GeneBLAzer® CALCR CRE-*bla* FREESTYLE293F Cells

Catalog Numbers – K1480

Target Description

Calcitonin is involved in decreasing circulating calcium through the inhibition of bone resorption by osteoclasts, and by promoting calcium excretion in urine. The calcitonin receptor (accession # NM_001742.2) was cloned from an expression library prepared from an ovarian small cell carcinoma cell line and belongs to the B family of G-protein-coupled receptors (1,2). The B-family of GPCRs recognize regulatory peptides and include the secretin, parathyroid, and glucagon receptors (1). Alternative splicing within the calcitonin receptor gene has led to the identification of numerous cDNA variants (2,3). The best characterized splice variants differ by the absence ($CT_{(a)}$/CTR_{I1-}/CTR2) or presence ($CT_{(b)}$ /CTR_{I1+}/CTR1) of 16 amino acids in the first intracellular loop (2,3). CTR2 is expressed in the CNS, skeletal muscle, lymphocytes, kidney, and testes while CTR1 is predominately expressed in the ovary, placenta, bone marrow, and lung (1). Despite little difference in the ability of these two isoforms to recognize peptides the CTR1/$CT_{(b)}$ receptor is poorly internalized with altered coupling to G proteins leading to a decrease and/or loss of second messenger signaling (4).

Calcitonin is the primary agonist for the CALC receptor and one of five members of the calcitonin peptide family. These members include calcitonin (CT), amylin (AMY), two calcitonin gene-related peptides (αCGRP and βCGRP), and adrenomedulliin (AM). These peptides do not share homology in their primary sequences but all form 6- or 7- amino acid ring structures formed by intramolecular disulfide bonds close to the N-terminus (1). These amino acid rings are followed by a region of potential amphipathic α-helixes and are C-terminally amidated. The calcitonin receptor has high affinity for the CT peptide and relatively low affinity for the CGRP, AMY, and AM peptides (2,3). The discovery of receptor activity modifying proteins (RAMPs) (15) and their interactions with the calcitonin receptor identified a mechanism for altering CGRP, AMY, and AM affinities for the calcitonin receptor (5,6,7,12).

Three separate RAMPs (RAMP-1, -2, and -3) have been identified and they share less than 30% sequence identity (1). RAMPs are membrane proteins that consist of an extracellular domain of approximately 100 amino acids, a transmembrane domain, and a short intracellular domain of approximately 10 amino acids (1). The calcitonin receptor does not require RAMPs to translocate to the cell membrane, bind, and respond to CT, while RAMPs are incapable of translocating to the cell membrane or binding CT, AMY, AM, and CGRP in the absence of the calcitonin receptor (5,14). Co-expression of the calcitonin receptor with RAMPs (-1, -2, and -3) will result in a series of amylin specific receptors that have different affinities for CT, AMY, CGRP, and AM peptides (5,6,7,11).

Cell Line Description

The GeneBLAzer® CALCR-CRE-*bla* Freestyle293 cells contain the CALCR receptor isoform (CTR2/CT(a)/CTRI1-), accession # NM_001742.2, stably integrated into the CellSensor® CRE-*bla* Freestyle293 cell line. The CellSensor® CRE-*bla* Freestyle293 (Cat No. K1636)cells contain a beta-lactamase reporter gene under control of the cAMP (CRE) response element. CALCR-CRE-*bla* Freestyle293 cells have been tested for variable assay conditions, including DMSO concentration, cell number, stimulation time, and substrate loading time and functionally validated for Z' and EC_{50} concentrations of calcitonin. Additional testing data using alternate stimuli are also provided.

Validation Summary

Testing and validation of this assay was evaluated in a 384-well format using LiveBLAzer™-FRET B/G Substrate.

