Validation & Assay Performance Summary

GeneBLAzer® ERR-alpha DA Assay Kit

GeneBLAzer® ERR-alpha DA Cells

GeneBLAzer® ERR alpha-UAS-*bla* HEK 293T Cells

Cat. no. K1421, K1702

Target Description

The estrogen-related-receptor-alpha (ERR alpha) is a nuclear hormone receptor identified by a low-stringency screen of a cDNA library with the estrogen-receptor-alpha (ER alpha) DNA binding domain and ERR alpha shares 60% sequence identity with ER alpha (2). ERR alpha is one of three estrogen-related-receptors (ERRs). Despite sequence identity in the DNA-binding domain the ligand binding domains of ERR alpha and ER alpha are not as similar, such that ERR alpha does not bind estradiol.

ERR alpha binds as a homodimer to the estrogen response element (ERE) and SF1 DNA sequences (6). No endogenous agonist for ERR alpha has been identified, but ERR alpha transcriptional activation occurs independent of ligand binding (3). The amino acid, Phe-232, is important to ERR alpha constitutive activity, such that a F232A mutation results in a loss of constitutive activity and the creation of a dominant negative mutant (4). Despite the absence of ligand binding ERR alpah has been shown to interact with the transcriptional co-activators PGC-1a and GRIP1 (5,9).

ERR alpha is expressed in many tissues including: heart, skeletal muscle, heart, kidney, liver and adipose tissue. ERR alpha -/- mice are resistant to high-fat diet induced obesity along with reduced lipogenesis in adipose tissue resulting in a lean phenotype, indicating a potential role in regulating metabolic homeostasis (2,7). ERR alpha has recently been shown to be an unfavorable biomarker for breast tumors due to its ability to activate estrogen-responsive genes in the absence of estrogen (10).

There have been no endogenous ligands identified for ERR alpha. Two organochlorine pesticides (toxaphene and chlordane) have been shown to decrease ERR alpha constitutive activity (8). These compounds may disrupt the interaction of ERR alpha with its coactivators in the micromolar range but were toxic to cells at concentrations greater than 10 µM (8). A recently identified compound, XCT790, has been shown to act as an inverse agonist for ERR alpha (9). XCT790 disrupts the binding of GRIP1 and PGC-1a transcriptional co-activators to ERR alpha (9). The treatment of ERR alpha F232A mutants with XCT790 resulted in XCT790 functioning as an agonist increasing ERR alpha interaction with transcriptional co-activators (9).

Cell Line Description

GeneBLAzer® ERR alpha DA(Division Arrested) cells and ERR alpha-UAS-*bla* HEK 293T cells contain the ligand-binding domain (LBD) of the human Estrogen related receptor alpha (ERR alpha) fused to the DNA-binding domain of GAL4 stably integrated in the GeneBLAzer® UAS-*bla* HEK 293T cell line. GeneBLAzer® UAS-*bla* HEK 293T cells stably express a beta-lactamase reporter gene under the transcriptional control of an upstream activator sequence (UAS). When an agonist binds to the LBD of the GAL4 (DBD)-ERR alpha (LBD) fusion protein, the protein binds to the UAS, resulting in expression of beta-lactamase. Division Arrested (DA) cells are available in two configurations- an Assay Kit (which includes cells and sufficient substrate to analyze 1 x 384-well plate), and a tube of cells sufficient to analyze 10 x 384-well plates.

DA cells are irreversibly division arrested using a low-dose treatment of Mitomycin-C, and have no apparent toxicity or change in cellular signal transduction. Both ERR alpha DA cells and ERR alpha-UAS-*bla* HEK 293T cells are functionally validated for Z' and EC_{50} concentrations of XCT790, an inverse agonist (Figure 1). In addition, ERR alpha-UAS-*bla* HEK 293T cells have been tested for assay performance under variable conditions, including DMSO concentration, cell number, stimulation time, and substrate loading time (data available upon request). Additional testing data using alternate stimuli are also available.

Validation Summary

Performance of this assay was validated under optimized conditions in 384-well format using LiveBLAzer™-FRET B/G Substrate.

