Chapter 34

Detection of Endogenous Selective Estrogen Receptor Modulators such as 27-Hydroxycholesterol

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Abstract

The estrogen receptors (ERs) belong to the nuclear receptor superfamily, and as such act as ligand inducible transcription factors, mediating the effects of estrogens. However, their pharmacology is complex, having the ability to be differentially activated by ligands. Such ligands possess the ability to behave as either ER-agonists or ER-antagonists, depending on the cellular and tissue context, and have been termed Selective Estrogen Receptor Modulators (SERMs). Several SERMs have been identified with clinical relevance such as tamoxifen and raloxifene. Recently, 27-hydroxycholesterol has been characterized as the first identified endogenous SERM leading to the notion that other endogenous SERMs may exist, each having potential pathophysiological functions. This, coupled with the historic pharmaceutical interest as well as growing concern over chemicals in the environment with the ability to behave like SERMs, has increased the demand for assays to detect SERM-like activity. Here, we describe a common, straightforward in vitro assay investigating the induction of classic ER-target genes in MCF7 breast cancer cells, allowing one to identify ligands with SERM-like activity.

Key words Estrogenreceptor, Selective estrogen receptor modulator (SERM), Estradiol, 27-Hydroxycholesterol, Tamoxifen, Gene expression, Quantitative PCR, MCF7 cells, Pharmacology

1 Introduction

The estrogen receptors (ERs) are intracellular transcription factors belonging to the large nuclear receptor superfamily, and are responsible for mediating the majority of the biological activity ascribed to estrogens. Nuclear receptors are characterized by containing a ligand binding domain (LBD) and a DNA binding domain (DBD). There are two forms of the ER, ERα and ERβ [1, 2]. The expression of ERα is fairly ubiquitous, while ERβ is the major form expressed in the ovary, lung and prostate [3]. 17β-Estradiol (E2) is the most potent endogenous agonist for the ERs. However, the well-defined LBD has allowed for the pharmacological development of agonists and antagonists for the treatment of various disorders such as osteoporosis or breast cancer. Intriguingly, it has been

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found that certain ligands have the capacity to antagonize the receptor in some contexts, while behaving as an agonist in other contexts. The best studied example of this is tamoxifen, which behaves as an ER-antagonist in ER-positive breast cancers, but agonizes the receptor in the uterus and bone tissues. These type of dual-activity ligands has been termed Selective Estrogen Receptor Modulators (SERMs) [4].

As a transcription factor, ligand-bound receptor dimers in the nucleus interact directly with DNA on hormone response elements [5, 6], or indirectly via other transcription factors such as AP1, NFκB or Sp1 [7]. Once bound to DNA, the receptors interact with a large assembly of coregulator proteins termed coactivators and corepressors. Different ligands result in the association of unique sets of coregulators, and it is generally understood that it is the composition of these coregulators that determine whether a ligand behaves as an agonist, antagonist, or SERM. With over 300 proteins having the ability of interacting with the ER [8], the potential complexity of the coregulator complex is immense. In a further layer of complexity, the ER can also function to regulate several other signaling pathways. In this regard, the ER has been demonstrated to interact with the c-Src protein kinase complex, the regulatory subunit of phosphoinositide-3 kinase (p85), MAPK, caveolins and the adaptor protein Shc, modulating the activity of the their associated signaling cascades [9, 10]. It is important to note that the assays described below will not be able to distinguish between ligands that directly bind to and activate the ER as opposed to indirect activation of the ER.

Perhaps due to its rather 'flexible' LBD, coupled with the number of signaling pathways that converge on the ER, many ligands have been identified as having SERM activity. These include drugs designed to modulate the ER such as diethylstilbestrol, tamoxifen, raloxifen or fulvestrant, as well as a growing number of anthropogenically derived chemicals such as dichlorodiphenyltrichloroethane (DDT) and its breakdown products, and bisphenol A (BPA). These chemicals, termed xenoestrogens or environmental estrogens, are a class of endocrine disrupting chemicals with SERM activity. Intriguingly, certain cholesterol metabolites such as 27-hydroxycholesterol (27HC) and 25-hydroxycholesterol have been recently identified as endogenous SERMs [11–13]. Importantly, the ability of 27HC to modulate ER activity may have important health consequences as it has been shown to inhibit the cardio-protective effects of estradiol [13], promote atherosclerosis [14], decrease bone quality [15, 16], and promote the growth of ER+ breast cancer [17, 18].