1. **Primary agonist dose response under optimized conditions(n=3)**

sCT EC_{50}	= 4.56 pM
Z'-Factor (EC_{100})	= 0.82
Response Ratio	= 8.1
Recommended cell no.	= 10K cells/well
DMSO Tolerance	= up to 1.0%
Recommended Stim. Time	= 5 hours
Max. [Stimulation]	= 1 nM

2. **Alternate agonist dose response**

hCT (EC_{50})	= 15.4 pM
Amylin (EC_{50})	= 3.0 nM
αCGRP (EC_{50})	= 6.9 nM
βCGRP (EC_{50})	= 3.9 nM

3. **Antagonist dose response**

sCT(8-32) (IC_{50})	= 15 nM

4. **Agonist 2nd messenger dose response**

sCT (EC_{50})	= 3.0 pM
hCT (EC_{50})	= 5.1 pM
Amylin (EC_{50})	= 7.9 nM
αCGRP (EC_{50})	= 5.0 nM
βCGRP (EC_{50})	= 3.8 nM

Assay Testing Summary

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

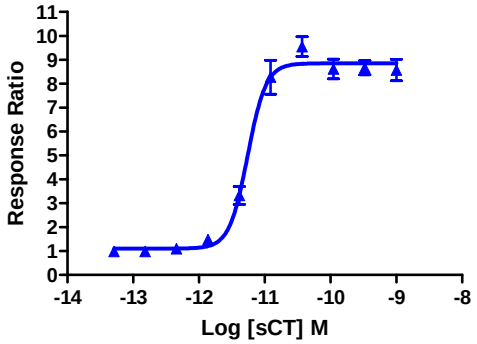
6. **Assay performance with variable stimulation time**

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

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

Primary Agonist Dose Response

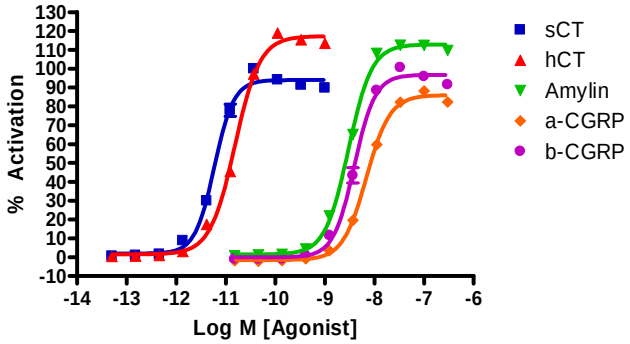
Figure 1 — GeneBLAzer® CALCR-CRE-*bla* Freestyle293F dose response to salmon calcitonin (sCT) under optimized conditions



GeneBLAzer® CALCR-CRE-*bla* Freestyle293 cells (10,000 cells/well) were plated in a 384-well format and incubated at 37°C for 16-20 hours. Cells were stimulated with a dilution series of salmon calcitonin (sCT) (Bachem cat# H-2260) in the presence of 0.5% DMSO for 4 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 2 hours. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and % Activation plotted for each replicate against the concentrations of sCT (n=16 for each data point).

Alternate Agonist Dose Response

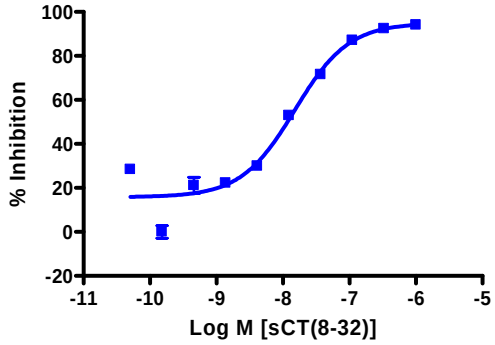
Figure 2 — GeneBLAzer® CALCR-CRE-*bla* Freestyle293F dose response to alternate agonists under optimized conditions



GeneBLAzer® CALCR-CRE-*bla* Freestyle293 cells were plated at 10,000 cells/well in a 384-well plate and incubated for 16-20 hours. Cells were stimulated with a dilution series of salmon CT (Bachem cat# H-2260), human CT (Bachem cat# H-3072), amylin (Bachem cat# H-7905), αCGRP (Bachem cat# H-1470), and βCGRP (Bachem cat# H-6730) in the presence of 0.5% DMSO for 4 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 2 hours. Fluorescence emission values at 460 nm and 530 nm were obtained and the % Activation is plotted for each cell number against the concentrations of the agonists (n=8 for each data point).