1. **Primary agonist dose response under optimized conditions (n=6)**

	DA	Dividing
XCT790 EC_{50}	368nM	293nM
Z'-Factor (EC_{100})	0.80	0.74

Response Ratio	= 3.1
Optimum cell no.	= 20K cells/well
DMSO Tolerance	= up to 1%
Stimulation Time	= 20-24 hours
Max. [Stimulation]	= 5 μM

2. **Alternate agonist dose response**

Chlordane EC_{50}	= N/A
Toxaphene EC_{50}	= N/A
4-hydroxytamoxifen EC_{50}	= N/A

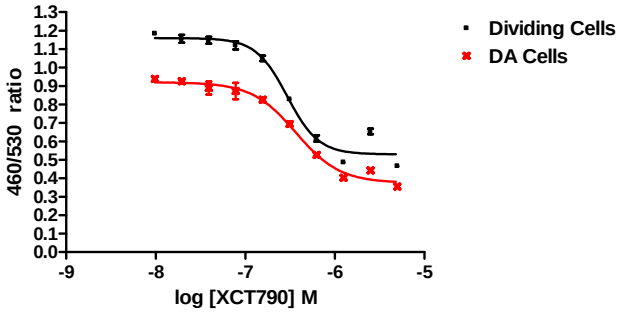
3. **Cell culture and maintenance**
   See Cell Culture and Maintenance Section and Table 1

Assay Testing Summary

4. **Assay performance with variable cell number**
5. **Assay performance with variable stimulation time**
6. **Assay performance with variable substrate loading time**
7. **Assay performance with variable DMSO concentration**
8. **Transfection with RNAi**

Primary Agonist Dose Response

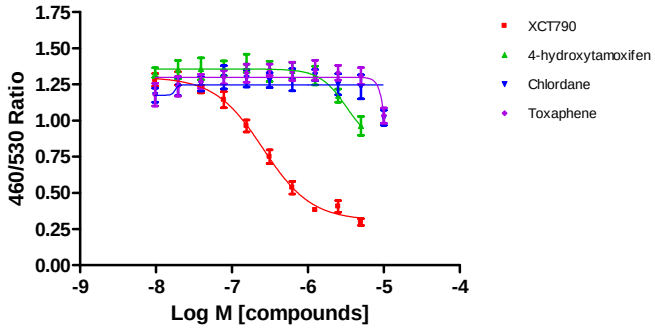
Figure 1 — ERR alpha DA and ERR alpha-UAS-*bla* HEK 293T dose response to XCT790 under optimized conditions



ERR alpha DA cells and ERR alpha-UAS-*bla* HEK 293T cells (20,000 cells/well) were plated in poly-D lysine coated plates in a 384-well format and stimulated with a dilution series of XCT790 in the presence of 0.1% DMSO for 20 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate (1μM final concentration of CCF4-AM) for 2 hours. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and plotted for each replicate against the concentrations of XCT790 (n=6 for each data point).

Alternate Agonist Dose Response

Figure 2 — XCT790, chlordane, toxaphene, and 4-hydroxytamoxifen dose response



ERR alpha-UAS-*bla* HEK 293T cells (20,000 cells/well) were plated the day of the assay in a poly-D-lysine 384-well plate. Cells were stimulated with either XCT790 (Sigma X4753), chlordane (Supelco cat#4-0089), toxaphene (Supelco cat# 4-0111) and 4-hydroxytamoxifen (Sigma cat# H7904) over the indicated concentration range in the presence of 0.1% DMSO for 20 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 2 hours. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the ratios were plotted against the indicated concentrations of the agonists (n= 8 for each data point).

