Validation & Assay Performance Summary

CellSensor® LEF/TCF-*bla* FreeStyle™ 293F Cell Line

Cat. no. K1677

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 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-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.

Cell Line Description

The CellSensor® LEF/TCF-*bla* FreeStyle™ 293F 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 FreeStyle™ 293F cells. FreeStyle™ 293F are human embryonal kidney cells derived from parental 293F cells (fast growing variant). This cell line has also been tested for assay performance under variable conditions, including DMSO concentration, cell number, stimulation time and validated for Z' and EC_{50} concentrations of mWnt3a. Additional testing information using Stealth™ RNAi is also provided.

Validation Summary

Testing and validation of this assay was evaluated using LiveBLAzer™-FRET B/G Substrate.

1. **Primary agonist dose response under optimized conditions**

mWnt3a EC_{50}	= 41.9 ng/mL
Z'-Factor (EC_{100})	= 0.73
Response Ratio	= 3.9
Recommended cell no.	= 10K cells/well
Recommended [DMSO]	= up to 1%
Recommended Stim. Time	= 5 hours
Max. [Stimulation]	= 450 ng/mL

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

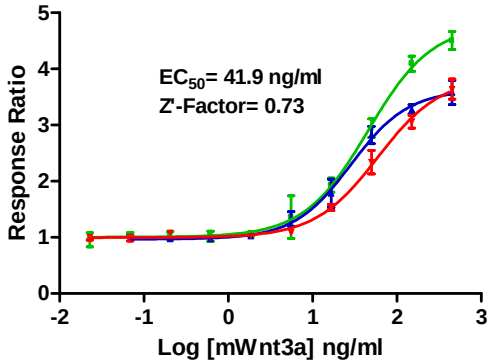
5. **Assay performance with variable pre-treatment concentrations of LiCl**

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

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

Primary Agonist Dose Response

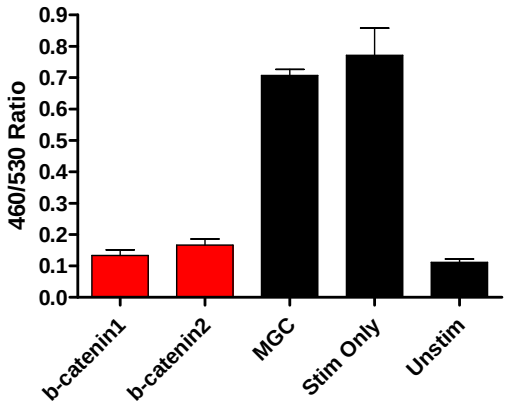
Figure 1 -LEF/TCF-*bla* FreeStyle™ 293F dose response to mWnt3a under optimized conditions



LEF/TCF-*bla* FreeStyle™ 293F cells (10,000 cells/well) were assayed on three separate days, represented by the three curves shown on the graph. Cells were plated in a 384-well plate (BD CellWare™ Poly-D-Lysine) and pre-treated with 10mM LiCl for 15 hours. Cells were then stimulated with mWnt3a (R&D Systems # 1324-WN-002) over the indicated concentration range in the presence of 0.5% DMSO for 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 and the Response Ratios plotted for the indicated concentrations of mWnt3a (n=5 for each data point).

Stealth™ RNAi Testing

Figure 2 — LEF/TCF-*bla* FreeStyle™ 293F response to various RNAis



LEF/TCF-*bla* FreeStyle™ 293F cells (7,500 cells/well) were plated in a 96-well format and treated with the listed Stealth™ RNAi duplexes for 52 hours. Cells were then treated with 10mM LiCl for 15 hours, followed by mWnt3a (R&D Systems # 1324-WN-002) treatment in 0.5% DMSO for 5 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 2.5 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.

Cell Culture and Maintenance

Cells should be thawed in Growth Medium without Blasticidin and grown in Growth Medium with Blasticidin. Cells should be passed or fed at least twice a week and maintained in a 37°C/5% CO_2 incubator. Cells should be maintained between 10% and 90% confluency. Do not allow cells to reach confluence.

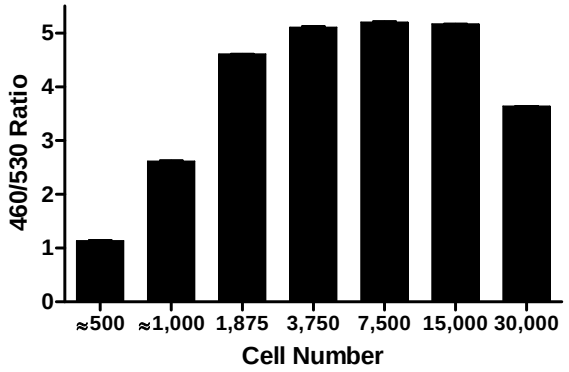
Note: We recommend passing cells for three passages after thawing before using them in the beta-lactamase assay. For optimal cell line recovery and performance, use dialyzed FBS (Invitrogen #26400-036). For more detailed cell growth and maintenance directions, please refer to protocol.