Antagonist Dose Response

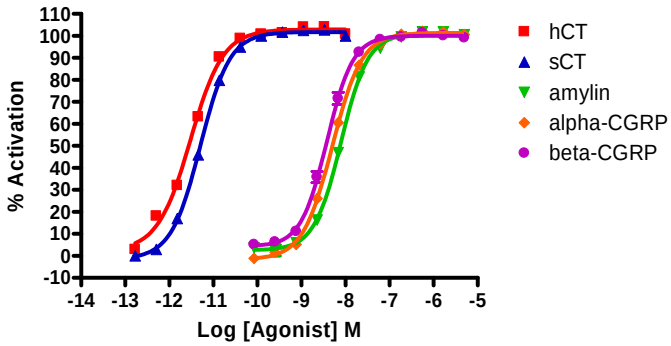
Figure 3— GeneBLAzer® CALCR-CRE-*bla* Freestyle293F 2nd messenger dose response to sCT(8-32) under optimized conditions



GeneBLAzer® CALCR-CRE-*bla* Freestyle293 cells were plated at 10,000 cells/well in a 384 well black walled poly-D-lysine coated plate (Corning cat# 3664) 16-20 hours prior to the assay in assay media and incubated at 37°C/5% CO_2 until time of assay. The cells were treated with a dilution series of sCT(8-32) (Bachem cat# H-5502) in the presence of 0.5% DMSO and placed at for 30 minutes prior to the addition of the EC_{80} [6 pM] of the sCT (Bachem cat# H-2260) peptide agonist. The cells were incubated at 37°C/5% CO2 for an additional 4 hours. Following stimulation, the cells were loaded 2 hours at room temperature in the dark 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 % Inhibition is shown plotted against the concentrations of the antagonist. (N=8 for each data point).

Agonist 2nd Messenger Dose Response

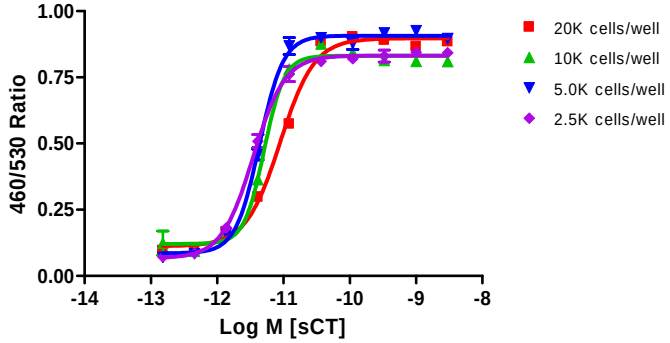
Figure 4— GeneBLAzer® CALCR-CRE-*bla* Freestyle293F 2nd messenger dose response to Agonist under optimized conditions



GeneBLAzer® CALCR-CRE-*bla* Freestyle293F cells were tested for a response to various agonists with a TR-FRET cAMP assay.

Assay Performance with Variable Cell Number

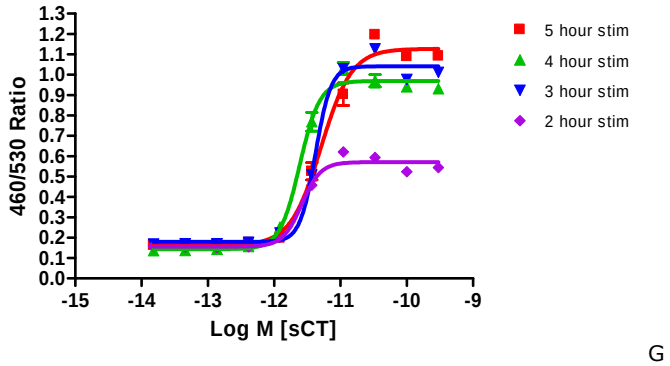
Figure 5– sCT dose response with 2.5, 5, 10, and 20K cells/well



GeneBLAzer® CALCR-CRE-*bla* Freestyle293 cells were plated at 2,500 5,000 10,000 or 20,000 cells/well in a black wall, clear bottom 384-well plate and incubated for 16-20 hours. Cells were stimulated with a dilution series of sCT (Bachem cat# H-2260) in the presence of 0.5% DMSO for 5 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 2 hours. Fluorescence emission values at 460 nm and 530 nm were obtained and the 460/530 Emission Ratios are shown plotted for each cell number against the concentrations of sCT (n=8 for each data point).

