

# Determining Endocrine Receptor Activity Quantitatively using Automated Image Analysis and Redistribution® Technology

Raymond A. Lewis, Amy M. Peters, Jeff R. Haskins, and Yuriy Fedorov  
Thermo Fisher Scientific, Inc. • Pittsburgh, PA 15219 • USA

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## Abstract

Endocrine hormone function has been shown to regulate numerous oncogenic processes from tumor progression, promotion, and dependence to prevention, diagnosis, and treatment. Notably, estrogen and androgen activity in breast and prostate cancers have been used to diagnose steroid hormone dependence and determine therapy options. Endocrine disruptors are compounds that mimic estrogen or androgen steroid activity and can be either agonists or antagonists. Identifying endocrine disruptors is a possible therapy for endocrine-dependent cancers. Screening complex chemical libraries for endocrine disruptors and investigating the molecular and cellular effects of existing treatment strategies is accelerated by using high throughput procedures to assess endocrine activity and potential cancer therapeutic efficacy.

The Thermo Scientific Redistribution® technology can be used to monitor the localization of GFP-tagged proteins in response to extra-cellular stimuli such as treatment with steroid compounds or activation of signaling cascades. Redistribution GFP-tagged steroid receptors such as estrogen receptor alpha (ERα), estrogen receptor beta (ERβ), and androgen receptor (AR) form nuclear foci in response to hormone stimulation that can be enumerated and quantified by automated image analysis. This technology can be used to screen for endocrine disruptors and antagonists of ER and AR activation. Additionally, multiplexed cell and nuclear phenotypes such as receptor kinetics, cellular health, and cell cycle effects can be monitored simultaneously following direct or indirect receptor stimulation.

Here we utilize Redistribution® cell lines and known endocrine stimulating chemicals to establish a screen for ER and AR disruptors. For ER screens, cells were treated with agonists including 17β-estradiol and bisphenol A with and without fulvestrant (ICI 162,780), a known ER antagonist. AR cells were treated with dihydrotestosterone (5α-androstan-17β-diol-3-one) as a positive control and other potential AR disruptors. Following fixation and Hoechst staining, plates were analyzed using a quantitative automated image analysis system. ER and AR nuclear foci intensity and nuclear morphology on a cell-by-cell basis. EC50 values were determined from dose-dependent response curves. The treatments to establish hierarchical receptor activation comparisons for both ER and AR. Z-factors for these features indicate that using this technology gives a robust assay with high reproducibility. This data suggests that ER and AR Redistribution cell lines can be effectively utilized with high throughput automated image analysis to screen for potential steroid hormone disruptors that would increase cancer therapeutic efficacy and specificity.

## Introduction

Endocrine receptors are members of the nuclear receptor family that function in response to hormone stimulation [1] and can be activated by natural or synthetic ligands. Activation of endocrine receptors results in nuclear translocation and transcriptional activation of endocrine-responsive genes involved in normal physiological function including reproduction, growth, and sexual maturation [3]; specifically, androgen receptors (AR) are activated by testosterone derivatives and two estrogen receptors (ERα and ERβ) are activated by estrogen and related hormones to regulate distinct aspects of human development and homeostasis. Not surprisingly, misregulation of endocrine receptors plays a major role in common cancers along with developmental and reproductive defects and thus have been heavily researched as therapeutic targets [4]. Additionally, the environmental impact of chemicals on both humans and wildlife endocrine functionality is a widely studied and disputed topic [5].

The molecular mechanism following receptor activation has been well defined and distinct steps in the hormone receptor lifecycle have been studied. The ER nuclear foci formed in response to stimulation have been hypothesized to be transcriptional ligand binding and nuclear responsive genes [6]. Following ligand binding and nuclear accumulation, ERα is phosphorylated at multiple sites and targeted for ubiquitination and degradation [7,8]. The ER nuclear foci are also studied with a small subset of ligands mainly using ERα and ERβ alterations in the ER lifecycle by endocrine disruptors and anti-cancer chemotherapies may differentially alter ERα and ERβ activity and function.

