Ecologiena®

Supersensitive

BPA ELISA KIT

(Microplate)

User's Guide

Japan EnviroChemicals, Ltd.

Supersensitive BPA ELISA KIT (Microplate)
- TABLE OF CONTENTS -

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

- ✧ BPA monoclonal antibody binds exclusively with BPA 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 0.05μg/L to 10μg/L (ppb), sensitive enough to detect BPA in field specimens.
- ✧ With ease of handling, the total time for measurement is only 2.5 hours.
- ✧ The ELISA measurement is highly reproducible; the coefficient of variation (CV) is generally under 10%.
- ✧ The kit, a 96-well microplate format, enables simultaneous measurement of multiple samples at more reasonable cost.

Measuring Principle
(Competitive ELISA)

1. Competitive Reaction

The test is based on the recognition of BPA by specific monoclonal antibodies. BPA present in the sample and a BPA-enzyme conjugate (i.e. BPA 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 BPA concentration is higher relative to the enzyme conjugate, the BPA will predominantly bind the antibody and vice versa.

2. Chromogenic Reaction

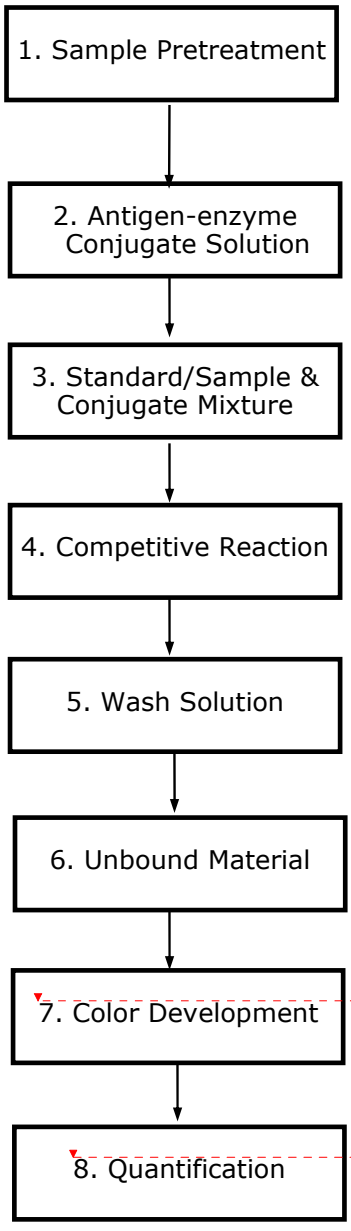
Unbound BPA and excess BPA-enzyme conjugates are washed out. The presence of BPA is detected by adding a chromogenic substrate. The enzyme-labeled BPA bound to the BPA antibody in the plate, catalyzes the conversion of the substrate to a colored product. After an incubation period, the reaction is stopped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

Flowchart for BPA Measurement

<Please follow the steps described in Test Protocol (PP 6-9)>



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.

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

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

Tap off the reaction liquid and 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, brown vessel) into each well and incubate it for 30 minutes at room temperature (18-25°C). Then, add 100µL of Stop Solution (#7, black cap) to terminate reaction.

Deleted: 9

Measure the absorbance at 450nm for each standard solution and generate a standard curve. Calculate the quantity of BPA in a sample from an absorbance reading and interpolation from the standard curve.

