

Validation & Assay Performance Summary



CellSensor[®] LEF/TCF-*bla* SW480 Cell Line

Cat. no. K1675

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

Wnt signaling via β -catenin plays a central role in development and homeostasis. This pathway is invariably disrupted in colorectal tumors and commonly affected by mutation in other cancers. Wnt ligand binding and activating the Frizzled transmembrane receptors (Fz) transduced the signal to a cytoplasmic protein, known as disheveled protein, which then inhibits the serine/threonine kinase Glycogen Synthase-3 β (GSK-3 β). This signal leads to functional inactivation and dissociation of a multi-protein β -catenin destruction complex, which is made up of the tumor suppressor protein Adenomatous Polyposis Coli (APC), GSK-3 β and a scaffold of Axin. This results in dephosphorylation and dissociation of β -catenin. The unphosphorylated β -catenin is stabilized and accumulates in the cytoplasm of the cell. β -catenin then associates with the T-Cell Factor (TCF)/Lymphoid Enhancer Factor (LEF) family of transcription factors in the nucleus leading to transcription and expression of target genes, such as c-Myc, c-jun, Fra and cyclin D1.

SW480 is a colon cancer cell line which expresses a truncated form of APC. This truncation of APC leads to a loss of function of APC and prevents the proper assembly of the β -catenin destruction complex resulting in the accumulation of non-complexed β -catenin and constitutive activation of this signaling pathway.

Cell Line Description

CellSensor[®] LEF/TCF-*bla* SW480 contains a beta-lactamase reporter gene under the control of 7 copies of LEF/TCF consensus binding sequences. The construct was transduced into SW480 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 β -catenin. The expression of the truncated version of APC in SW480 cells results in the constitutive activation of beta-lactamase in this CellSensor[®] line, which is not susceptible to further stimulation with Wnt ligand. In addition we also tested a recently published small molecule inhibitor for this pathway, to further validate the pathway.

Due to the constitutive activity of this pathway in this CellSensor[®] cell line all optimization studies were performed using the beta-lactamase inhibitor clavulonic acid. The treatment with clavulonic acid mimics the complete inhibition of the signaling pathway and allows the determination of the maximum assay window.

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 (EC₁₀₀)* = 0.85
Response Ratio (max inhib.) = 16
*EC₁₀₀ for Clavulonic acid treatment

Recommended cell no. = 10000 cells/well
Recommended [DMSO] = 0.5-1%
Recommended Stim. Time = n.a.
Max. [Stimulation] = n.a.

2. Alternate Stimuli

n.a.

3. Stealth™ RNAi Testing

> 75 % inhibition with two different RNAi duplexes targeted against β-catenin

4. Small molecule inhibitor Testing

Currently no inverse agonist / inhibitor commercially available

5. Cell culture and maintenance

See Cell Culture and Maintenance Section and Table 1

Assay Testing Summary

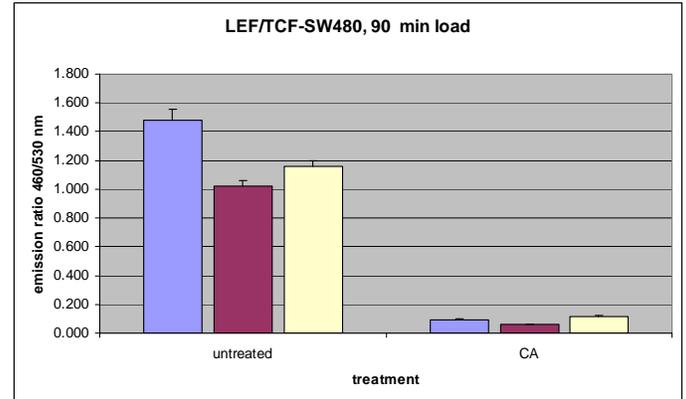
6. Assay performance with variable cell number

7. Assay performance with variable substrate loading time

8. Assay performance with variable DMSO concentration

Determination of maximum assay window

Figure 1 — LEF/TCF-*bla* SW480 response to clavulonic acid treatment under optimized conditions



LEF/TCF-*bla* SW480 cells (10,000 cells/well) were assayed on three separate days represented by the three columns shown on the graph. Cells were plated the day prior to the assay in a 384-well format and treated with 200 μM clavulonic acid (CA) in the presence of 0.5% DMSO for 2 hours. Cells were then loaded with LiveBLazer™-FRET B/G Substrate for 90 minutes. 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 treatment (n=16 for each data point).