Due to the pharmaceutical interest in the therapeutic development of SERMs, the environmental concerns of endocrine disruptors, and the potential pathological roles of endogenous SERMs, there is strong demand for assays that identify SERM like activity.

There are several approaches that can be taken. While each approach has strong merit in and of itself, in actuality, one would desire a comprehensive workup including several, if not all of these approaches.

The most traditional approach is to determine whether a ligand binds the ER, and/or whether it has the ability to compete for estradiol occupancy [19]. Another conventional method to identify estrogen-like activity is the use of a receptor-reporter assay such as the luciferase reporter assay. A version of this assay was used to identify 27HC as an endogenous SERM [11, 13]. Harnessing the ability of different ligands to shift the ERs into unique conformations, peptide interactions with the ligand-bound ER could potentially be used to predict the SERM like activity of a given ligand [20]. This technique was used to show that 27HC shifted the ER into a conformation similar to, but still distinct from that of tamoxifen or estradiol [11]. Furthermore, this technique has been used to accurately predict the relative agonist or antagonistic properties of different androgen receptor ligands, indicating the powerful nature of this style of assay [21]. One fairly straightforward method to determine whether a ligand modulates ER expression is to determine whether it regulates the gene expression of ER-target genes. This method has the advantage of investigating endogenous gene transcription in a well-defined system. While it can be adapted to high-throughput screening with a one or two gene readout, differential gene expression can also be used to determine the SERM-like nature of ligands [22]. This chapter describes a protocol for assessing ER-regulated gene expression in response to estradiol and known agonists and antagonists of ER, as well as proposed new potential ligands using qRT-PCR as the read out of gene expression.

2 Materials

Prepare all solutions using sterile, ultrapure water (resistivity 18.2 MΩ) and molecular grade reagents.

1. MCF7 cells (*see* **Note 1**).
2. Sterile DMEM/F-12 media with phenol red. This can be purchased from any commercial vendor or made as follows: 1:1 mix of high glucose DMEM (4.5 g/L glucose) and standard Ham's F-12.
3. Sterile, phenol red free DMEM/F-12 (*see* **Note 2**).
4. Fetal Bovine Serum (FBS) (*see* **Note 3**).
5. Culture media: DMEM/F-12 with phenol red, 8 % FBS, 1 mM sodium pyruvate, 1× nonessential amino acid mix, 100

U/mL penicillin–streptomycin (optional). Nonessential amino acid mix can be purchased commercially at 100×.

6. Charcoal stripped FBS (CFBS) (*see* **Note 4**).
7. Experimental media: phenol red free DMEM/F-12, 8 % CFBS, 1 mM sodium pyruvate, 1× non-essential amino acids mix, 100 U/mL penicillin–streptomycin mix (optional).
8. Sterile Phosphate Buffer Saline (PBS).
9. Phenol red free 1× Trypsin–EDTA solution.
10. Trypan blue dye.
11. 10 cm cell culture plates (*see* **Note 5**).
12. 6-well cell culture plates (*see* **Note 5**).
13. 17β-estradiol.
14. 27-hydroxycholesterol (27HC; (25R)-Cholest-5-ene-3β, 26-diol).
15. Fulvestrant (ICI 182,780).
16. Vehicle: dimethyl sulfoxide (DMSO).
17. Alternate vehicle: ethanol.
18. RNA purification kit (*see* **Note 6**).
19. cDNA synthesis kit (*see* **Note 7**).
20. SYBR Green supermix (*see* **Note 8**).
21. siRNA delivery kit and siRNA against ERα.
22. Laminar flow hood (sterile cell culture hood), cell culture incubator, swinging bucket centrifuge, hemocytometer.
23. Real-time thermal cycler capable of reading SYBR Green.

