Validation & Assay Performance Summary

CellSensor® Gli-*bla* 22Rv1 Cell Line

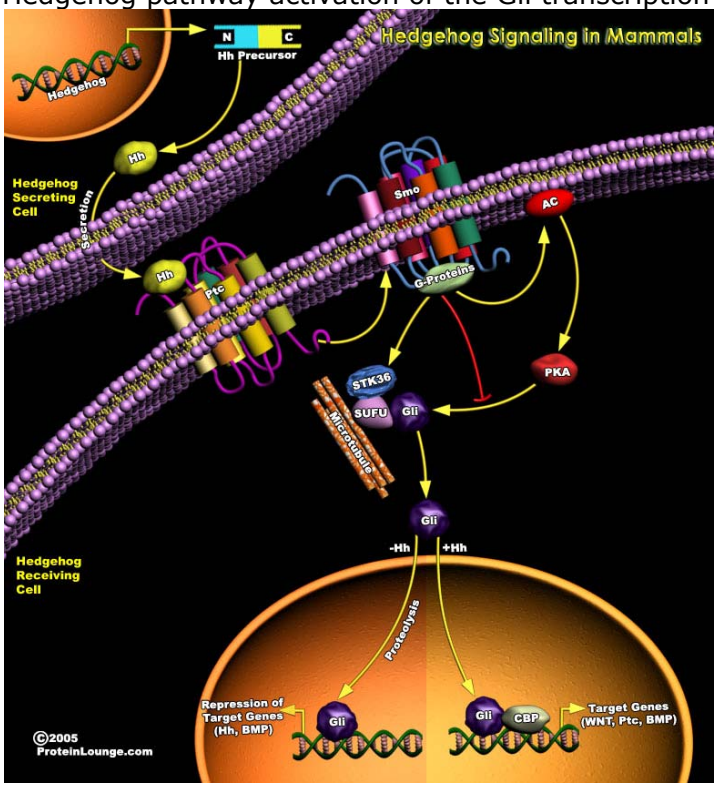
Cat. no. K1838

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* 22Rv1 cell line contains a beta-lactamase reporter gene under control of the Gli response element stably integrated into 22Rv1 cells. This cell line is a clonal population isolated by flow cytometry. The clone was selected based on inhibition of the pathway with KAAD-Cyclopamine. This cell line has also been tested for assay performance under variable conditions, including DMSO concentration, cell number, compound incubation time, and substrate loading time and validated for Z′ under maximal inhibition of β-lactamase activity with Clavulanic Acid.

Validation Summary

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

1. **KAAD-Cyclopamine inhibition under optimized conditions (n=3)**

Average Z'-Factor	= 0.61
Average Response Ratio	= 3.2
Recommended cell no.	= 15,000 cells/well
Recommended [DMSO]	= 0.25 to 1%
Recommended Inhib. Time	= 24 hrs
Max. [Inhibition]	= 20 µM
KAAD-Cyclopamine IC_{50}	≈ 6.1 µM

2. **Stealth™ RNAi Testing**
   See Stealth™ RNAi Testing

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

Assay Testing Summary

4. **Assay performance with variable plating cell density**

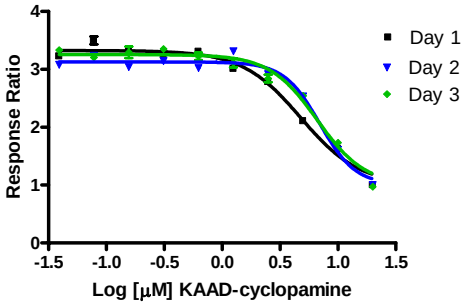
5. **Assay performance with variable compound incubation time**

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

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

Inhibition by KAAD-Cyclopamine

Figure 1 — Inhibition of constitutive β-lactamase under optimized conditions

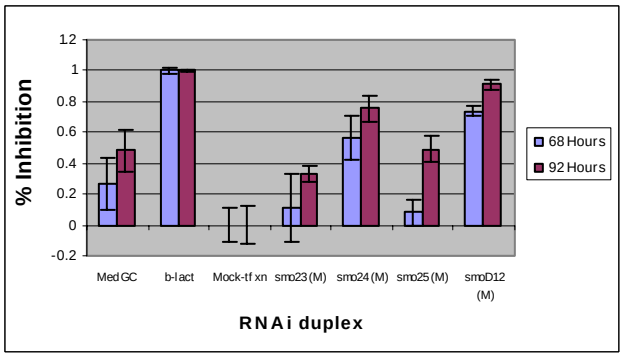


	Day 1	Day 2	Day 3
Best-fit values			
BOTTOM	1.000	1.000	1.000
TOP	3.325	3.127	3.255
LOGEC50	0.6798	0.8432	0.8209
HILLSLOPE	-1.713	-2.810	-2.117
EC50	4.784	6.970	6.621

