1.0 INTRODUCTION

The Estrogen Receptor-alpha (ERα) Coactivator Assay is designed as a screening assay for profiling known ERα ligands or test compounds. This kit contains the necessary reagents to perform assays:

1. To assess the ligand *dose dependency* for the recruitment of coactivator peptides to human ERα or
2. Alternatively, to perform *equilibrium binding* to measure the affinity of ERα for the coactivator peptide in the presence of saturating amounts of ligand.

The assays use a fluorescent coactivator peptide (D22), which is a rhodamine-labeled coactivator-like peptide containing an LXXLL motif and flanking sequences that resemble known coactivators (1).

When used to assess ligand *dose dependency*, ERα and D22 are added to increasing concentrations of ligand (or test compound), which results in either the formation or disruption of the ERα/D22 complex. Agonist-bound ERα can recruit D22, resulting in a larger fraction of bound D22 and therefore a higher polarization value (expressed as millipolarization units, mP) than the no-ligand control. In contrast, antagonist-bound ERα has a lower affinity for D22, yielding a smaller fraction of bound D22 and therefore a lower polarization value than the no-ligand control. The EC_{50} value of the ligands, either to promote or to disrupt the ERα/D22 interaction, provides a means to classify test compounds as antagonists, agonists, or selective modulators.

An alternative protocol (for *equilibrium binding*) is provided to measure the affinity of the ERα/D22 interaction in the presence of saturating amounts of ligand (or test compound) with increasing concentrations of ERα. This equilibrium binding format offers another method to classify test compounds as antagonists, agonists, or selective modulators. This method provides a larger dynamic range for antagonist ligands.

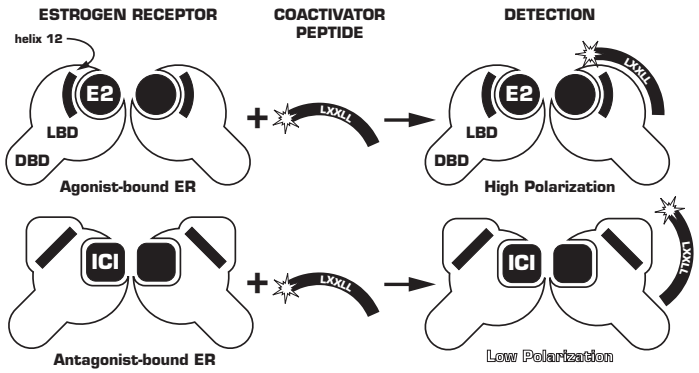

Figure 1. Principle of the ERα Coactivator Assay. E2 = agonist; ICI = antagonist.

Please see PanVera®'s website for more information about:

- ERα Coactivator Assay Kit, Red (**www.panvera.com/catalog/P3071.html**).
- ERβ Coactivator Assay Kit, Red (**www. panvera.com/catalog/P2994.html**).
- Fluorescence polarization theory and techniques in PanVera®'s on-line Fluorescence Polarization Applications Guide (http://www.panvera.com/tech/appguide/index.html).

2.0 SAFETY PRECAUTIONS

Exercise normal precautions, such as using gloves, lab coats, eye protection and a fume hood, when working with any chemical reagents. Although the 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.

3.0 DESCRIPTION

3.1 Materials Supplied

Description	Composition	Amount	Part No.
10X D22 Coactivator peptide, Red (LPYEGSLLLKLLRAPVEEV) (1)	10X in buffer (pH 7.5) containing peptide stabilizing agents	2 × 1 mL	P2993
Estrogen Receptor-alpha (ERα), Human Recombinant	50 mM Tris•HCl (pH 8), 500 mM KCl, 2 mM DTT, 1 mM EDTA, 1 mM sodium orthovanadate and 10% glycerol	2 × 750 pmol	P2187
ERα Coactivator Assay Buffer	Buffer (pH 7.5) containing protein stabilizing agents	20 mL	P3070
1 M DTT	Aqueous	1 mL	P2325

3.2 Materials Required But Not Supplied

- Fluorescence polarization (FP) instrument with suitable 535 nm excitation and 590 nm emission interference filters
- Black, round-bottom 384-well plates
- Red (FP) Standardization Kit (PanVera® Part No. P2888), recommended for verifying FP instrument performance. The Red Polarization Standard (RPS) may be used to determine whether the instrument is measuring polarization values accurately.
- Control Agonist: 17β-estradiol is recommended; 250 µM stock in DMSO, ethanol (EtOH) or methanol (MeOH) suggested
- Control Antagonists: 4-OH-tamoxifen or ICI 182,780 is recommended; 250 µM stock in DMSO, EtOH or MeOH suggested

