Validation & Assay Performance Summary

CellSensor® T-REx™ NICD CSL-*bla* HeLa Cell Line

Cat. no. K1487

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

Through direct cell-to-cell interactions, ligand-induced Notch signaling dictates many cell-fate decisions that underlie normal organ development and tissue homeostasis, including neurogenesis, myogenesis, hematopoiesis, angiogenesis, cellular proliferation and apoptosis. Consequently, aberrant Notch pathway function is associated with a wide array of developmental disorders, cancers, and neurodegenerative diseases. For example, among the four mammalian Notch receptors (Notch1-4) and five ligands (Delta1,3,4 and Jagged1,2), germline loss-of-function mutations have been described that result in congenital aortic valve disorder (Notch1), the adult onset vasculopathy called CADASIL (Notch3), and the developmental disorder referred to as Alagille syndrome (Jagged1). Moreover, somatic gain-of-function mutations in Notch1 have been detected in more than 50% of human T cell acute lymphoblastic leukemias (T-ALL) while additional reports document contributions of deregulated Notch signaling to breast, gut, and skin neoplasias.

Notch signaling is mediated by two sequential cleavage events following ligand binding. First, the extracytoplasmic domain of Notch is cleaved near the plasma membrane by a metalloprotease of the ADAM/TACE/Kuzbanian family. Next, regulated intramembrane proteolysis is catalyzed by the gamma-secretase activity of the presenilin-nicastrin-Aph1-Pen2 protein complex, thereby releasing the Notch intracellular domain (NICD). NICD translocates to the nucleus where it interacts with the transcription factor, CSL (for CBF1, suppressor of hairless, and Lag-1), and coactivator Mastermind-like-1 (MAML1) to activate transcription of downstream target genes such as tissue-specific transcription factors of the hairy enhancer of split (HES) and HES-related families.

Cell Line Description

CellSensor® T-REx™ NICD CSL-*bla* HeLa cell line was engineered by lentiviral transduction of HeLa cervical cancer cells with a Notch-response element driving beta-lactamase reporter gene expression (CSL-*bla*) along with tetracycline repressor and tetracycline (or the tetracycline analog, doxycycline)-inducible NICD (Notch intracellular domain) constructs. This cell line is a clonal population isolated by flow cytometry. Addition of doxycycline to these cells allows for regulated NICD transcription factor expression and subsequent beta-lactamase expression.

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)**

   Average Doxycycline EC_{50} = 1.2 ng/ml
   Average Z'-Factor (EC_{100}) = 0.81
   Average Response Ratio = 6.3

   Recommended cell no. = 6,000 cells/well
   Recommended [DMSO] = up to 0.5 %
   Stimulation Time = 16-20 hours
   Max. [Stimulation] = 316 ng/ml Dox

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

3. **Stealth™ RNAi Testing**

4. **Small molecule inhibitor Testing**
   Currently no 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**

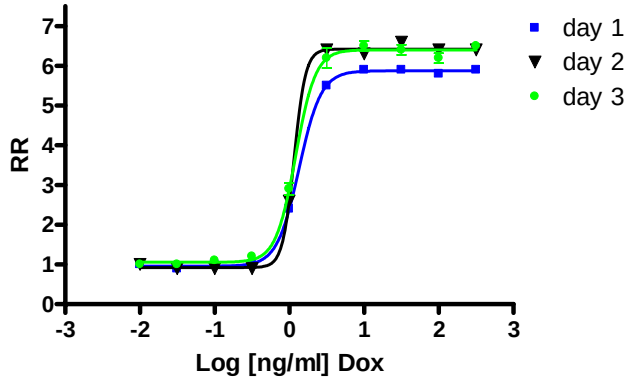
7. **Assay performance with variable DMSO concentration**

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

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

Primary Agonist Dose Response

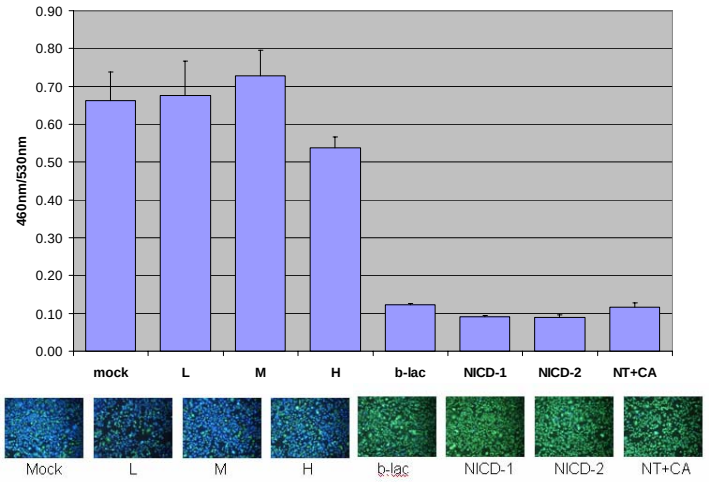
Figure 1 — Doxycycline-induced NICD dose response under optimized conditions



