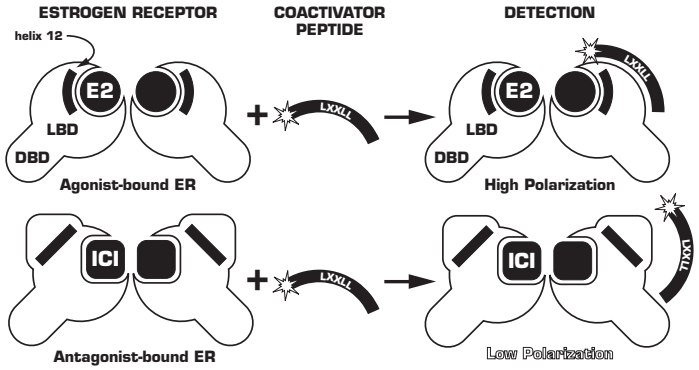
1.0 INTRODUCTION

This kit contains the reagents necessary to perform an assay to assess the ligand dose dependency for the recruitment of coactivator peptides to human Estrogen Receptor-beta. Increasing concentrations of ligand or test compound are added to Estrogen Receptor-beta (ERβ) and a fluorescent peptide (D22) which results in either the formation or disruption of the ERβ/D22 complex. D22 is a coactivator-like peptide containing an LXXLL motif and flanking sequences that resemble known coactivators (1). Agonist-bound ERβ is able to recruit D22, resulting in a larger fraction of bound D22 and a larger polarization value. Antagonist-bound ER has a lower affinity for D22, yielding a larger fraction of unbound D22 and a lower polarization value. The EC_{50} value of the ligands to either promote or disrupt the ERβ/D22 interaction provides a means to classify the test compound as an antagonist, agonist, or selective modulator.

An alternate equilibrium binding protocol is also provided to measure the affinity of the ERβ and D22 interaction in the presence of saturating amounts of ligand or test compound. This format is another method to classify a test compound as antagonist, agonist, or selective modulator.



If you would like more information on fluorescence polarization theory and techniques, please see our on-line Fluorescence Polarization Applications Guide at:

http://www.panvera.com/tech/appguide/index.html

If you would like more information about this specific product, please see our website at:

http://www.panvera.com/catalog/P2994.html

2.0 SAFETY PRECAUTIONS

Normal precautions exercised in handling laboratory reagents should be followed. All reagents in this kit are considered non-hazardous according to 29 CFR 1910.1200. The chemical, physical, and toxicological properties of these products may not, as yet, have been thoroughly investigated. We recommend using gloves, lab coats, eye protection, and a fume hood when working with any chemical reagents.

3.0 DESCRIPTION

3.1 Materials Supplied

Description	Composition	Amount	Part #
10X D22 peptide, Red (LPYEGSLLLKLLRAPVEEV) (1)	10X in ERβ Coactivator Dose Dependence Assay Buffer	1 mL	P2993
Estrogen Receptor-beta (ERβ), Human Recombinant	50 mM Bis-Tris-Propane (pH 9.0), 400 mM KCl, 2 mM DTT, 1 mM EDTA, 10% glycerol	2 × 750 pmol	P2466
ERβ Coactivator Dose Dependence Assay Buffer	Buffer (pH 7.5) containing protein stabilizing agents.	10 mL	P3038
ERβ Coactivator Equilibrium Assay Buffer	Buffer (pH 7.5) containing protein stabilizing agents.	10 mL	P3039

3.2 Materials Required but Not Supplied

- Fluorescence polarization instrument with suitable 535 nm excitation and 590 nm emission interference filters
- Pipetting devices P20, P200, and P1000; multi-channel pipettor
- Reagent reservoir
- Black, round-bottom multiwell plates for use in the fluorescence polarization instrument
- Laboratory timer
- Beacon® Red (FP) Standardization Kit (PanVera® Part No. P2888), recommended for standardizing fluorescence polarization instrumentation and data
- 17β-estradiol, required for agonist effect; 250 µM stock in DMSO or ethanol recommended.
- 4-OH-tamoxifen or ICI 182,780, required for antagonist effect (optional control); 250 µM stock in DMSO or ethanol recommended.

