Validation & Assay Performance Summary

GeneBLAzer® T-REx™ ROR gamma DA Assay Kit

Cat. no. K1883

GeneBLAzer® T-REx™ ROR gamma UAS-*bla* HEK 293T Cell Line

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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

ROR gamma is a member of the retinoic acid receptor-related orphan receptor (ROR) subfamily of nuclear receptors. This subfamily consists of ROR alpha, ROR beta, and ROR gamma. There are two main isoforms of ROR gamma that differ only in the N-terminus, the LBDs are conserved. The longer ROR gamma isoform is expressed in many tissues including muscle and liver, whereas the ROR gamma T isoform has a truncated N-terminus and is expressed exclusively in thymocytes and lymphoid tissue inducer (LTi) cells where it is involved in regulating T cell development and lymphoid organogenesis ([1,2]). ROR gamma T regulates the differentiation of Th17 cells, which is a subset of T helper cells that produce IL-17 and have been implicated in autoimmune and inflammatory diseases, cancer, and elimination of extracellular bacteria ([3,4]). This orphan nuclear receptor has no known ligands, though all-*trans* retinoic acid (ATRA) has been suggested to inhibit ROR gamma activity at high concentrations ([5]).

Cell Line Description

The GeneBLAzer® T-REx™-ROR gamma-UAS-*bla* HEK293T cells contain tetracycline-inducible Gal4 DNA-binding domain/human ROR gamma ligand binding domain (Accession #NM_001001523.1) fusion and tetracycline repressor constructs stably integrated in the CellSensor® UAS-*bla* HEK293T cell line. CellSensor® UAS-*bla* HEK293T cells (#K1711) stably express a beta-lactamase reporter gene under the transcriptional control of a 7x Upstream Activator Sequence (UAS). Transcription from the 7xUAS is activated by the binding of the Gal4 transcription factor DNA-binding-domain (DBD). Addition of doxycycline, a tetracycline analog, allows for Gal4 DBD/ROR gamma LBD expression and subsequent beta-lactamase expression. This cell line is a clonal population isolated by flow cytometry that has been tested for robust assay performance.

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 T-Rex™-ROR gamma DA cells and T-REx™-ROR gamma-UAS-*bla* HEK 293T cells are functionally validated for Z' and assay window. In addition, T-REx™-ROR gamma-UAS-*bla* HEK 293T cells have been tested for assay performance under variable conditions, including DMSO concentration, cell number, substrate loading time, and stimulation time.

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)**

   Doxycycline EC_{50} = 0.75 ng/ml
   Z'-Factor (EC_{100}) = 0.78
   Response Ratio = 4.5

   Optimum cell no. = 20K cells/well
   Optimum [DMSO] = up to 1%
   Stimulation Time = 16-18 hours
   Max. [Stimulation] = 50 ng/ml

2. **All-*trans* retinoic acid dose response**

3. **Stealth RNAi™ transfection**
   See RNAi 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**

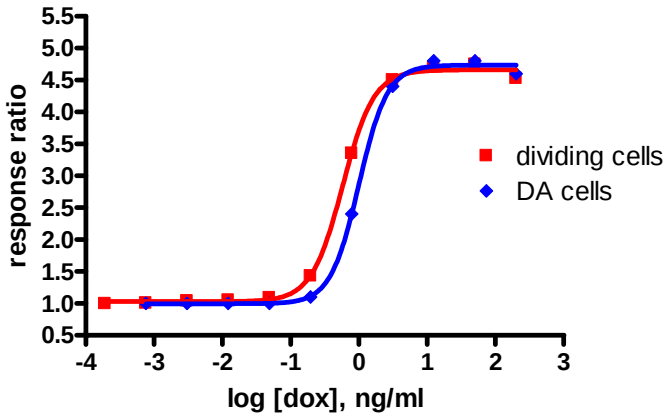
6. **Assay performance with variable stimulation time**

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

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

Primary Agonist Dose Response

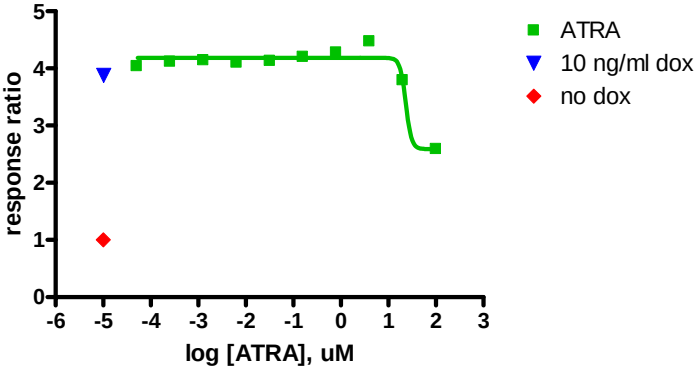
Figure 1 —Doxycycline dose response under optimized conditions



T-REx™-ROR gamma DA cells and T-REx™-ROR gamma-UAS-*bla* HEK 293T cells were were plated at 20,000 cells/well in a 384-well poly-D-lysine assay plate and treated with doxycycline (MP Biomedicals #195044) over the indicated concentration range for ~16-18 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 in bottom-read mode, and the background-corrected Response Ratios plotted against the indicated concentrations of doxycycline (n= 16 for each data point).