Assay Performance with Variable Stimulation Time

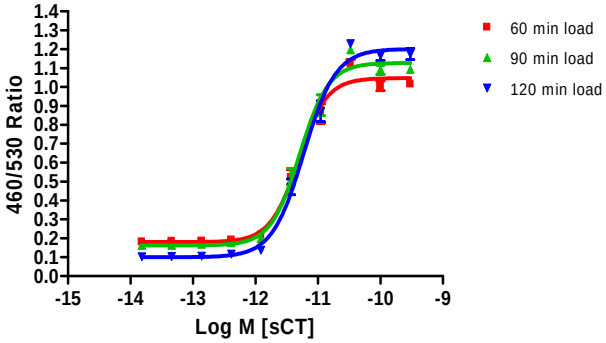
Figure 6– sCT dose response with 2, 3, 4 and 5 hr stimulation times



GeneBLAzer® CALCR-CRE-*bla* Freestyle293 cells were plated in a black walled, clear bottom 384-well plate at 10,000 cells/well, and incubated for 16-20 hours. Cells were stimulated with a dilution series of sCT (Bachem cat# H-2260) for 2, 3, 4, or 5 hrs in the presence of 0.5% DMSO. Cells were then loaded for 1.5 hours with LiveBLAzer™-FRET B/G Substrate. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard florescence plate reader and the 460/530 Emission Ratios are shown plotted for each stimulation time against the concentrations of sCT (n=8 for each data point).

Assay Performance with Variable Substrate Loading Times

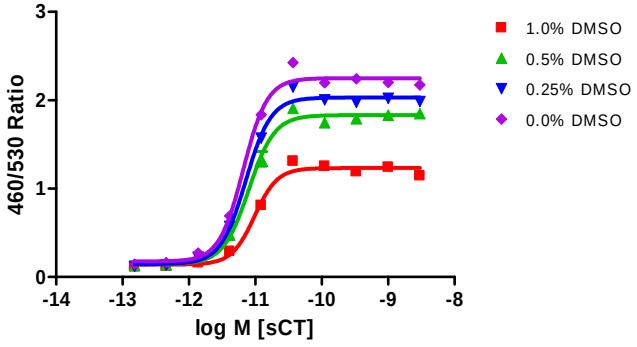
Figure 7– sCT dose response with 1, 1.5, and 2 hour substrate loading times.



GeneBLAzer® CALCR-CRE-*bla* Freestyle293 cells (10,000 cells/well) were plated in a black walled, clear bottom 384-well plate and incubated for 16-20 hours. Cells were stimulated with a dilution series of sCT (Bachem cat# H-2260) in the presence of 0.5% DMSO for 5 hours. Cells were then loaded for either 60, 90, or 120 minutes with LiveBLAzer™-FRET B/G Substrate. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard florescence plate reader and the 460/530 Emission Ratios are shown plotted for each substrate loading time against the concentrations of sCT (n=8 for each data point).