T-REx™ NICD CSL-*bla* HeLa cells were assayed on three separate days. Cells were plated in 384-well assay format in Assay Medium at 6000 cells/well. Cells were stimulated overnight with a serial dilution of doxycycline in the presence of 0.1 % DMSO prior to loading the wells 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 response ratios (460/530 ratio of the Dox-treated points divided by the 460/530 ratios of the untreated control) were plotted (n = 16 for each data point).

Target validation with RNAi

Figure 2 — RNAi knockdown of NICD



T-REx™ NICD CSL-*bla* HeLa cells were plated in 384-well assay format at 3000 cells/well and reverse-transfected using Lipofectamine™ RNAiMAX and 20 nM of the following panel of RNAi duplexes: mock transfected (no RNAi duplex), L (Stealth™ RNAi Negative Control LO GC), M (Stealth™ RNAi Negative Control Med GC), H (Stealth™ RNAi Negative Control Hi GC), b-lac (Beta-lactamase RNAi positive control duplex), NICD-1 (Notch1 Stealth™ Select RNAi duplex #HSS107249), NICD-2 (custom-designed Stealth™ RNAi against Notch1), or NT+CA (non-transfected control cells + 316 uM potassium clavulonate, which was added at 24 hours post-transfection). Doxycycline (0.5 ug/ml final) was added to the cells at 24 hours post-transfection. At 48 hours post-transfection, the wells were loaded with LiveBLAzer™ substrate plus probenecid for 2 hours. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the 460/530 ratios were plotted for each treatment (n = 4 for each data point).

Cell Culture and Maintenance

Thaw cells in Growth Medium without selection (Blasticidin, Hygromycin, or Zeocin) and culture them in Growth Medium with selection. Pass or feed cells 2-3 times a week and maintain them in a 37°C/5% CO_2 incubator. Maintain cells between 25% and 95% confluence.

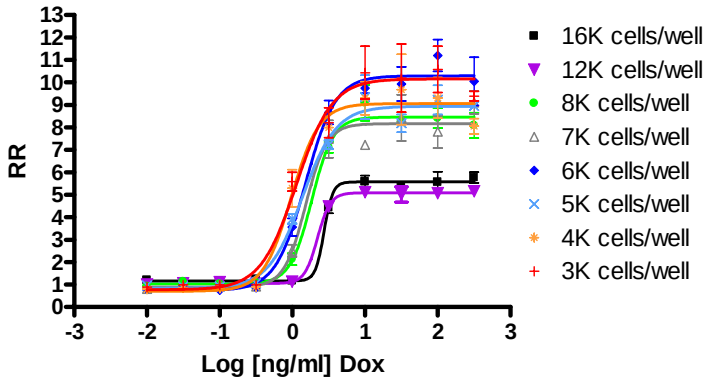
Note: We recommend passing cells for three passages after thawing before using them in the beta-lactamase assay. For more detailed cell growth and maintenance directions, please refer to protocol.

Table 1 – Cell Culture and Maintenance

Component	Growth Medium (–)	Growth Medium (+)	Assay Medium	Freeze Medium
DMEM with GlutaMAX™	500 mL	500 mL	500 mL	—
Dialyzed FBS (dFBS) **Do not substitute!**	50 mL	50 mL	50 mL	—
HEPES (1 M)	12.5 mL	12.5 mL	12.5 mL	
NEAA (100x)	5 mL	5 mL	5 mL	—
Pen/Strep (100x)	5 mL	5 mL	5 mL	—
Blasticidin	—	5 µg/mL	—	—
Hygromycin B	—	125 µg/mL	—	—
Zeocin	—	75 µg/mL	—	—
Recovery™ Cell Culture Freezing Medium	—	—	—	100%

Assay Performance with Variable Cell Number

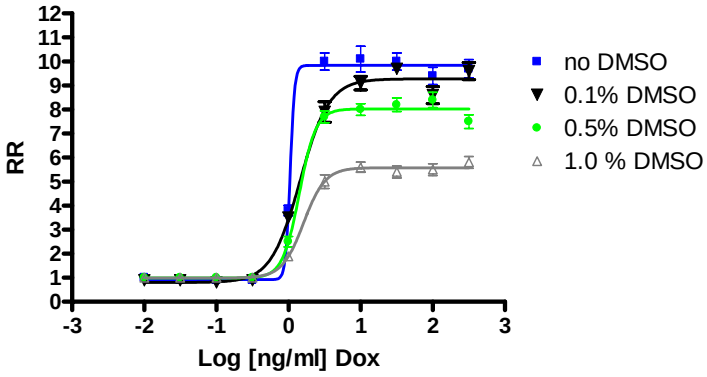
Figure 3 — Doxycycline-induced NICD dose response with varying cell plating density



