

Validation & Assay Performance Summary



GeneBLazer® FXR-UAS-*bla* HEK 293T Validated Assay

Cat. no. K1239

This cell-based assay has been thoroughly tested and validated by Invitrogen and is suitable for immediate use in a screening application. The following information illustrates the high level of assay testing completed and the validation of assay performance under optimized conditions.

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.

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 that 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 hepatocytes 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-UAS-*bla* HEK 293T cells contain the ligand-binding domain (LBD) of the human farnesoid X receptor 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 (catalog #K1104) 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, it translocates to the nucleus where it binds to the UAS inducing transcription of beta-lactamase. FXR-UAS-*bla* HEK 293T cells have been tested for assay performance using variable assay conditions, including DMSO concentration, cell number, stimulation time, substrate loading time and have been validated for Z' and EC₅₀ concentrations of chenodeoxycholic acid. Additional testing data using alternate stimuli are also provided.

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=3)

Chenodeoxycholic acid EC_{50} = 31 μ M
Z'-Factor (EC_{100}) = 0.82
Response Ratio = 14.2

Optimum cell no. = 20K cells/well
Optimum [DMSO] = up to 1%
Stimulation Time = 16 hours
Max. [Stimulation] = 100 μ M

2. Alternate agonist dose response

Cholic acid EC_{50} = 348 μ M
Deoxycholic acid EC_{50} = 24 μ M

3. Antagonist dose response

See antagonist dose response section

4. Cell culture and maintenance

See Cell Culture and Maintenance Section and Table 1

Assay Performance & Testing Summary

5. Assay performance with variable cell number

6. Assay performance with variable stimulation time

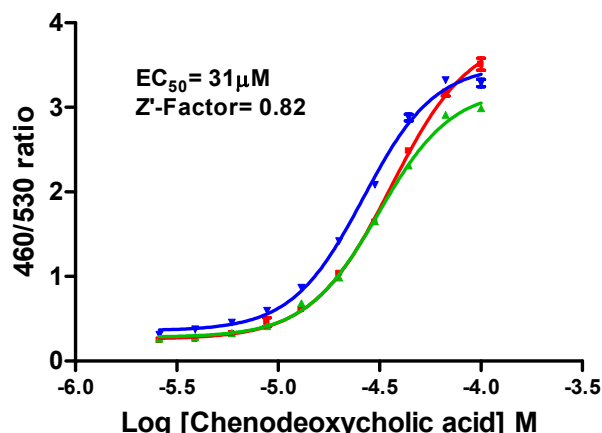
7. Assay performance with variable substrate loading time

8. Assay performance with variable DMSO concentration

9. Toxicity of chenodeoxycholic acid at high concentrations.

Primary Agonist Dose Response

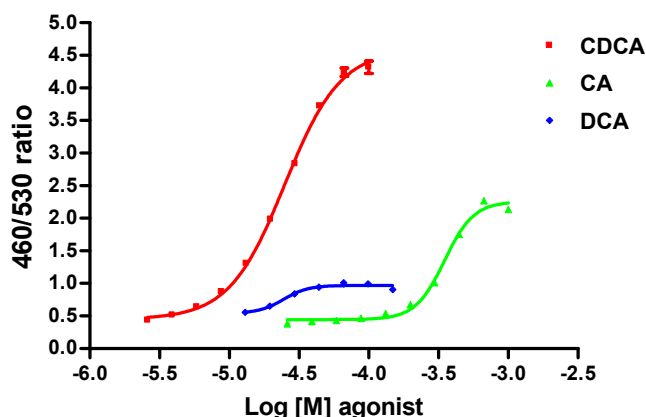
Figure 1 — FXR-UAS-*bla* HEK 293T dose response to Chenodeoxycholic acid under optimized conditions



FXR-UAS-*bla* HEK 293T cells (20,000 cells/well) were assayed on three separate days. Cells 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 LiveBLazer™-FRET B/G Substrate (1 μ M 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 each replicate plotted against the indicated concentrations of chenodeoxycholic acid (n = 16 for each data point).