Gli-*bla* 22Rv1 cells (15,000 cells/well) were assayed on three separate days represented by the three dose response curves on the graph. Cells were plated the day prior to the assay in a poly-D-lysine coated 384-well plate in growth medium. 20 hours later, cells were washed once with assay medium and then treated KAAD-Cyclopamine (Toronto Research Chemicals K171000) in Assay Medium (the final DMSO concentration was 0.25%.) 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. The response ratios in relation to cells at maximum inhibition with KAAD-Cyclopamine are plotted for the indicated concentrations of the inhibitor (n=16 for each data point).

Stealth™ RNAi Testing

Figure 2 — Inhibition of Gli pathway with Stealth™ RNAi

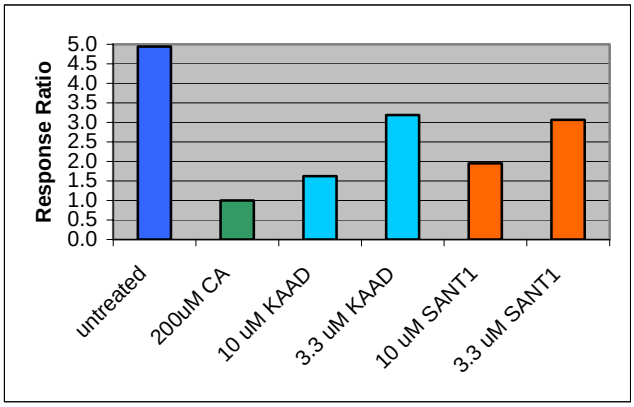


Gli-*bla* 22Rv1 cells (10,000 cells/well) were plated in a 96-well format and transfected with a Stealth™ Select RNAi duplex targeting Smo (Invitrogen, 1299003), a negative control duplex with a Medium GC content, and a duplex targeting β-lactamase. The cells were incubated with the duplexes for 68, or 92 hours, at which point the growth media was replaced with assay media for 20 hours. The cells were 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. The % Inhibition compared to the Medium GC content negative control and β-lactamase positive

control is shown for the three duplexes and controls. (n=4 for each data point).

Known Compound Testing

Figure 3 — Inhibition of Gli pathway with Smoothened inhibitors



Gli-*bla* 22Rv1 cells (10,000 cells/well) were plated on the 384-well assay plate the day prior to the assay in growth medium. Medium was replaced with assay medium and cells were left untreated or treated with Clavulanic Acid (Sigma-Aldrich P3494) or KAAD-Cyclopamine (Toronto Research Chemicals K171000) and SANT1 (EMD 559303) at indicated concentrations for 24 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 4 hours. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader. The response ratio was calculated as the ratio of blue/green ratio of cells untreated or treated vs the blue/green ratio of Calvulanic Acid treated cells.

Cell Culture and Maintenance

Thaw and culture cells in Growth Medium without Blasticidin. Pass or feed cells at every two to three days and maintain them in a 37°C/5% CO_2 incubator. Maintain cells between 10% and 75% confluency. Do not allow cells to reach confluence.

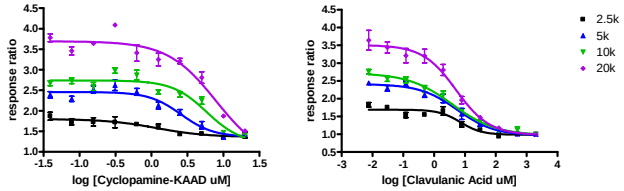
Note: We recommend passing cells for three passages after thawing before using them in the β-lactamase assay. For more detailed cell growth and maintenance directions, please refer to protocol.