High Content Analysis (HCA) combines automated imaging with image analysis to provide multiple measures of morphological and biological outputs thus providing a robust alternative to classic methods. The EPA has been challenged to develop *in vitro* protocols to screen for endocrine receptor disruptors that are fast, sensitive, reduce animal usage, are amenable to automation, and directly monitors receptor function [9]. Compelling Redistribution technology with HCA to screen for endocrine disruptors provides distinct advantages over other screening methods. In addition to meeting the EPA requirements, this cell-based fluorescence reporter approach provides multiple readouts including fluorescence and receptor activation on a cell-by-cell basis. Measuring the cellular activity of individual nuclear receptor subtypes following chemical treatment provides a visual and quantitative mechanism of action and may be predictive of endocrine side effects by elucidating off-target receptor interactions.

## Materials and Methods

### Cell lines and maintenance

Redistribution cell lines (Thermo Fisher Scientific, Pittsburgh, PA) were maintained in DMEM/F12 (Life Technologies) with the following instructions. All cell culture media, serum, and supplements were from Hyclone (Thermo Fisher Scientific, Pittsburgh, PA).

**AR cell line** – Recombinant U2OS cells stably expressing human androgen receptor (AR) (GenBank Acc. NC014373) were added to the C-terminus of enhanced green fluorescent protein (eGFP).  
**ERα cell line** – Recombinant U2OS cells stably expressing human estrogen receptor alpha (ERα) (GenBank Acc. NC014373) were added to the C-terminus of enhanced green fluorescent protein (eGFP).  
**ERβ cell line** – Recombinant U2OS cells stably expressing human estrogen receptor beta (ERβ) (GenBank Acc. NC014373) fused to the C-terminus of enhanced green fluorescent protein (eGFP).

**Chemicals, additional reagents, and microtiter plates**  
All chemicals purchased from Sigma (St. Louis, MO) and were dissolved in DMSO. Fulvestrant (ICI 162,780) was prepared in ethanol solution 4, 5, and 8 for chemical CASRN and abbreviations. Fixation solution, PBS wash buffer, and Hoechst nuclear counter stain were used for each assay. Cells were plated in 96-well View Plates (Perkin Elmer, Waltham, MA).

### Image acquisition and analysis

**The Thermo Scientific Toxinsight IVT Platform**  
The Thermo Scientific Toxinsight IVT Platform is an innovative combination of fluorescence imaging hardware, analysis software, and pre-validated panel of reagents (cartridges) for multiplexed assessment of toxicity targets. The integration of cartridges with image analysis is a powerful and robust tool for convenient, high-throughput *in vitro* testing. The design simplifies screening workflows by simultaneously measuring multiple parameters in each cell to provide a clearer picture of mechanism of action as well as potential side effects such as toxicity.

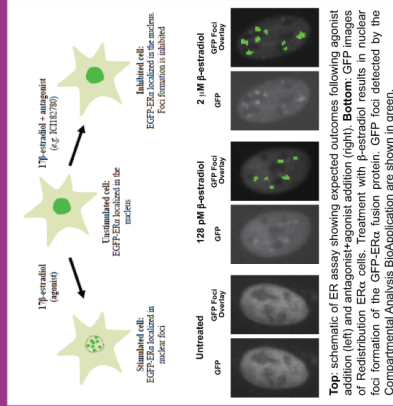
### The Thermo Scientific Compartmental Analysis BioApplication

The Thermo Scientific Compartmental Analysis BioApplication is a software algorithm that looks at various locations within the cell such as the primary object (circ), location around the primary object (ring), and cell within or around the primary object (circ spots and ring spots, respectively). For these assays, antibodies and object identification were obtained in channel 2 using nuclear masks derived from changes in channel 1. The software algorithm was used to identify cells to define the circ (nucleus) and ring (cytoplasm) signal. Nuclear GFP foci were identified as CircSpots. Object selection criteria were set to exclude apoptotic cells and cells with low GFP expression from analysis. For ER screens, the nuclear GFP total intensity was monitored for receptor turnover, nuclear GFP foci formation was monitored by foci total and average intensity, total and average area, and count. For AR assays, the same features from ER assays were used plus the cytoplasmic GFP total intensity and difference between the nuclear and cytoplasmic GFP average intensity were used to measure nuclear translocation.

