PolarScreen™ Estrogen Receptor-α Competitor Assay, Green

Catalog nos. P2614 and P2698 **Storage: See page 2**

Protocol part no. L0320 Revision date: 10 December 2009

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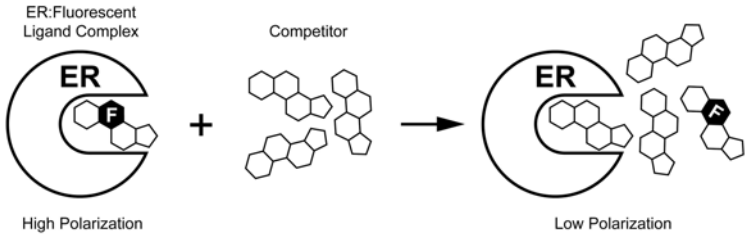
1. Introduction

Estrogen receptors (ER) act in an estrogen-dependent manner to control growth and differentiation of target tissues included in the reproductive, skeletal, nervous and cardiovascular systems. The classical ER, now known as ERα, has long been a drug target in the treatment of breast cancer due to its involvement in the pathogenesis of breast tumors (1, 2). The identification of a second estrogen-inducible transcription factor, ERβ, has added a new level of complexity to estrogen signaling (3). Although both ERs bind to 17β-estradiol with similar high affinities, they show differences in binding to some steroidal ligands, have distinct tissue distributions, regulate separate sets of genes and may oppose each other's actions in some instances.

The PolarScreen™ Estrogen Receptor Competitor Assays provide a sensitive and efficient method for high-throughput, fluorescence polarization-based screening of potential ERα and ERβ ligands. The PolarScreen™ Estrogen Receptor-α Competitor Assay kits use insect cell-expressed, full-length, untagged, human estrogen receptors and a novel, tight-binding, fluorescent estrogen ligand (Fluormone™ ES2) in a homogenous mix-and-read assay format.

2. Assay Theory



ER is added to a fluorescent estrogen ligand to form an ER/Fluormone™ ES2 complex. This complex is then added to individual test compounds in multiwell plates. If the test compounds do not compete with Fluormone™ ES2 for binding to the ER, then the ER/Fluormone™ ES2 complex will remain intact. Thus, the Fluormone™ ES2 will tumble slowly during its fluorescence lifetime, resulting in a high polarization value. Competing test compounds will displace the Fluormone™ ES2 ligand from ER, permitting it to tumble rapidly and result in a low polarization value. The change in polarization value in the presence of a test compound is used to determine relative affinity of that test compound for ER.

3. Kit Description

3.1 Materials Supplied

Each kit contains enough reagents to perform 500 assays (catalog no. P2698) or 2,000 assays (catalog no. P2614) in 100 µL volumes.

Description	Composition	P2698		P2614	
		Size	Part #	Size	Part #
Fluormone™ ES2	1800 nM in 80% methanol/20% water (see **Note** below)	125 µL	P2645	500 µL	P2613
Estrogen Receptor-alpha (ERα) Human Recombinant	Buffer: 50 mM Tris-HCl (pH 8.0), 500 mM KCl, 2 mM DTT, 1 mM EDTA, 1 mM Na_3VO_4, 10% glycerol	750 pmol*	P2187	4 × 750 pmol*	P2187
ES2 Screening Buffer	100 mM potassium phosphate (pH 7.4), 100 µg/mL BGG, 0.02% NaN_3	120 mL	P2616	2 × 120 mL	P2616
* The concentration of ERα is provided on the Certificate of Analysis, included with the product.					

Note on the Concentration of Fluormone

As of November 2009, we have updated our method for calculating the concentration of fluormone. We had been using a method of fluorescent intensity to ensure that FP instruments would be detecting 1 nM of fluormone with uniform intensity lot to lot. We have changed our method to absorbance, as this gives a much more accurate determination of the true concentration of fluormone in solution.

We have **not** changed the concentration of fluormone used in this kit. But we have determined that the actual concentration as determined by absorbance is different than what was determined using fluorescent intensity. To be as clear and as accurate as possible, we are therefore updating the listed concentrations to the values as determined by absorbance. You will notice that the final volumes used in your assays are not affected since the concentration of the reagent and the recommended concentration for the assay have both been updated.