3 Methods

3.1 Establishing MCF7 Cells

1. Remove a vial of MCF7 cells from cryopreservation.
2. Rapidly thaw.
3. Dilute cells in 10 mL of pre-warmed culture media.
4. Plate on 10 cm cell culture plate.
5. Incubate at 37 °C, 5 % CO_2.

3.2 Passaging MCF7 Cells

Passage MCF7 cells approximately twice/week. It is recommended that they are split 1:3 to 1:6 (*see* **Note 9**).

1. Aspirate media.
2. Rinse with PBS.
3. Aspirate PBS.
4. Add 2 mL of warmed trypsin–EDTA mix.

5. Incubate at 37 °C, 5 % CO_2 for approximately 5 min until cells start lifting.
6. Dilute with 8–10 mL of warmed culture media (*see* **note 10**).
7. Split at desired ratio into fresh 10 cm culture plates with 10 mL of culture media.

3.3 Setting Up the Dose Response Experiment

1. Aspirate media from several plates of MCF7 cells.
2. Rinse with PBS.
3. Aspirate PBS.
4. Add 2 mL of warmed phenol red free trypsin–EDTA mix.
5. Incubate at 37 °C, 5 % CO_2 for approximately 5 min until cells start lifting.
6. Dilute with 8–10 mL of warmed Experimental Media and pool cells from different plates.
7. Pellet the cells in a swinging bucket centrifuge (approximately $125 \times g$ for 5 min).
8. Aspirate supernatant and resuspend pellet in approximately 5 mL of Experimental Media (*see* **Note 10**).
9. Count cells:
   (a) Add 10 μL cells to 10 μL trypan blue dye.
   (b) Add 10 μL of the mix to a hemocytometer.
   (c) Count the cells based on trypan blue exclusion.
10. Dilute cells to 300,000/mL in Experimental Media.
11. Plate 1 mL of cells per well in 6-well culture dishes. The total concentration of MCF7 cells should be 300,000 cells/well.
12. Incubate overnight at 37 °C, 5 % CO_2.
13. Treat with ligands such as estradiol and 27HC (*see* **Notes 11–15**).
    (a) Aspirate media.
    (b) Replenish with 1 mL of experimental media already containing diluted ligands.
14. Incubate for 24 h at 37 °C, 5 % CO_2.
15. Aspirate media and lightly rinse with PBS.
16. Aspirate PBS.
17. Freeze at –80 °C, or continue processing as described in Quantitative, Reverse-Transcription PCR.

3.4 Determining Requirement of ER: Pharmacological Approach

Once it has been shown that your ligand of interest modulates the expression of ER target genes (experiment 3.3, 3.6 and 3.7), it is necessary to establish the requirement of the ER. This can be done by use of a pharmacological antagonist of the ER such as fulvestrant (ICI 182,780), or by siRNA against the ER.

1. Repeat Dose Response experiment, **steps 1–12**.
2. Treat with ligands as in Dose Response experiment, **step 13** (*see* **Note 16**).
3. Incubate for 24 h at 37 °C, 5 % CO_2.
4. Aspirate media and lightly rinse with PBS.
5. Aspirate PBS.
6. Freeze at −80 °C, or continue processing as described in Quantitative, Reverse Transcription PCR.

3.5 Determining Requirement of ER: RNAi Approach

Although fulvestrant has been validated as a 'pure' antiestrogen, potential off target effects are still possible. Likewise, RNA interference (RNAi) can also have off-target effects. Therefore, one should make use of both complementary strategies.

