Version 09-04/24

User's Manual

Bisphenol A ELISA Kit

REF **DEIA493**

Σ **96T**

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

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PRODUCT INFORMATION

Intended Use

This kit can be used for the determination of BPA in urine, serum, plasma, cells, and tissues following proper isolation and purification. Instructions are provided as to the proper isolation and purification in the following pages. The free BPA level in urine or cell culture media can be measured using the BPA ELISA, without ethyl acetate extraction after 4-fold dilution of the sample.

General Description

BPA is a phenolic environmental estrogen which disrupts endocrine activity. In human, a BPA glucuronide was a primary metabolite of BPA. In a recent study, the age group with highest BPA exposure was 6-11 years old with a mean total (free^{+} glucuronidated) BPA level of 4.33 ng/g of creatinine. Urinary BPA levels were correlated with cardiovascular diseases and diabetes. A recent study revealed that a 12-ounce serving of canned soup for 5 days increased urinary BPA level 12-fold due to BP A-containing epoxy resin lining of the cans.

Principles of Testing

This competitive ELISA kit, based on competition between the BPA epitope and BPA-HRP conjugate for a limited number of binding sites available from the anti-BPA antibody, which is coated on the bottom of the wells of the 96 well ELISA plate. The conjugate concentration is held constant in each well, while the concentration of the BPA is variable, based on the concentration of the sample or standard. Thus the amount of the BPA conjugate which is able to bind to each of the wells is inversely proportional to the concentration of BPA in the standard or sample. The amount of the conjugate which is bound to each well is then determined by the amount of color obtained, when TMB is added. The TMB reacts with the HRP available in the well. With the addition of sulfuric acid, the blue colored product is converted into a yellow colored product, which can be read on a plate reader at 450 nm.

Reagents And Materials Provided

1. BPA ELISA Plate, Solid 96-well plate coated with anti-BPA antibody in each well
2. BPA Standard (2 µL), Stock standard at a concentration of 1 mg/mL
3. BPA-HRP Conjugates (12 µL), 1000× concentrated solution
4. Sample Dilution Buffer (25 mL), 10× solution of Tris-buffered saline with preservatives
5. HRP Buffer (15 mL), 1× solution of Tris-buffered saline with preservatives
6. Wash Buffer Solution (25 mL), 10× solution of Tris-buffered saline with detergents and preservatives
7. TMB Substrate (24 mL), A solution of TMB (tetra methyl benzadine)

Materials Required But Not Supplied

1. Plate reader with a 450 nm filter

2. An 8-channel adjustable pipetter and an adjustable pipetter
3. Storage bottles
4. Cluster tubes (1.2 mL) and microcentrifuge tubes
5. Deionized water
6. 2N Sulfuric acid

Storage

All components are stored below -20°C and should not be re-frozen and thawed more than necessary. This kit will obtain optimal results if all of the components are stored at the proper temperature prior to use. Items should be stored at the designated temperatures upon receipt of this kit.

Specimen Collection And Preparation

There are different protocols for isolating BPA depending on the nature of the biological sample. Listed below are the different protocols. For optimal results follow the appropriate protocol based on the biological sample.

BPA measurement in urine

1. Dilute urine sample 4-fold with 1× sample dilution buffer. Centrifuge diluted urine sample to remove any precipitates.
2. Add 100 uL of the sample to a well in the 96-well plate and perform the ELISA for BPA (according to the instructions of the manufacturer).

BPA measurement in plasma or serum

1. Combine 1.0 mL of plasma (adjusted with acetic acid to pH 4) and 1.0 mL of ethyl acetate. Vortex thoroughly. Centrifuge at 2000 rpm for ten minutes at 22°C. Three phases should result:

   i. Upper organic phase - ethyl acetate phase (lipoproteins)