3.2 Materials Required but Not Supplied

- Single-tube or multi-well fluorescence polarization instrument with suitable 485 nm excitation and 530 nm emission interference filters
- Pipetting devices P20, P200, and P1000
- Reagent reservoir
- Black, round-bottom microtiter plates for use in the multi-well fluorescence polarization instrument, or 6 mm diameter borosilicate tubes
- Laboratory timer
- FP One-Step Reference Kit (Invitrogen Part No. P3088). This kit is recommended for validating instrument performance.
- Estradiol, required for the positive control

3.3 Storage and Stability

Description	Storage Temperature	Notes
Fluormone™ ES2	–20°C	The Fluormone™ ES2 may have estrogenic activity *in vivo* and therefore should be handled with caution. Avoid repeated freeze/thaw cycles.
Estrogen Receptor-alpha (ERα) Human Recombinant	–80°C	May aggregate with rough handling. Do not vortex. Do not expose the reagent to more than 3 freeze-thaw cycles. Once thawed, the ER must remain on ice.
ES2 Screening Buffer	20–30°C	

4. Estrogen Competition Assay

Adding 2X Fluormone™ ES2/ERα complex to a dilution series of the test compound will generate a competition curve. The polarization will be plotted against the concentration of test compound. The concentration of the test compound that results in a half-maximum shift in polarization equals the IC_{50} of the test compound.

4.1 General Considerations When Designing a Competition Assay

Controls:

Type of Control	Control Composition	Expected Result
High Polarization Control (No Competition)	1X Fluormone™ ES2/ER complex	mP_{max}
Low Polarization Control (100% Competition)	1X Fluormone™ ES2/ER complex + 1 µM (or more) estradiol (or other potent estrogenic compound)	mP_{min}†
Fluormone™ ES2 baseline	1X Fluormone™ ES2	tracer mP†
ES2 Screening Buffer background fluorescence	ES2 Screening Buffer	No fluorescence
ER background fluorescence‡	1X ER in ES2 Screening Buffer	No fluorescence

† Note that 100% competition and Fluormone™ ES2 baseline should have very similar mP values: there is essentially no non-specific binding in this assay, and therefore all Fluormone™ ES2 can be displaced.

‡ We have observed **no** fluorescence from the ER or the ES2 Screening Buffer.

If the final concentration of solvent exceeds 2%, then include the same concentration of solvent in the recommended controls.

Standards: We recommend using 1 nM Low Polarization Solution and 1X High Polarization Solution from the FP One-Step Reference Kit to determine if the instrument is measuring polarization accurately.

ER Handling: Thaw ER on ice, and leave on ice or at 4°C. **Never vortex ER.**

Solvent Tolerance: We recommend using a minimal amount of DMSO in the assay. The reaction will tolerate 1% DMSO, but 2% DMSO will result in a diminished dynamic range. You may use up to 2% methanol without any loss in dynamic range.

4.2 Competition Experiments

Design the fluorescence polarization competition experiments such that the ERα/K_d ratio is at least 1, so that the starting polarization value will represent at least 50% of the maximal shift. The K_d of the Fluormone™ ES2 equals 18 ± 9 nM. We recommend using 4.5 nM Fluormone™ ES2 and 15 nM ERα.

5. Procedure

5.1 Prepare Reagents

1. Remove ERα from the –80°C freezer and thaw on ice for at least 1 hour prior to use.
2. Prepare serial dilutions of the test compounds in tubes or directly on the microtiter plate. The test compounds will be diluted 2-fold in the final reaction.

5.2 Prepare the 2X Fluormone™ ES2/ ERα Complex

1. Prepare enough of the 2X Complex for all reactions being performed. Assuming the total reaction volume equals 100 µL, you need 50 µL of 2X Complex for each tube or well. The recommended concentration of the 2X Complex is 9 nM Fluormone™ ES2 and 30 nM ERα. The Fluormone™ ES2 is dissolved in 80% methanol and is therefore quite volatile. Keeping this reagent covered and on ice will aid in its stability and reduce its volatility.
2. Calculate the amount of Fluormone™ ES2 needed in the 2X Complex. In the equation below, write in the number of tubes or wells needed [A], the volume of 2X Complex needed per tube or well [B], the desired Fluormone™ ES2 concentration in pmol/µL (we recommend 9 nM or 0.009 pmol/µL) [C], and calculate the volume of Fluormone™ ES2 needed in µL [D]. For example, if you need 50 wells, with 50 µL of 2X Complex per well, and the desired Fluormone™ ES2 concentration is 0.009 pmol/µL; include 12.5 µL of Fluormone™ ES2 in the 2X Complex.

