Validation & Assay Performance Summary

GeneBLAzer® MR DA Cells & Assay Kit

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

Cat. no. K1409, K1696

Target Description

The Mineralocorticoid receptor (MR) is a validated drug target for hypertension and other disease states such as congestive heart failure.

Cell Line Description

GeneBLAzer® MR DA(Division Arrested) cells and MR-UAS-*bla* HEK 293T cells contain the ligand-binding domain (LBD) of the human Mineralcorticoid Receptor (MR) 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)-MR (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 MR DA cells and MR-UAS-*bla* HEK 293T cells are functionally validated for Z' and EC_{50} concentrations of aldosterone (Figure 1). In addition, MR-UAS-*bla* HEK 293T cells have been tested for assay performance under variable conditions, including DMSO concentration, cell number, and substrate loading time. 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
Aldosterone EC_{50}	0.4nM	0.4nM
Z'-Factor (EC_{100})	0.87	0.78

Response Ratio = 4.5
Optimum cell no. = 25K cells/well
Optimum [DMSO] = up to 1%
Stimulation Time = 16 hours
Max. [Stimulation] = 10 nM

2. **Alternate agonist dose response**
   See agonist dose response section

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

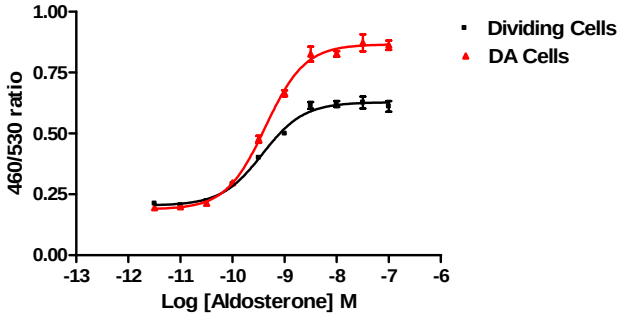
5. **Assay performance with variable cell number**

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

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

Primary Agonist Dose Response

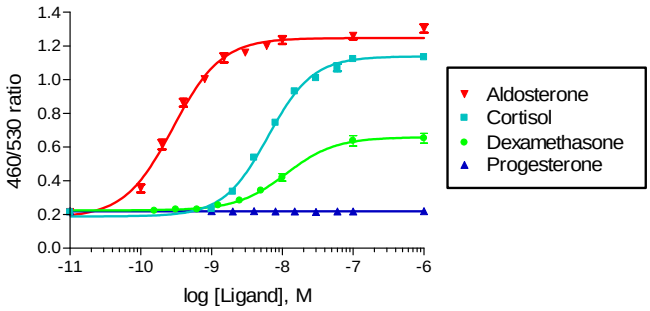
Figure 1 — MR DA and MR-UAS-*bla* HEK 293T dose response to aldosterone under optimized conditions



MR DA cells and MR-UAS-*bla* HEK 293T cells (25,000 cells/well) were plated in a 384-well format and stimulated with a dilution series of aldosterone 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 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 aldosterone (n=6 for each data point).

Alternate Agonist Dose Response

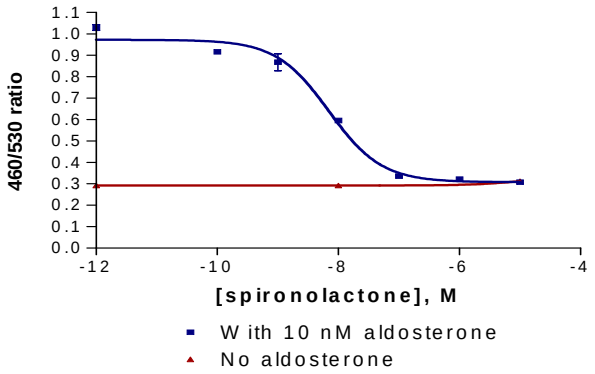
Figure 2 — MR-UAS-*bla* HEK 293T dose response to known agonists Cortisol and Dexamethasone



MR-UAS-*bla* HEK293T cells were starved overnight and then plated (25,000 cells/well) in a 384-well black-walled tissue culture assay plate. Cells were stimulated with Aldosterone, Cortisol, Dexamethasone, or Progesterone 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 2 hours. 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.

Antagonist Dose Response

Figure 3 — MR-UAS-*bla* HEK 293T dose response to known antagonist Spironolactone



MR-UAS-*bla* HEK293T cells were starved overnight and then plated (25,000 cells/well) in a 384-well black-walled tissue culture assay plate. Cells were treated with Spironolactone and incubated at 37 degrees C for 30 min., followed by 10 nM Aldosterone agonist stimulation for 16 hours in 0.5% DMSO. Cells were then loaded for 2 hours 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 ligand.

