Validation & Assay Performance Summary

CellSensor® TrkC-NFAT-*bla* CHO-K1 Cell Line

Cat. no. K1515A

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

Neurotrophins (NGF, BDNF, NT-3, and NT-4) and their transmembrane receptors (TrkA, TrkB, TrkC, and P75NTR) play important roles in the regulation of neuronal and non-neuronal cell proliferation, differentiation, survival, and death. Neurotrophin signaling also mediates higher-order neuronal activities, such as learning, memory, and behavior. Alterations in neurotrophin levels and their receptors have been implicated in neurodegenerative disorders, such as Alzheimer's disease and Huntington's disease, as well as psychiatric disorders. Neurotrophins propagate their signal through activating multiple signaling pathways. One of the signaling pathways of NT-3, the ligand for TrkC, activates phospholipase C, releasing DAG and IP3, increasing downstream intracellular calcium and activating protein kinase C, which in turn promotes the translocation of the transcription factor, nuclear factor of activated T-cells (NFAT), from the cytosol into the nucleus and results in NFAT-dependent transcription.

Cell Line Description

The TrkC-NFAT-*bla* CHO-K1 cell line was engineered by intergrating the human TrkC expression plasmid into the genome of existing CellSensor® NFAT-*bla* CHO-K1 cell line, which is engineered to express beta-lactamase under the control of NFAT. This cell line has been tested for assay performance under variable conditions, including DMSO concentration, cell number, stimulation time, substrate loading time and validated for Z' and EC_{50} under optimized conditions using NT-3. Additional testing information using various small molecule inhibitors, Stealth RNAi and alternate stimuli is 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(n=3)**

NT-3 EC_{50}	= 7.82 ng/mL
Z'-Factor (EC_{100})	= 0.86
Response Ratio	= 14.96
Optimum cell no.	= 10K cells/well
Optimum [DMSO]	= up to 1%
Optimum Stim.Time	= 5 hours
Max. [Stimulation]	= ~111 ng/mL

2. **Alternate stimuli**

EC_{50} NGF2.5s	> 1000 ng/mL
EC_{50} BDNF	= 732.9 ng/mL
EC_{50} NT-4	> 1000 ng/mL

3. **Small Molecule Inhibitors Dose Response**

IC_{50} AG 879	= 0.446 µM
IC_{50} GW 441756	=0.141 µM
IC_{50} K252a	= 0.01 µM

4. **Stealth™ RNAi Testing**

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

Assay Testing Summary

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

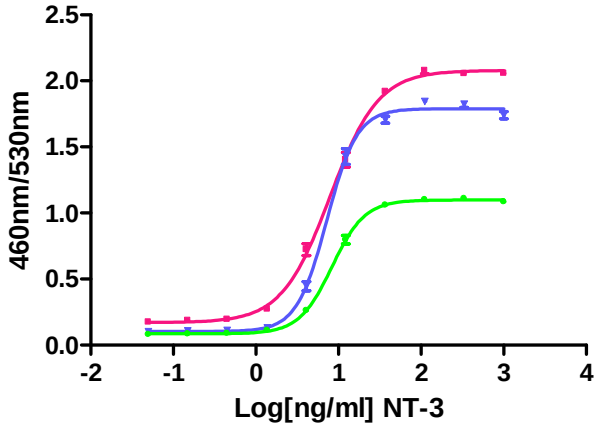
7. **Assay performance with variable stimulation time**

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

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

Primary Agonist Dose Response

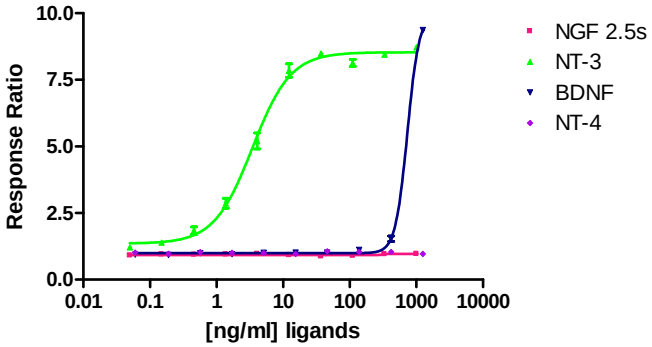
Figure 1 — TrkC-NFAT-*bla* CHO-K1 dose response to Neurotrophin-3 (NT-3) under optimized conditions