1. Repeat Dose Response experiment, **steps 1–9**.
2. Treat with siRNA as described in siRNA delivery kit. Remember to use Experimental Media (no phenol red, charcoal stripped serum) (*see* **Note 17**).
3. Incubate for 24 h at 37 °C, 5 % CO_2 (*see* **Note 18**).
4. Treat with ligands as in Subheading 3.3, **step 13**.
5. Incubate for 24 h at 37 °C, 5 % CO_2.
6. Aspirate media and lightly rinse with PBS.
7. Aspirate PBS.
8. Freeze at −80 °C, or continue processing as described in Quantitative, Reverse Transcription PCR.

3.6 Quantitative, Reverse-Transcription PCR

1. Isolate RNA using a standard RNA column purification kit as described in the instructions provided by the manufacturer (*see* **Note 6**).
2. Quantify RNA by measuring the absorbance at 260 nm.
3. Synthesize complementary DNA (cDNA) as described in the instructions provided by the manufacturer for reverse-transcription (*see* **Note 19**). Remember to include a no-RT negative control.
4. Dilute cDNA with sterile, RNAse and DNAse free water as appropriate (*see* **Note 20**).
5. Set up SYBR Green based quantitative, real-time PCR (qPCR) as described by the kit manufacturer and as optimized by your laboratory (*see* **Note 21**). Include primers for the following genes: pS2, PR, and one or more housekeeping genes (*see* Table 1 and **Notes 22–24**). Remember to create a standard curve to confirm the efficiency of each primer set.

Table 1
Primer sequences for SYBR Green based quantitative PCR

Gene	Forward sequence	Reverse sequence
PS2 (TFF1)	TCCCCTGGTGCTTCTATCCTAATAC	GCAGTCAATCTGTGTTGTGAGCC
PR	GCATCGTTGATAAAATCCGCAG	AATCTCTGGCTTAGGGCTTGGC
36B4	GGACATGTTGCTGGCCAATAA	GGGCCCGACCAGTGTT

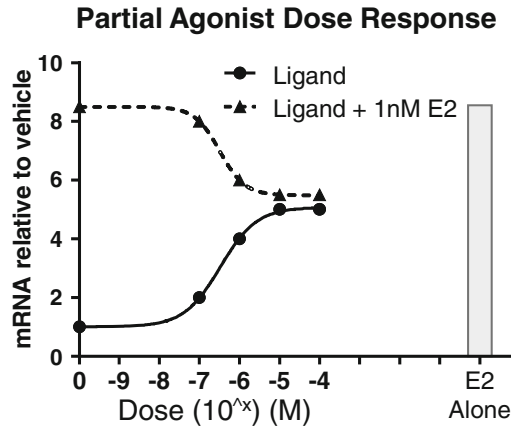

Fig. 1 Example dose response depicting fictitious data from an experiment similar to that in the Dose Response Experiment. This is similar to the results that would be expected from a classic partial ER agonist, such as 27-hydroxycholesterol [11, 13]. The ligand induces ER-target gene expression in a dose related manner, but never reaches the induction level of 1 nM of 17β-estradiol (E2). Furthermore, increasing doses of the ligand decrease the ability of E2 to induce the gene, until the induction reaches the same point as the maximal induction by the ligand alone

3.7 Analysis and Interpretation of Results

1. Ensure that your primers amplify only one product by consulting the melt-curve data generated at the end of the qPCR run.
2. Ensure that each primer set amplified genes in an equally efficient manner.
3. Use the Comparative Ct Method of qPCR analysis (2 – [delta][delta]Ct method) as follows:
   (a) Expression = $2^{(-(\text{Ctgene of interest} - \text{Cthouse keeping gene}))}$.
   (b) Normalize expression to that of vehicle alone (i.e., divide all values by the vehicle expression, so that vehicle becomes set at 1).
   (c) Plot values in graphical format (*see* Figs. 1, 2, and 3).

