Validation & Assay Performance Summary

CellSensor® :Myc-*bla* HCT116 Cell Line

Cat. no. K1467

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

Increased wild-type MYC expression occurs frequently in human cancers. Myc up-regulation occurs as a consequence of activation of one or more signaling pathways that induce MYC expression and function as a regulator of gene transcription. These include MAPK, PI3K and Wnt-β-catenin pathways. The target genes regulated by Myc are involved in the many biological activities attributed to Myc, including growth, transformation, proliferation and angiogenesis.

HCT116 is a colon cancer cell line which expresses a mutated form of β-catenin. This form of β-catenin leads to the accumulation of β-catenin and constitutive activation of downstream genes such as MYC.

Cell Line Description

CellSensor® Myc-*bla* HCT116 contains a beta-lactamase reporter gene under the control of Myc binding sequences. The construct was transduced into HCT116 cells by lentivirus. This cell line is a clonal population isolated by flow cytometry. It has been validated for cell plating density and DMSO tolerance. The signaling pathway has been validated using RNAi against c-MYC and ICG-001, an inhibitor of the wnt-β-catenin pathway. The expression of the mutated version of β-catenin in HCT116 cells results in the constitutive activation of beta-lactamase in this CellSensor® line, which can be knocked down by Myc RNAi and ICG-001.

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)**

   Z'-Factor = 0.66
   Response Ratio = 3.4

   Recommended cell no. cells/well = 8000
   Recommended [DMSO] = 0.5-1%
   Recommended compound incubation time = 24 hours

2. **Alternate Stimuli**
   n.a.

3. **Stealth™ RNAi Testing**
   See below

4. **Small molecule inhibitor Testing**
   See below

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

Assay Testing Summary

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

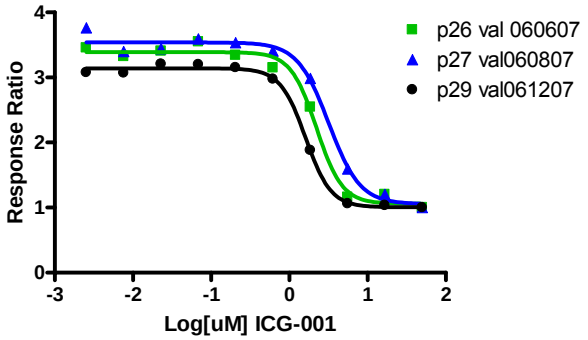
7. **Assay performance with variable substrate loading time**

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

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

Determination of assay window

Figure 1 — Myc-*bla* HCT-116 response to ICG-001 under optimized conditions



	p26 val 060607	p27 val060807	p29 val061207
BOTTOM	1.061	1.054	1.006
TOP	3.388	3.538	3.139
LOGEC50	0.3461	0.5036	0.2072
HILLSLOPE	-2.638	-2.177	-2.684
EC50	2.219	3.189	1.611

Myc-*bla* HCT116 cells (8000 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 treated with indicated amount of ICG-001 in the presence of 0.1% DMSO in Assay Medium for 24 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 120 minutes. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the response ratio was calculated as the 460/530 Emission Ratio of untreated cells divided by the 460/530 Emission Ratio of cells treated with 50 μM ICG-001. The response ratio was plotted for the indicated treatment (n=16 for each data point).

Target validation with RNAi

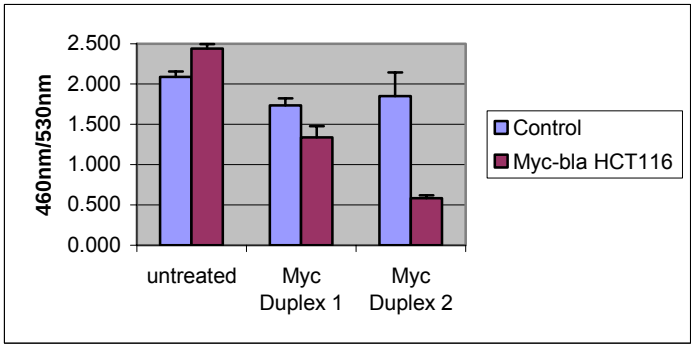


Figure 2 — Myc-*bla* HCT116 response to treatment with Myc RNAi
Myc-*bla* HCT116 or control cells (10000 cells/well) were plated the day prior to the transfection in a 96-well format in growth medium. The cells were transfected with the indicated RNAi duplexes (20 nM final concentration, Invitrogen, #12936-50) using Lipofectamine™RNAiMax (Invitrogen, #13778-075) according to manufacturer instructions, and incubated for 72 hours with the RNAi. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 120 minutes. 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.

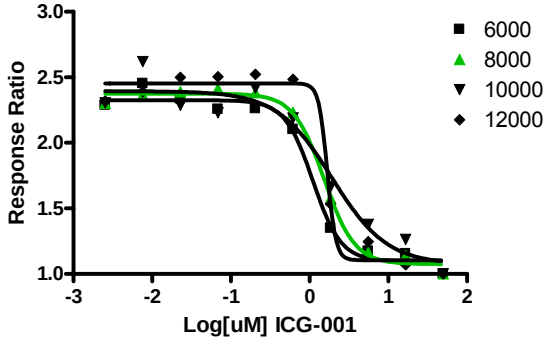
Note: We recommend passing cells for three passages after thawing before using them in the beta-lactamase assay. For more detailed cell growth and maintenance directions, please refer to protocol.