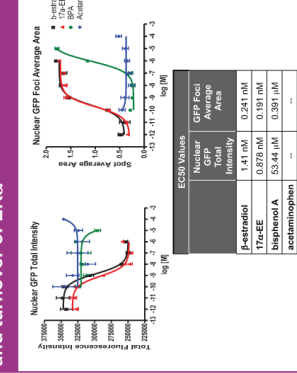
### Dose Response curves, EC50 values, and Data Analysis

Data was imported into GraphPad Prism v4 software and analyzed by non-linear regression (Sigmoidal, Dose-Response fitting) to generate EC50 and EC50 values. Comparisons of EC50 values between cell lines were graphed in Microsoft Office Excel.

## Figure 1: Detection of nuclear ER foci in the Redistribution ER assay

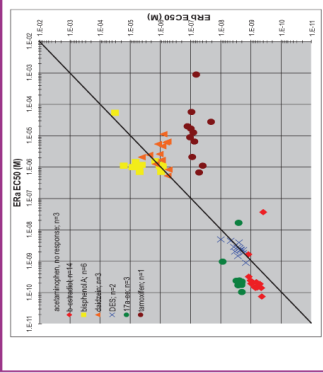


## Figure 2: Chemical-dependent activation and turnover of ERα



Dose response curves and EC50 values were calculated following treatment of ERα cells (triplicate dose points). GFP foci formation was monitored by foci average area while GFP turnover was monitored by nuclear GFP total intensity. Each chemical differentially activates ERα. Acetaminophen does not activate ERα, ERα degradation occurs following receptor activation as the nuclear GFP total intensity decreases with increased concentration and had a higher EC50 value compared to foci formation.

## Figure 3: Endocrine disruptors differentially activate ERα and ERβ



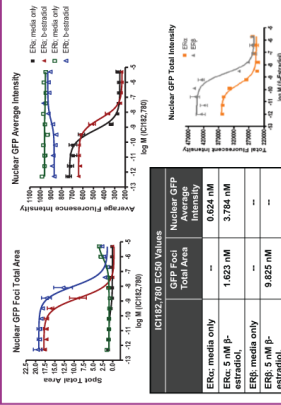
Comparison of EC50 values between ERα (X axis, top) and ERβ (Y axis, right) demonstrates the relative activation of each receptor by a chemical. Points above the diagonal line represent activation of ERα at a lower concentration than ERβ and vice versa. Clustering of chemicals such as DES, 17α-ene, and β-estradiol may signify similarity in the mechanism of receptor activation that is distinguishable from other clusters (bisphenol A, diethylstilbestrol, tamoxifen). n = number of experiments with each dose of response curve performed in triplicate.

## Figure 4: Chemical comparison via ER-GFP nuclear foci formation

| Chemical                 | CASRN      | ERα EC50 (nM) | ERβ EC50 (nM) | RBA      |
|--------------------------|------------|---------------|---------------|----------|
| β-estradiol              | 50-29-2    | 0.18          | 1.00          | 0.73     |
| bisphenol A (BPA)        | 80-05-7    | 1123.05       | 0.016         | 4278.02  |
| diethylstilbestrol (DES) | 486-65-8   | 3205.84       | 0.010         | 1291.43  |
| 17β-allyl-estradiol      | 56-53-1    | 2.57          | 7.90          | 2.65     |
| Acetaminophen            | 10540-29-1 | NR            | NR            | 0.04     |
| Acetaminophen            | 103-90-2   | NR            | NR            | negative |

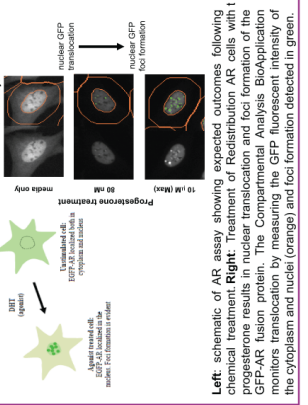
EC50 and Relative Binding Affinity (RBA) values were averaged from multiple experiments (see in the figures 5) and GFP foci formation determined by the Thermo Scientific Compartmental Analysis BioApplication (RBA = EC50β/EC50α, n = 100). NR = no response.