   ii. Interphase - proteins

   iii. Lower phase - aqueous phase
2. Collect the upper organic phase (a) and set aside.
3. Discard the interphase. Transfer the lower phase with a glass pipette to a new tube, and repeat the ethyl acetate extraction step 2 more times.
4. Evaporation of pooled organic phase: There should be approximately 3 mL of the ethyl acetate phase (a). Dry the pooled organic phase in a Speedvac to get the extracted sediment (b).
5. Store the sediment (e) at-20 ^{O}C if performing assay later. For ELISA assay, dissolve the sediment (e) in 20 µL of ethanol or DMF, then add 130 µL of 1× Sample Dilution Buffer.
6. For the competitive BPA ELISA, the above 150 µL sample may need to be further diluted. When calculating the concentration, consider any dilution factors.
7. Perform the ELISA for BPA (according to the instructions of the manufacturer).

BPA measurement in cells

1. Collect and homogenize and/or sonicate the cells.
2. Acidify the whole homogenized cells with acetic acid to a pH of approximately 3-4. Measure using standard

pH paper.

3. Extraction with ethyl acetate. Add an equal volume of ethyl acetate to the homogenized cells and vortex very well. Place the upper organic phase into a fresh clean tube after centrifugation. Then add another equal volume of ethyl acetate to the homogenized cells to start the second-time extraction. It is strongly recommended that extraction is performed three times.
4. Evaporate the pooled ethyl acetate from the extractions until all has dried up under argon or nitrogen gas.
5. Add 10 µL to 20 µL ethanol, or N, N-dimethyl-formamide (DMF), to reconstitute the dried-up residue from above step #4. Add 0.5 mL of 1× Sample Dilution Buffer (provided in kit). Load 100 µL in each well, in triplicates, on the ELISA plate. (Note: We recommend measuring a different dilution of sample in attempt to fit the results to the standard curve. e.g., add 3 wells with 50 µL of the rest of sample plus 50 µL 1× Sample Dilution Buffer, and 3 wells with 10 µL of the rest of sample plus 90 µL of 1× Sample Dilution Buffer.)
6. Perform the ELISA for BPA (according to the instructions of the manufacturer).

BPA measurement in tissues

1. Homogenize 1 g of tissue, 4 ml of H_2O.
2. Acidify the homogenate by adding 8 µL of acetic acid to each homogenate.
3. Extract with an equal amount of ethyl acetate, vortex thoroughly, spin down, and collect the organic phase. Repeat this extraction twice more and combine all of the organic phases.
4. Dry the organic phase with argon or nitrogen gas.
5. Dissolve the dried residue from above step #4 with ethanol or DMF. (Add approximately 20 µL of ethanol or DMF to reconstitute the dried-up residue.)
6. Dilute further with 1× Sample Dilution Buffer: Add approximately 0.5 mL of 1× Sample Dilution Buffer and centrifuge at 10,000 rpm for five minutes at room temperature. The supernatant will be used for ELISA.
7. Perform the ELISA for BPA (according to the instructions of the manufacturer).

Reagent Preparation

The solid 96-well plate and TMB solution are provided ready to use. The preparations of other assay reagents are detailed below.

Wash Buffer: Mix the solution with a stir bar, applying low heat until a clear colorless solution is obtained. Dilute the entire contents of the Wash Buffer Concentrate (25 mL) with 225 mL of deionized water to yield a final volume of 250 mL of 1× Wash Buffer. This can then be refrigerated for the entire life of the kit.

HRP Conjugate: Dilute 1 vial of the BPA-HRP conjugate (0.012 mL) with 12.00 mL of 1× HRP buffer. One vial makes enough conjugate for one plate. The conjugate must be used the same day and should not be stored for later use.

Standards: Label 5 microtubes as Standard 1 through Standard 5. Dilute the entire contents of Sample Dilution Stock buffer (25 mL) with 225 mL deionized water to yield a final volume of 250 mL of 1× Sample Dilution Buffer. Add 0.9 mL of the Sample Dilution Buffer to the microtubes for Standards 1 to 5. Spin down the enclosed BPA standard vial (2 µL, filled with inert gas) and add 1.998 mL of Sample Dilution Buffer to obtain 2 mL of solution. Label this Standard 6. Add 0.1 mL of the Standard 6 to the microtube labeled Standard 5 and mix thoroughly. Next, add 0.1 mL of Standard 5 into the microtube labeled Standard 4 and mix thoroughly. Continue to serially dilute the standards using 1:10 dilutions for the remaining standards.

