Validation & Assay Performance Summary

CellSensor® Gli-*bla* NIH3T3 Cell Line

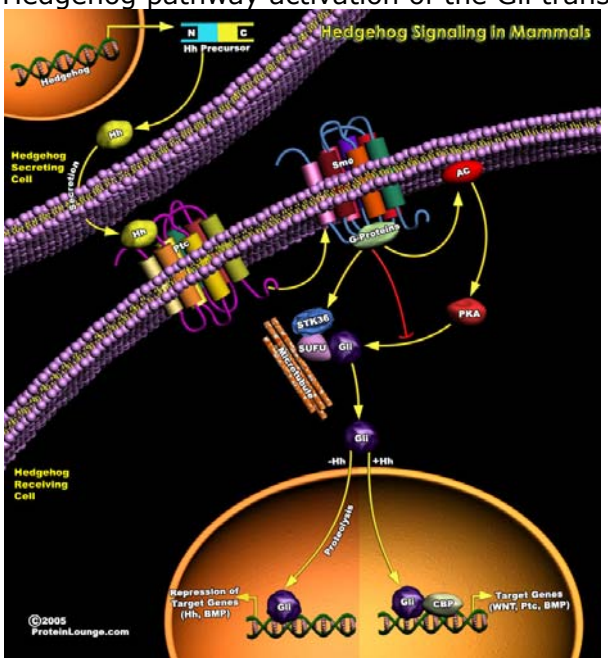
Cat. no. K1642

CellSensor® Cell-Based Assay Validation Packet

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

The morphogenic signal Shh provides in the developing CNS induces proliferation of neuronal precursor cells in the developing cerebellum and other tissues. Proliferative signaling by Shh is involved in the development of cancer, including specific brain and skin cancers such as basal cell carcinomas. Signaling takes place through a Patched (PTC-1)/Smoothened (SMO) Receptor complex. The activation of Patched by Shh releases the inhibition of Patched on Smoothened leading to Sonic Hedgehog pathway activation of the Gli transcription factor to induce downstream gene expression.



Cell Line Description

The CellSensor® Gli-*bla* NIH3T3 cell line contains a beta-lactamase reporter gene under control of the Gli response element stably integrated into NIH3T3 cells. This cell line is a clonal population isolated in response to Shh by flow cytometry. This cell line has also been tested for assay performance under variable conditions, including DMSO concentration, cell number, stimulation time, and substrate loading time and validated for Z' and EC_{50} concentrations of Shh. Additional testing information using known inhibitors or activator of the pathway 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)**

mShh EC_{50}	= 0.4 µg/ml
Z'-Factor (EC_{100})	= 0.67
Response Ratio	= 4.9
Recommended cell no. cells/well	= 7500
Recommended [DMSO]	= 0.5-1%
Recommended Stim. Time	= 24 to 48 hrs
Max. [Stimulation]	= 3 µg/ml

2. **Alternate Stimuli**
   See Compound Panel Section

3. **Stealth™ RNAi Testing**
   Pending

4. **Small molecule inhibitor Testing**
   See Compound Panel

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

Assay Testing Summary

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

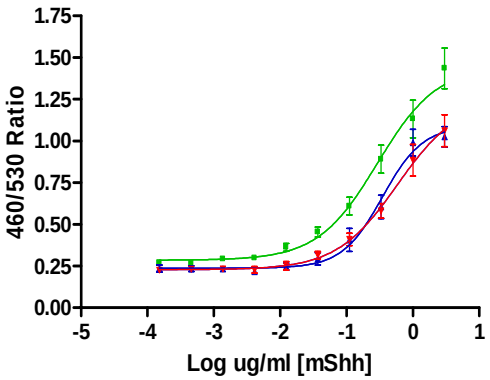
7. **Assay performance with variable stimulation time**

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

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

Primary Agonist Dose Response

Figure 1 — mShh dose response under optimized conditions



Gli-*bla* NIH3T3 cells (7500 cells/well) were assayed on three separate days represented by the three curves shown on the graph. Cells were plated the day prior to the assay in a 384-well format and stimulated with mShh (R&D Systems # 461-SH-025) over the indicated concentration range in the presence of 0.5% DMSO for 48hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 3 hours. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the 460/530 Emission Ratios plotted for the indicated concentrations of mShh (n=16 for each data point).