## Figure 5: ICI162,780 antagonizes β-estradiol induced foci formation of both ERα and ERβ but protects only ERβ from degradation

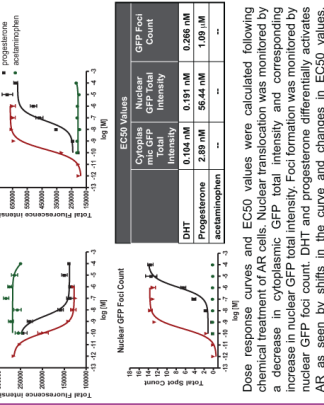


Dose response curves and EC50 values were calculated following treatment of ERα and ERβ cells with both ICI162,780 (CASRN 129453-61-8) dilutions and 5 nM β-estradiol. ICI162,780 is a more potent inhibitor of foci formation at a lower concentration (top left, red) and inhibition of foci formation at a lower concentration (top left, red) and receptor without hormone stimulation (top left, green and black curves). ICI162,780 treatment accelerates ERα degradation in the absence of β-estradiol (right, black and red curves) but does not alter ERβ levels (top right, blue and green curves). ICI162,780 protects ERβ from β-estradiol-induced degradation as ERβ is degraded in the presence of β-estradiol alone (bottom right).

## Figure 6: Detection of nuclear translocation and foci in the Redistribution AR assay



## Figure 7: Chemical-dependent AR activation



Dose response curves and EC50 values were calculated following chemical treatment of AR cells. Nuclear translocation was monitored by a decrease in cytoplasmic GFP total intensity and corresponding increase in nuclear GFP total intensity. Foci formation was monitored by nuclear GFP foci count. DHT and progesterone differentially activates AR as seen by shifts in the curve and changes in EC50 values. Acetaminophen does not activate AR.

## Figure 8: Chemical comparison via AR-GFP nuclear translocation and foci formation

| Compound                          | CASRN      | ERα EC50 (nM) | ERβ EC50 (nM) |
|-----------------------------------|------------|---------------|---------------|
| DHT (5α-androstan-17β-diol-3-one) | 521-18-6   | 0.2           | 0.3           |
| progesterone                      | 57-83-0    | 120.9         | 1090.0        |
| corticosterone                    | 50-22-6    | 774.6         | 14860.0       |
| β-estradiol                       | 50-29-2    | 19.0          | 54.2          |
| Mifepristone                      | 84371-65-3 | 7.4           | 29960.0       |
| Vinclozolin                       | 50471-44-8 | 1348.0        | 8122.0        |
| acetaminophen                     | 103-90-2   | NR            | NR            |

EC50 values were derived from the difference between the nuclear and cytoplasm average GFP intensity for nuclear translocation and nuclear GFP foci count for foci formation. NR = no response.

## Conclusions

Combining the Toxinsight IVT Platform, corresponding image analysis, and the Compartmental Analysis BioApplication allows easy and efficient detection and quantification of multiple fluorescent cell-by-cell basis and can be utilized to screen for potential endocrine disruptors while simultaneously elucidating the pharmacokinetics of the receptor activation pathway.

Utilizing Redistribution estrogen and androgen receptor cell lines in endocrine disruptor screens with the Toxinsight IVT Platform provides distinct advantages over amenable to automation:

- limited hands-on time and turnaround to automation
- fluorescence-based, direct visualization of the receptor
- individual steps in the receptor pathway can be distinguished and monitored for each chemical
- receptor specific mechanism of action comparisons
- multiple outputs to detect chemical side effects such as cytotoxicity and cell cycle effects
- gating and cell-by-cell analysis to eliminate GFP expression variability effects

The methods and assays described in this study can simplify endocrine disruptor screening workflows during high-throughput *in vitro* testing and provide a more in-depth evaluation of receptor activity for everyday research and chemotherapy optimization.

## References

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