[A]_____ × [B]_____ × [C]_____ ÷ Fluormone™ ES2 concentration 1.8 pmol/µL = µL of ES2 needed [D]_____

3. Calculate the amount of ERα needed in the 2X Complex: In the equation below, write in the number of tubes or wells needed [A], the volume of 2X Complex needed for each tube or well [B], the desired ERα concentration (we recommend 30 nM or 0.030 pmol/µL) [E], the concentration of the ERα (taken from the Certificate of Analysis) [F], and calculate the volume of ERα in µL needed [G]. For example, if you need 50 wells, with 50 µL of ERα per well, and the concentration of ERα is 6 pmol/µL, include 12.5 µL of ERα in the 2X Complex. Make up the rest of the volume with ES2 Screening Buffer. Protect the 2X Complex from light.

[A]_____ × [B]_____ × [E]_____ ÷ [F]_____ = µL of ER needed [G]_____

The following table contains the recipe for making 2X Complex assuming the Fluormone™ ES2 concentration is 1800 nM and the ERα concentration is 6 pmol/µL [F]. Use the empty rows in the table as a worksheet for your own experiments.

Number of wells [A]	Final Volume per Reaction	2X Complex per well [B]	2X Complex Final Volume [A] x [B]	Fluormone™ ES2 [D]	ERα [G]	ES2 Screening Buffer [A] x [B] – ([D] + [G])
50	100 µL	50 µL	2500 µL	12.5 µL	12.5 µL	2475 µL
50	200 µL	100 µL	5000 µL	25 µL	25 µL	4950 µL
384	100 µL	50 µL	19200 µL	96 µL	96 µL	19008 µL

5.3 Perform the Competition Assay

1. Add 50 µL of 2X Complex to the tubes or microtiter plate wells (already containing the serial dilutions of test compounds) and mix well by shaking on a plate shaker. Include a control that contains 50 µL ES2 Screening Buffer and 50 µL of 2X Complex. This is a negative control to determine the polarization value with no competitor present, and represents 0% competition (maximum theoretical polarization).
2. Incubate the tubes or plates in the dark at room temperature (20–30°C) for 2 hours. Read the polarization values within 7 hours of the time when the reaction was started.
3. Measure polarization values in each tube or well.

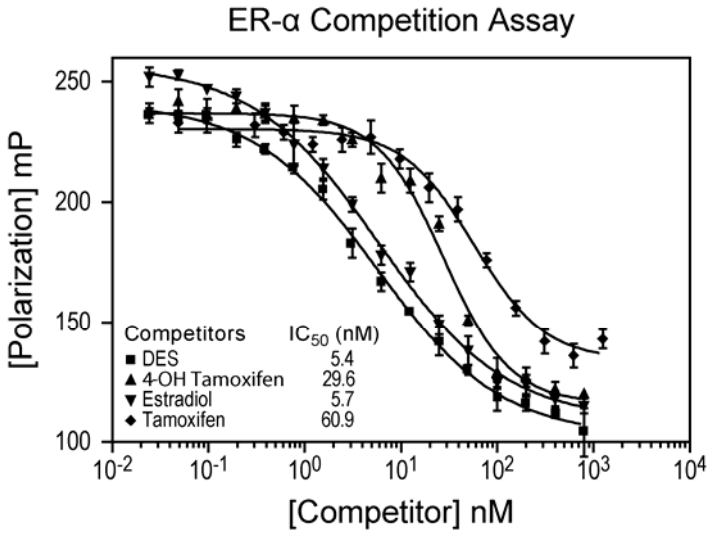
6. Results and Discussion

Below is an example of competition data generated using the PolarScreen™ Estrogen Receptor-α Competitor Assay. The concentration of the test compound that results in a half-maximum shift in polarization equals the IC_{50} of the test compound, which is a measure of the relative affinity of the test compound for ERα. Error bars represent the mean of three separate experiments ± 1 standard deviation. This curve was plotted using the following equation:

$$Y = mP_{100\%} + (mP_{0\%} - mP_{100\%})/1 + 10^{((LogIC50-X) \times Hillslope)}$$

Where: Y = mP, X = Log [inhibitor], $mP_{100\ \%}$ = 100 % inhibition, and $mP_{0\ \%}$= 0 % inhibition.

Curve fitting was performed using GraphPad Prism® software from GraphPad™ Software Inc.



7. References

1. Green *et al.* (1986) *Nature* **320:**134-9
2. Greene *et al.* (1986) *Science* **231:**1150-4
3. Mosselman *et al.* (1996) *FEBS Lett.* **392:**49-53