4.0 STORAGE AND STABILITY

Description	Storage Temp.	Notes	Part #
10X D22 peptide, Red (LPYEGSLLLKLLRAPVEEV) (1)	–20°C		P2993
Estrogen Receptor-beta (ERβ), Human Recombinant	–80°C	Avoid repeated freeze-thaw cycles with ERβ (no more than 3 freeze-thaw cycles).	P2466
ERβ Coactivator Dose Dependence Assay Buffer	20-30°C	Thaw upon receipt and store at room temperature.	P3038
ERβ Coactivator Equilibrium Assay Buffer	20-30°C	Thaw upon receipt and store at room temperature.	P3039

5.0 General Considerations for the Assays

- **Controls:** Use 17β-estradiol as a control agonist and 4-OH-tamoxifen or ICI 182,780 as a control antagonist in each experiment for comparison to test compounds. In addition, include control wells that contain the appropriate buffer (either Dose Dependence or Equilibrium Buffer) as blank, 1X D22 in the appropriate buffer (either Dose Dependence or Equilibrium Buffer), 1X D22/ERβ complex, and a 1:10 dilution of Red Polarization Standard (PanVera® Part No. P2889, which is found in the Red (FP) Standardization kit, PanVera® Part No. P2888) as a polarization standard.
- **Handle ERβ gently:** For best results, thaw ERβ on ice before use. **Never vortex ERβ.**
- **DMSO:** We recommend using a minimal amount of DMSO in the assay. Both assay formats will tolerate 2% DMSO.
- **Instrument Calibration:** To set the K- or G-factor on the instrument, or to determine if the instrument is measuring polarization accurately, we recommend using the Low Polarization Standard and High Polarization Standard from the Beacon® FP One-Step Standardization Kit (PanVera® Part No. P2581) with suitable 485 nm excitation and 530 nm emission interference.

6.0 DOSE DEPENDENCY ASSAY PROCEDURE

6.1 Introduction

This kit is supplied with enough reagents to perform 100 reactions in 100 µL volumes in the dose dependency format in a 96-well plate. Each well of a set will contain constant amounts of D22 (C_f = 1X) and ERβ (C_f = 75 nM) with varying amounts of test ligand. In general, there should be at least 16 wells (ligand dilutions) per set. Each set will test a different ligand. The final concentration of ligand in the starting well should be 5 µM (after dilutions).

6.2 Prepare Reagents

1. Remove ERβ from the –80°C freezer and thaw on ice prior to use.
2. Remove D22 from the –20°C freezer and thaw on ice.

6.3 Dose dependency Protocol

1. Add 50 µL of ERβ Coactivator Dose Dependence Assay Buffer to the wells of each set in a 96-well plate, except for the first well that will contain the highest ligand concentration. There should be at least 16 wells (ligand dilutions) per set.
2. To the first well of the set, add 100 µL of 10 µM test ligand (diluted in ERβ Coactivator Dose Dependence Assay Buffer). If a different starting concentration of test ligand is desired, adjust accordingly by adding the test compound to the desired concentration in ERβ Coactivator Dose Dependence Assay Buffer in a final volume of 100 µL.
3. Perform 2-fold serial dilutions of 50 µL test ligand from well 1 through well 16.
4. Prepare a 2X Master Mix containing ERβ and D22 as follows (per 16-well set). The empty boxes underneath each example equation are for your calculations:

First calculate the total volume of Master Mix needed:

50 µL/well × 16 wells × 1.1 (for pipetting error) = 880 µL (total volume)

[ ] µL/well × [ ] wells × 1.1 (for pipetting error) = [ ] µL (total volume)

Calculate the volume of ERβ stock needed:

[880 µL × 150 nM] ÷ 2000 nM ERβ stock = 66 µL ERβ

[[ ] µL × 150 nM] ÷ [ ] nM ERβ stock = [ ] µL ERβ

Calculate the volume of D22 needed:

[880 µL × 2X stock] ÷ 10X stock = 176 µL of ERβ

[[ ] µL × 2X stock] ÷ 10X stock = [ ] µL of ERβ

Calculate volume of ERβ Coactivator Dose Dependence Assay Buffer needed:

880 µL – 66 µL of ERβ – 176 µL of D22 = 638 µL of ERβ Coactivator Dose Dependence Assay Buffer