Table 1 – Cell Culture and Maintenance

Component	Growth Medium	Assay Medium	Freezing Medium
RPMI 1640	500 mL bottle	—	—
OptiMEM	—	500 mL bottle	—
Dialyzed FBS	50 mL	2.5 mL	—
NEAA (10 mM)	5 mL	5 mL	—
Sodium Pyruvate (100 mM)	5 mL	5 mL	—
Penicillin (10,000 U/mL) & Streptomycin (10,000 µg/mL)	5 mL	5 mL	—
Recovery™ Cell Culture Freezing Medium	—	—	100%

Assay Performance with Variable Plating Cell Density

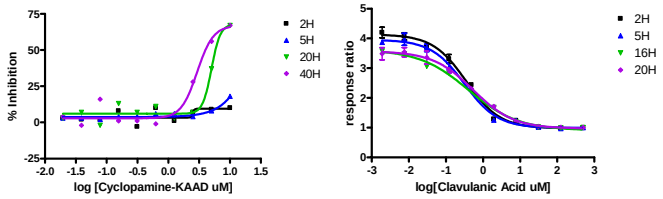
Figure 3 — Clavulanic Acid and KAAD-Cyclopamine dose response with different plating cell numbers/well



Gli-*bla* 22Rv1 cells were plated the day prior to the assay at indicated plating cell density in a 384-well format in growth medium. 4 hours later, medium was replaced with assay medium and cells were treated with Clavulanic Acid (Sigma-Aldrich P3494) or KAAD-Cyclopamine (Toronto Research Chemicals K171000) for 20 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. The response ratios in relation to cells at maximum inhibition with Clavulanic Acid are plotted for each cell plating density. (n=4 for each data point).

Assay Performance with Variable Compound Incubation Time

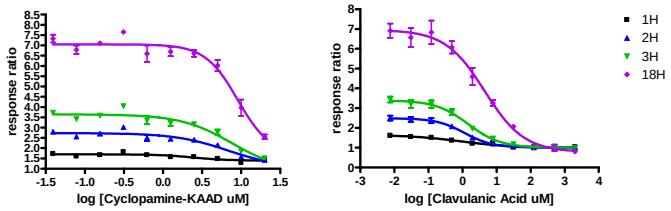
Figure 4 – Clavulanic Acid and KAAD-Cyclopamine dose response with various incubation times



Gli-*bla* 22Rv1 cells were plated the day prior to the assay at 15,000 cells/well in a 384-well format in growth medium. 4 hours later, medium was replaced with assay medium and the 16H, 20H, and 40H plates were treated with Clavulanic Acid (Sigma-Aldrich P3494) or KAAD-Cyclopamine (Toronto Research Chemicals K171000) for 16, 20 or 40 hours. The 2H and 5H plates were incubated for 16 hours and subsequently treated with the inhibitors for either 2 or 5 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. The response ratios in relation to maximum inhibition with Clavulanic Acid are plotted for each incubation time. (n=3 for each data point).

Assay Performance with Variable Substrate Loading Time

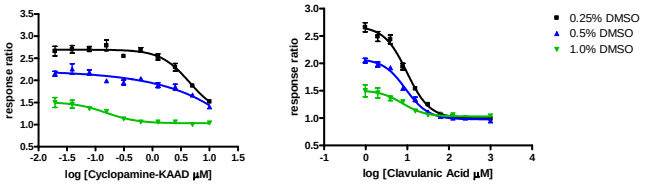
Figure 5 — Clavulanic Acid and KAAD-Cyclopamine dose response with various substrate loading times



Gli-*bla* 22Rv1 cells were plated the day prior to the assay at 20,000 cells/well in a 384-well format in growth medium. 4 hours later, medium was replaced with assay medium and cells were treated with Clavulanic Acid (Sigma-Aldrich P3494) or KAAD-Cyclopamine (Toronto Research Chemicals K171000) for 20 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for indicated hours. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader. The response ratios in relation to maximum inhibition with Clavulanic Acid are plotted for each loading time. (n=4 for each data point).

Assay Performance with Variable DMSO Concentration

Figure 6 – Clavulanic Acid and KAAD-Cyclopamine dose response with 0.25, 0.5 and 1% DMSO



Gli-*bla* 22Rv1 cells were plated the day prior to the assay at indicated plating cell density in a 384-well format in growth medium. 4 hours later, medium was replaced with assay medium and cells were treated with Clavulanic Acid (Sigma-Aldrich P3494) or KAAD-Cyclopamine (Toronto Research Chemicals K171000) in the presence of indicated amount of DMSO for 20 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. The response ratios in relation to maximum inhibition with Clavulanic Acid are plotted for each incubation time. (n=8 for each data point).