Assay Performance with Variable DMSO Concentration

Figure 8– sCT dose response with 0, 0.1, 0.5 and 1% DMSO

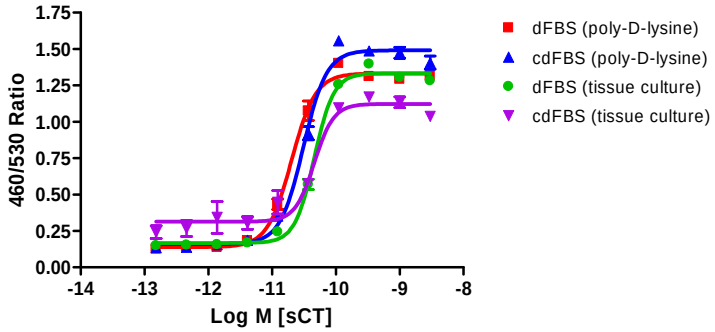


GeneBLAzer® CALCR-CRE-*bla*-Freestyle293 cells (10,000 cells/well) were plated in a black walled, clear bottom 384-well plate and incubated for 16-20 hours. DMSO was added to the cells at concentrations from 0% to 1%. Cells were stimulated with a dilution series of sCT (Bachem cat# H-2260) for 5 hours. Cells were then loaded for 1.5 hours with LiveBLAzer™-FRET B/G Substrate. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard florescence plate reader and the 460/530 Emission Ratios are shown plotted for each DMSO concentration against the concentrations of sCT (n=8 for each data point).

ADDITIONAL ASSAYS

Test of assay performance with 10% dFBS and 10% cdFBS.

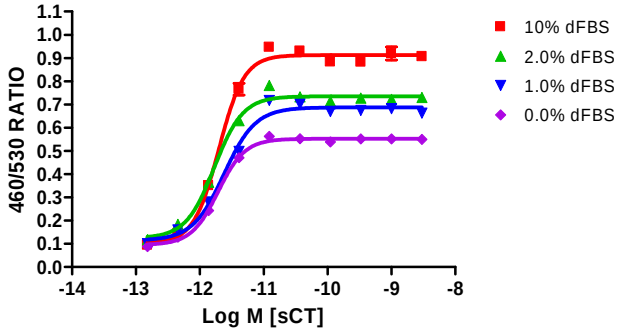
To test the effect of charcoal treated FBS (cdFBS) versus dialyzed FBS (dFBS) on assay performance cells were seeded in either a 384 well black walled tissue culture treated plate (Corning cat#) or a 384 well black walled poly-D-lysine coated plate (Corning cat# 3664) at 10,000 cells/well in DMEM supplemented with either 10% dFBS or 10% cdFBS. The cells were incubated at 37°C/5% CO_2 until time of assay (~16-20 hours). At the time of the assay cells were stimulated with a dilution series of sCT (performed in assay media supplemented with either 10% dFBS or 10% cdFBS) and incubated for 4 hours at 37°C/5% CO_2. Following stimulation, the cells were loaded for 2 hours at room temperature in the dark with LiveBLAzer™-FRET B/G loading solution containing 1µM substrate. Cells were assayed in the presence of 0.5% DMSO. Results are shown in Figure 20. When in poly-D-lysine plates were used the assay performed well in both dFBS and cdFBS. If the assay is to be performed with tissue culture plates dFBS should be used. The use of cdFBS in tissue culture plates resulted in a significantly lower response ratio and poor Z' value.



FBS	Plate	EC50 [pM]	Response Ratio	Z'
dFBS	Poly-D-lysine	20.2	10.6	0.87
cdFBS	Poly-D-lysine	30.9	10.2	0.65
dFBS	Tissue Culture	45.8	9.1	0.85
cdFBS	Tissue Culture	45.9	3.9	0

Test of assay performance with different dFBS concentrations

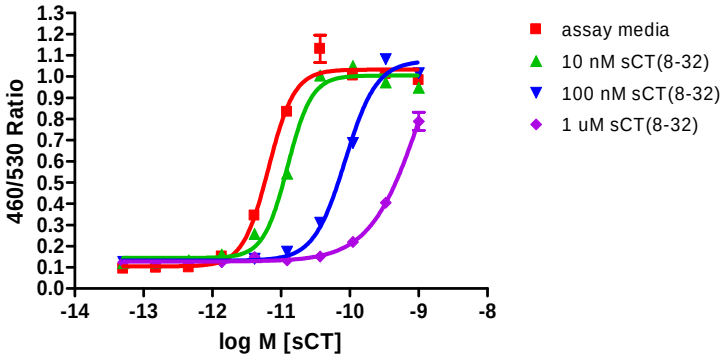
To determine how the assay performs in the presence of different concentrations of dFBS cells were plated at 10,000 cells/well in DMEM with 10%, 2%, 1%, or no dFBS 16-20 hours prior to the assay in a 384 well black walled poly-D-lysine plate (Corning cat# 3664). The cells were incubated at 37°C/5% CO_2 until time of assay. At the time of the assay cells were stimulated with a dilution series of sCT (performed in assay media supplemented with either 10%, 2%, 1%, or 0% dFBS) and incubated for 4 hours at 37°C/5% CO_2. Following stimulation, the cells were loaded for 2 hours at room temperature in the dark with LiveBLAzer™-FRET B/G loading solution containing 1µM substrate. Cells were assayed in the presence of 0.5% DMSO. The assay performed in the presence of 10% dFBS had the highest response ratio compared to the other dFBS concentrations. The EC_{50} and Z' values were similar for all conditions.