Alternate Agonist Dose Response

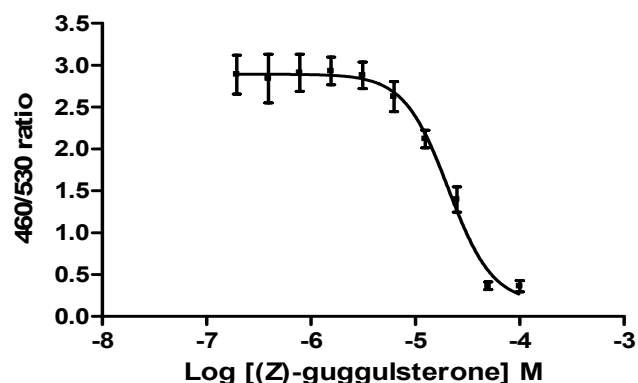
Figure 2 — FXR-UAS-*bla* HEK 293T dose response to Cholic acid, deoxycholic acid, and chenodeoxycholic acid



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 (1 μ M 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 460/530 Ratios plotted against the indicated concentrations of the agonists (n = 8 for each data point).

Antagonist Dose Response

Figure 3 — FXR-UAS-*bla* HEK 293T dose response to (Z)-guggulsterone



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 μ M 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.

Cell Culture and Maintenance

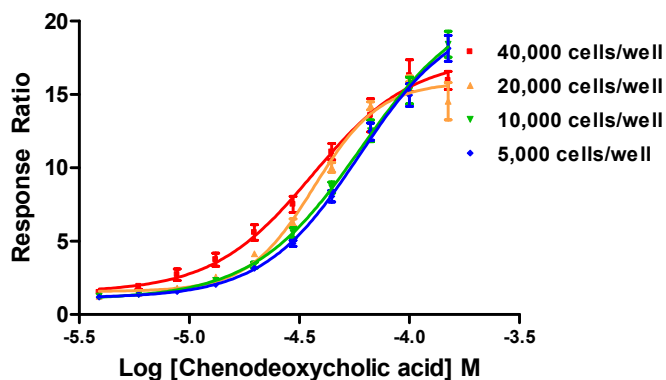
Cells should be maintained at between 5 and 90% confluency in complete growth media and in a humidified incubator at 37°C and 5% CO₂. Split cells at least twice a week. Do not allow cells to reach confluence. For optimal cell line performance, use dialyzed FBS (Invitrogen # 26400-036).

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 μ g/mL	—	—
Zeocin™	—	100 μ g/mL	—	—
Penicillin	100 U/mL	100 U/mL	100 U/mL	—
Streptomycin	100 μ g/mL	100 μ g/mL	100 μ g/mL	—
Sodium Pyruvate	—	—	1 mM	—
Recovery™ Cell Culture Freezing Medium	—	—	—	100%

Assay Performance with Variable Cell Number

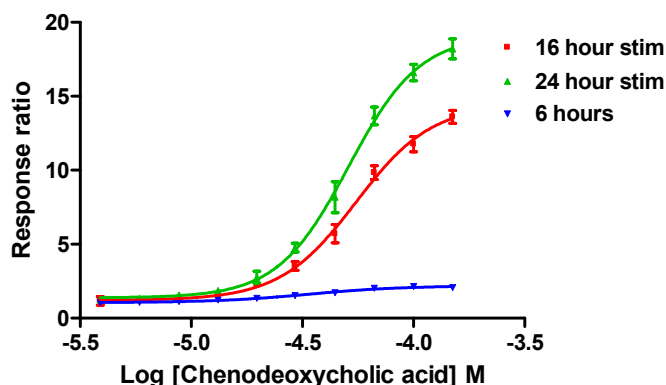
Figure 4— FXR-UAS-*bla* HEK 293T dose response to Chenodeoxycholic acid with 5, 10, 20, and 40K cells/well



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 (1 μ M 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 for each cell number plotted against the indicated concentrations of chenodeoxycholic acid (n=8 for each data point).