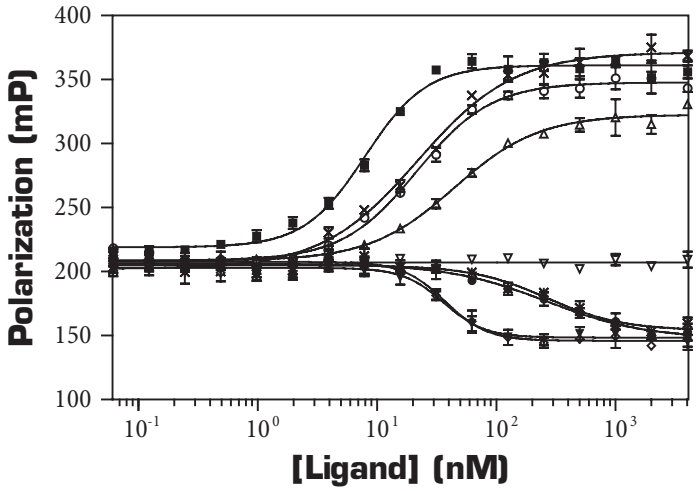
[ ] µL – [ ] µL of ERβ – [ ] µL of D22 = [ ] µL of ERβ Coactivator Dose Dependence Assay Buffer

Gently mix the ERβ Coactivator Dose Dependence Assay Buffer, 10x D22, and ERβ in a tube (microcentrifuge or conical).

5. Add 50 µL of 2X Master Mix to each well.
6. Also include control wells (3-5 wells of each) per plate containing: 100 µL of 1x D22; 100 µL of ERβ Coactivator Dose Dependence Assay Buffer as a blank; 100 µL of a 1:10 dilution of RPS; and 100 µL of 1X D22/ERβ (50 µL of ERβ Coactivator Dose Dependence Assay Buffer and 50 µL of 2X Master Mix).
7. Mix components in multi-well plate by gentle agitation and then incubate for 1 hour at room temperature (20-30°C) in the dark.
8. Measure polarization values in each well.

7.0 RESULTS AND DISCUSSION – DOSE DEPENDENCY FORMAT

Below is an example of the dose dependency data generated using the Estrogen Receptor-β Coactivator Assay. The concentration of the ligand that results in a half-maximum increase or decrease in polarization equals the ligand EC_{50} for the ERβ/D22 interaction. The EC_{50} values obtained in this example are listed after each ligand. The curve was fit using the sigmoidal dose response curve (variable slope) with Prism® software from GraphPad™. ND, not determined. DMSO, unliganded ERβ with DMSO solvent.



■	17β-Estradiol	7.9 nM
×	Estriol	22.0 nM
○	Genistein	21.5 nM
△	Estrone	44.5 nM
▽	DMSO	ND
✱	Clomiphene	265.7 nM
●	Tamoxifen	261.6 nM
▼	4-OH Tamoxifen	36.3 nM
◇	ICI	37.2 nM

8.0 EQUILIBRIUM BINDING ASSAY PROCEDURE

8.1 Introduction

In this alternate protocol, an equilibrium binding curve will be generated by adding increasing amounts of ERβ to a constant amount of D22 (C_f = 1X) in the presence of saturating amounts of ligand (C_f = 5 µM). The polarization will be plotted against the concentration of ERβ. The concentration of ERβ that results in a half-maximum shift in polarization equals the EC_{50} of the ERβ/D22 interaction.

8.2 Prepare Reagents

1. Remove ERβ from the –80°C freezer and thaw on ice prior to use.
2. Remove D22 from the –20°C freezer and thaw on ice.

8.3 Equilibrium Binding Protocol

1. In a 96-well plate, add 50 µL of ERβ Coactivator Equilibrium Assay Buffer to the wells of each set, except for the first well that will contain the highest ERβ concentration. In general, there should be at least 16 wells (ERβ dilutions) per set.
2. To the first well of each set, add ERβ to a concentration of 2 to 4 µM. If necessary, bring the volume to 100 µL using ERβ Coactivator Equilibrium Assay Buffer.
3. Perform 2-fold serial dilutions of 50 µL ERβ from well 1 through well 16.
4. To one set, add 2 µL of a 250 µM test ligand stock to each well for a final concentration of 5 µM. **Note:** It may be necessary to add higher concentrations of test compound if its affinity for ERβ is low, but do not exceed 2% DMSO per well.
5. Prepare a 2.1X working stock of the 10X D22 stock supplied. The empty boxes underneath each example equation are for your calculations:

Calculate the amount of 2.1X D22 needed:

16 wells × 48 µL/well × 1.1 (for pipetting error) = 845 µL (total volume)

☐ wells × 48 µL/well × 1.1 (for pipetting error) = ☐ µL (total volume)

Then calculate the dilution:

[845 µL × 2.1X D22] ÷ 10X D22 = 177 µL 10x D22

[☐ µL × 2.1X D22] ÷ 10X D22 = ☐ µL 10x D22

Calculate the amount of ERβ Coactivator Equilibrium Assay Buffer:

845 µL – 177 µL 10X D22 – 668 µL of ERβ Coactivator Equilibrium Assay Buffer

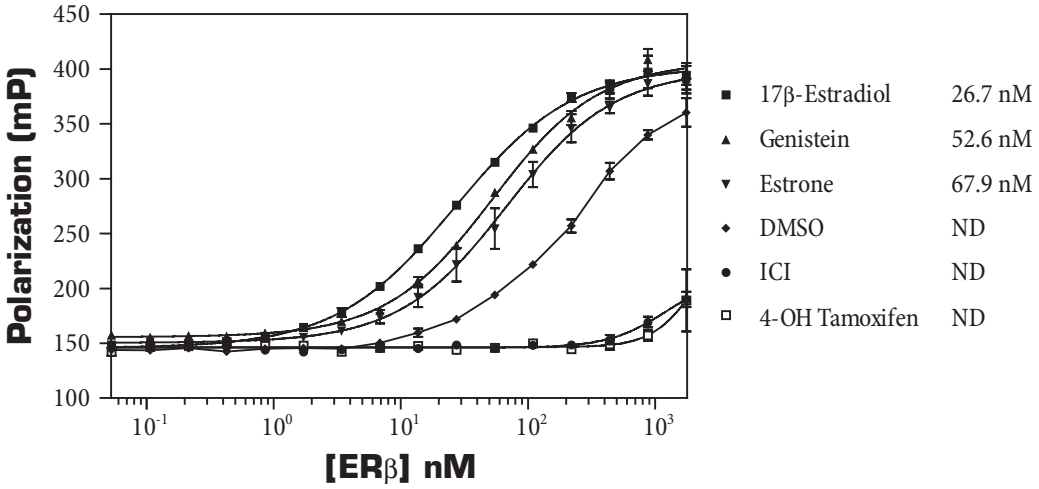
[ ] µL – [ ] µL 10X D22 – [ ] µL of ERβ Coactivator Equilibrium Assay Buffer

Prepare the 2.1X D22 working stock by mixing 10X D22 and ERβ Coactivator Equilibrium Assay Buffer. Add 48 µL of 2.1X D22 to each well.

6. Also include 3-5 control wells per plate containing each of the following: 100 µL of 1X D22; 100 µL of ERβ Coactivator Equilibrium Assay Buffer containing 2% DMSO (or other C_f of solvent) as a blank; 100 µL of a 1:10 dilution of RPS.
7. Mix components in multiwell plate by gentle agitation and then incubate for 1 hour at room temperature (20-30°C) in the dark.
8. Measure polarization values in each well.

9.0 RESULTS AND DISCUSSION – EQUILIBRIUM BINDING FORMAT

Below is an example of the equilibrium binding data generated using the Estrogen Receptor-β Coactivator Assay with the D22 peptide (D22). The concentration of the ERβ that results in a half-maximum shift in polarization equals the EC_{50} of the ERβ/D22 interaction. The curve was fit using the sigmoidal dose response curve (variable slope) with Prism® software from GraphPad™. The EC_{50} values can be converted mathematically to equilibrium binding constants (K_d) by plotting bound ERβ vs. log(free ERβ) also known as a Klotz plot. The K_d values for the ERβ/D22 interaction are listed after each ligand. Agonists promote interaction of ERβ with D22, but no interaction is detected in the presence of antagonists. ND, not determined. DMSO, unliganded ERβ in DMSO solvent (C_f = 2%).



10.0 REFERENCES

1. Chang, C.-Y. *et al.* (1999) *Mol. Cell. Biol.* **19:**8226-39.

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