Validation & Assay Performance Summary

GeneBLAzer® TR alpha DA Assay Kit

GeneBLAzer® TR alpha DA Cells

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

Cat. no. K1385, K1684

Target Description

The thyroid hormone receptors are members of nuclear hormone receptor superfamily. Nuclear hormone receptors function as ligand inducible transcription factors capable of acting as co-repressors and/or co-activators 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) (1). The TR DNA binding domain recognizes and interacts with the thyroid hormone response elements (TREs) (2). TR can bind to this half-site, AGGTCA, as a monomer, a homodimer, or a heterodimer with the RXR (3). The TR/RXR heterodimer is associated with corepressor proteins at the TRE in the absence of ligand (4). Upon binding of thyroid hormone, TR undergoes a conformational change, releasing corepressor proteins and allowing for the interaction with coactivator proteins that enhance TRE-driven gene transcription (5).

Thyroid hormones (TH) have important effects on development, growth, and metabolism. They are encoded by two different genes, alpha and beta. Their effect is mediated principally through T_3, which regulates gene expression by binding to the TH receptors (TR)-α and –β. Thyroid hormones affect most mammalian tissues. In excess, these hormones may cause weight loss, tachycardia, atrial arrhythmias, and heart failure. Studies of TR isoform-specific knockout mice and patients with resistance to thyroid hormone syndrome suggest that TR-α mediates the effects of thyroid hormone on heart rate (7).

Cell Line Description

GeneBLAzer® TR alpha DA(Division Arrested) cells and TR alpha-UAS-*bla* HEK 293T cells contain the ligand-binding domain (LBD) of the human Thyroid hormone receptor alpha(TR 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)-TR 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 TR alpha DA cells and TR alpha-UAS-*bla* HEK 293T 293 cells are functionally validated for Z' and EC_{50} concentrations of T3 Thryoid hormone (Figure 1). In addition, TR alpha-UAS-*bla* HEK 293T 293 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.

Thyroid hormones affect most mammalian tissues, and in excess may cause weight loss, tachycardia, atrial arrhythmias, and heart failure. TR isoforms have distinct, nonredundant, and tissue-specific functions. TR is known as an important regulator of heart function, while TR appears to be a key regulator of hypothalamus-pituitary-thyroid feedback regulation and plasma cholesterol levels. Mutations in the gene encoding human TR have been associated with general resistance to thyroid hormone (GRTH). This disorder is associated with significant behavioral abnormalities.

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
T3 EC_{50}	0.1nM	0.1nM
Z'-Factor (EC_{100})	0.91	0.90

Response Ratio	= 20-27
Optimum cell no.	= 10K cells/well
Optimum [DMSO]	= up to 0.5%
Stimulation Time	= 24 hours
Max. [Stimulation]	= 4 nM

2. **Alternate agonist dose response**

T4 EC_{50}	= 6.08 nM
Tetra EC_{50}	= 32.4 nM

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**

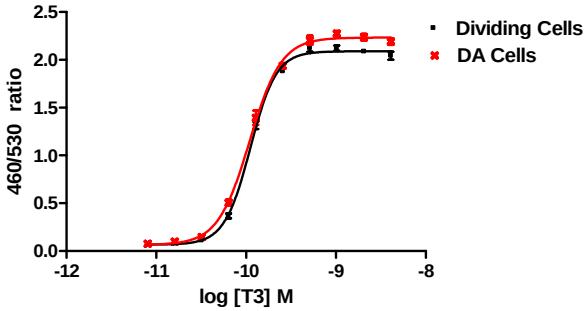
6. **Assay performance with variable stimulation time**

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

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

Primary Agonist Dose Response

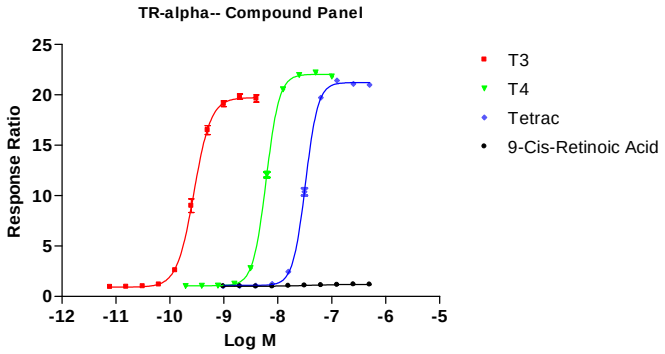
Figure 1 — TR alpha DA and TR alpha-UAS-*bla* HEK 293T dose response to T3 Thryoid hormone under optimized conditions



TR alpha DA cells and TR alpha-UAS-*bla* HEK 293T cells (10,000 cells/well) were plated in a 384-well format and stimulated with a dilution series of T3 Thryoid hormone in the presence of 0.5% DMSO for 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 and plotted for each replicate against the concentrations of T3 Thryoid hormone (n=6 for each data point).

