COSMO BIO CO., LTD.
Inspiration for Life Science

EnBio Estrogen Receptor (alpha)/Coactivator, Ligand Assay system

Code: ERA-SRC

STORAGE :

Store at 2 - 8 ℃

EXPIRY :

The expiry date is stated on the package and will be at least 3 months from the date of dispatch.

Warning

For research use only.　Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

EnBioTec Laboratories Co., Ltd.

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1. INTRODUCTION

Ligand (hormone) binding to estrogen receptor (ER) and the following cascade reaction including dimerization of the receptors, binding Coactivator to the receptor and binding the receptor complex to DNA plays an important role of reproduction in physiological functions. To study chemical binding to estrogen receptor is very useful for research of endocrine disrupting chemicals (EDCs) and drug screening.

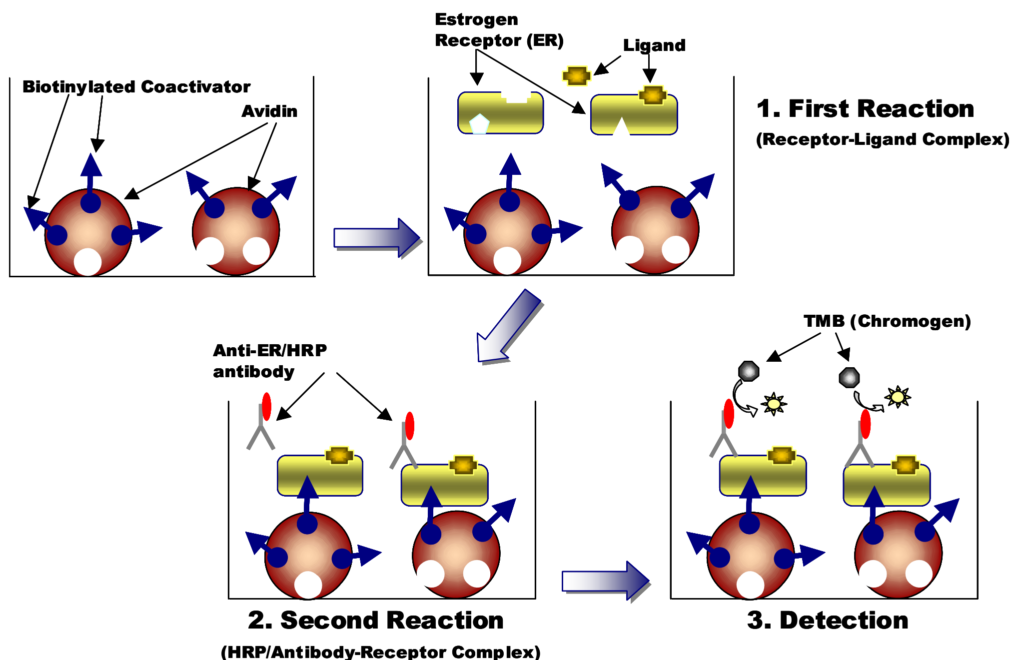
Since EnBio Estrogen Receptor (alpha) / Coactivator Ligand Assay system contains Coactivator peptide and the Coactivator join the reaction followed by receptor- ligand binding, both agonist and antagonist for Estrogen Receptor (alpha) can be detected.

1. Detection of agonist and antagonist
2. High sensitivity
3. Total assay time: within 3 hours

2. PRINCIPLE

The EnBio Estrogen Receptor (alpha) / Coactivator Ligand Assay system use avidin coated 96 wells microtiter plate. Biotinylated Coactivator peptide bound to avidin coated plate. Assay sample (ligand) and recombinant human estrogen receptor (ER) are incubated in the well, and a complex of ER and ligand bound to Coactivator on the assay plate. After washing the plate, the complexes of ER, ligand and Coactivator are detected by HRP conjugated anti-ER antibody. The HRP activity is increased depending on the concentration of estrogenic ligand in the sample.

HRP activity is determined by the addition of TMB substrate solution. The reaction is stopped by addition of an acid solution and the resultant color read at 450 nm using a microtiter plate spectrophotometer. The reactivity of the sample to the receptor can be determined by calculation of EC50 using the absorbance data.



