Validation & Assay Performance Summary

CellSensor® SBE-*bla* A375 Cell Line

Cat. no. K1514

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.

Pathway Description

Bone morphogenetic proteins (BMPs), members of the transforming growth factor β (TGF-β) superfamily, play important roles in the development of the heart, central nervous system and cartilage. Disruption of BMP signaling affects the body plan of the developing embryo. BMPs propagate their signal by activating Activin receptor-like kinase (ALK), which in turn mediates the phosphorylation of Smads. Phosphorylated Smad associates with Smad4 and then translocates to the nucleus regulating gene expression.

Cell Line Description

A375 cells are human malignant melanoma cells containing endogenous Smad signaling pathway. SBE-*bla* A375 cell line was engineered to express beta-lactamase under the control of Smad binding element. This cell line has been validated for Z' and EC_{50} under optimized conditions using BMP-4. This cell line has also responded to Nodal and was tested for assay performance under variable conditions, including DMSO concentration, cell number, stimulation time, substrate loading time. Additional information using various small molecule inhibitors and Stealth™ RNAi is 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)**

   BMP-4 EC_{50} = 65.7 ng/mL (1.9 nM)
   Z'-Factor (EC_{100}) = 0.75
   Response Ratio = 4.5

   Optimum cell no. = 10K cells/well
   Optimum [DMSO] = up to 1%
   Optimum Stim.Time = 5 hours
   Max. [Stimulation] = 5 µg/mL (147 nM)

2. **Alternate Stimuli**

   Nodal EC_{50} = 3.8 µg/mL (147 nM)

3. **Small Molecule Inhibitors Dose Response**

   IC_{50} SB431542 = 191.4 nM
   IC_{50} TGF-β R1 kinase inhibitor = 89.8 nM
   IC_{50} TGF-β R1 inhibitor III = 121.3 nM

4. **Stealth™ RNAi Testing**

5. **Cell culture and maintenance**
   See Cell Culture and Maintenance Section and Table 1

Assay Testing Summary

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

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

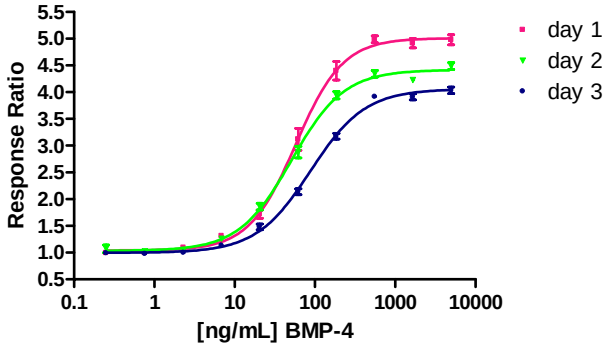
8. **Assay performance with variable substrate loading time**

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

10. **Assay performance with cryo-preserved cells**

Primary Agonist Dose Response

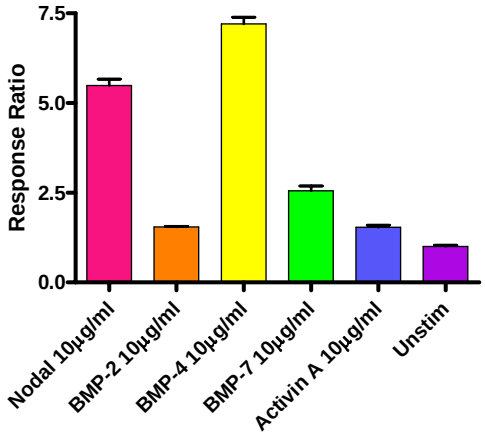
Figure 1 – SBE-*bla* A375 dose response to BMP-4 under optimized conditions



SBE-*bla* A375 cells (passage# 14,15,16, 10,000 cells/well) were assayed on three separate days represented by the three curves shown on the graph. Cells were plated the day before the assay in a 384-well format and then stimulated with BMP-4 (Invitrogen # PHC7914) over the indicated concentration range in the presence of 0.1% 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 using a standard fluorescence plate reader and the Response Ratios were plotted for the indicated concentrations of BMP-4 (n= 12 for each data point).

