Validation & Assay Performance Summary

CellSensor® LEF/TCF-*bla* HCT-116 Cell Line

Cat. no. K1676

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 is commonly affected by a mutation in other cancers. Wnt ligand binding and activation of the Frizzled transmembrane receptors (Fz) transduces 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-3β, 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.

Cell Line Description

The CellSensor® LEF/TCF-*bla* HCT-116 cell line contains a beta-lactamase reporter gene under control of the Lymphoid Enhancer Factor/ T-Cell Factor (LEF/TCF) response element stably integrated into HCT-116 cells. HCT-116 is a colon cancer cell line carrying a gain-of-function mutation in the beta-catenin gene (a deletion of amino acid Serine 45), which prevents beta-catenin protein degradation, thus leading to constitutive activation of downstream genes. This cell line has been tested for assay performance under variable conditions, including DMSO concentration and cell number, and validated for Z' and EC_{50} concentrations of mWnt3a. Additional testing information using Stealth™ RNAi 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**

mWnt3a EC_{50}	= 4.6 ng/mL
Z'-Factor (EC_{100})	= 0.75
Response Ratio	= 2.1
Optimum cell no.	= 10K cells/well
Optimum [DMSO]	= up to 1%
Optimum Stim. Time	= 4.5 hours
Max. [Stimulation]	= ~300 ng/mL

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

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

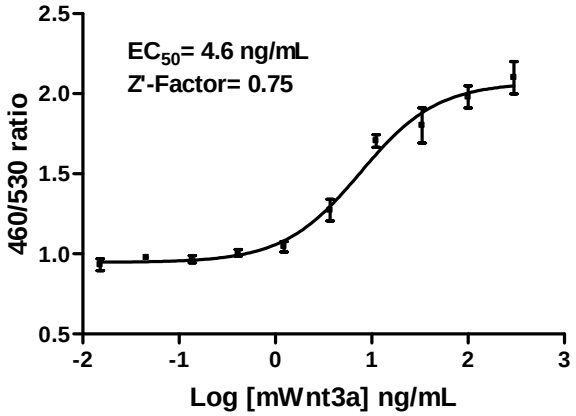
Assay Testing Summary

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

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

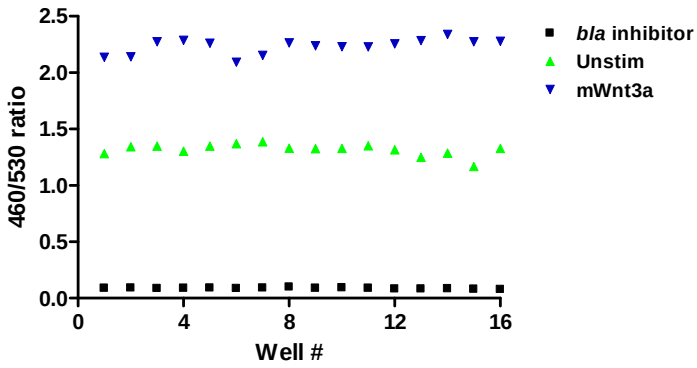
Primary Agonist Dose Response

Figure 1 -LEF/TCF-*bla* HCT-116 dose response under optimized conditions



LEF/TCF -*bla* HCT-116 cells (10,000 cells/well) were plated the day of the assay in a 384-well format and stimulated with mWnt3a (R&D Systems # 1324-WN-002) over the indicated concentration range in the presence of 0.5% DMSO for 4.5 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 460/530 Emission Ratios plotted for the indicated concentrations of mWnt3a (n=16 for each data point).

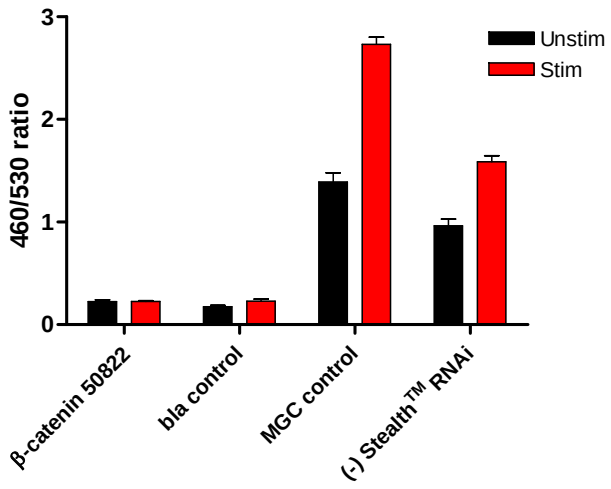
Figure 2 -LEF/TCF-*bla* HCT-116 constitutive activity can be further stimulated by mWnt3a and inhibited by an inhibitor of beta-lactamase.