Dividing Cell Culture and Maintenance

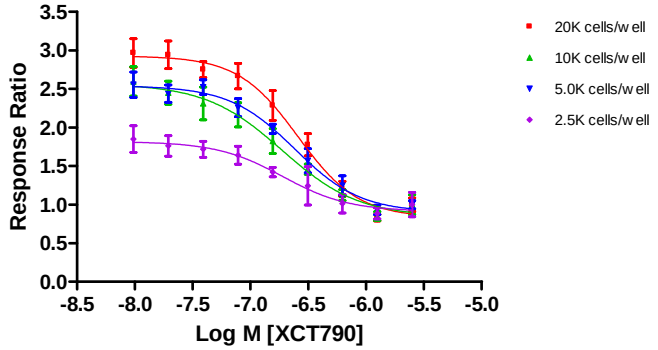
Dividing cells should be maintained at between 5 and 90% confluency in complete growth media and in a humidified incubator at 37ºC and 5% CO_2. Split dividing cells at least twice a week. Do not allow dividing cells to reach confluence.

Table 1 – Dividing Cell Culture and Maintenance

Component	Growth Medium (–)	Growth Medium (+)	Assay Medium	Freeze Medium
DMEM, w/ GlutaMAX™	90%	90%	—	—
Phenol Red free DMEM	—	—	98%	—
Dialyzed FBS **Do not substitute!**	10%	10%	—	—
Charcoal/Dextran FBS	—	—	2%	
NEAA	0.1 mM	0.1 mM	0.1 mM	—
HEPES (pH 7.3)	25 mM	25 mM	—	—
Hygromycin B	—	100 µg/mL	—	—
Zeocin™	—	100 µg/mL	—	—
Penicillin	100 U/mL	100 U/mL	100 U/mL	—
Streptomycin	100 µg/mL	100 µg/mL	100 µg/mL	—
Sodium Pyruvate	—	—	1 mM	
Recovery™ Cell Culture Freezing Medium	—	—	—	100%

Assay Performance with Variable Cell Number

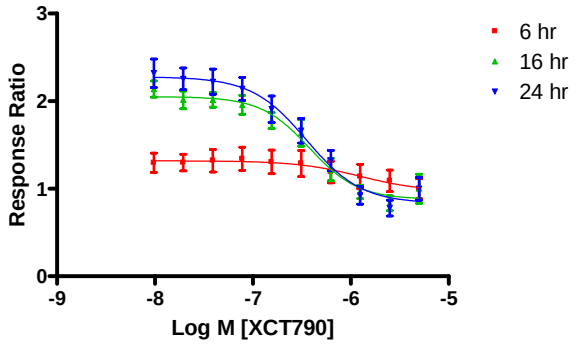
Figure 3— XCT790 dose response with 2.5, 5.0, 10, and 20K cells/well



ERR alpha-UAS-*bla* HEK 293T cells were plated at 2,500, 5,000, 10,000 or 20,000 cells/well in a 384-well poly-D-lysine treated black-walled, clear bottom plate the day of the assay. Cells were stimulated with XCT790 (Sigma #X4753) for 20 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 120 min. Fluorescence emission values at 460 nm and 530 nm for the various cell numbers were obtained using a standard fluorescence plate reader and the Response Ratios plotted against the indicated concentrations of XCT790 (n=8 for each data point).

Assay performance with Variable Stimulation Time

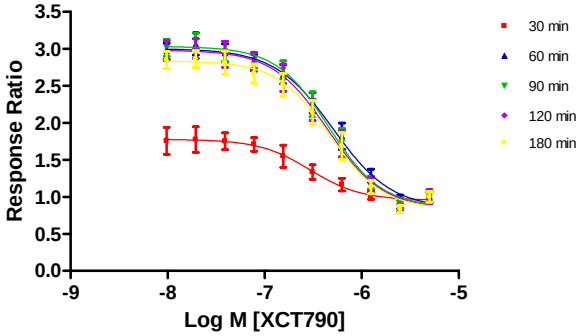
Figure 4 – XCT790 dose response with 6, 16, and 24 hour stimulation times



ERR alpha-UAS-*bla* HEK 293T cells (20,000 cells/well) were plated the day of the assay in a 384-well poly-D-lysine treated black-walled, clear bottom assay plate. Cells were stimulated with a serial dilution of XCT790 (Sigma #X4753) for 6, 16, and 24 hours in 0.1% DMSO and then loaded for 120 minutes with LiveBLAzer™-FRET B/G Substrate. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader. Response Ratios were plotted against the indicated concentrations of XCT790 (n=16 for each data point)