4.0 STORAGE AND STABILITY

Description	Storage Temperature	Notes	Part No.
10X D22 Coactivator peptide, Red (1)	–20°C		P2993
Estrogen Receptor-alpha (ERα), Human Recombinant	–80°C	Do not expose to more than four freeze/thaw cycles.	P2187
ERα Coactivator Assay Buffer	4°C	Store at 4°C after first use.	P3070
1 M DTT	–20°C		P2325

5.0 GENERAL CONSIDERATIONS FOR THE ASSAYS

- **Controls:** Use a control agonist, such as 17β-estradiol, and a control antagonist, such as 4-OH-tamoxifen or ICI 182,780, in each experiment for comparison to test compounds. In addition, include the controls specified under the specific assay experimental procedure for comparison to test compounds.
- **Solvents:** We recommend using a minimal amount of solvent in the assay. However, both assay formats will tolerate a final concentration of up to 5% DMSO, 4% EtOH or 4% MeOH.
- **Handle ERα gently:** For best results, thaw ERα on ice before use. **Never vortex ERα.**
- **Verify Instrument Calibration:** Verify that the instrument is calibrated properly and has suitable 535 nm excitation and 590 nm emission interference filters.

6.0 DOSE DEPENDENCY ASSAY PROCEDURE

6.1 Introduction

This kit provides sufficient reagents to perform 400 (40-µL) reactions (dose dependency format) in a 384-well plate. Each well of a set contains a constant concentration of D22 (1X) and ERα (75 nM) with serially diluted ligand. Each set will assay a different ligand and should contain at least 16 wells (ligand dilutions). Alternatively, the assay may be performed in a 96-well plate (100-µL reactions) by proportionally scaling up the volume of reagents used per well.

6.2 Dose Dependency Protocol (384-well format)

1. Place ERα, 10X D22, DTT and ERα Coactivator Assay Buffer on ice until ready to use.
2. Prepare the Complete ERα Coactivator Assay Buffer by adding sufficient 1 M DTT to the ERα Coactivator Assay Buffer to yield a final DTT concentration of 10 mM.
3. Dispense 20 µL of Complete ERα Coactivator Assay Buffer into the wells of each ligand set in a 384-well plate, except for the first well. This first well will contain the highest ligand concentration. Use at least 16 wells (ligand dilutions) per set.
4. Prepare a 2X concentration of ligand in Complete ERα Coactivator Assay Buffer. Add 40 µL of 2X ligand to the first well of the set.

   NOTE: Although a recommended starting 2X concentration of 10 µM is suggested for known ERα ligands, using higher concentrations of a ligand may be necessary if its affinity for ERα is low.

5. Perform two-fold serial dilutions of 20 µL of ligand in Complete ERα Coactivator Assay Buffer from Well #1 through Well #16.
6. Prepare a 2X Master Mix containing 2X D22 and 150 nM ERα, diluted in Complete ERα Coactivator Assay Buffer, as shown below. The empty boxes underneath each example equation are for your calculations. The volumes given below are appropriate for 16 (40-µL) reactions.

First calculate the total volume of 2X Master Mix needed:

20 µL/well × 16 wells × 1.1 (for pipetting error) = 352 µL (total volume)

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

Calculate the volume of ERα stock needed:

$$[\ 352\ \mu L \times 150\ \frac{nM}{1} \div 2000\ nM\ ER\alpha\ stock^{*} = 26.4\ \mu L\ ER\alpha$$

$$[\ [\ ]\ \mu L \times 150\ \frac{nM}{1} \div [\ ]\ nM\ ER\alpha\ stock^{*} = [\ ]\ \mu L\ ER\alpha$$

*The ERα concentration is lot specific. Use the concentration provided on the ERα tube or Certificate of Analysis to calculate the appropriate volume of concentrated ERα.

Calculate the volume of D22 needed:

[352 µL × 2X Stock] ÷ 10X stock = 70.4 µL D22

[ ] µL × 2X Stock] ÷ 10X stock = [ ] µL D22

Calculate volume of Complete ERα Coactivator Assay Buffer needed:

[352 µL – 26.4 µL ERα* – 70.4 µL D22 = 255.2 µL Complete ERα Coactivator Assay Buffer

[ ] µL – [ ] µL ERα* – [ ] µL D22 = [ ] µL Complete ERα Coactivator Assay Buffer

*The ERα concentration is lot specific. Use the concentration provided on the ERα tube or Certificate of Analysis to calculate the appropriate volume of concentrated ERα.