Samples: Samples can be directly diluted into the 1× Sample Dilution Buffer if it is in solution. For extracted

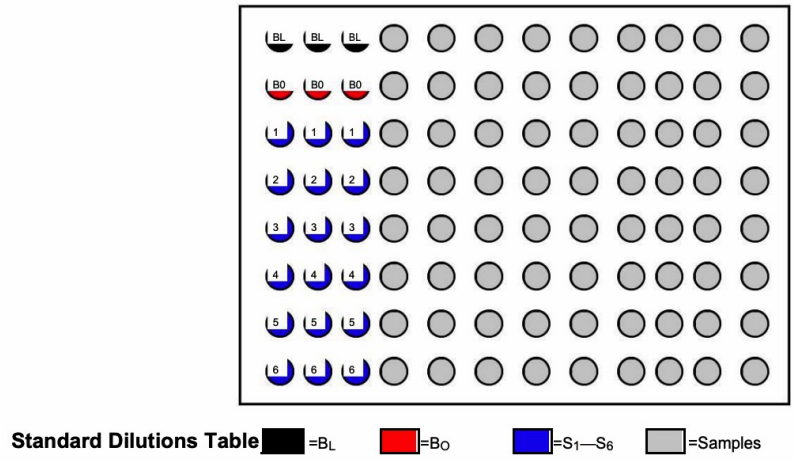
and dried samples, it is recommended to dissolve the dried-up samples with a minimal amount of ethanol of N, N-dimethyl-formanmide (DMF, 10 µL to 20 µL) and vortex well. Before ELISA assay, add 100 µL of 1× Sample Dilution Buffer to make the stock sample solution ready for quantification with ELISA. The stock sample solution can be further diluted to a proper range of concentration for ELISA test.

Assay Procedure

Procedural Notes

1. Remove all of the reagents required, including the TMB, and allow them to equilibrate to room temperature before proceeding with the assay.
2. It is necessary to thoroughly mix the concentrated buffer solutions. A stir bar is contained within each buffer solution.

Plate Setup: Each plate must contain a minimum of three blank wells (B_L), three maximum binding wells (B_O), and a six point standard curve (S_1-S_6). Each sample should be assayed in triplicate. A suggested plate format is shown below:



Standards	Final Concentration (pg/mL)	Add Sample Dilution Buffer (mL)	Serial Dilutions Procedure
No. 6	100,000	1.998	2 µL of stock solution.
No. 5	10,000	0.9	Add 0.1 mL of No. 6
No. 4	1,000	0.9	Add 0.1 mL of No. 5
No. 3	100	0.9	Add 0.1 mL of No. 4
No. 2	10	0.9	Add 0.1 mL of No. 3
No. 1	1	0.9	Add 0.1 mL of No. 2

Step 1: Load 200 microliters of Sample Dilution Buffer into the blank (B_L) wells and 100 microliters of Sample Dilution Buffer into the maximum binding (B_O) wells.

Step 2: Load 100 microliters of each of the standards into the appropriate wells.

Step 3: Load 100 microliters of each of the samples into the appropriate wells.

Step 4: Load 100 microliters of the diluted BPA-HRP conjugate in the B_O wells, the standard wells, and the sample wells. Do NOT add HRP conjugate into the B_L wells.

Step 5: Incubate the plate at room temperature for two hours.

Step 6: Wash the plate three times with 400 microliters of the diluted Wash Buffer per well.

Step 7: After the last of the three wash cycles pat the plate dry onto some paper toweling.

Step 8: Add 200 microliters of the TMB substrate to all of the wells (including B_L wells).

Step 9: Incubate the plate at room temperature for 15-30 minutes.

Step 10: Add 50 micoliters of 2 N sulfuric acid to all of the wells.

Step 11: Read the plate at 450 nm.