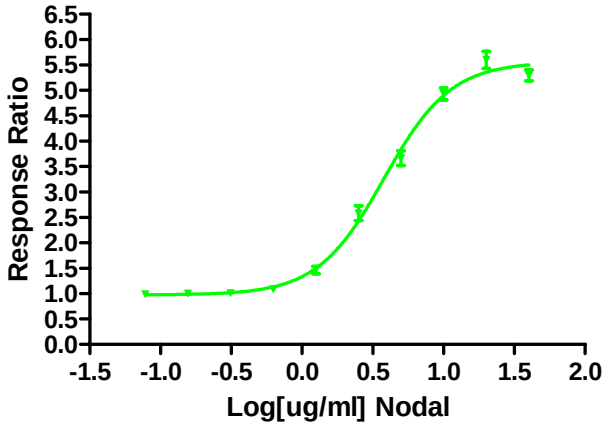
Alternate Stimuli

Figure 2 – SBE-*bla* A375 response to various stimuli



SBE-*bla* A375 cells (10,000 cells/well) were plated the day before the assay in a 384-well format and treated with Nodal (R&D system # 3218-ND), BMP-2 (Invitrogen # PHC7094), BMP-4 (Invitrogen # PHC7914), BMP-7 (Invitrogen # PHC7204) or Activin A (Invitrogen # PHG9014) at the indicated concentrations in the presence of 0.1% DMSO for 5 hours, and 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 the Response Ratios were plotted for each stimulus (n=4 for each data point).

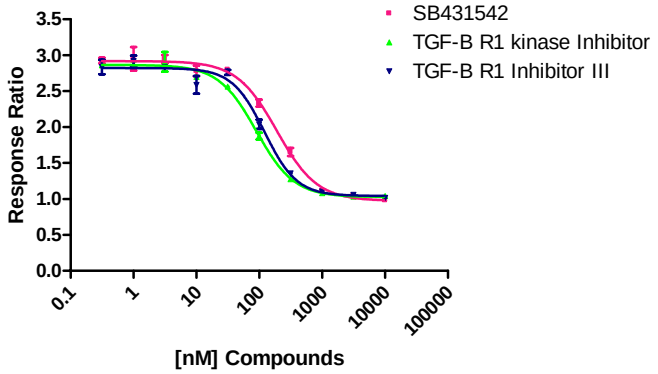
Figure 3 –SBE-*bla* A375 dose response to Nodal



SBE-*bla* A375 cells (10,000 cells/well) were plated the day before the assay in a 384-well format and then stimulated with Nodal (R&D system # 3218-ND) over the indicated concentration range in the presence of 0.1% 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 using a standard fluorescence plate reader and the Response Ratio was plotted for the indicated concentrations of Nodal (n= 6 for each data point).

Small Molecule Inhibitors Dose Response

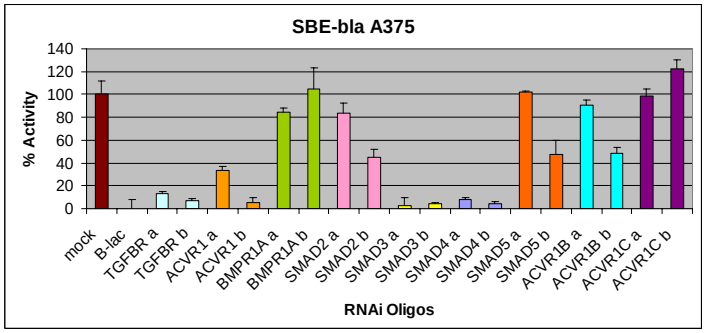
Figure 4 – SBE- *bla* A375 dose response to various small molecule inhibitors under optimized conditions



SBE-*bla* A375 cells (10,000 cells/well) were plated in a 384-well plate in the absence of BMP-4 for 16 hrs, followed by pre-treatment with the indicated concentrations of SB431542 (Tocris bioscience # 1614), TGF-β R1 kinase inhibitor (EMD/Calbiochem # 616451) and TGF-β R1 Inhibitor III (EMD/Calbiochem # 616453) for 30 min. Cells were then stimulated with BMP-4 (Invitrogen # PHC7914) at 200 ng/ml in the presence of 0.1% DMSO for 5 hours, and 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 the Response Ratios were plotted for the indicated concentrations of each Inhibitor (n=4 for each data point).