7. Gently mix the appropriate volumes of 10X D22, ERα and Complete ERα Coactivator Assay Buffer in a tube.
8. Dispense 20 µL of 2X Master Mix into each well.

9. Prepare the following set of controls and standards (3–5 replicates per control or standard, 40 µL per well), as follows:
   - 1X D22 in Complete ERα Coactivator Assay Buffer
   - 1X ERα/D22 Coactivator peptide (20 µL of 2X Master Mix and 20 µL of Complete ERα Coactivator Assay Buffer)
   - Complete ERα Coactivator Assay Buffer as a blank
   - 1:10 dilution of RPS in RPS buffer
   - RPS buffer as a blank for RPS
10. Mix components in 384-well plate by gentle agitation. Protect the plate from light and evaporation by covering with foil and then incubate for 1 to 5 hours at room temperature (20–25°C).
11. Measure polarization values.

7.0 RESULTS AND DISCUSSION – DOSE DEPENDENCY FORMAT

Below is an example of the dose dependency data generated using the ERα Coactivator Assay. In these experiments, 10 µM (2X) of ligand was used as the starting concentration. The concentration of the ligand that results in a half-maximal increase or decrease in polarization equals the ligand EC_{50} for the ERα/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 (GraphPad Software, Inc., San Diego, CA).

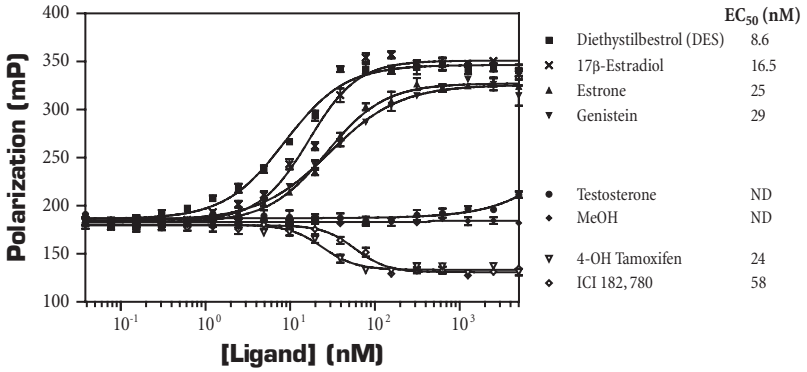

Figure 2. Dose dependency of the ERα/D22 interaction. Ligand was serially diluted in Complete ERα Coactivator Assay Buffer in a 384-well plate with final reaction conditions containing 75 nM ERα and 1X D22. The samples were incubated for 1 hour at room temperature. Values reported are ligand EC_{50} values for the ERα/D22 interaction. ND = not determined; MeOH = unliganded ERα in solvent.

8.0 EQUILIBRIUM BINDING ASSAY PROCEDURE

8.1 Introduction

In this alternative protocol, an equilibrium binding curve can be generated by adding a constant concentration of D22 (1X) to increasing concentrations of ERα in the presence of a saturating concentration (typically 5 µM) of ligand. Polarization values will be plotted against the concentration of ERα. The concentration of ERα that results in a half-maximal shift in polarization equals the EC_{50} of the ERα/D22 interaction. The assay may be performed in a 96-well plate (100-µL reactions) by proportionally scaling up the volume of reagents used per well.

8.2 Equilibrium Binding Protocol (384-well format)

1. Place ERα, 10X D22, DTT and ERα Coactivator Assay Buffer on ice until ready to use.
2. Prepare the Complete ERα Coactivator Assay Buffer by adding sufficient 1 M DTT to the ERα Coactivator Assay Buffer to yield a final DTT concentration of 10 mM.
3. Dispense 20 µL of Complete ERα Coactivator Assay Buffer into each well set in a 384-well plate except for the first well of each ligand. This first well will contain the highest concentration of ERα. Use at least 16 wells (ERα dilutions) per set.
4. Prepare a 2X concentration of ERα (typically 1–4 µM) in Complete ERα Coactivator Assay Buffer. Add 40 µL of 2X ERα to the first well of the set.
5. Perform two-fold serial dilutions of 20 µL of ERα from Well #1 through Well #16.

6. Prepare a 2X Master Mix containing a saturating concentration of ligand (2X, typically 10 µM) and 2X D22, diluted in Complete ERα Coactivator Assay Buffer, as shown below. The empty boxes underneath each example equation are for your calculations. The volumes given in the table below are appropriate for 16 (40-µL) reactions

   NOTE: It may be necessary to use higher concentrations of a test compound if its affinity for ERα is low.