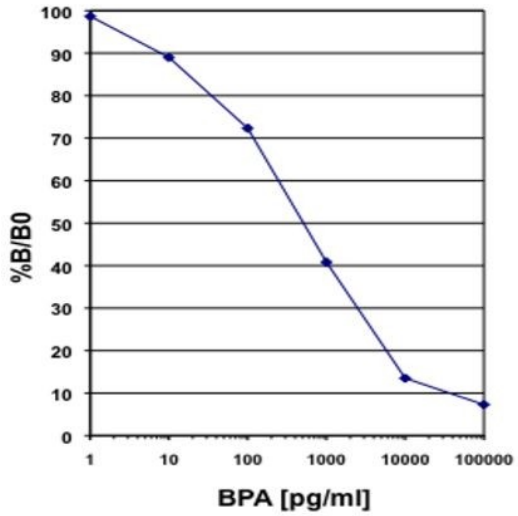
Calculation

Most plate readers provide data reduction software that can be used to plot the standard curve and determine the sample concentrations. If your plate reader does not have this option, then a data reduction program can be used (4 parameter of log-log curve fit).

If you do not have these options, the results can be obtained manually as follows:

1. Average the absorbance readings from the blanks and subtract that value from each well of the plate to obtain the corrected readings. (Note: Some plate readers do this automatically. Consult the user manual of your plate reader.)
2. Average the corrected absorbance readings from the Bo wells. This is your maximum binding.
3. Calculate the %B/Bo for Standard 1 by averaging the corrected absorbance of the two S, wells, divide the average by the maximum binding, then multiply by 100. Repeat this formula for the remaining standards.
4. Plot the %B/Bo versus the concentration of BPA from the standards using semi-log paper.
5. Calculate the %B/Bo for the samples and determine the concentrations, utilizing the standard curve.
6. Multiply the concentrations obtained for each of the samples by their corresponding dilution factor.

Typical Standard Curve



The data shown here is an example of typical results obtained using the BPA ELISA kit. These results are only a guideline, and should not be used to determine values from your samples. The user must run their own standard curve every time.

B_L wells = 0.052

B_O wells = 2.870

Standard	Concentration	O.D.	%B/Bo
No. 1	1 pg/mL	2,834	98.7
No. 2	10 pg/mL	2.555	89.0
No. 3	100 pg/mL	2.077	72.3
No. 4	1000 pg/mL	1.172	40.8
No. 5	10,000 pg/mL	0.388	13.5
No. 6	100,000 pg/mL	0.209	7.3

Detection Range

10-100,000 pg/mL

Sensitivity

10 pg/mL

Specificity

The specificity of the BPA ELISA was investigated using authentic BPA and a panel of bisphenols and related chemicals.

BPA: 100%

BPF: <0.01%

BPS: <0.01%

Resveratrol: <0.01%

Precautions

1. Please read all instructions carefully before beginning the assay.
2. The reagents in this kit have been tested and formulated to perform optimally. This kit may not perform correctly if any of the reagents are replaced or any of the procedures are modified.
3. This kit is intended for research use only and is not to be used as a diagnostic.

Limitations

Troubleshooting

1. No color present in standard wells.

   The HRP conjugate was not added. Redo the assay and add the conjugate at the proper step.

   The HRP conjugate was not incubated for the proper time. Redo the assay and incubate for the proper time.

2. No color in any wells, including the TA wells.

   The TMB substrate was not added. Add substrate.

The TMB substrate was not incubated for the proper time. Continue incubation until desired color is reached.

3. The color is faint.

   One or all of the incubation times were cut short. Redo the assay with the proper incubation times.

   The TMB substrate was not warmed up to room temperature. Redo the assay making sure all reagents are at room temperature.

   The lab is too cold. Be sure the lab temperature is between 21-27°C and redo the assay.

4. The background color is very high.

   The TMB substrate has been contaminated. Redo the assay with a fresh bottle of substrate.

5. Scattered O.D. obtained from the sample.

   Redo assay using an 8-channel pipetman making sure that 8 channels are equal volume while loading.

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