Compound Panel

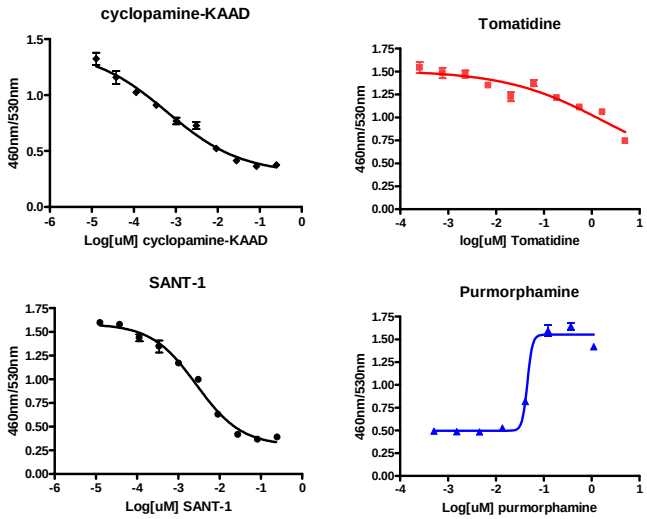


Figure 2 — Gli-*bla* NIH3T3 response to various known compound treatment.
Gli-*bla* NIH3T3 cells (7500 cells/well) were plated the day prior to the assay in a 384-well format in complete growth medium. On the day of the assay, growth medium was replaced with assay medium containing 0.5% FCS and cells were treated with cyclopamine-KAAD (EMD, #239804), Tomatidine (EMD, #614350) SANT-1 (EMD, #559303) or Purmorphamine (EMD, #540220) at the indicated concentrations for 0.5 hours before mShh was added at EC80 (1 µg/ml) to all treated cells except cells treated with Purmorphamine. Cells were stimulated with mShh for 24 hours and then loaded with LiveBLAzer™-FRET B/G Substrate for 3 hours. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader

and the 460/530 Ratios plotted for each treatment (n=4 for each data point).

Cell Culture and Maintenance

Thaw cells in Growth Medium without Blasticidin and culture them in Growth Medium with Blasticidin. Pass or feed cells at least twice a week and maintain them in a 37°C/5% CO_2 incubator. Maintain cells between 10% and 85% confluency. Do not allow cells to reach confluence.

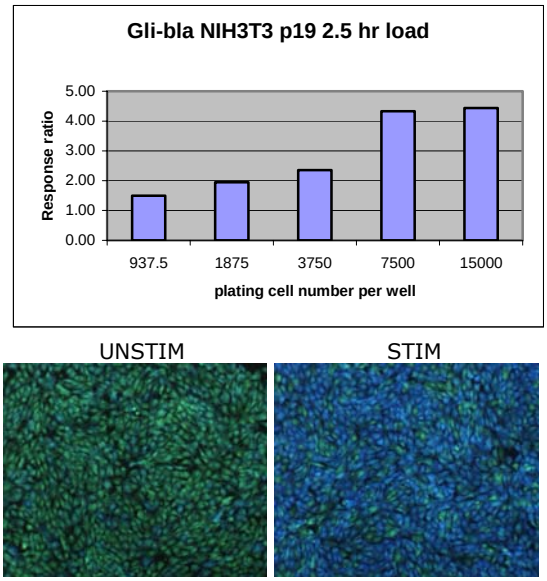
Note: We recommend passing cells for three passages after thawing before using them in the beta-lactamase assay. For more detailed cell growth and maintenance directions, please refer to protocol.