Alternate Agonist Dose Response

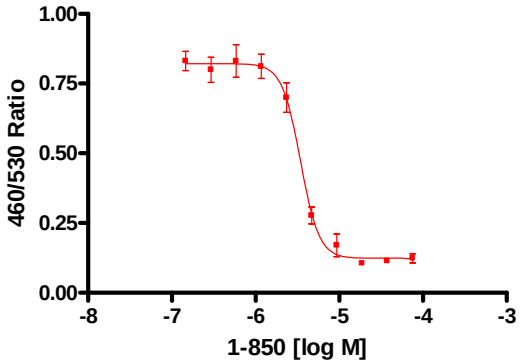
Figure 2 —T3, T4, Tetrac, and 9-cis-Retinoic Acid Dose Response



TR alpha-UAS-*bla* HEK 293T cells (10,000 cells/well) were plated the day of the assay in a 384-well format. Cells were stimulated with either T3 (Calbiochem #64245), T4-thyroxine (Sigma # T1775), Tetrac (Sigma #T3787), or 9-cis-retinoic acid (Biomol GR-101) over the indicated concentration range in the presence of 0.5% DMSO for 24 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for 120 minutes. Fluorescence emission values at 445 nm and 515 nm were obtained using a Safire^2 plate reader and the Ratios plotted against the indicated concentrations of the agonists (n= 8 for each data point).

Antagonist Dose Response

Figure 3 — Thyroid Hormone Receptor Antagonist, 1-850



TR alpha-UAS-*bla* HEK 293T cells (10,000 cells/well) were plated the day of the assay in a 384-well black-walled tissue culture assay plate in 0.5%DMSO. Cells were treated with Thyroid Hormone Receptor Antagonist, 1-180 (Calbiochem # 609315) and incubated at 37 degrees C for 45 min., followed by 0.5nM T3 agonist stimulation for 24 hours. Cells were then loaded for 2 hours with LiveBLAzer™-FRET B/G Substrate. Fluorescence emission values at 445 nm and 515 nm were obtained using a Safire^2 plate reader and the Ratios are shown plotted against the indicated concentrations of 1-180.

Dividing Cell Culture and Maintenance

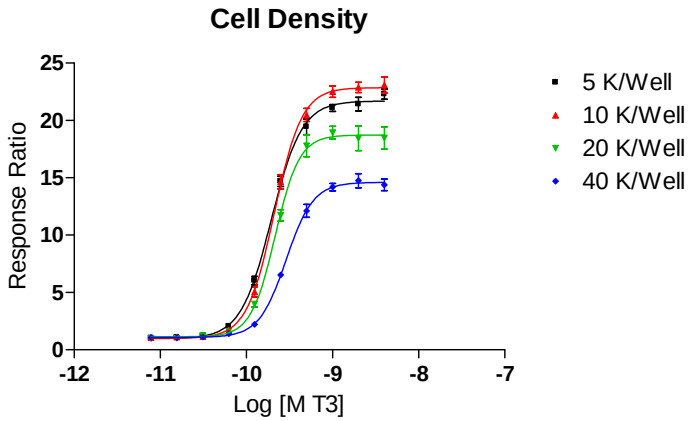
Dividing cells should be maintained at between 5 and 85% confluency in complete growth media and in a humidified incubator at 37ºC and 5% CO_2. Split dividing cells at least twice a week. Do not allow dividing cells to reach confluence.

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	—	80 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

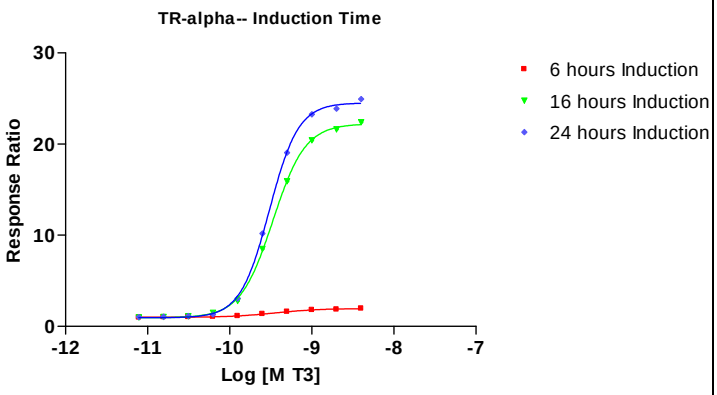
Figure 4— T3 dose response with 5, 10, 20 and 40K cells/well



TR alpha-UAS-*bla* HEK 293T cells were plated at 5000, 10,000, 20,000, or 40,000 or cells/well in a 384-well format the day of the assay in 0.5%DMSO. Cells were stimulated with T3 (Calbiochem #64245) for 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 445 nm and 515 nm for the various cell numbers were obtained using a Safire^{2}plate reader and the Response Ratios plotted against the indicated concentrations of T3 (n=8 for each data point).