For dose response data, nonlinear regression can be performed using a 4-parameter fit (variable slope model) (*see* Figs. 1 and 2). If the dose response looks similar to Fig. 1, then it is likely that the

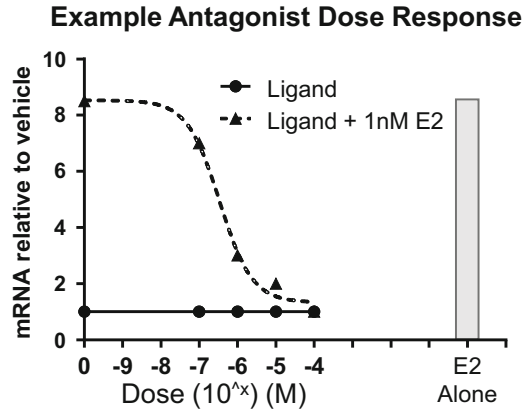

Fig. 2 Example dose response depicting fictitious data from an experiment similar to that in the Dose Response Experiment. This is similar to what would be expected from an antagonistic SERM, such as tamoxifen or fulvestrant. The ligand fails to induce the expression of an ER target gene at any dose tested. However, the ligand antagonizes the activity of estradiol (E2)

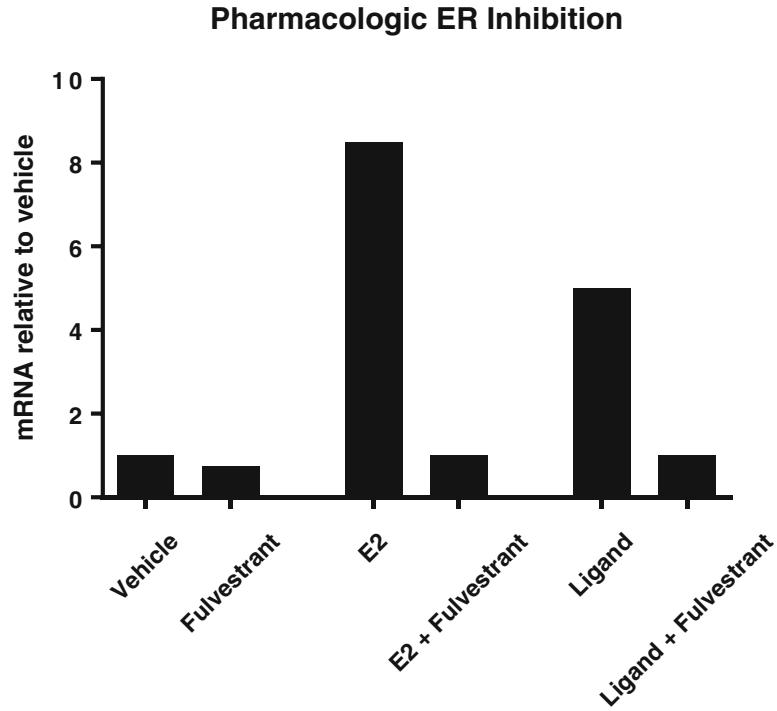

Fig. 3 Example of fictitious data from an experiment designed to determine if the ligand of interest induces gene expression in an ER-dependent manner. Fulvestrant is a "pure" ER antagonist which also leads to the depletion of ER protein. It is common to observe that fulvestrant treatment alone will decrease the expression of ER-target genes slightly. Note that when fulvestrant is co-treated, it attenuates the gene induction by either E2 or the ligand of interest. This is similar to data that would be obtained from a partial ER-agonist such as 27-hydroxycholesterol [11, 17]