Deleted: 10

Kit Content

#	Contents	Volume	Quantity	Storage
1	MoAb-Coated Microplate	96 Wells	1 Plate	2-8°C
2	BPA Standard 0µg/L (10%MeOH)	1.5mL each	1 Vial each	2-8°C
	BPA Standard 0.05µg/ (10%MeOH)			
	BPA Standard 0.3µg/L (10%MeOH)			
	BPA Standard 1.0µg/L (10%MeOH)			
	BPA Standard 10µg/L (10%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

Common to both environmental / biological analysis

1. Glass disposable test tubes (e.g. ASAHI TECHNO GLASS, item No. 9831-1207)
   *Be sure to use disposable tubes to avoid BPA adsorption.
2. Micropipettes (20µL - 200µL and 100µL - 1000µL, e.g. Gilson Pipetman P-200, P-1000) and tips (e.g. ICN Superpack 96NS)
3. Multichannel pipettes (50µL - 300µL e.g. LabSystems Finnpipette Digital 8-channel Pipettor) and tips (e.g. ICN Superpack 96NS)
4. Microplate reader (450nm wavelength) (e.g. TECAN Sunrise Remote)
5. Stop watch
6. Strip ejector (e.g. COSTAR, No.2578)
7. Methanol (HPLC grade)

For environmental samples

When Sample Concentration is NOT Required.

1-7. Same as above
8. Glass fiber filters (e.g. ADVANTEC Co., item No. 36481047 Φ47mm) and filtering equipment

When Sample Concentration through SPE is Required.

1-7. Same as above
9. Solid phase extraction cartridge (e.g. NEXUS SPE Cartridge Producer: VARIAN PART#:1210-3102 ABS ELUT-NEXUS,200MG 6ML,30/PK)
10. Dichloromethane (HPLC grade)

For biological samples

Sample Concentration through SPE (Isolute M-M) is Required.

1-7. Same as above

11. Solid phase extraction cartridge (e.g. Isolute M-M Cartridge Producer: ISOLUTE M-M 500mg/3ml PART#:904-0050-B INTERNATIONAL SORBENT TECHNOLOGY

12. β–glucronidase (example)

Distributor	Product No.	Origin	Optimum pH	Activity (Fishman units)
NIPPON BIOTEST	type-AI	Pomacea canalculata	5.0	22,000 units/mL
Sigma-Aldrich	G0751	Helix pomatia	5.0	>300,000 units/g solid

13. Acetic acid Buffer solution (pH 5.0)

Sodium Acetate	1.2 g
Acetic Acid	1 ml
L-Ascorbic Acid	0.15 g
EDTA-2Na	0.01 g
Distilled Water	100 ml

IMPORTANT

- Comparative tests should be performed if an alternate supplier is used for specified reagents or materials.

Sample Clean-up

For Field Samples

Filter raw water samples through the specified glass fiber filter (1µm pore diameter).

If the BPA concentrations in samples are over the LOQ (0.05µ g/L), add methanol to the filtrate to be at a final methanol concentration of 10% (v/v), and perform the ELISA as in Test Protocol.

If the BPA concentrations in samples are lower the LOQ (0.05µ g/L), concentrate the analyte with NEXUS cartridge, and perform the ELISA as in Test Protocol. An example of the SPE procedure is described as follows;

[Example of analyte concentration with a NEXUS cartridge]

1) Pour the filtrate, prepared above, through a NEXUS cartridge preconditioned with dichloromethane (10mL), methanol (5mL) and distilled water (5mL).
2) Wash the cartridge with distilled water and distilled water/methanol=1:1. Dry the cartridge for 45 minutes. Drying time can be shortened once a recovery of BPA is verified.
3) Elute the analyte with dichloromethane (6mL), then evaporate the solvent with nitrogen.
4) Add 100% methanol to the residue and stir the mixture with a vortex. Terminate the mixing and pour distilled water to adjust the content at 10% methanol (v/v).

For Biological Samples Only for Serum Samples

<Analysis of free BPA>
Clean-up the sample with Isolute M-M cartridge, and perform the ELISA as in Test Protocol. An example of the clean-up procedure with the cartridge is described as follows;

[Example of the sample clean-up with a Isolute M-M cartridge]

1) Pour the biological sample through an Isolute M-M cartridge preconditioned with methanol (10mL) and distilled water (6mL).
2) Wash the cartridge with 35% methanol solution (water/methanol=65:35, 6mL).
3) Elute the analyte with methanol (2.5mL), then evaporate the solvent with nitrogen.
4) Add 100% methanol to the residue and stir the mixture with a vortex. Terminate the mixing and pour distilled water to adjust the content at 10% methanol (v/v).