3. CONTENTS

No	Content	Amount
1	Avidin coated microtiter plate (96 wells, 12 strips)	1 plate
2	Biotinylated Coactivator peptide (SRC1) solution in DMSO	1 vial (0.14 mL in 1.6 mL plastic tube)
3	Recombinant human estrogen receptor alpha (ER)	2 vial (lyophilized in 10 mL-glass tube)
4	Anti-ER/HRP antibody solution	1 vial (0.14 mL in 1.6 mL - plastic tube)
5	Assay buffer	1vial (15 mL in 30 mL - PP bottle)
6	Wash buffer concentrate (X10)	1vial (50 mL in 60 mL – PP bottle)
7	TMB substrate	1 vial
8	Stop solution	1 vial (13 mL in 15mL-glass)
9	Blank : Dimethyl Sulfoxide (DMSO)	1 vial (5 mL in 10 mL - glass tube)
10	E2 standard : 400 nM Estradiol in DMSO	1 vial (1.0 mL in 1.6 mL - glass tube)
11	Plate sealer	2 sheets

4. SAFETY WARNINGS AND PRECAUTION

Warning: For research use only. Not for clinical diagnostic use. Do not use internally or externally in humans or animals. Avoid contact with skin or eyes.

We recommend that this product and components are handled only by those persons who have been trained in laboratory techniques. As all chemicals should be considered as potentially hazardous, it is advisable when handling chemical reagents to wear suitable protective clothing, such as laboratory overalls, safety glasses and gloves.

5. GENERAL NOTES

1. Read the complete procedure before starting the assay.
2. Allow all reagents to reach room temperature prior to performing the assays.
3. Avoid handling the tops and bottoms of the wells both before and after filling.
4. Blank, standard and samples should be assayed in duplicate.
5. The total dispensing time for each plate should not exceed 20 minutes.
6. Use only coated wells from the same reagents batch for each assay. Also do not mix reagents from different kit lots.
7. New pipette tips should be used for each standard and sample.
8. It is important that the wells are washed thoroughly and uniformly. If using automatic washer check operation of head before starting. If washing by hand ensure that all wells are completely filled at each wash.

6. ADDITIONAL MATERIALS AND EQUIPMENTS REQUIRED

The following materials and equipments are required:

1. Pipettes or pipetting equipment with disposable tips (1-20 µL, 20-200 µL and 100-1, 000 µL).
2. Multiple pipette (8 channels)
3. Disposable polypropylene test tubes
4. Disposable glass test tubes
5. 500mL measuring cylinder
6. Distilled or de-ionized water
7. Plate reader capable of reading at 450 nm
8. Plate shaker
9. DMSO

7. ASSAY PROCEDURE

(1) Reagent preparation

Note: All reagents must be allowed to equilibrate to room temperature before use. This is particularly important for the enzyme substrate (TMB).

1. Either distilled or deionized water may be used for reagent preparation.
2. The microtiter plate, substrate and stop reagent are supplied ready to use when equilibrated to room temperature.
3. All reagents (Biotinylated Coactivator solution, Anti-ER/HRP antibody solution and Assay buffer) should be mixed well prior to use.

Wash buffer

Stand the Wash buffer concentrate (x10) at room temperature, and mix it well by inversion. Transfer 50 mL of the Wash buffer concentrate (x10) to a 500 mL measuring cylinder. Adjust the final volume to 500mL with deionized water and mix thoroughly. Store at 2 - 8 ℃ for up to one month.

x100 Coactivator solution

Mix Coactivator (SRC1) solution and the wash buffer with the mixture ratio 1:100. (i.e. preparation of the Coactivator mixture for 1 well, Coactivator solution: Wash buffer= 1.2 μL: 118.8 μL, containing pipetting error)

anti-ER/HRP working solution

Mix anti-ER/HRP solution and the wash buffer with the mixture ratio 1:100. (i.e. preparation of the Coactivator mixture for 1 well, anti-ER/HRP solution: Wash buffer= 1.2 μL: 118.8 μL, containing pipetting error)

(2) Preparation of Coactivator binding plate

Decide the number of strips you wish to run, leaving the strips to be used in the frame. Remove the unnecessary strips and store them with plate cover in the foil pouch at 2- 8 ℃, making sure the foil pouch is sealed tightly. After running the assay, retain the plate frame for the second partial plate.

