Validation & Assay Performance Summary

GeneBLAzer® FXR DA Assay Kit

GeneBLAzer® FXR DA Cells

GeneBLAzer® FXR-UAS-*bla* HEK 293T Cells

Cat. no. K1397, K1691

Target Description

The farnesoid X receptor (FXR) is a nuclear hormone receptor and can function as a ligand inducible transcription factor capable of acting as a co-repressor and/or co-activator for gene expression. Nuclear receptors contain a series of conserved domains or regions. These domains/regions include a variable NH_2-domain (A/B region), a conserved DNA-binding domain (DBD or region C), a linker region (region D), a ligand binding domain (LBD or region E), and in some receptors a variable COOH-terminal (region F) (3).

The FXR nuclear receptor forms a heterodimer with RXR (retinoid X receptor) that recognizes an inverted repeat of the AGGTCA sequence with no spacing (4) and 1 base-spacing (5). The FXR-RXR heterodimer can recognize additional direct repeats with different binding affinities (7). When bound the FXR-RXR heterodimer can function as a transcription activator or inhibitor. When a ligand interacts with the FXR ligand binding domain the receptor undergoes conformational changes. These conformational changes lead to a decrease in the affinity of transcription co-repressors and the interaction with transcription co-activators. These co-activators and co-repressors regulate gene transcription by interacting with the transcriptional pre-initiation complex and histone acetyl transferases (3). The interaction of nuclear receptors and FXR with these co-activators and co-repressors may be ligand specific (8).

FXR is activated by bile acids and regulates the expression of genes involved in bile acid synthesis, cholesterol metabolism, and plasma triglyceride concentrations (3). The primary agonist for FXR is chenodeoxycholic acid (CDCA) (1,2). Additional bile acids function as partial agonists for FXR including: deoxycholic acid (DCA), cholic acid (CA), and ursodeoxycholic acid (UDCA) (1). FXR is expressed in the liver, intestine, kidney and adrenal cortex (3). FXR reduces bile acid concentration in heptocytes by repressing genes involved in the bile acid biosynthetic pathway (CYP7A1, CYP8B1, and CYP27A1), and regulates triglyceride and lipoprotein metabolism by increasing the expression of apolipoprotein and lipoprotein enzymes (PLTP and APOCII) (reviewed in 3).

Cell Line Description

GeneBLAzer® FXR DA (Division Arrested) cells and FXR-UAS-*bla* HEK 293T 293 cells contain the ligand-binding domain (LBD) of the human farnesoid X receptor (FXR) 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)-FXR (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 FXR DA cells and FXR-UAS-*bla* HEK 293T cells are functionally validated for Z' and EC_{50} concentrations of chenodeoxycholic acid (CDCA); (Figure 1). In addition, FXR-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
Chenodeoxycholic acid EC_{50}	19uM	27uM
Z'-Factor (EC_{100})	0.89	0.89

Response Ratio = 14.2
Optimum cell no. = 20K cells/well
Optimum [DMSO] = up to 1%
Stimulation Time = 16 hours
Max. [Stimulation] = 100 μM

2. **Alternate agonist dose response**

Cholic acid EC_{50} = 348 μM
Deoxycholic acid EC_{50} = 24 μM

3. **Antagonist dose response**
See antagonist dose response section

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

Assay Testing Summary

5. **Assay performance with variable cell number**

6. **Assay performance with variable stimulation time**

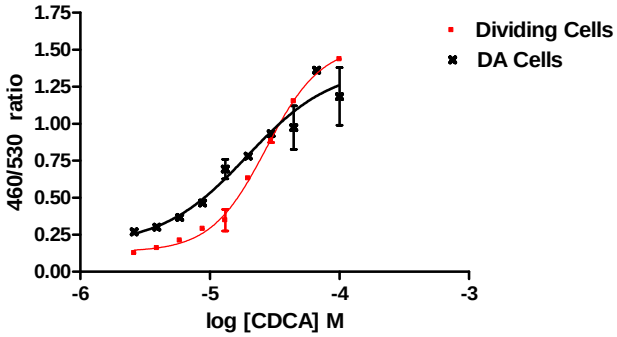
7. **Assay performance with variable substrate loading time**