Table 1 – Cell Culture and Maintenance

Component	Growth Medium	Assay Medium	Freezing Medium
McCoy's 5A Medium	90%	—	—
OPTI-MEM	—	90%	
Dialyzed FBS **Do Not Substitute!**	10%	0.5%	—
NEAA	—	0.1 mM	—
HEPES (pH 7.3)	—	10 mM	—
Sodium Pyruvate	—	1 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

Figure 3 — ICG-001 inhibition with different plating cell numbers / well

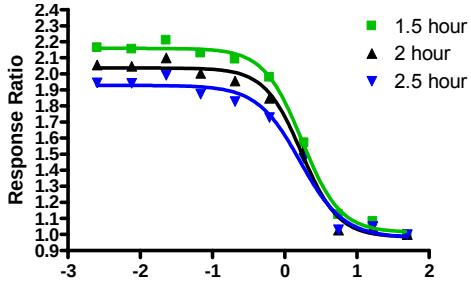


	6000	8000	10000	12000
BOTTOM	1.097	1.074	1.072	1.105
TOP	2.325	2.375	2.394	2.453
LOGEC50	0.04235	0.1848	0.2848	0.2263
HILLSLOPE	-2.432	-2.227	-1.240	-8.016
EC50	1.102	1.530	1.927	1.684

Myc-*bla* HCT116 cells were plated the day prior to the assay at the indicated number of cells/well in a 384-well format in growth medium and treated with indicated amount of ICG-001 in Assay Medium for 24 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 120 minutes. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the response ratio was calculated as the 460/530 Emission Ratio of untreated cells divided by the 460/530 Emission Ratio of cells treated with 50 μM ICG-001. The response ratio was plotted for the indicated treatment (n=8 for each data point).

Assay Performance with Variable Substrate Loading Time

Figure 4 — ICG-001 inhibition with various substrate loading times

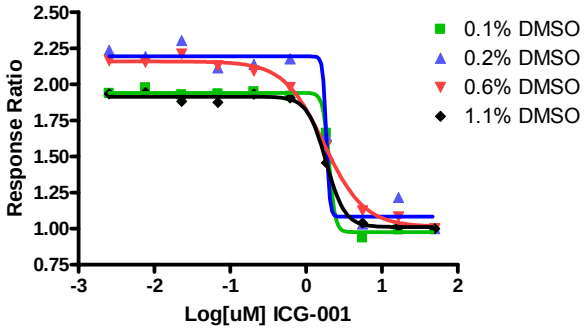


	1.5 hour	2 hour	2.5 hour
BOTTOM	1.013	0.9840	0.9808
TOP	2.159	2.037	1.928
LOGEC50	0.2380	0.2328	0.2150
HILLSLOPE	-1.641	-1.703	-1.456
EC50	1.730	1.709	1.641

Myc-*bla* HCT116 cells were plated the day prior to the assay at 8000 cells/well in a 384-well format in growth medium and treated with indicated amount of ICG-001 in Assay Medium for 24 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 1.5, 2 and 2.5 hours. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the response ratio was calculated as the 460/530 Emission Ratio of untreated cells divided by the 460/530 Emission Ratio of cells treated with 50 μM ICG-001. The response ratio was plotted for the indicated treatment (n=8 for each data point).

Assay Performance with Variable DMSO Concentration

Figure 5 – ICG-001 inhibition with various DMSO concentrations

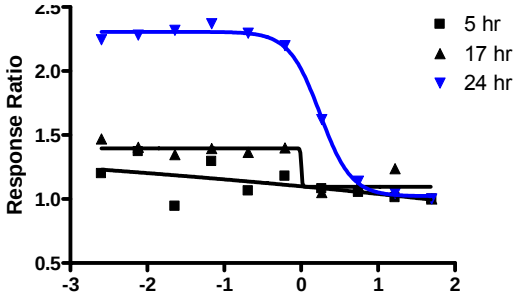


	0.1% DMSO	0.2% DMSO	0.6% DMSO	1.1% DMSO
BOTTOM	0.9756	1.083	1.013	1.011
TOP	1.941	2.194	2.159	1.915
LOGEC50	0.3042	0.2680	0.2380	0.2646
HILLSLOPE	-10.74	-19.22	-1.641	-3.513
EC50	2.015	1.853	1.730	1.839

Myc-*bla* HCT116 cells (8000 cells/well) were plated the day prior to the assay in a 384-well format and treated with indicated amount of ICG-001 in the presence of indicated concentrations of DMSO in Assay Medium for 24 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 120 minutes. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the response ratio was calculated as the 460/530 Emission Ratio of untreated cells divided by the 460/530 Emission Ratio of cells treated with 50 μM ICG-001. The response ratio was plotted for the indicated treatment (n=8 for each data point).

Compound Incubation Time

Figure 6 – Compound incubation time



	5 hr	17 hr	24 hr
BOTTOM	-1663	1.097	1.022
TOP	1.692	1.396	2.306
LOGEC50	82.95	0.003941	0.2471
HILLSLOPE	-0.04157	-64.78	-2.150
EC50	Value too large	1.009	1.766

Myc-*bla* HCT116 cells were plated at 8000 cells/well in a 384-well format in growth medium and treated with indicated amount of ICG-001 in Assay Medium for 5, 17 and 24 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 calculated as the 460/530 Emission Ratio of untreated cells divided by the 460/530 Emission Ratio of cells treated with 50 μM ICG-001. The response ratio was plotted for the indicated treatment (n=8 for each data point).