1. Set up the microtiter plate with sufficient wells for running all blank (zero standard), E2 standard and samples as required. We recommend that all standards and samples are assayed duplicate.
2. Pre-wash your plate with 3 x 300 μL /well wash buffer (see section 7 (1)).
3. Add x100 Coactivator solution (see section 7 (1)) to the each well (100 μL /well).
4. Cover the plate with the plate cover sheet and incubate at room temperature (20- 28 ℃) for 1 hour with moderate shaking on a plate shaker.
5. (During the plate incubation, prepare reaction mixtures (see section 7 (3)-(6).)
6. Aspirate and wash all wells 3 times with wash buffer, ensuring that the wells are completely filled and emptied at each wash. After third wash, blot the plate by tapping briskly on tissue paper.

(3) Preparation of blank and E2 standard solution

Thaw Blank and E2 standard solution (400 nM) at room temperature and mix well by pipetting.

Note: Blank and E2 standard solution can be stored at 4 ℃ after use.

(4) Preparation of sample solution

Dilute samples to be tested in DMSO.

Note: All samples should be prepared in glass tube since chemicals may be absorbed to the surface of plastic tube.

(5) Preparation of reaction mixture for agonist assay

1. Add 6 mL of the Assay buffer to lyophilized ER bottle. Mix well by pipetting gently. (This ER solution should be kept on ice until analysis.)
2. Mix sample solution (see section 7 (4)) and ER solution with the mixture ratio, 5 : 95. (Do not Vortex! Mix gently by pipetting or inversion)
   (i.e. preparation of the reaction mixture for 1 well, sample solution: ER solution= 6 µL: 114 µL, containing pipetting error)

   Note: Remaining ER solution can be stored at -80 ℃ . Do not freezing and thawing cycles.

(6) Preparation of reaction mixture for antagonist assay

1. Prepare 40 nM of E2 solution (10-folds dilution of E2 standard (400 nM) with DMSO).
2. Mix prepared E2 (40 nM; final concentration is 1 nM) solution and sample with the ratio, 1:1.
3. Add the ER solution into the sample mixture contained E2. Mixture ratio of ER solution : sample mixture is 95 : 5.
   (i.e. preparation of the reaction mixture for 1 well, E2-sample mixture: ER working solution= 6 µL: 114 µL, containing pipetting error)

(7) Assay protocol

When adding the TMB substrate reagent, pour out from the bottle only the amount needed to run the plate. Care must be taken to ensure that the remaining TMB substrate reagent is not contaminated. If the substrate reagent is bright blue prior to use, it has been contaminated. DO NOT USE.

<u>STEP 1: Sample-ER Reaction</u>

1. Apply 100 μL of the reaction mixtures (see section 7 (5)(6)) to the wells of the Coactivator binding plate (see section 7 (2)).
2. Cover the plate with the plate cover sheet and incubate at room temperature (20- 28 ℃) for 1 hour with moderate shaking on a plate shaker.

<u>STEP 2: anti-ER/HRP antibody Reaction</u>

1. Aspirate and wash all wells 3 times with wash buffer (see section 7 (1)), ensuring that the wells are completely filled and emptied at each wash. After third wash, blot the plate by tapping briskly on tissue paper.
2. Apply 100 μL of anti-ER/ HRP working solution (see section 7 (1)) to each well.
3. Incubate the plate with gentle agitation for 30 min at room temperature.

<u>STEP 3: Detection</u>

1. Aspirate and wash all wells 3 times with wash buffer, ensuring that the wells are completely filled and emptied at each wash. After third wash, blot the plate by tapping briskly on tissue paper.
2. Add 100 μL of TMB substrate to all wells.
3. Incubate at room temperature for 20 min (no shaking!).
4. Add 100 μL of Stop solution to all wells.
5. Read the absorbance at 450 nm.

8. DATA PROCESSING

(1) Evaluation of analytical precision

When the difference of absorbance value between blank and E2 standard (400 nM; final concentration is 20 nM) is less than 0.5, the assay values of the samples may not be correct. Retest is recommended.

(2) Typical assay data

Typical assay data for agonist (E2, DES and Genistein) and antagonist (Tamoxifen) are shown in Figure 1. The EC50 or IC50 value of test chemicals will be calculated by logistic 4-parameter curve fit using computer software such as Prism (GraphPad).