8. **Assay performance with variable DMSO concentration**

9. **Toxicity of chenodeoxycholic acid at high concentrations.**

Primary Agonist Dose Response

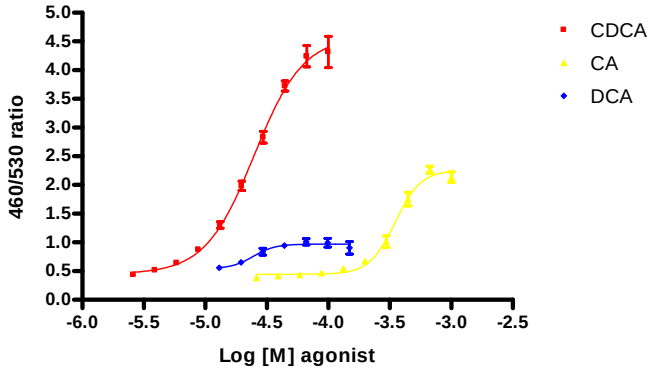
Figure 1 — FXR DA and FXR-UAS-*bla* HEK 293T dose response to chenodeoxycholic acid (CDCA) under optimized conditions



FXR DA cells and FXR-UAS-*bla* HEK 293T cells (20,000 cells/well) were plated in a 384-well format and stimulated with a dilution series of chenodeoxycholic acid (CDCA) in the presence of 0.5% DMSO for 16 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate (1μM final concentration of CCF4-AM) for 1.5 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 chenodeoxycholic acid (CDCA); (n=6 for each data point).

Alternate Agonist Dose Response

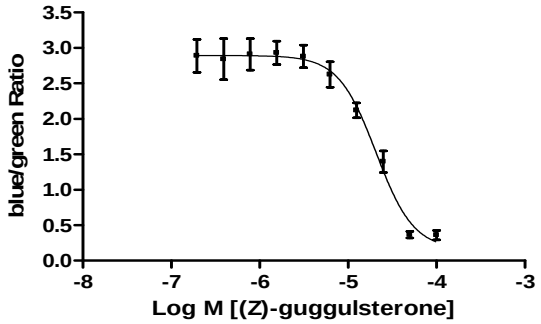
Figure 2 —Cholic acid, deoxycholic acid, and chenodeoxycholic acid agonist dose Response



FXR-UAS-*bla* HEK 293T cells (20,000 cells/well) were plated the day of the assay in a 384-well format. Cells were stimulated with either chenodeoxycholic acid (Sigma #C9377), cholic acid (Sigma #C9282), and deoxycholic acid (Sigma #D2510) over the indicated concentration range in the presence of 0.5% DMSO for 16 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate (1μM final concentration of CCF4-AM) for 90 minutes. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the Ratios plotted against the indicated concentrations of the agonists (n= 8 for each data point).

Antagonist Dose Response

Figure 3 — (Z)-guggulsterone dose response



FXR-UAS-*bla* HEK 293T cells (20,000 cells/well) were plated the day of the assay in a 384-well black-walled tissue culture assay plate. Cells were treated with (Z)-guggulsterone (Sigma #G5168) and incubated at 37 degrees C for 30 min., followed by 60 µM chenodeoxycholic acid agonist stimulation for 16 hours in 0.5% DMSO. Cells were then loaded for 90 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 Ratios are shown plotted against the indicated concentrations of (Z)-guggulsterone.