Table 1 – Cell Culture and Maintenance

Component	Growth Medium	Assay Medium	Freezing Medium
DMEM	90%	-	-
Opti-MEM®	-	96.5%	-
Dialyzed FBS	10%	0.5%	-
NEAA	0.1 mM	0.1 mM	-
Sodium pyruvate	-	1 mM	-
HEPES (pH 7.3)	25 mM	10 mM	-
Penicillin	100 U/ml	100 U/ml	-
Streptomycin	100 µg/ml	100 µg/ml	-
Blasticidin antibiotic	5 µg/ml	-	-
Recovery™ Cell Culture Freezing Medium	-	-	100%

Assay Performance with Variable Cell Number

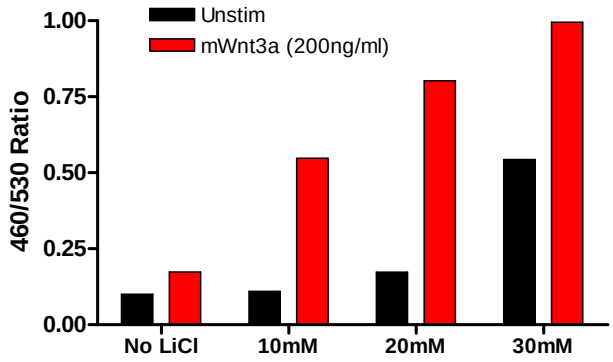
Figure5 — LEF/TCF-*bla* FreeStyle™ 293F response to mWnt3a using ~500, ~1000 , 1875, 3750, 7500, 15000, or 30,000K cells/well



LEF/TCF-*bla* FreeStyle™ 293F cells were plated at ~500, ~1000, 1875, 3750, 7500, 15000, or 30,000 cells/well in a 384-well format. Cells were pre-treated with 10mM LiCl for 15 hours then stimulated with mWnt3a (R&D Systems # 1324-WN-002) at 150 ng/ml in the presence of 0.5% DMSO for 5 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 460/530 Ratios plotted for each cell number against the indicated concentrations of mWnt3a.

Assay Performance with Variable Pre-treatment concentrations of LiCl

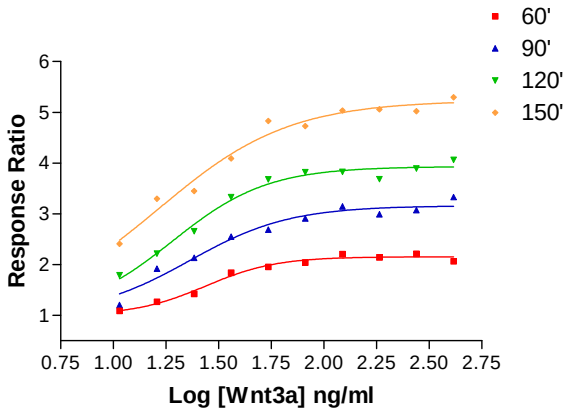
Figure 6 – LEF/TCF-*bla* FreeStyle™ 293F response to mWnt3a using 0, 10, 20 and 30mM pre-treatment concentrations of LiCl



LEF/TCF-*bla* FreeStyle™ 293F cells (10,000 cells/well) were plated the in a 384-well assay plate and then pre-treated with the listed concentrations of LiCl for 15 hours. mWnt3a (R&D Systems # 1324-WN-002) was then added to the plate at 200 ng/ml final concentration. Plates were stimulated for 5 hrs with mWnt3a in 0.5% DMSO and then loaded for 2.5 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 plotted for each concentration of LiCl.

Assay Performance with Variable Substrate Loading Time

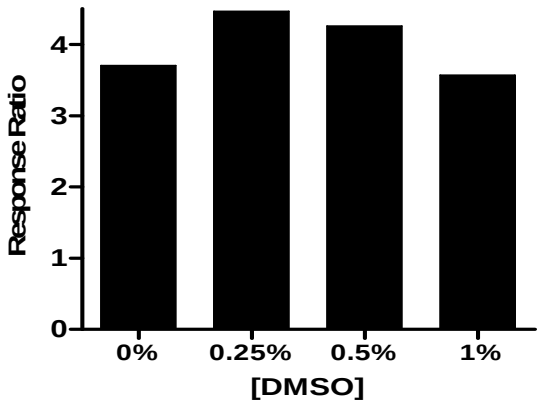
Figure 7 — LEF/TCF-*bla* FreeStyle™ 293F response to mWnt3a with 1, 1.5, 2 and 2.5 hour substrate loading times



LEF/TCF-bla FreeStyle™ 293F cells were plated at 10,000 cells/well in a 384-well format. Cells were pre-treated with 10mM LiCl then stimulated with mWnt3a (R&D Systems # 1324-WN-002) over the indicated concentration range in the presence of 0.5% DMSO for 15 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for either 1, 1.5, 2 or 2.5 hours. Fluorescence emission values at 460 nm and 530 nm for the various substrate loading times were obtained using a standard fluorescence plate reader and the Response Ratios plotted for each substrate loading time against the indicated concentrations of mWnt3a.

Assay Performance with Variable DMSO Concentration

Figure 8 – LEF/TCF-*bla* FreeStyle™ 293F response to mWnt3a using 0, 0.25, 0.5 and 1% DMSO



LEF/TCF-*bla* FreeStyle™ 293F cells (10,000 cells/well) were plated t in a 384-well plate and pre-treated with 10mM LiCl for 15 hrs. 150 ng/ml mWnt3a (R&D Systems # 1324-WN-002) was then added to the plate with final DMSO concentrations ranging from 0% to 1%. Plates were stimulated for 5 hrs and loaded for 2.5 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 Response Ratios plotted for each DMSO concentration.