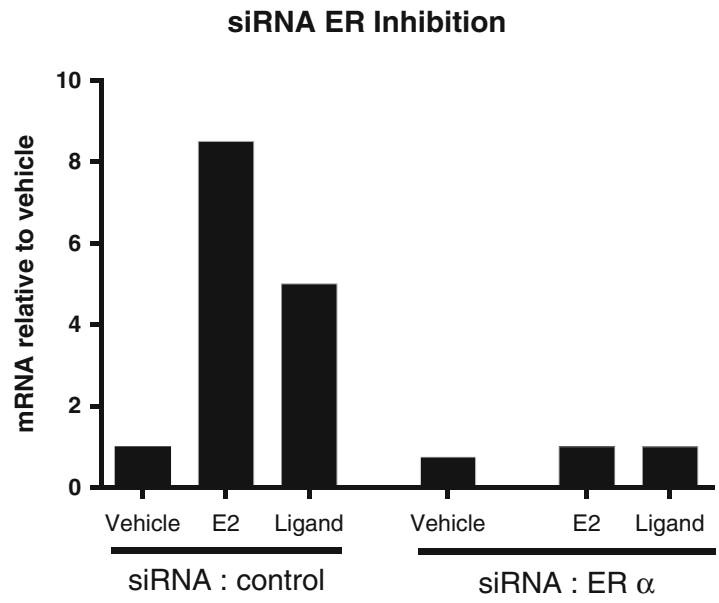

Fig. 4 Example of fictitious data from an experiment designed to determine if the ligand of interest induces gene expression in an ER-dependent manner. Use of siRNA against ERα will deplete the receptor. It is common to observe that siRNA against ERα will decrease the expression of ER-target genes slightly. Note that when siRNA against ERα is present, the E2 or ligand induction of the ER target gene is attenuated. This is similar to data that would be obtained from a partial ER-agonist such as 27-hydroxycholesterol [11, 17]

ligand of interest is a partial ER agonist, similar to 27HC. On the other hand, if the dose response looks similar to Fig. 2, then it is likely that the ligand of interest is an ER antagonist, similar to tamoxifen or fulvestrant. If your data looks similar to Fig. 1, then Determining Requirement of ER experiments should be performed in order to determine if the observed induction of ER target genes requires the presence of the ER. If it is, then you should obtain data similar to Figs. 3 and 4.

4 Notes

1. The Amterican Type Culture Collection (ATCC) supplies MCF7 cells. Many different sublines of MCF7 cells have evolved over time in different labs. While they all typically continue to express ERα, the magnitude of response to estradiol varies significantly between sub-lines. Furthermore, it has been our experience that response to estradiol decreases as MCF7 cells are passaged or if they are overgrown. For more on this, please refer to **Note 9**.

2. Phenol red is a weak 1agonist of the ER. Therefore, to eliminate this confounding effect, and to increase the window of response, use phenol red free media when studying ER gene regulation.
3. FBS lots very greatly with respect to growth factor and steroid concentrations. We test several lots for estrogenic activity using a receptor-reporter assay and select the one with the least activity. We then buy this in bulk. To reduce variation, all experiments should be done with one lot of FBS.
4. FBS is charcoal stripped to remove any steroids, thereby eliminating their confounding effects on the ER, and increasing the window of response. CFBS should be from the same lot of nonstripped FBS. The effectiveness of charcoal stripping should be confirmed in an ER receptor-reporter assay.
5. Although the data is perhaps more anecdotal in nature, plastics can leach chemicals with estrogen like activity. We typically select a high quality plastic and use the same plastic (and manufacturer) throughout all of our studies.
6. A number of commercially available RNA column purification kits are suitable for this assay. We commonly use the Bio-Rad Aurum Total RNA Mini Kit. Guanidinium thiocyanate–phenol–chloroform extraction can also be used [23]. Regardless of the method selected, it is important to include a DNAse step to remove any contaminating genomic DNA. Column purification kits facilitate this by allowing in-column DNAse digestion and subsequent purification from DNAse. If you use the chloroform-extraction method we recommend the Ambion TURBO DNA-free kit (Life Technologies) as this technology completely removes the DNAse prior to subsequent cDNA synthesis.
7. A number of commercially available kits are suitable for this assay. We commonly use Bio-Rad iScript Reverse Transcription Supermix for RT-qPCR. Random hexamer oligo dNTPs are commonly used to anchor the reverse transcriptase, although oligo dT approach can also be used. This step can be done without a kit as well, just be sure to select a reverse transcriptase that can overcome some secondary structure.
8. SYBR green supermix can be purchased from several different vendors. We commonly use Bio-Rad iTaq Universal SYBR Green Supermix. The mix can be made in-house, but requires independent validation and optimization [24].
9. MCF7 cells should be split when they are ~80–90 % confluent. Overcrowding should be avoided. Cells should form a monolayer; cells growing on top of one another are detrimental to this assay. MCF7 cells lose their responsiveness to estradiol

through passaging. We recommend only using MCF7 cells passaged up to maximum of 20–25 times.