LEF/TCF -*bla* HCT-116 cells (10,000 cells/well) were plated the day of the assay in a 384-well format and stimulated with 50 ng/mL mWnt3a (R&D Systems # 1324-WN-002) or a beta-lactamase inhibitor in the presence of 0.5% DMSO for 4.5 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 460/530 Emission Ratios plotted for the unstimulated cells and those treated with mWnt3a and the bla inhibitor.

Stealth™ RNAi Testing

Figure 2 — LEF/TCF-*bla* HCT-116 response to various RNAis



LEF/TCF-*bla* HCT-116 cells (8,000 cells/well) were plated the day of the assay in a 96-well format and treated with the listed Stealth™ RNAi duplexes for 60 hrs. Cells were then treated with mWnt3a (R&D Systems # 1324-WN-002) at 50 ng/mL for 4.5 hrs., then loaded with LiveBLAzer™-FRET B/G Substrate for 2 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 RNAi (n=3 for each data point).

Cell Culture and Maintenance

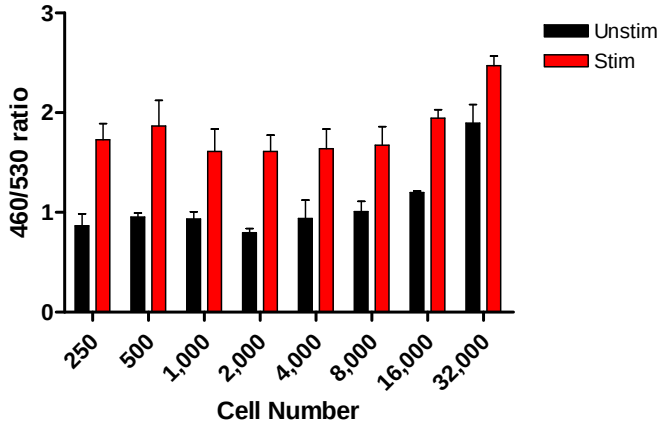
Note: We recommend passing cells for three passages after thawing before using them in the beta-lactamase assay. For optimal cell line recovery and performance, use dialyzed FBS (Invitrogen #26400-036). For more detailed cell growth and maintenance directions, please refer to protocol.

Table 1 – Cell Culture and Maintenance

Component	Growth Medium (-)	Growth Medium (+)	Assay Medium	Freezing Medium
McCoy's 5A	90%	90%	—	85%
OPTI-MEM®	—	—	99%	—
Dialyzed FBS Do not Substitute!	10%	10%	0.5%	10%
NEAA	—	—	0.1 mM	—
Sodium pyruvate	—	—	1 mM	—
Penicillin	—	100 U/ml	100 U/ml	—
Streptomycin	—	100 µg/ml	100 µg/ml	—
Blasticidin	—	5 µg/ml	—	—
DMSO	—	—	—	5%

Assay Performance with Variable Cell Number

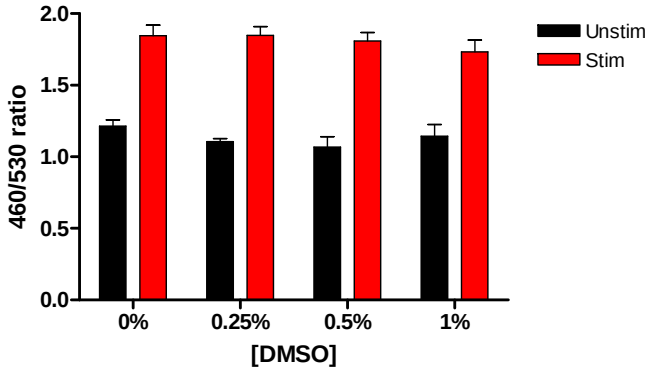
Figure 3 — LEF/TCF-*bla* HCT-116 response with 250, 500, 1K, 2K, 4K, 8K, 16K and 32K cells/well



LEF/TCF-*bla* HCT-116 cells were plated the day of the assay at 250, 500, 1000, 2000, 4000, 8000, 16000 or 32,000 cells/well in a 384-well format. Cells were stimulated with 50ng/mL mWnt3a (R&D Systems # 1324-WN-002) in the presence of 0.5% DMSO for 16 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 460/530 Ratios plotted for each cell number against the indicated concentrations of mWnt3a (n=4 for each data point).

Assay Performance with Variable DMSO Concentration

Figure 4 – LEF/TCF-*bla* HCT-116 response with 0, 0.25, 0.5 and 1% DMSO



LEF/TCF-*bla* HCT-116 cells (10,000 cells/well) were plated the day of the assay in a 384-well black-walled tissue culture assay plate. mWnt3a (R&D Systems # 1324-WN-002) was then added to the plate over the indicated concentration range. DMSO was then added to the assay at final concentrations from 0% to 1%. Plates were stimulated for 5 hrs and 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 460/530 Ratios for each DMSO concentration were plotted against the indicated concentrations of mWnt3a (n=4 for each data point).