Stealth™ RNAi Testing

Figure 5 — SBE-*bla* A375 response to various RNAi oligos



SBE-*bla* A375 cells (5,000 cells/well) were plated with growth medium in a 96-well format and incubated at 37°C overnight. Cells were then treated with RNAiMax mixtures containing the listed Stealth™ RNAi oligos (TGFBR, Invitrogen # 12938-075; ACVR1, Invitrogen # 12937-01; BMPR1A, Invitrogen # 12937-04; SMAD2, Invitrogen # 12937-09; SMAD3, Invitrogen # 12937-10; SMAD4, Invitrogen # 12938-126; SMAD5, Invitrogen # 12937-11; ACVR1C, Invitrogen # 12937-02) for 32 hrs. Following an Assay Media exchange and a 37°C incubation for 16 hours, cells were then stimulated with BMP-4 (200 ng/mL) for 5 hours, and 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 the percentage of β-lactamase activity was plotted for each RNAi Oligos.

Cell Culture and Maintenance

Thaw cells in Growth Medium without selection and culture them in Growth Medium with Blasticidin. Passage or feed cells at least twice a week and maintain them in a 37°C/5% CO_2 incubator. Maintain cells between 5% and 80% confluence. Do not allow cells to reach confluence.

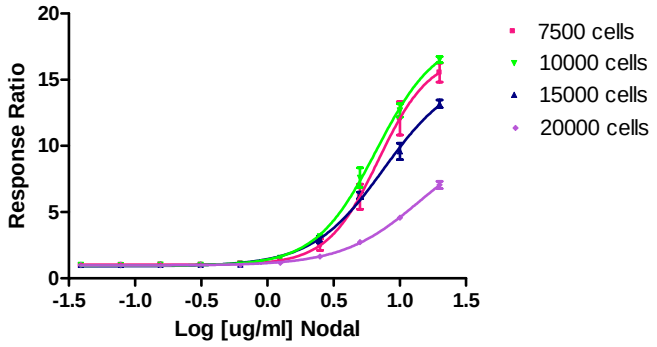
Note: For optimal cell line performance, use dialyzed FBS (Invitrogen# 26400-036). For more detailed cell growth and maintenance directions, please refer to the protocol.

Table 1 – Cell Culture and Maintenance

Component	Growth Medium (+)	Growth Medium (-)	Assay Medium	Freezing Medium
DMEM w/ GlutaMAX	90%	90%	--	--
OPTI-MEMI	--	--	99.5%	--
Dialyzed FBS **DO NOT SUBSTIUTE!**	10%	10%	0.5%	--
NEAA	--	--	0.1 mM	--
Sodium Pyruvate	--	--	1mM	--
Penicillin	100 U/mL		100 U/mL	--
Streptomycin	100 µg/mL		100 µg/mL	--
Blasticidin	5 µg/mL	--	--	--
Recovery™ Cell Culture Freezing Medium	--	--	--	100%

Assay Performance with Variable Cell Number

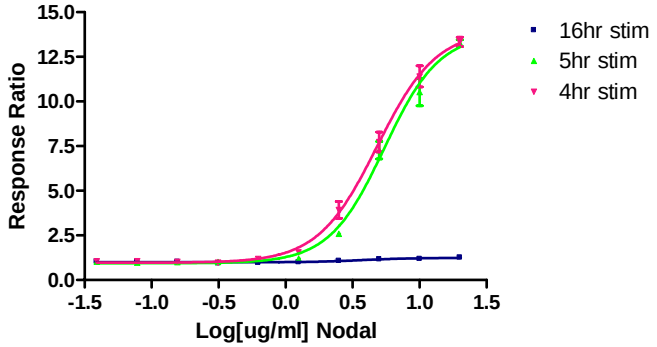
Figure 6 — SBE-*bla* A375 response to Nodal using ~10000, 15000, 20000 or 30000 cells/well



SBE-*bla* A375 cells were plated at ~ 7500, 10000, 15000 or 20000 cells/well in a 384-well format. Cells were then stimulated with Nodal (R&D system # 3218-ND) over the indicated concentration range in the presence of 0.1% 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 for the various cell numbers were obtained using a standard fluorescence plate reader and the Response ratios plotted for each cell number against the indicated concentrations of Nodal. (n=4 for each data point).