TrkC-NFAT-*bla* CHO-K1 cells (passage# 12-14, 10,000 cells/well) were assayed on three separate days represented by the three curves shown on the graph. Cells were plated the day before the assay in a 384-well format and then stimulated with NT-3 (EMD/Calbiochem # 480875) over the indicated concentration range in the presence of 0.1% DMSO for 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 Ratios plotted for the indicated concentrations of NT-3 (n=16 for each data point).

Alternate Stimuli

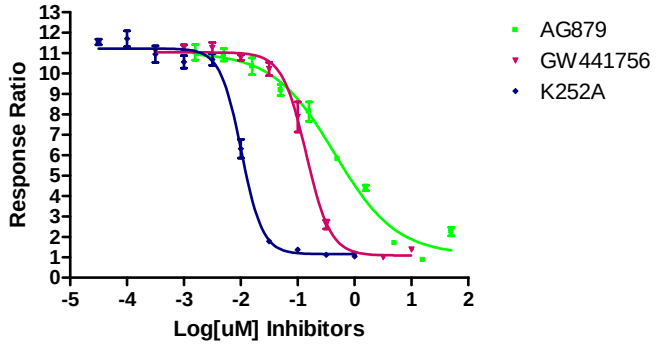
Figure 2– Cryo-preserved TrkC-NFAT-*bla* CHO-K1 dose response to various stimuli



Cryo-preserved TrkC-NFAT-*bla* CHO-K1 cells were thaw and resuspended in the assay medium, plated (10,000 cells/well) in a 384-well format and then stimulated with NGF 2.5s (Invitrogen # 13257-019), NT-3 (Invitrogen # PHC7034), BDNF (Invitrogen # PHC7074) and NT-4 (Invitrogen # PHC7024) over the indicated concentration range in the presence of 0.1% DMSO for 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 Response Ratio plotted for the indicated concentrations of each stimuli (n=5 for each data point).

Small Molecule Inhibitor Dose Response

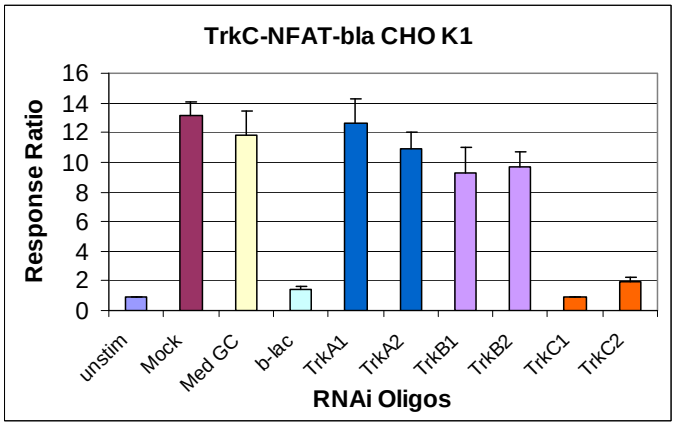
Figure 3–TrkC-NFAT-*bla* CHO-K1 dose response to various small molecule inhibitors under optimized conditions



TrkC-NFAT-*bla* CHO-K1 cells (10,000 cells/well) were plated in a 384-well plate in the absence of NT-3 for 16 hrs, followed by pre-treatment with the indicated concentrations of AG 879 (EMD/Calbiochem # 658460), GW441756 (Tocris bioscience # 2238) and K252a Inhibitor (Invitrogen # PHZ1131) for 30 min. Cells were then stimulated with NT-3 (EMD/Calbiochem # 480875) at 20 ng/ml in the presence of 0.1% DMSO for 5 hours, and 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 Ratios were plotted for the indicated concentrations of each Inhibitor (n=7 for each data point).