<Analysis of total BPA>

Before clean-up the sample with Isolute M-M cartridge, BPA conjugate should be hydrolyzed by the enzyme. An example of the clean-up procedure for the enzymatic hydrolysis is described as follows;

[Example of the Enzymatic Hydrolysis]

1) Mix the biological sample (0.1mL), acetic acid buffer solution (pH5.0, 0.2mL) and β-Glucuronidase(0.01mL).
2) Incubate the mixed sample above at 37ºC for 18hr (or more).
3) Clean-up the sample with Isolute M-M cartridge as in this section above, and perform the ELISA as in Test Protocol.

When using β-glucuronidase except for NIPPON BIOTEST, please adjust the activity of enzyme solution to approximately 22,000 Fishman units / mL.

For urine samples as well as other biological samples, interferences in the samples are not effectively eliminated with this procedure. The procedure for biological samples except serum is under development.

IMPORTANT

- Dichloromethane is a possible carcinogen, classified as Group B in NTP and as Group 2B in IARC. Follow the applicable regulation when you use it.
- Keep the methanol concentration to be 10%. Higher methanol content may result in inaccurate readings.
- Use a new cartridge for each filtrate.

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)

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. 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.

2. Conjugate Solution and Mixture of Standard / Sample

Transfer 100µL of conjugate solution, and then transfer 100µL of BPA standard or 100µL of sample, prepared as 10 % (v/v) methanol 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 10% methanol as a blank.

3. Competitive Reaction

Formatted: Bullets and Numbering

Dispense 100µL aliquots of the mixture, prepared in the above Section 2, 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.

4. Wash Solution

Formatted: Bullets and Numbering

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.

5. Unbound Material

Formatted: Bullets and Numbering

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.

6. Color Development

Formatted: Bullets and Numbering

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

Deleted: mixture

- 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 color solution will turn yellow once the stop solution is added.

7. Quantification

Formatted: Bullets and Numbering

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 0.05µg/L and 10µg/L. Samples of concentration beyond 10µg/L must be diluted with 10% methanol and re-tested. If the concentration of BPA 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 BPA 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-Linear (or Log-Log) Graph Paper (Section Paper) Fitting.
X-axis : BPA concentration
Y-axis : Optical Density(OD) or Inhibition Rate(B/B0%)

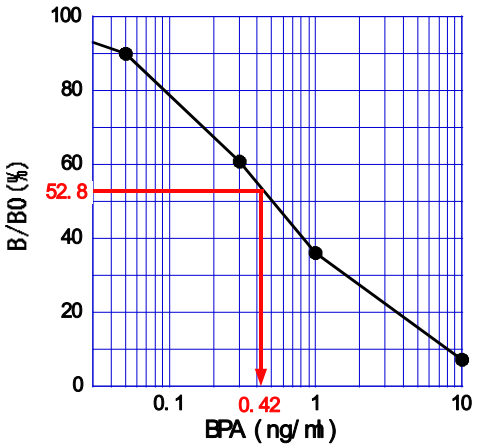
Inhibition Rate(B/Bo%) = (Sample or standard OD)/(OD at BPA standard=0)

(Example)

Standard OD or B/B0%	Log-Linear Graph Paper Calculation
	BPA=0.42(ug/L) from B/B0%=52.8%

BPA (μ g/L)	OD	B/B0%
0	1.319	100
0.05	1.187	90.0
0.30	0.802	60.8
1.0	0.475	36.0
10.0	0.094	7.1

BPA (μ g/L)	OD	B/B0%
0.42	(0.696)	52.8



APPENDIX

1. Plate Layout

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

Example 1) Full Plate Format

Five different concentrations of BPA standards (0, 0.05, 0.3, 1.0, 10μg/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.