All-*trans* retinoic acid Dose Response

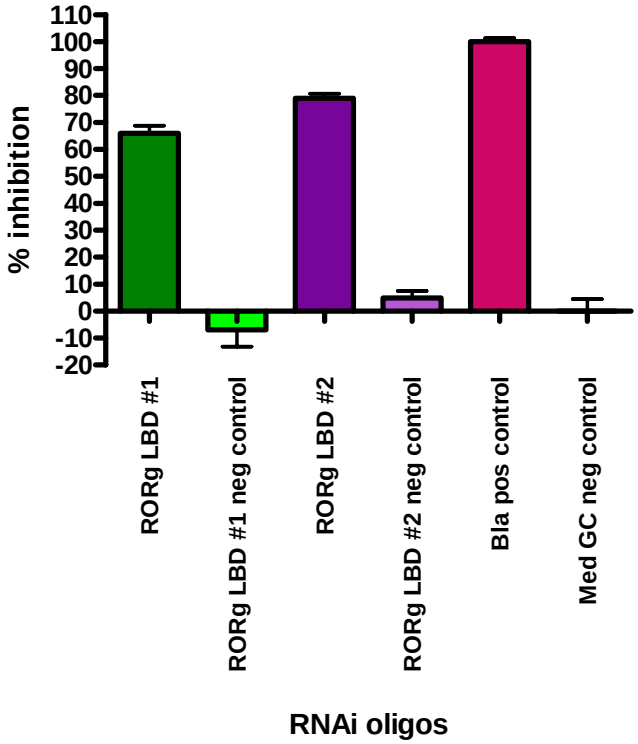
Figure 2 —ATRA dose response



T-REx™-ROR gamma-UAS-*bla* HEK 293T cells were plated at 20,000 cells/well in a 384-well poly-D-lysine assay plate and treated with all-trans retinoic acid (ATRA) (Sigma #R2625) over the indicated concentration range. Doxycycline (MP Biomedicals #195044) was added at a final concentration of 10 ng/ml, and the cells were incubated for ~16-18 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 in bottom-read mode, and the background-corrected Response Ratios plotted against the indicated concentrations of doxycycline (n= 16 for each data point).

T-REx™-ROR gamma-UAS-*bla* HEK 293T cells transfected with Stealth RNAi™

Figure 3 —Stealth RNAi™ transfection



T-Rex™-ROR gamma-UAS-*bla* HEK 293T cells were plated the day of transfection in a 96-well poly-D-lysine assay plate. Cells were transfected with 20nM of RNAi using Lipofectamine2000, following the reverse transfection protocol provided with the Stealth RNAi. Several RNAi oligos directed against the LBD of ROR gamma were tested along with multiple controls. Two custom RNAi oligos were tested with sequences against the LBD of ROR gamma (LBD #1 and 2). Included with these custom oligos was a negative control for each with a similar but slightly scrambled sequence. We also chose the medium-GC content universal negative control, and a beta-lactamase positive control. Cells were incubated with transfection complexes for ~48 hours, and then treated with 100 ng/ml doxycycline (MP Biomedicals #195044) for ~18 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 in bottom-read mode, and normalized data of % inhibition was plotted, where the beta-lactamase positive control is 100% inhibition and the medium GC negative control is 0% inhibition (n= 8 for each data point).

Cell Culture and Maintenance

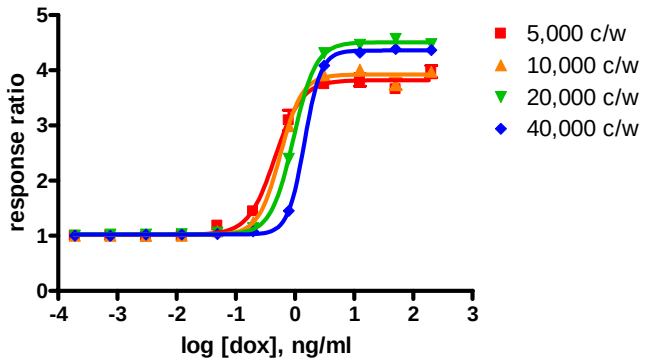
Cells should be maintained at between 5 and 95% confluency in complete growth media and in a humidified incubator at 37ºC and 5% CO_2. Split cells at least twice a week. Do not allow cells to reach confluence.