Dividing Cell Culture and Maintenance

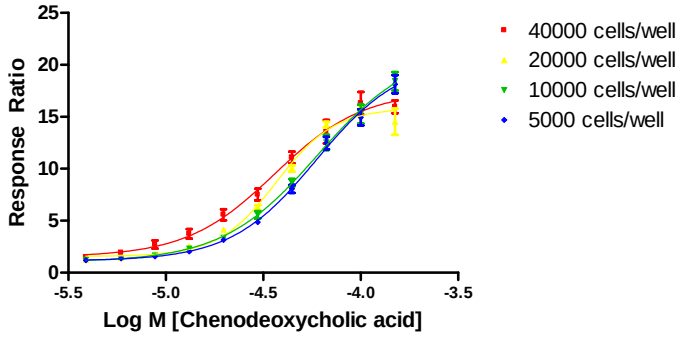
Dividing cells should be maintained at between 5 and 90% confluency in complete growth media and in a humidified incubator at 37ºC and 5% CO_2. Split dividing cells at least twice a week. Do not allow dividing cells to reach confluence.

Table 1 – 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

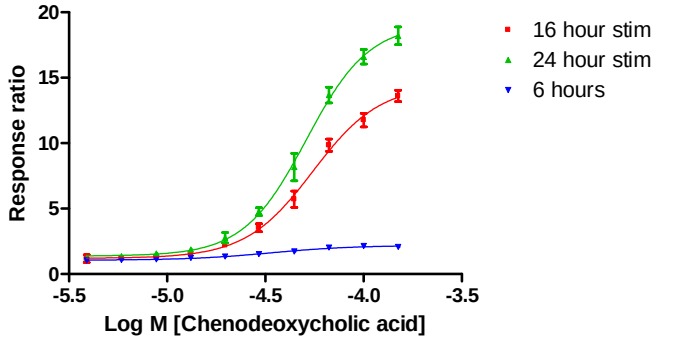
Figure 4— Chenodeoxycholic acid dose response with 5, 10, 20, and 40K cells/well



FXR-UAS-*bla* HEK 293T cells were plated at 5000, 10,000, 20,000 or 40,000 cells/well in a 384-well format the day of the assay. Cells were stimulated with chenodeoxycholic acid (Sigma #C9377) for 16 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 for the various cell numbers were obtained using a standard fluorescence plate reader and the Response Ratios plotted against the indicated concentrations of chenodeoxycholic acid (n=8 for each data point).

Assay performance with Variable Stimulation Time

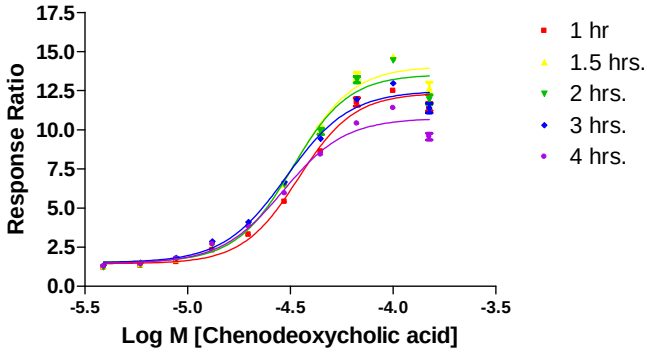
Figure 5 – Chenodeoxycholic acid dose response with 6, 16, and 24 hour stimulation times



FXR-UAS-*bla* HEK 293T cells (20,000 cells/well) were plated the day of the assay in a 384-well black-walled tissue culture assay plate. Chenodeoxycholic acid (Sigma #C9377) was then added to the plate over the indicated concentration range for 6, 16, and 24 hours in 0.5% DMSO and then loaded for 90 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 Response Ratios plotted (n=8 for each data point).