Target validation with RNAi

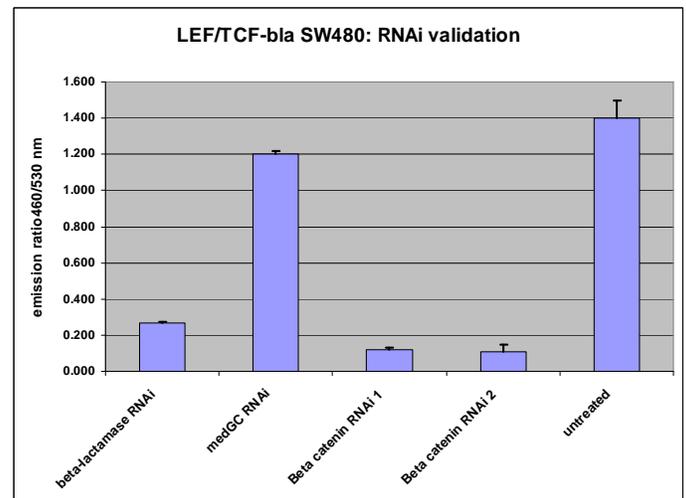


Figure 2 — LEF/TCF-*bla* SW480 response to treatment with beta-catenin RNAi

LEF/TCF-*bla* SW480 cells (25,000 cells/well) were plated the day prior to the transfection in a 96-well format in assay medium. The cells were transfected with the indicated RNAi duplexes (20 nM final concentration) using Lipofectamine™RNAiMax (Invitrogen, #13778-075) according to manufacturers instructions, and incubated for 72 hours with the RNAi. Cells were then loaded with LiveBLazer™-FRET B/G Substrate for 90 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). Beta-lactamase RNAi and medium GC RNAi were used as positive and negative control respectively.

Cell Culture and Maintenance

Thaw cells in Growth Medium without Blastcidin and culture them in Growth Medium with Blastcidin. Pass or feed cells at least twice a week and maintain them in a 37°C/5% CO₂ incubator. Maintain cells between 10% and 85% confluency. Do not allow cells to reach confluence.

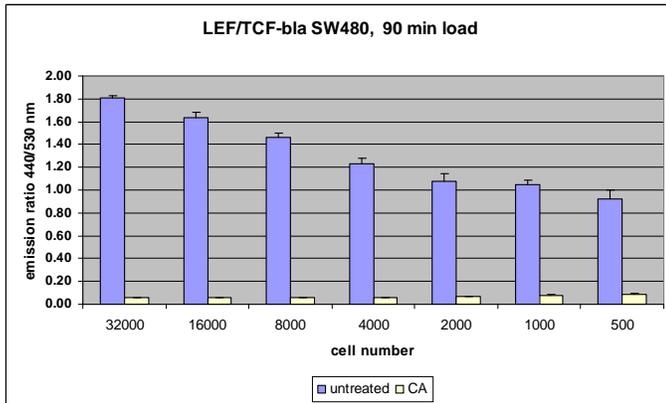
Note: We recommend passing cells for three passages after thawing before using them in the beta-lactamase assay. For more detailed cell growth and maintenance directions, please refer to protocol.

Table 1 – Cell Culture and Maintenance

Component	Growth Medium	Assay Medium	Freezing Medium
RPMI with GlutaMAX™	90%	90%	—
FBS Do Not Substitute!	10%	10%	—
NEAA	0.1 mM	0.1 mM	—
HEPES (pH 7.3)	25 mM	25 mM	—
Sodium Pyruvate	1 mM	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

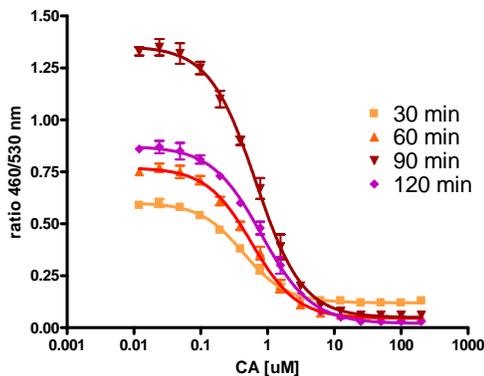
Figure 3 — Clavulonic acid inhibition of beta-lactamase with different plating cell numbers/well