%FBS	EC50 [pM]	Response Ratio	Z'
10%	1.99	10.54	0.77
2.0%	1.69	9.14	0.77
1.0%	2.4	8.13	0.86
0.0%	1.93	8.65	0.81

Test of sCT serial dilution in the presence of sCT(8-32)

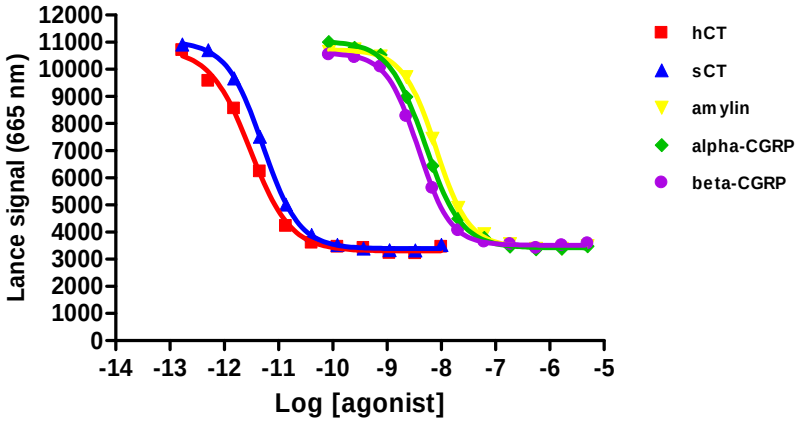
To determine how the assay performs in the presence of increasing concentrations of sCT(8-32). Cells were plated 16-20 hours prior to the assay in a 384 well black walled poly-D-lysine plate (Corning cat# 3664) at 10,000 cells/well in DMEM + 10% dFBS. The cells were incubated at 37°C/5% CO_2 until time of assay. The cells were treated with increasing concentrations of sCT(8-32) in the presence of 0.5% DMSO and placed at for 30 minutes prior to the addition of a serial dilution of the sCT peptide agonist. The cells were incubated at 37°C/5% CO2 for an additional 4 hours. Following stimulation, the cells were loaded 2 hours at room temperature in the dark with LiveBLAzer™-FRET B/G loading solution containing 1µM substrate. Cells were assayed in the presence of 0.5% DMSO.



sCT(8-32)	EC50 [pM]	Response Ratio
0 nM	6.5	11.6
10 nM	12.4	8.6
100 nM	85.5	9.2
1 µM	~1208	

Test of calcitonin agonist in 2nd messenger cAMP assay

To determine how the CALCR-CRE-bla-Freestyle293 cells perform in a cAMP 2nd messenger assay. CALCR-CRE-bla Freestyle293 cells were used in the Lance cAMP (Perkin Elmer cat# AD0263). 12,000 cells were assayed per well, and were treated with increasing concentrations of sCT (Bachem cat# H-2260), human CT (Bachem cat# H-3072), amylin (Bachem cat# H-7905), αCGRP (Bachem cat# H-1470), and βCGRP (Bachem cat# H-6730) using the provided protocol



Agonist	EC50
sCT	3.0 pM
hCT	5.1 pM
amylin	7.9 nM
αCGRP	5.0 nM
βCGRP	3.8 nM

References:

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