Assay performance with Variable Stimulation Time

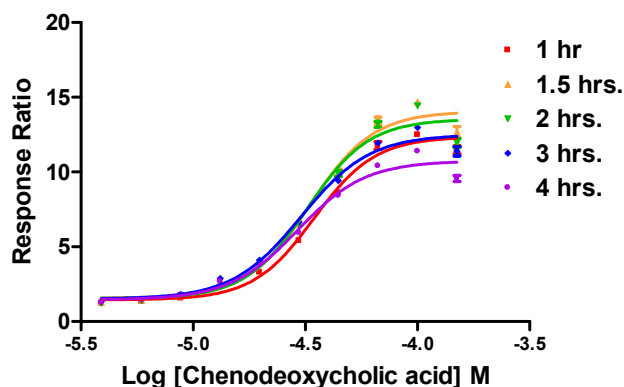
Figure 5 – FXR-UAS-*bla* HEK 293T dose response to Chenodeoxycholic acid with 6, 16, and 24 hour stimulation times



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 (1 μ M 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 of each stimulation time plotted against the indicated concentrations of chenodeoxycholic acid (n=8 for each data point).

Assay performance with Variable Substrate Loading Time

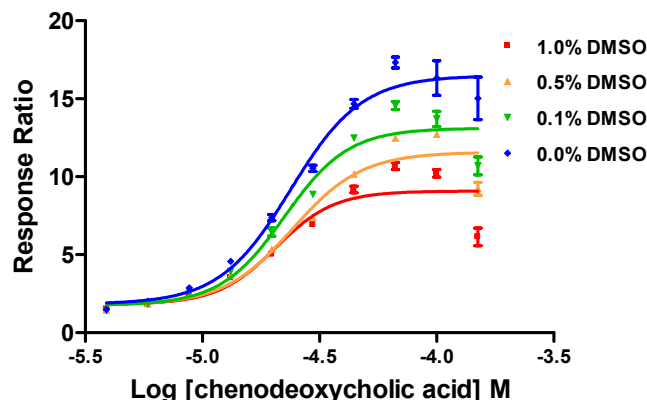
Figure 6 – FXR-UAS-*bla* HEK 293T dose response to Chenodeoxycholic acid with 1, 1.5, 2, 3, and 4 hour loading times



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 (1 μ M 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 for each substrate load time plotted against the indicated concentrations of chenodeoxycholic acid (n=8 for each data point).

Assay Performance with variable DMSO concentration

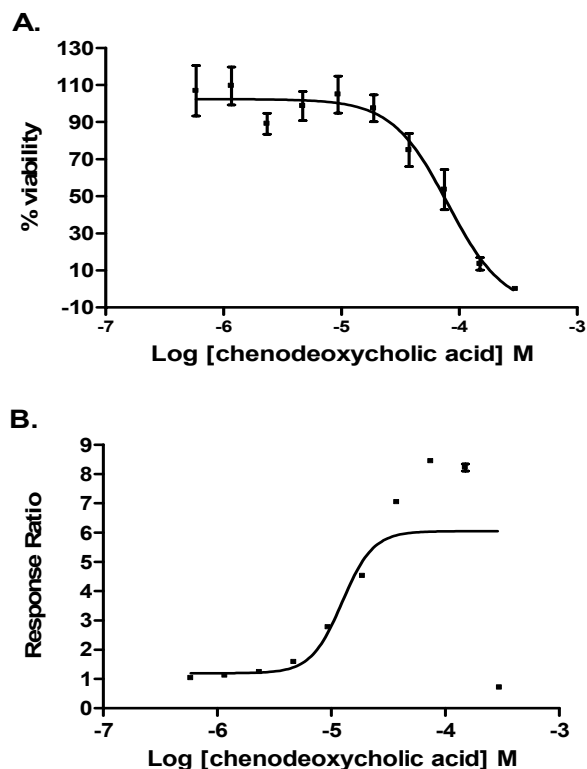
Figure 7 – FXR-UAS-*bla* HEK 293T dose response to Chenodeoxycholic acid with 0, 0.1, 0.5 and 1% DMSO.



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 (1 μ M 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 for each DMSO concentration plotted against the indicated concentrations of chenodeoxycholic acid (n=8 for each data point).

Toxicity of Chenodeoxycholic acid at high concentrations

Figure 8 – FXR-UAS-*bla* HEK 293T dose response to Chenodeoxycholic acid using 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 for 90 minutes. Panel A: Fluorescence emission values at 650 nm (excitation at 600 nm) were obtained using a standard fluorescence plate reader and each replicate plotted against the indicated concentration of chenodeoxycholic acid. Panel 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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