Stealth™ RNAi Testing

Figure 4 — TrkC-NFAT-*bla* CHO K1 response to various RNAi oligos



TrkC-NFAT-*bla* CHO K1 cells (6,000 cells/well) were plated with growth medium in a 96-well format and incubated at 37°C overnight. Cells were then treated with RNAiMax mixtures containing the listed Stealth™ RNAi oligos for 32 hrs. Following an Assay Media exchange and a 37°C incubation for 16 hours, cells were then stimulated with NT-3 (20 ng/mL) for 5 hours, and 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 Ratios were plotted for each RNAi Oligos.

Cell Culture and Maintenance

Thaw cells in Growth Medium without selection and culture them in Growth Medium with Blasticidin and Zeocin . Passage or feed cells at least twice a week and maintain them in a 37°C/5% CO_2 incubator. Maintain cells between 5% and 80% confluence. Do not allow cells to reach confluence.

Note: For optimal cell line performance, use dialyzed FBS (Invitrogen# 26400-036). For more detailed cell growth and maintenance directions, please refer to the protocol.

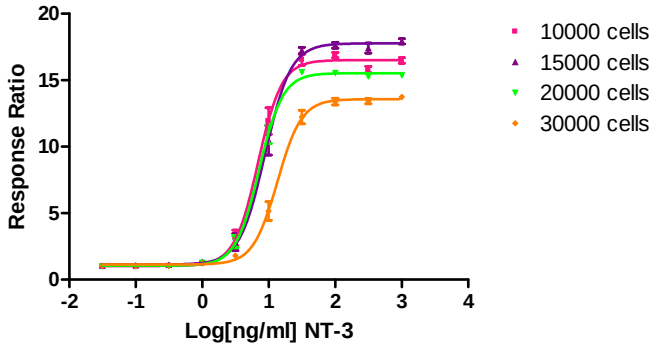
Table 1 – Cell Culture and Maintenance

Component	Growth Medium (+)	Growth Medium (-)	Assay Medium*	Freezing Medium
DMEM w/ GlutaMAX	90%	90%	--	--
OPTI-MEMI	--	--	99.5%	--
Dialyzed FBS **DO NOT SUBSTIUTE!**	10%	10%	0.5%	--
NEAA	0.1 mM	0.1 mM	0.1 mM	--
HEPES (PH 7.3)	25mM	25mM	--	--
Sodium Pyruvate	--	--	1mM	--
Penicillin	100 U/mL	100 U/ml	100 U/mL	--
Streptomycin	100 µg/mL	100 µg/mL	100 µg/mL	--
Blasticidin	5 µg/mL	--	--	--
Zeocin	200 µg/mL	--	--	--
Recovery™ Cell Culture Freezing Medium	--	--	--	100%

* ***Note:*** One additional assay medium formulation containing lower serum concentration have also been tested and shown to perform comparably well with this cell line: DMEM + GlutaMax™-1 (500 mL bottle) supplemented with 0.5mL dFBS, 5 mL NEAA (10 mM stock), 12.5 mL HEPES (pH7.3, 1 M stock), and 5 mL of Penicillin (10,000 U/mL) / Streptomycin (10,000 µg/mL).

Assay Performance with Variable Cell Number

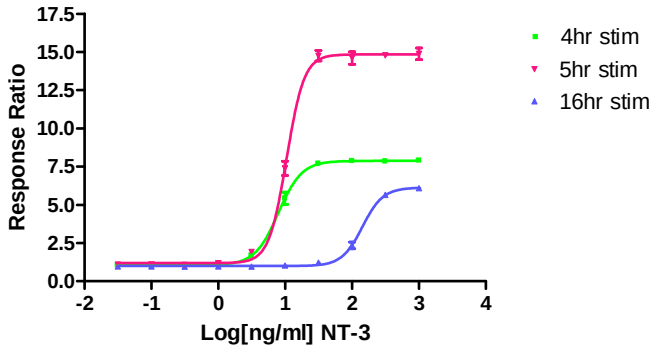
Figure 5 — TrkC-NFAT-*bla* CHO-K1 response to NT-3 using ~10000, 15000, 20000 or 30000 cells/well



TrkC-NFAT-*bla* CHO-K1 cells were plated at ~10000, 15000, 20000 or 30000 cells/well in a 384-well format. Cells were then stimulated with NT-3 (EMD/Calbiochem # 480875) over the indicated concentration range in the presence of 0.1% DMSO for 5 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 Response ratios were plotted for each cell number against the indicated concentrations of NT-3. (n=8 for each data point).