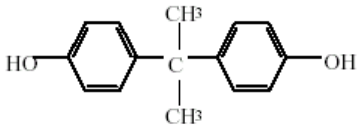
	1	2	3	4	5	6	7	8	9	10	11	12
A	**0**	**0**	S04	S04	S12	S12	S20	S20	S28	S28	S36	S36
B	**0.05**	**0.05**	S05	S05	S13	S13	S21	S21	S29	S29	S37	S37
C	**0.3**	**0.3**	S06	S06	S14	S14	S22	S22	S30	S30	S38	S38
D	**1.0**	**1.0**	S07	S07	S15	S15	S23	S23	S31	S31	S39	S39
E	**10**	**10**	S08	S08	S16	S16	S24	S24	S32	S32	S40	S40
F	S01	S01	S09	S09	S17	S17	S25	S25	S33	S33	S41	S41
G	S02	S02	S10	S10	S18	S18	S26	S26	S34	S34	S42	S42
H	S03	S03	S11	S11	S19	S19	S27	S27	S35	S35	S43	S43

Example 2) Partial Plate Format

Five different concentrations of BPA 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.

	1	2	3	4	5	6	7	8	9	10	11	12
A	**0**	**0**	S04	S04	S12	S12	**0**	**0**	S04	S04	S12	S12
B	**0.05**	**0.05**	S05	S05	S13	S13	**0.05**	**0.05**	S05	S05	S13	S13
C	**0.3**	**0.3**	S06	S06	S14	S14	**0.3**	**0.3**	S06	S06	S14	S14
D	**1.0**	**1.0**	S07	S07	S15	S15	**1.0**	**1.0**	S07	S07	S15	S15
E	**10**	**10**	S08	S08	S16	S16	**10**	**10**	S08	S08	S16	S16
F	S01	S01	S09	S09	S17	S17	S01	S01	S09	S09	S17	S17
G	S02	S02	S10	S10	S18	S18	S02	S02	S10	S10	S18	S18
H	S03	S03	S11	S11	S19	S19	S03	S03	S11	S11	S19	S19

2. Chemical Structure of BPA Standard

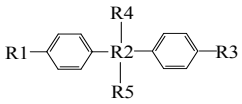


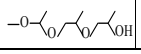
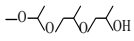
3. Cross-reactivity Pattern

1) Endocrine Disrupters, Estrogens, Surfactants and Humic Substance

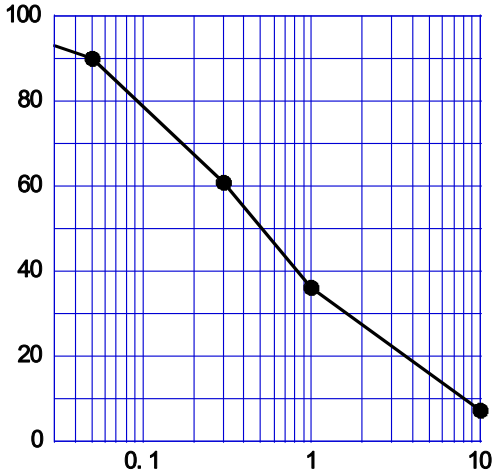
Category	Compounds	CR(%)
Endocrine Disruptors	**Bisphenol A (BPA)**	**100**
	Diethylhexylphthalate (DEHP)	<0.05
	Nonylphenol (NP)	0.19
Estrogen	17β-estradiol (E2)	<0.05
	Estrone (E1)	<0.05
Surfactant	linear-Alkylbenzene sulphonate (LAS)	<0.05
	Alkylphenolethoxylate (APE)	<0.05
	Alkylethoxylate (AE)	<0.05
Humic Substance	Humic acid Na	<0.05