Table 1 – Cell Culture and Maintenance

Component	Growth Medium	Assay Medium	Freezing Medium
DMEM with GlutaMAX™	90%	--	—
OPTI-MEM	--	96%	
NCS **Do Not Substitute!**	10%	0.5%	—
NEAA	0.1 mM	0.1 mM	—
HEPES (pH 7.3)	25 mM	25 mM	—
Penicillin (antibiotic)	100 U/ml	100 U/ml	—
Streptomycin (antibiotic)	100 µg/ml	100 µg/ml	—
Blasticidin (antibiotic)	5 µg/ml	—	—
Recovery™ Cell Culture Freezing Medium	—	—	100%

Assay Performance with Variable Cell Number

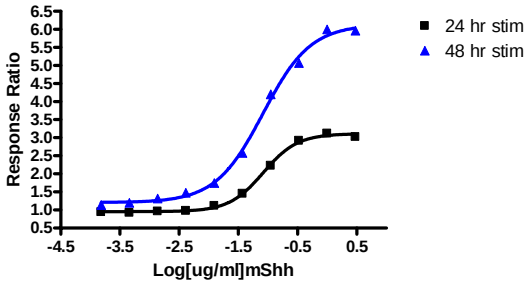
Figure5 — mShh dose response with different plating cell numbers/well



Gli-*bla* NIH3T3 cells were plated the day prior to the assay at 7500 cells/well in a 384-well format in growth medium. 24 hours later, medium was replaced with assay medium and Cells were stimulated with mShh (R&D Systems #461-SH) in for 48 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 2.5 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. (n=4 for each data point). Images of unstimed and stimulated cells at 7500 cells/well plating density are shown in the bottom.

Assay Performance with Variable Stimulation Time

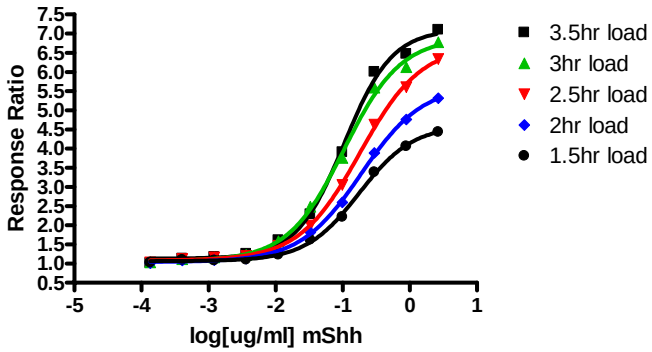
Figure 6 – mShh dose response with 24 and 48 hour stimulation times



Gli-*bla* NIH3T3 cells were plated the day prior to the assay at 7500 cells/well in a 384-well format in growth medium. 24 hours later, medium was replaced with assay medium and Cells were stimulated with mShh (R&D Systems #461-SH) in for 24 or 48 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 3 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 mShh (n=8 for each data point).

Assay Performance with Variable Substrate Loading Time

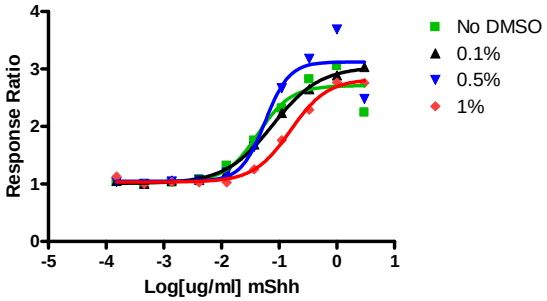
Figure 7 — mShh dose response with various substrate loading times



Gli-*bla* NIH3T3 cells were plated the day prior to the assay at 7500 cells/well in a 384-well format in growth medium. 24 hours later, medium was replaced with assay medium and Cells were stimulated with mShh (R&D Systems #461-SH) in for 48 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for indicated 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 mShh (n=16 for each data point).

Assay Performance with Variable DMSO Concentration

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



Gli-*bla* NIH3T3 cells were plated the day prior to the assay at 7500 cells/well in a 384-well format in growth medium. 24 hours later, medium was replaced with assay medium and Cells were stimulated with mShh (R&D Systems #461-SH) in the presence of indicated amount of DMSO in for 24 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 3 hours. 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 mShh (n=8 for each data point).