LEF/TCF-*bla* SW480 cells were plated the day prior to the assay at the indicated number of cells/well in a 384-well format in growth medium. 24 hours later cells were treated with 200 μ M clavulonic acid (CA) for 2 hrs. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 90 minutes. Fluorescence emission values at 460 nm and 530 nm for the various cell numbers were obtained using a standard fluorescence plate reader and the emission ratios plotted for each cell number. (n=6 for each data point).

Assay Performance with Variable Substrate Loading Time

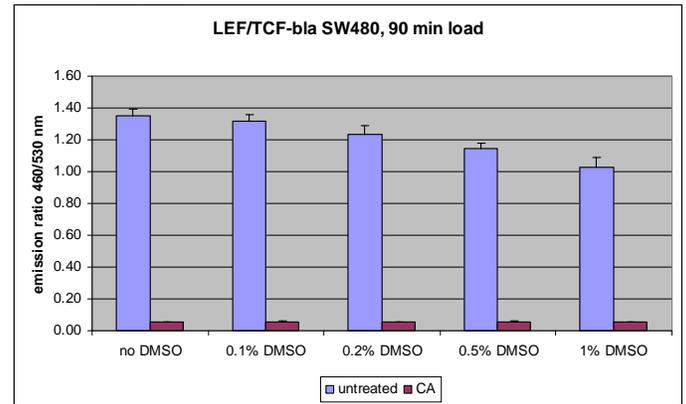
Figure 4 — Clavulonic acid inhibition of beta-lactamase with various substrate loading times



LEF/TCF-*bla* SW480 cells were plated the day prior to the assay at 10,000 cells/well in a 384-well format in growth medium. 24 hours later, cells were treated with the indicated concentration of clavulonic acid (CA) for 2 hrs. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for indicated time. Fluorescence emission values at 460 nm and 530 nm for the various loading times were obtained using a standard fluorescence plate reader and the emission ratios plotted for each loading time against the indicated concentrations of clavulonic acid (n=6 for each data point).

Assay Performance with Variable DMSO Concentration

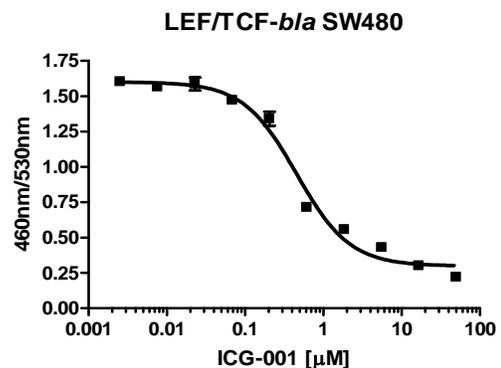
Figure 5 — Clavulonic acid inhibition of beta-lactamase with 0, 0.1, 0.2, 0.5 and 1% DMSO



LEF/TCF-*bla* SW480 cells were plated the day prior to the assay at 10,000 cells/well in a 384-well format in growth medium. 24 hours later, cells were treated with 200 μ M of clavulonic acid (CA) for 2 hrs in the presence of the indicated concentration of DMSO. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 90 minutes. Fluorescence emission values at 460 nm and 530 nm for the various DMSO concentrations were obtained using a standard fluorescence plate reader and the emission ratios plotted for each DMSO concentration (n=6 for each data point).

Inhibition of constitutive active Wnt / β -catenin pathway with ICG-001

Figure 6 — Dose dependent inhibition of constitutive β -catenin pathway with small molecule inhibitor ICG-001



LEF/TCF-*bla* SW480 cells were plated at 40,000 cells/well in a 96-well plate in Assay Medium (OPTIMEM plus 0.5% dialyzed FBS). Cells were treated with the indicated concentration of ICG-001 for 17 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 loading times were obtained using a standard fluorescence plate reader and the emission ratios plotted for each loading time against the indicated concentrations of ICG-001 (n=3 for each data point).