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	1X Matrigel Matrix	Freeze Medium
DMEM, w/ GlutaMAX™	90%	90%	—	99.75%	—
DMEM, Phenol red-free	-	-	98%	-	-
FBS, Charcoal-stripped	—	—	2%	-	—
Dialyzed FBS **Do not substitute!**	10%	10%	-	-	—
NEAA	0.1 mM	0.1 mM	-	-	—
Sodium Pyruvate	1mM	1mM	-	-	—
HEPES (pH 7.3)	25 mM	25 mM	—	-	—
Hygromycin	—	80 µg/mL	—	-	—
Zeocin®	—	80 µg/mL	—	-	—
Penicillin	100 U/mL	100 U/mL	100 U/mL	-	—
Streptomycin	100 µg/mL	100 µg/mL	100 µg/mL	-	—
Matrigel Matrix	-	-	-	0.25%	-
Recovery™ Cell Culture Freezing Medium	—	—	—	-	100%

Assay Performance with Variable Cell Number

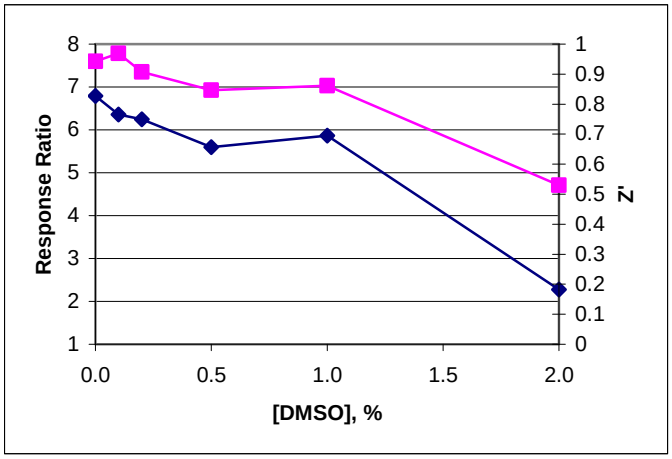
Figure 4 – MR-UAS-*bla* HEK 293T response to aldosterone with 12, 25 and 50K cells per well

Cells/well	[DMSO]	Response Ratio	Z'
12,500	0	6.3	0.91
25,000	0	6.6	0.95
50,000	0	5.8	0.83
12,500	0.5%	4.6	0.81
25,000	0.5%	4.9	0.83
50,000	0.5%	4.7	0.94

MR-UAS-*bla* HEK293T cells were starved overnight and then plated (25,000 cells/well) in a 384-well black-walled tissue culture assay plate. Cells were stimulated with 9 nM Aldosterone for 16 hours either in the absence or presence of 0.5% 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 and the Response Ratios and Z' values calculated.

Assay Performance with variable DMSO concentration

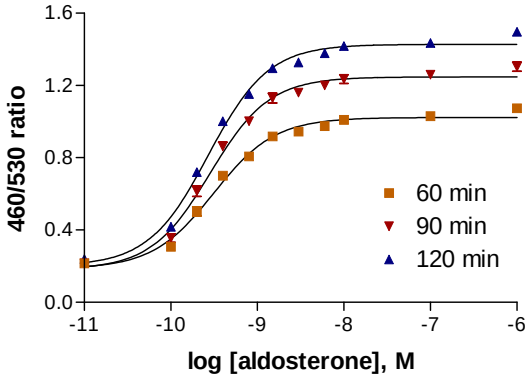
Figure 5 – MR-UAS-*bla* HEK 293T response to Aldosterone with 0 - 2% DMSO.



MR-UAS-*bla* HEK293T cells were starved overnight and then plated (25,000 cells/well) in a 384-well black-walled tissue culture assay plate. Cells were stimulated with 9 nM Aldosterone, and DMSO was added to the assay at concentrations from 0% to 2%. Cells were stimulated for 16 hrs with agonist and loaded for 2 hours 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 and Z' values are shown.

Assay Performance with variable substrate loading time

Figure 6 – MR-UAS-*bla* HEK 293T dose response to aldosterone with 60, 90 and 120 minute substrate loading time



MR-UAS-*bla* HEK293T cells were starved overnight and then plated (25,000 cells/well) in a 384-well black-walled tissue culture assay plate. Cells were stimulated with aldosterone 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 60, 90, or 120 minutes. Fluorescence emission values at 460 nm and 530 nm for the various loading times were obtained using a standard fluorescence plate reader and the Ratios plotted against the indicated concentrations of aldosterone.