Assay performance with Variable Stimulation Time

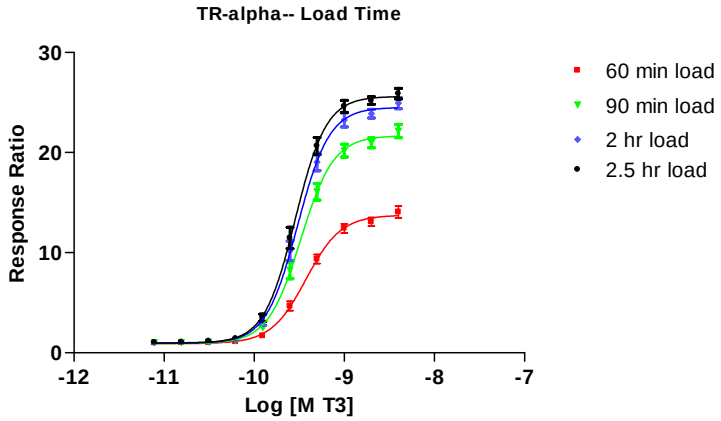
Figure 5 – T3 dose response with 6, 16, and 24 hour stimulation times



TR alpha-UAS-*bla* HEK 293T cells (10,000cells/well) were plated the day of the assay in a 384-well black-walled tissue culture assay platein 0.5% DMSO. T3 (Calbiochem #64245) was then added to the plate over the indicated concentration range for 6, 16, and 24 hours and then loaded for 2 hours with LiveBLAzer™-FRET B/G Substrate(1µM final concentration of CCF4-AM). Fluorescence emission values at 445 nm and 515 nm were obtained using a Safire^2 plate reader and the Response Ratios plotted (n=8 for each data point)

Assay performance with Variable Substrate Loading Time

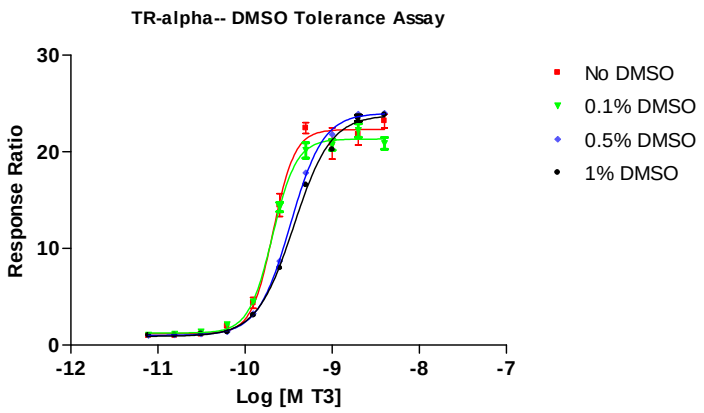
Figure 6 – T3 dose response with 30, 60, 90, 120, 180 and 240 minutes loading times



TR alpha-UAS-*bla* HEK 293T cells were plated at 10,000 cells/well in a 384-well format the day of the assay in 0.5%DMSO. Cells were stimulated with T3 (Calbiochem #64245) for 24 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for either 60, 90, 120, and 180 minutes. Fluorescence emission values at 445 nm and 515 nm for the various loading times were obtained using a Safire^2 plate reader and the Response Ratios plotted against the indicated concentrations of T3 (n=8 for each data point).

Assay Performance with variable DMSO concentration

Figure 7 – T3 dose response with 0, 0.1, 0.5 and 1% DMSO.



TR alpha-UAS-*bla* HEK 293T cells (10,000 cells/well) were plated the day of the assay in a 384-well black-walled tissue culture assay plate. T3 (Calbiochem #64245) 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 24 hrs with agonist and loaded for 2 hours with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM). Fluorescence emission values at 445 nm and 515 nm were obtained using a Safire^2 plate reader and the Response Ratios are shown plotted for each DMSO concentration against the indicated concentrations of T3 (n=8 for each data point).

References

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