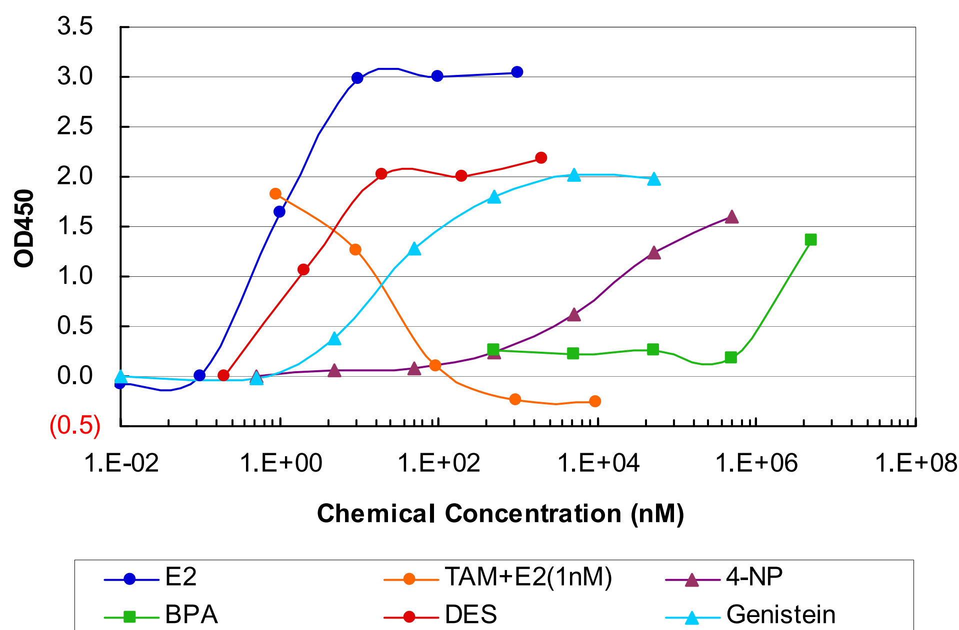


Figure 1. Dose dependent curve of 17beta-estradiol (E2), Diethylstilbestrol (DES), Genistein, 4-Nonylphenol (NP), Bisphenol A (BPA) and Tamoxifen (TMX). Intra-assay variations, which were generated by assaying 6 replicates of 7 different concentrations E2 samples, were less than 15%.

9. TROUBLESHOOTING GUIDE

1. Low optical densities

1.1) Check the reader wavelength at 450nm.

1.2) Check reagents have been correctly reconstituted.

1.3) Check reagents have been stored under the recommended conditions.

1.4) Check incubation time and temperatures.

1.5) Ensure all reagents have been equilibrated to room temperature before use.

1.6) Ensure that the plate is read within 30 minutes of adding the stop reagent.

2. High optical density

2.1) Check point 1.2.

2.2) Check point 1.4.

2.3) Ensure the every wash step in the assay procedure is carried out completely.

3 Poor replication or precision

3.1) Ensure automatic washers are working correctly, or that each well is completely filled and emptied at every wash step when hand washing.

3.2) Check pipette calibration

3.3) Ensure troughs used with multi-channel pipettes are dedicated to individual components.

3.4) Ensure that plates have been carefully placed into the shaking incubator and the plate reader, to avoid splashing and resultant cross contamination of the wells.

3.5) Check standard dilution procedure.

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Quick Guide

STEP 1: Plate preparation

1. Pre-wash your plate with 3 x 300 µL/well wash buffer.
2. Add x100 Coactivator solution diluted with assay buffer to the each well (100 µL/well).
3. Incubate the plate with gentle agitation for 1 hr at room temperature.
4. (During the plate incubation), prepare sample-ER alpha mixtures.

STEP 2: First Reaction (Ligand-Receptor-Coactivator Complex)

1. (After washing the plate with 3 x 300 µL/well of wash buffer), apply 100 µL of the reaction mixtures to the wells.
2. Incubate the plate with gentle agitation for 1 hr at room temperature.

STEP 3: Second Reaction (HRP/ Antibody-Receptor binding)

1. (After washing the plate with 3 x 300 µL/well of wash buffer), apply 100 µL of anti-ER/ HRP antibody (x100) to the wells.
2. Incubate the plate with gentle agitation for 30 min at room temperature.

STEP 4: Detection

1. (After washing the plate with 3 x 300µL/well of wash buffer), add 100 µL of TMB substrate to all wells.
2. Incubate at room temperature for 10 min (no shaking!).
3. Add 100 µL of Stop solution to all wells.
4. Read the absorbance at 450 nm.
5. Calculate the results.

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