Assay performance with Variable Substrate Loading Time

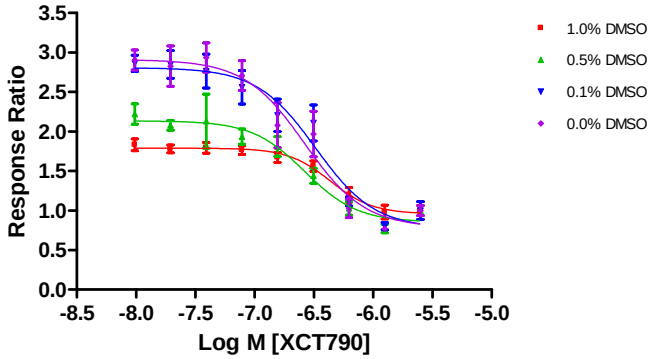
Figure 5 – XCT790 dose response with 0.5, 1, 1.5, 2, and 3 hour loading times



ERR alpha-UAS-*bla* HEK 293T cells were plated at 20,000 cells/well in a 384-well poly-D-lysine treated black-walled, clear bottom assay plate the day of the assay. Cells were stimulated with a dilution series of XCT790 (Sigma #X4753) for 20 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for either 0.5, 1, 1.5, 2, or 3 hours. Fluorescence emission values at 460 nm and 530 nm for the various loading times were obtained using a standard fluorescence plate reader and the Response Ratios were plotted against the indicated concentrations of XCT790 (n=16 for each data point).

Assay Performance with variable DMSO concentration

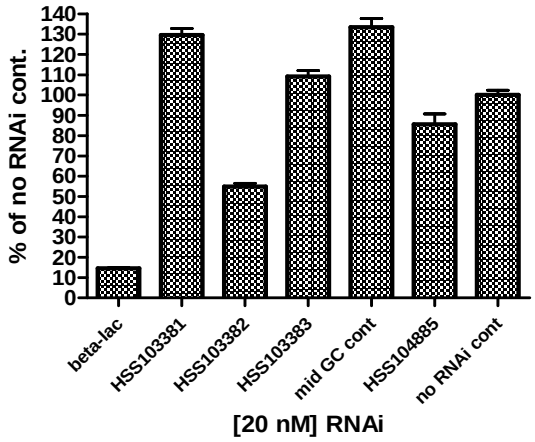
Figure 6 – XCT790 dose response with 0, 0.1, 0.5 and 1% DMSO.



ERR alpha-UAS-*bla* HEK 293T cells (20,000 cells/well) were plated the day of the assay in a 384-well poly-D-lysine black-walled, clear bottom assay plate. DMSO was added to the cells at concentrations from 0% to 1%. Cells were stimulated with a serial dilution series of XCT790 (Sigma cat# X4753) for 20 hours at 37ºC. Cells were loaded for 120 minutes with LiveBLAzer™-FRET B/G Substrate. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the Response Ratios are shown plotted for each DMSO concentration against the indicated concentrations of XCT790 (n=8 for each data point).

ERR alpha-UAS-*bla* HEK 293T cells transfected with Stealth RNAi

Figure 7 – Transfection of ERR alpha-UAS-bla HEK 293T cells with RNAi.



ERR alpha-UAS-*bla* HEK 293T cells (20,000 cells/well) were plated the day of the transfection in 96 well poly-D-lysine coated plates. Cells were transfected with 20 nM of RNAi with Lipofectamine2000 using the provided reverse transfection protocol. ERR alpha cells were treated with Lipofectamine2000 in the absence of RNAi oligos, ERR alpha specific Stealth RNAi oligos HSS103381, HSS103382, and HSS103383 (Invitrogen), HNF4 gamma Stealth RNAi oligo HSS104885, beta-lactamase positive control (Invitrogen), and Mid GC negative control (Invitrogen). Cells were incubated at 37ºC for ~48 hours. Cells were loaded for 120 minutes with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM). Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the percent of No RNAi controls were plotted (n=12 for each data point).

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