Assay performance with Variable Substrate Loading Time

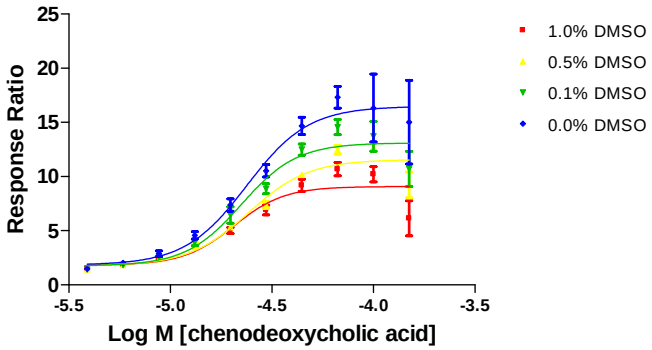
Figure 6 – Chenodeoxycholic acid dose response with 1, 1.5, 2, 3, and 4 hour loading times



FXR-UAS-*bla* HEK 293T cells were plated at 20,000 cells/well in a 384-well format the day of the assay. Cells were stimulated with Chenodeoxycholic acid (Sigma #C9377) in the presence of 0.5% DMSO for 16 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for either 1, 1.5, 2, 3, and 4 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 plotted against the indicated concentrations of Chenodeoxycholic acid (n=8 for each data point).

Assay Performance with variable DMSO concentration

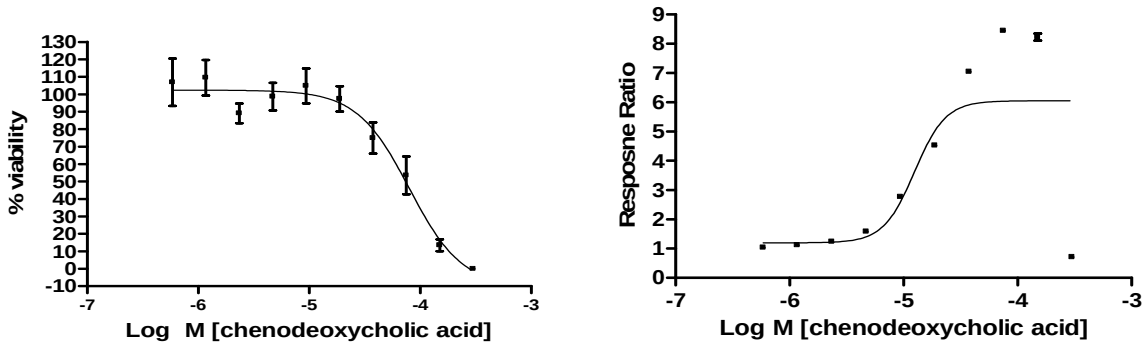
Figure 7 – Chenodeoxycholic acid dose response with 0, 0.1, 0.5 and 1% DMSO.



FXR-UAS-*bla* HEK 293T cells (20,000 cells/well) were plated the day of the assay in a 384-well black-walled tissue culture assay plate. Chenodeoxycholic acid (Sigma #C9377) was then added to the plate over the indicated concentration range. DMSO was added to the assay at concentrations from 0% to 1%. Cells were stimulated for 16 hrs with agonist and loaded for 90 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 Response Ratios are shown plotted for each DMSO concentration against the indicated concentrations of chenodeoxycholic acid (n=8 for each data point).

Toxicity of Chenodeoxycholic acid at high concentrations

Figure 8. – Chenodeoxycholic acid dose response with ToxBLAzer™ Dual Screen for (a) cell viability and (b) beta-lactamase response



FXR-UAS-*bla* HEK 293T cells (20,000 cells/well) were plated the day of the assay in a 384-well format and stimulated with chenodeoxycholic acid (Sigma #C9377) over the indicated concentration range in the presence of 0.5% DMSO for 16 hours. Cells were then loaded with ToxBLAzer™ Dual Screen Substrate (1µM final concentration) for 90 minutes. (a) Fluorescence emission values at 650 nm (excitation at 600 nnm) were obtained using a standard fluorescence plate reader and each replicate plotted against the indicated concentration of chenodeoxycholic acid. (b) Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and each replicate plotted against the indicated concentrations of chenodeoxycholic acid (n= 16 for each data point).

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