pipette 0.1 mL of the hexane supernatant (top layer) into a clean vial 4 mL glass vial.
5. Evaporate extract on a heat block or hot plate in fume hood.

Note: Extract should be evaporated using heat only. The use of nitrogen in evaporation may contaminate samples with Phthalates from the plastic tubing.

6. Add 1 mL of methanol. Vortex thoroughly to re-dissolve.
7. Add 1 mL of deionized or distilled water. Vortex thoroughly.
8. Analyze as sample (Assay Procedure, step 1).

The Phthalates concentration contained in concentrated sample is determined by dividing the ELISA result by the concentration factor of 3. Highly contaminated samples (those outside of the calibration range of the assay) must be diluted further in 1:1 methanol/deionized water and re-analyzed.

E. Working Scheme

The microtiter plate consists of 12 strips of 8 wells, which can be used individually for the test. The standards must be run with each test. Never use the values of standards, which have been determined in a test performed previously.

Std 0-Std 5: Standards
0; 15; 50; 150; 400; 1000 ppb

Samp1, Samp2, etc.: Samples

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 0	Std 4	etc.									
B	Std 0	Std 4	etc.									
C	Std 1	Std 5										
D	Std 1	Std 5										
E	Std 2	Samp1										
F	Std 2	Samp1										
G	Std 3	Samp2										
H	Std 3	Samp2										

F. Assay Procedure

1. **Add 50 µL of the standard solutions and samples or sample extracts** into the wells of the test strips according to the working scheme given. Analysis in duplicate or triplicate is recommended.
2. **Add 50 µL of reconstituted enzyme conjugate solution** to the individual wells successively using a multi-channel pipette or a stepping pipette.
3. **Add 50 µL of antibody solution** to the individual wells successively using a multi-channel pipette or a stepping pipette. Cover the wells with aluminum foil and mix the contents by moving the strip holder in a circular motion on the benchtop for 60 seconds. Be careful not to spill the contents. **Incubate for 30 minutes at room temperature.**
   Note: The use of aluminum foil to cover the microtiter plate during incubations is recommended as the use of adhesive plastic plate covers or parafilm may contaminate the test wells, effecting assay performance.
4. Remove the covering, decant the contents of the wells into a sink, and blot the inverted plate on a stack of paper towels. **Wash the strips six times using the diluted wash buffer.** Please use at least a volume of 250 µL of 1X wash buffer for each well and each washing step. Blot the inverted plate after each wash step on a stack of paper towels. After the last wash/blot, check the wells for any remaining buffer in the wells, and if necessary, remove by additional blotting.
5. **Add 150 µL of substrate (color) solution** to the wells using a multi-channel pipette or a stepping pipette. Cover the wells with aluminum foil and mix the contents by moving the strip holder in a circular motion on the benchtop for 60 seconds. **Incubate for 20 minutes at room temperature.** Protect the strips from direct sunlight.
   Note: The use of aluminum foil to cover the microtiter plate during incubations is recommended as the use of adhesive plastic plate covers or parafilm may contaminate the test wells, effecting assay performance.
6. **Add 100 µL of stop solution** to the wells using a multi-channel pipette or a stepping pipette in the same sequence as for the substrate solution.
7. Read the absorbance at 450 nm using a microplate ELISA photometer within 15 minutes after the addition of the stopping solution.

G. Evaluation

The evaluation of the ELISA can be performed using commercial ELISA evaluation programs (4-Parameter (preferred) or Logit/Log). For manual evaluation, calculate the mean absorbance value for each of the standards. Calculate the $\%B/B_0$ for each standard by dividing the mean absorbance value for each standard by the Zero Standard (Standard 0) mean absorbance. Construct a standard curve by plotting the $\%B/B_0$ for each standard on the vertical linear (y) axis versus the corresponding Phthalates concentration on the horizontal logarithmic (x) axis on graph paper. $\%B/B_0$ for samples will then yield levels of Phthalates (in ppb) by interpolation using the standard curve; results for water samples (30 ppb limit of detection) are determined by multiplying the concentration determined by the curve by the dilution factor of 2, results for concentrated water samples (5 ppb limit of detection) are determined by dividing the concentration determined by the curve by the concentration factor of 3. Samples showing lower concentrations of Phthalates compared to Standard 1 (15 ng/mL) should be reported as containing < 30 ng/mL of Phthalates for water samples or < 5 ng/mL for concentrated water samples. Samples showing a higher concentration than Standard 5 (1000 ng/mL) should be reported as containing > 2000 ng/mL for water samples or > 333.3 ng/mL for concentrated water samples. If a quantitative result is necessary, samples must be diluted further and re-analyzed to obtain accurate results.