First calculate the total volume of Master Mix needed:

20 µL/well × 16 wells × 1.1 (for pipetting error) = 352 µL (total volume)

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

Calculate the volume of D22 needed:

[352 µL × 2X Stock] ÷ 10X stock = 70.4 µL D22

[ ] µL × 2X Stock] ÷ 10X stock = [ ] µL D22

Calculate the volume of ligand needed:

[352 µL × 10 µM ligand*] ÷ 250 µM ligand* = 14 µL ligand

[ ] µL × [ ] µM ligand*] ÷ [ ] µM ligand* = [ ] µL ligand

NOTE: Although a recommended starting 2X concentration of 10 µM is suggested for known ERα ligands, using higher ligand concentrations may be necessary if the ligand has low affinity for ERα.

Calculate volume of Complete ERα Coactivator Assay Buffer needed:

352 µL – 70.4 µL D22 – 14 µL ligand = 267.6 µL ERα Complete Coactivator Assay Buffer

[ ] µL – [ ] µL D22 – [ ] µL ligand = [ ] µL ERα Complete Coactivator Assay Buffer

7. Gently mix the appropriate volumes of 10X D22, ligand and Complete ERα Coactivator Assay Buffer in a tube.
8. Dispense 20 µL of 2X Master Mix into each well.
9. Prepare the following set of controls and standards (3–5 replicates per control or standard, 40 µL per well), as follows:
   - 1X D22 in Complete ERα Coactivator Assay Buffer
   - 1X ligand/D22 in Complete ERα Coactivator Assay Buffer (20 µL of 2X Master Mix and 20 µL of Complete ERα Coactivator Assay Buffer)
   - Complete ERα Coactivator Assay Buffer as a blank
   - 1:10 dilution of RPS in RPS buffer
   - RPS buffer as a blank for RPS
10. Mix components in 384-well plate by gentle agitation. Protect plate from light and evaporation by covering with foil and then incubate for 1 to 5 hours at room temperature (20–25°C).
11. Measure polarization values.

9.0 RESULTS AND DISCUSSION – EQUILIBRIUM BINDING FORMAT

Below is an example of the equilibrium binding data generated using the ERα Coactivator Assay with D22. In this experiment, the final concentration of each ligand was 5 µM. The concentration of the ERα that results in a half-maximal shift in polarization equals the EC_{50} of the ERα/D22 interaction. The EC_{50} values for the ERα/D22 interaction are listed after each ligand. The EC_{50} values can be converted mathematically to equilibrium binding constants (K_d) by plotting bound ERα vs. log(free ERα), also known as a Klotz plot. Agonists promote interaction of ERα with D22, but no interaction is detected in the presence of antagonists. The curve was fit using the sigmoidal dose response curve (variable slope) with Prism® software.

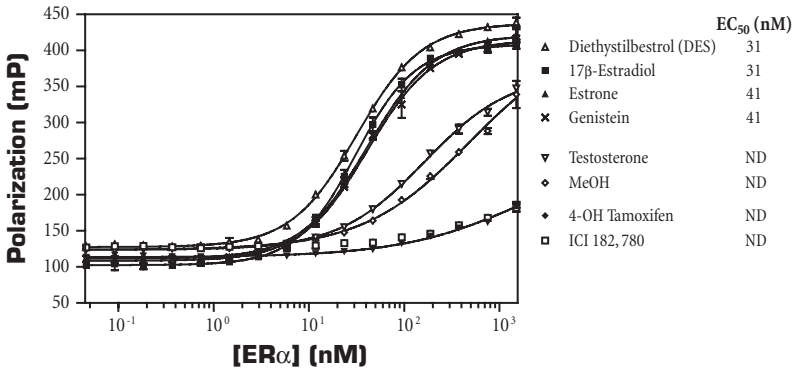

Figure 3. Affinity of ERα for D22 in the presence of various ligands. Purified ERα was serially diluted in Complete ERα Coactivator Assay Buffer in a 384-well plate with final reaction conditions containing 5 µM ligand, 1X D22 and 0.5% MeOH. The samples were incubated for 1 hour at room temperature. Values reported are the EC_{50} values for the interaction of ligand-occupied ERα and D22. ND = not determined; MeOH = unliganded ERα in solvent.

10.0 REFERENCES

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

The performance of this product is guaranteed for six months from the date of purchase if stored and handled properly.