Assay Performance with Variable Stimulation Time

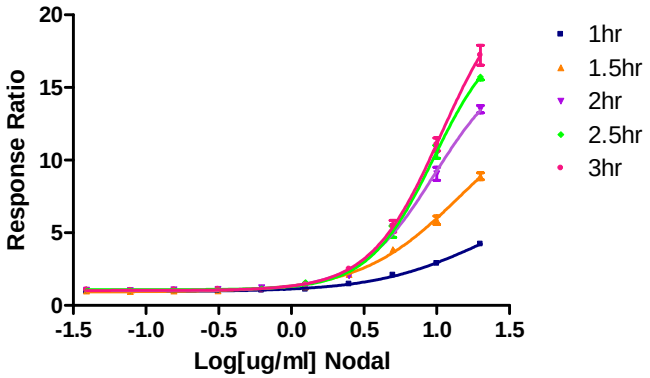
Figure 7 – SBE-*bla* A375 dose response to Nodal with 4, 5 and 16 hour stimulation times



SBE-*bla* A375 cells (10,000 cells/well) were plated in a 384-well assay plate. For 4hr and 5hr stimulation experiments, cells were plated the day before the assay; for 16hr stimulation experiment, cells were plated the day of assay. Nodal (R&D system # 3218-ND) was then added to the plate over the indicated concentration range. Plates were treated for 4, 5 or 16 hrs with NODAL in 0.1% DMSO and 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 Response Ratios plotted for each stimulation time against the indicated concentrations of Nodal (n=4 for each data point).

Assay Performance with Variable Substrate Loading Time

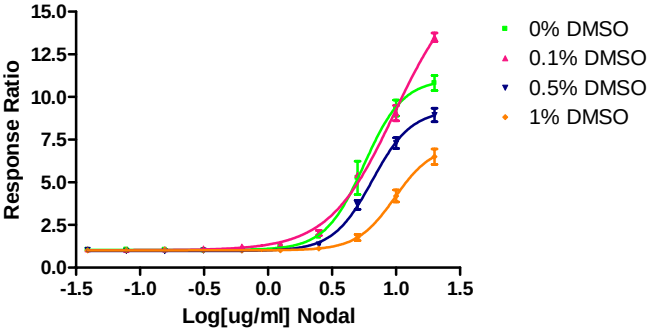
Figure 8 — SBE-*bla* A375 dose response to Nodal with 1, 1.5, 2, 2.5 and 3 hour substrate loading times



SBE-*bla* A375 cells were plated the day before the assay at 10,000 cells/well in a 384-well format. Cells were treated with Nodal (R&D system # 3218-ND) over the indicated concentration range in the presence of 0.1% DMSO for 5 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for either 1, 1.5, 2, 2.5 or 3 hours. Fluorescence emission values at 460 nm and 530 nm for the various substrate loading times were obtained using a standard fluorescence plate reader and the Response Ratios plotted for each substrate loading time against the indicated concentrations of Nodal (n=4 for each data point).

Assay Performance with Variable [DMSO]

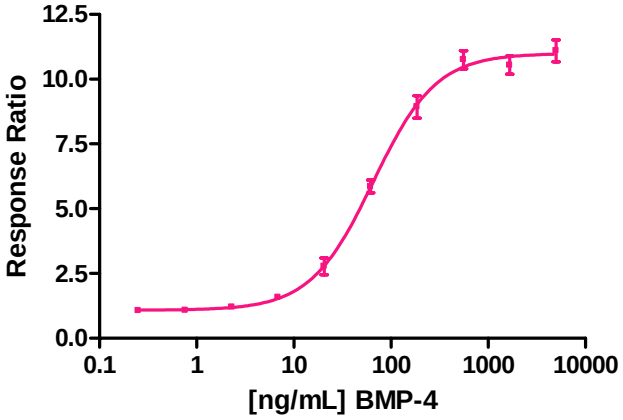
Figure 9 – SBE-*bla* A375 dose response to Nodal with 0, 0.1, 0.5 and 1% DMSO



SBE-*bla* A375 cells (10,000 cells/well) were plated the day before the assay in a 384-well assay plate. Nodal (R&D system # 3218-ND) was then added to the plate over the indicated concentration range with 0, 0.1, 0.5 or 1% final DMSO concentrations. 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 Response Ratios for each DMSO concentration were plotted against the indicated concentrations of Nodal (n=4 for each data point).

Assay performance with cryo-preserved cells

Figure 10 – Cryo-preserved SBE-*bla* A375 dose response to BMP-4



Cryo-preserved SBE-*bla* A375 cells (passage# 5) were thawed, resuspended with assay medium and plated (10,000 cells/well) the day before the assay in a 384-well format. Next morning, cells were stimulated with BMP-4 (Invitrogen # PHC7914) over the indicated concentration range in the presence of 0.1% 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 using a standard fluorescence plate reader and the Response Ratio plotted for the indicated concentrations of BMP-4 (n=6 for each data point).