Table 1 – Cell Culture and Maintenance

Component	Growth Medium (–) And Assay Medium	Growth Medium (+)	Freeze Medium
DMEM, w/ GlutaMAX™	90%	90%	—
Dialyzed FBS **Do not substitute!**	10%	10%	—
HEPES (pH 7.3)	25 mM	25 mM	—
NEAA	0.1 mM	0.1 mM	—
Puromycin	—	1 µg/mL	—
Hygromycin B	—	80 µg/mL	—
Zeocin™	—	80 µg/mL	—
Penicillin	100 U/mL	100 U/mL	—
Streptomycin	100 µg/mL	100 µg/mL	—
Recovery™ Cell Culture Freezing Medium	—	—	100%

Assay Performance with Variable Cell Number

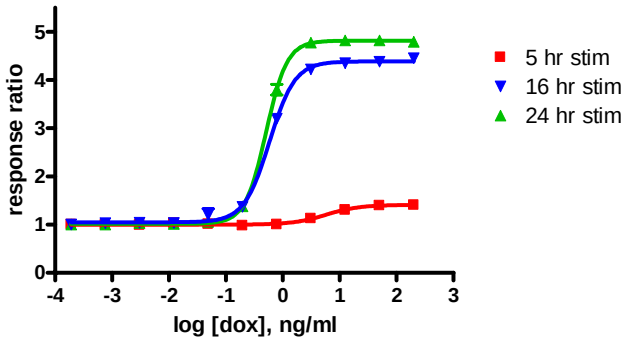
Figure 4— Doxycycline dose response with 5, 10, 20, and 40K cells / well



T-REx™-ROR gamma-UAS-*bla* HEK 293T cells were plated at 5000, 10,000, 20,000, or 40,000 cells/well in a 384-well poly-D-lysine assay plate the day of the assay. Cells were stimulated with doxycycline (MP Biomedicals #195044) for 16-18 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 in bottom-read mode, and the background-corrected Response Ratios plotted against the indicated concentrations of doxycycline (n=8 for each data point).

Assay performance with Variable Stimulation Time

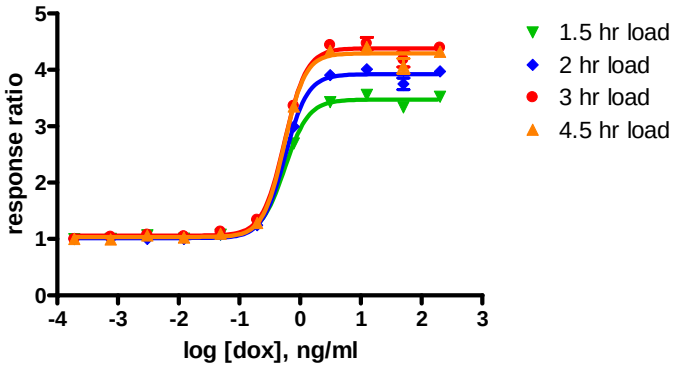
Figure 5 – Doxycycline dose response with 5, 16, and 24 hour stimulation times



T-REx™-ROR gamma-UAS-*bla* HEK 293T cells were plated at 20,000 cells/well in a 384-well poly-D-lysine assay plate the day of the assay. Cells were stimulated with doxycycline (MP Biomedicals #195044) for 5, 16, or 24 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 in bottom-read mode, and the background-corrected Response Ratios plotted against the indicated concentrations of doxycycline (n=16 for each data point).

Assay performance with Variable Substrate Loading Time

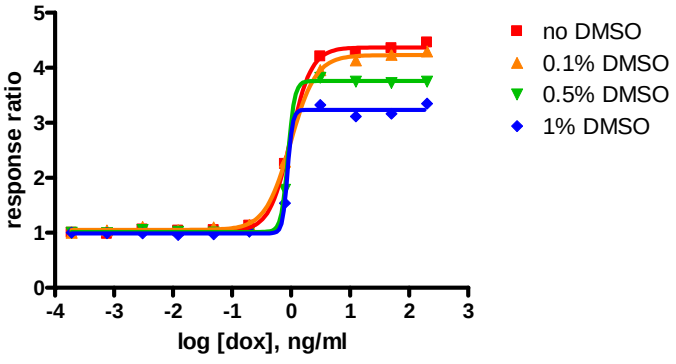
Figure 6 – Doxycycline dose response with 1.5, 2, 3, and 4.5 hour substrate loading times



T-REx™-ROR gamma-UAS-*bla* HEK 293T cells were plated at 10,000 cells/well in a 384-well poly-D-lysine assay plate the day of the assay. Cells were stimulated with doxycycline (MP Biomedicals #195044) for 16-18 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for either 1.5, 2, 3, or 4.5 hours. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader in bottom-read mode, and the background-corrected Response Ratios plotted against the indicated concentrations of doxycycline (n=8 for each data point).

Assay Performance with variable DMSO concentration

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



T-REx™-ROR gamma-UAS-*bla* HEK 293T cells were plated at 20,000 cells/well in a 384-well poly-D-lysine assay plate the day of the assay. Cells were stimulated with doxycycline (MP Biomedicals #195044) for 16-18 hours in the presence of 0%, 0.1%, 0.5%, or 1% DMSO. 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 in bottom-read mode, and the background-corrected Response Ratios plotted against the indicated concentrations of doxycycline (n=8 for each data point).

References

1. Jetten AM, Joo JH. **Retinoid-related Orphan Receptors (RORs): Roles in Cellular Differentiation and Development.** *Adv Dev Biol.* **2006,** *16,* 313-55.

2. Dzhagalov I, Zhang N, He Y-W. **The Roles of Orphan Nuclear Receptors in the Development and Function of the Immune System.** *Cell Mol Immunol.* **2004,** *1(6):* 401-7.

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