T-REx™ NICD CSL-*bla* HeLa cells were assayed for Doxycycline-induced NICD dose response while varying cell plating density. Cells were plated in 384-well assay format in Assay Medium at the indicated cell densities. The cells were stimulated overnight with a serial dilution of doxycycline in the presence of 0.1 % DMSO prior to loading the wells with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for 3 hours. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the response ratios (460/530 ratio of the Dox-treated points divided by the 460/530 ratios of the untreated control) were plotted (n = 4 for each data point).

Assay Performance with variable DMSO concentration

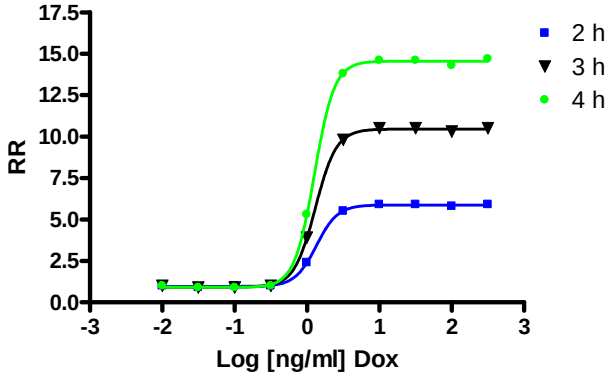
Figure 4 – Doxycycline-induced NICD dose response with 0, 0.1, 0.5 and 1% DMSO.



T-REx™ NICD CSL-*bla* HeLa cells were assayed for Doxycycline-induced NICD dose response with variable final DMSO concentration. Cells were plated in 384-well assay format in Assay Medium at 6000 cells/well. The cells were stimulated overnight with a serial dilution of doxycycline in the presence of indicated final DMSO concentrations prior to loading the wells with LiveBLAzer™-FRET B/G Substrate (1µM final concentration of CCF4-AM) for 3 hours. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the response ratios (460/530 ratio of the Dox-treated points divided by the 460/530 ratios of the untreated control) were plotted (n = 8 for each data point).

Assay performance with Variable Substrate Loading Time

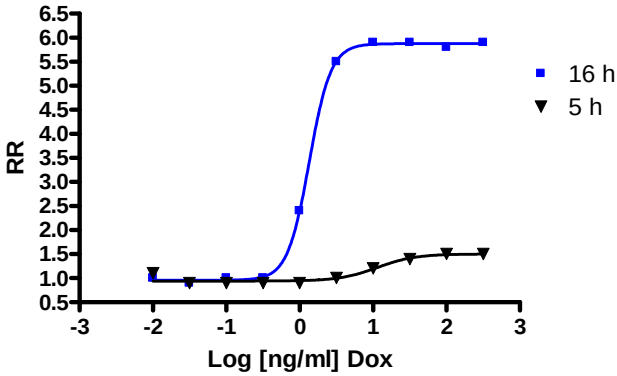
Figure 5 – Doxycycline-induced NICD dose response with 2, 3, and 4 hour loading times



T-REx™ NICD CSL-*bla* HeLa cells were assayed for Doxycycline-induced NICD dose response while varying substrate loading time. Cells were plated in 384-well assay format in Assay Medium at 6000 cells/well. Cells were stimulated overnight with a serial dilution of doxycycline in the presence of 0.1 % DMSO prior to loading the wells with LiveBLAzer™-FRET B/G Substrate for 2, 3, and 4 hours. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the response ratios (460/530 ratio of the Dox-treated points divided by the 460/530 ratios of the untreated control) were plotted (n = 16 for each data point).

Assay performance with Variable Stimulation Time

Figure 6 – Doxycycline-induced NICD dose response with 5 and 16 hour stimulation times



T-REx™ NICD CSL-*bla* HeLa cells were assayed for Doxycycline-induced NICD dose response while varying Doxycycline stimulation time. Cells were plated in 384-well assay format in Assay Medium at 6000 cells/well. Cells were stimulated for 5 h or 16 h with a serial dilution of doxycycline in the presence of 0.1 % DMSO prior to loading the wells 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 response ratios (460/530 ratio of the Dox-treated points divided by the 460/530 ratios of the untreated control) were plotted (n = 16 for each data point).