2) Substances of Similar Structure to BPA



compound	R1	R2	R3	R4	R5	Cross-Reactivities (%)
Bisphenol A (BPA)	OH	C	OH	CH_3	CH_3	100
Bisphenol E (BPE)	OH	C	OH	H	CH_3	6.0
Bis(p-hydroxyphenyl)methane	OH	C	OH	H	H	1.8
Bisphenol B (BPB)	OH	C	OH	CH_3	C_2H_5	15.6
2,2'-Bis(4-hydroxyphenyl)-1-propanol	OH	C	OH	CH_3	CH_2OH	1.7
BPA Diacetate	$OOCCH_3$	C	$OOCCH_3$	CH_3	CH_3	0.2
1,2-Bis(4-hydroxyphenyl)-2-propanol	OH	CH_2C	OH	OH	CH_3	0.4
4,4'-Bis(p-hydroxyphenyl) pentanoic acid	OH	C	OH	CH_3	C_2H_4COOH	<0.1
4,4'-dihydroxydiphenyl ether	OH	O	OH	-	-	0.2
p, p'-dihydroxybenzophenone	OH	C	OH	-	O	<0.1
Bisphenol S (BPS)	OH	SO_2	OH	-	-	0.2
Bis[4-(2-hydroxyethoxy)phenyl]sulfone	$O(CH_2)_2OH$	SO2	$O(CH_2)_2OH$	-	-	<0.1
BPA Dimethacrylate		C		CH_3	CH_3	0.7
BPA Diglycidyl Ether		C		CH_3	CH_3	<0.1
BPX-33		C		CH_3	CH_3	<0.1

4. BPA Standard Curve

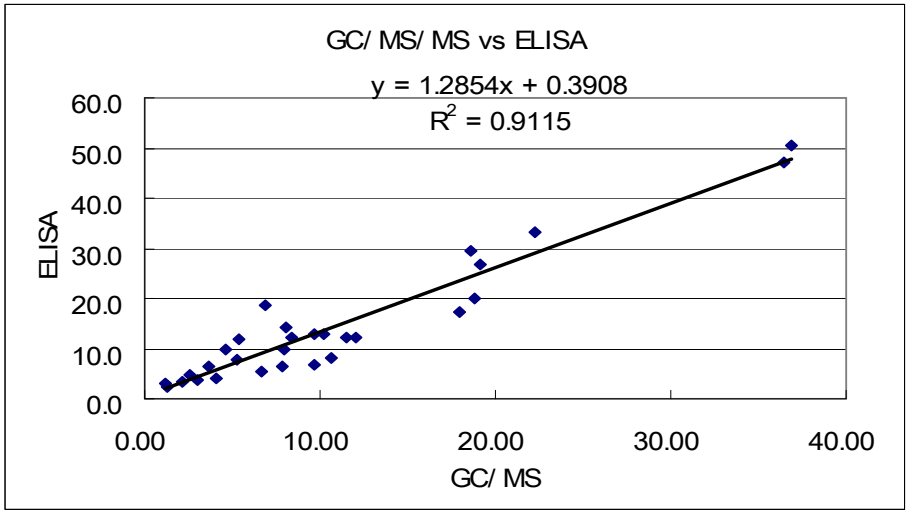


This test kit has a wide detection range between 0.05µg/L and 10µg/L. Samples within this range can be directly applied to the assay only after filtration.

Samples with BPA content outside of the range must be either diluted with 10% methanol or extracted with solid phase concentration prior to analysis.

5. Comparison with Traditional Method

The BPA ELISA shows an extremely high correlation with GC/MS/MS.



Shiraishi et al. (2002), Report of Special Research from the National Institute for Environmental Studies JAPAN (SR-46, p22)

Japan EnviroChemicals, Ltd.

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

Ver.0604

Distributed in North America by:

PN 590023

Abraxis LLC
54 Steamwhistle Drive
Warminster, PA 18974
Phone: (215) 357-3911
FAX: (215) 357-5232

Email: info@abraxiskits.com