Assay Performance with Variable Stimulation Time

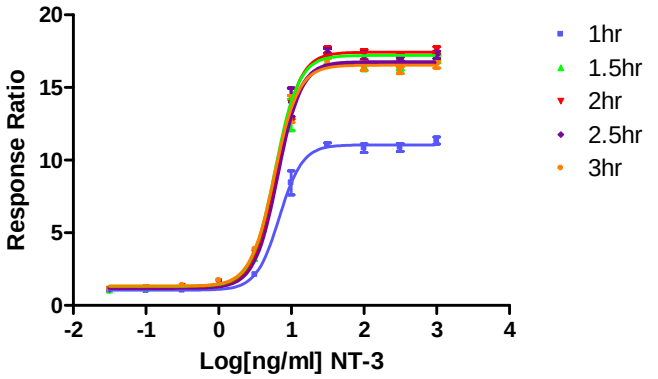
Figure 6 – TrkC-NFAT-*bla* CHO-K1 dose response to NT-3 with 4, 5 and 16 hour stimulation times



TrkC-NFAT-*bla* CHO-K1 cells (10,000 cells/well) were plated in a 384-well assay plate. For 4hr and 5hr stimulation experiments, cells were plated the day before the assay; for 16hr stimulation experiment, cells were plated the day of assay. NT-3 (EMD/Calbiochem # 480875) was then added to the plate over the indicated concentration range. Plates were treated for 4, 5 or 16 hrs with NT-3 in 0.1% DMSO and then 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 Response Ratios were plotted for each stimulation time against the indicated concentrations of NT-3 (n=8 for each data point).

Assay Performance with Variable Substrate Loading Time

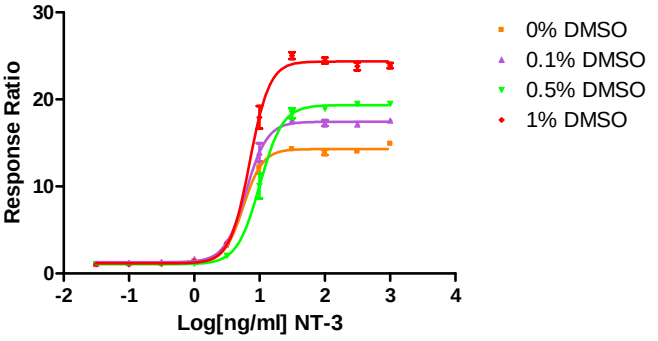
Figure 7 — TrkC-NFAT-*bla* CHO-K1 dose response to NT-3 with 1, 1.5, 2, 2.5 and 3 hour substrate loading times



TrkC-NFAT-*bla* CHO-K1 cells were plated the day before the assay at 10,000 cells/well in a 384-well format. Cells were treated with NT-3 (EMD/Calbiochem # 480875) over the indicated concentration range in the presence of 0.1% DMSO for 5 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for either 1, 1.5, 2, 2.5 or 3 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 were plotted for each substrate loading time against the indicated concentrations of NT-3 (n=8 for each data point).

Assay Performance with Variable [DMSO]

Figure 8 – TrkC-NFAT-*bla* CHO-K1 dose response to NT-3 with 0, 0.1, 0.5 and 1% DMSO



TrkC-NFAT-*bla* CHO-K1 cells (10,000 cells/well) were plated the day before the assay in a 384-well assay plate. NT-3 (EMD/Calbiochem # 480875) was then added to the plate over the indicated concentration range with 0, 0.1, 0.5 or 1% final DMSO concentrations. Cells were then 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 Response Ratios for each DMSO concentration were plotted against the indicated concentrations of NT-3(n=8 for each data point).