10. MCF7 cells are rather sticky and tend to clump together. When diluting from trypsin, run the culture media / cell mixture up and down through a 10 mL pipette several times to help dislodge the cells from one another.
11. To avoid toxicity, it is best to keep the vehicle volume (either DMSO or ethanol) to a minimum. Therefore, we usually keep our ligands at a 1000× stock.
12. Avoid adding ligands directly to the cells. A better approach is to pre-dilute ligands into 1 mL of experimental media, and using this to replace the existing media.
13. Avoid letting cells dry out. One approach is to aspirate and replenish one well at a time.
14. We suggest the following treatment groups:
    (a) Vehicle alone.
    (b) 17β-Estradiol at 1 nM. This will serve as a positive control.
    (c) 4–8 different doses of your ligand of interest.
    (d) 17β-Estradiol in combination with 4–8 different doses of your ligand of interest. This group will allow you to determine whether your ligand of interest is a "partial agonist" or antagonist, with the ability to compete with full agonist potential of estradiol.
15. Remember to control for the total volume of vehicle added (i.e.: the estradiol in combination with your ligand of interest treatment groups will receive vehicle for both treatments. Thus all other groups must have extra vehicle to equal this volume).
16. We suggest the following treatment groups:
    (a) Vehicle alone.
    (b) 17β-Estradiol at 1 nM. This will serve as a positive control.
    (c) Fulvestrant at 1 μM. This is a 'pure' antiestrogen and leads to the degradation of the ER. It is also known as Faslodex or ICI-182,780.
    (d) 17β-Estradiol (1 nM) in combination with fulvestrant (1 μM). This will serve as a positive control for the efficacy of fulvestrant.
    (e) Your ligand of interest at a dose which gives the maximal induction as determined in experiment 3.5.
    (f) Your ligand of interest at the above dose in combination with fulvestrant (1 μM). This dose of fulvestrant has been

chosen as it is sufficient to inhibit the effects of 1 nM estradiol. It may be necessary to optimize this dose, depending on the affinity of your ligand for the ER.

17. ERα is the predominant, if not the only ER subtype expressed in MCF7 cells. Therefore, RNAi knockdown of only this subtype is sufficient to inhibit the estradiol induction of ER target genes.
18. 24 h of siRNA treatment should be enough time to achieve a significant decrease in ER protein. However, this should be confirmed, as different siRNA delivery kits or siRNA targeting sequences may exhibit different kinetics.
19. We typically synthesize 1 μg of total RNA into cDNA.
20. The dilution factor must be optimized for the particular kits and qPCR apparatus you are using. We typically dilute by sixfold.
21. qPCR is a science unto itself and requires optimization and standardization. It is necessary to optimize the conditions for SYBR Green qPCR in your own laboratory as described in the many good manuals, webpages, and advice from the manufacturers of real-time thermocyclers for details.
22. pS2 is also known as Trefoil factor 1 (TFF1), PR is progesterone receptor.
23. Many good housekeeping genes are available. We have found that GAPDH is not reliable and can be regulated by ER. 36B4, RPS18, and tata binding protein have been reliable in our hands. Furthermore, many groups are now advocating that more than one housekeeping gene be used.
24. PS2 and PR have been chosen as they are robustly induced by estradiol in MCF7 cells. However, one can also look at other ER-target genes such as WISP2 and SDF-1. Furthermore, genes specifically regulated by different SERMs in MCF7 cells have been described [22, 25, 26]. We encourage several genes to be included in order to provide a comprehensive overview of the SERM-like activity of the ligand in question.

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