Validation & Assay Performance Summary

CellSensor® dhfr(E2F)-*bla* NIH3T3 Cell Line

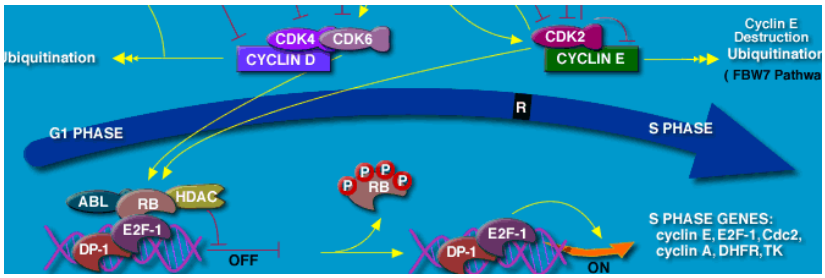
Cat. no. K1639

CellSensor® Cell-Based Assay Validation Packet

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

The G1/S cell cycle checkpoint controls the passage of eukaryotic cells from the first 'gap' phase (G1) into the DNA synthesis phase (S). Two cell cycle kinases, CDK4/6-cyclin D and CDK2-cyclin E, and the transcription complex that includes Rb and E2F are pivotal in controlling this checkpoint. During G1 phase, the Rb-HDAC repressor complex binds to the E2F-DP1 transcription factors, inhibiting the downstream transcription. Phosphorylation of Rb by CDK4/6 and CDK2 dissociates the Rb-repressor complex, permitting transcription of S-phase genes encoding for proteins that amplify the G1 to S phase switch and that are required for DNA replication. Many different stimuli exert checkpoint control including TGFb, DNA damage, contact inhibition, replicative senescence, and growth factor withdrawal.



Disease Relevance:

- RB is a tumor suppressor protein.
- Germline mutations in RB predispose humans to retinoblastoma and sarcoma.
- Somatic mutations in RB leading to pRB inactivation have been described in a variety of adult tumors including SCLC, osteosarcomas, glioblastomas, breast cancer, etc.

Cell Line Description

The CellSensor® dhfr(E2F)-*bla* NIH3T3 cell line contains a beta-lactamase reporter gene under control of the E2F/DP1 binding sequence found in DHFR gene promoter stably integrated into NIH3T3 cells. This cell line is a clonal population isolated in response to 10% Newborn Calf Serum by flow cytometry. This cell line has also been tested for assay performance under variable conditions, including DMSO concentration, cell number, stimulation time, and substrate loading time and validated for Z'. Additional testing information using known inhibitors or activator of the pathway 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 response under optimized conditions (n=3)**

   Z'-Factor (EC100) = 0.70

   Response Ratio = 4.9

   Seeding Density = 3750 cells/well

   Recommended [DMSO] = 0.0-1.0%

   Recommended Stim. Time = 16 hrs

   [Stimulation] = 10% FCS

2. **Alternate Stimuli**
   N/A

3. **Stealth™ RNAi Testing**
   Pending

4. **Small molecule inhibitor Testing**
   See Inhibitor profile panel

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

Assay Testing Summary

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

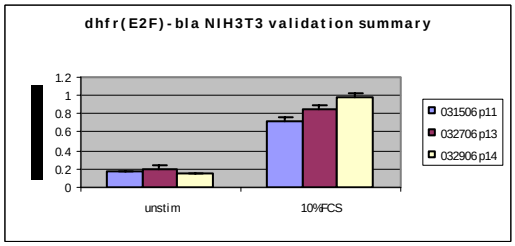
7. **Assay performance with variable stimulation time**

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

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

Primary Agonist Response

Figure 1 — Newborn Calf Serum response under optimized conditions



Dhfr(E2)-*bla* NIH3T3 cells (3750 cells/well) were assayed on three separate days represented by the three different colored columns shown on the graph. Cells were plated in a 384-well format in assay medium and stimulated with Newborn Calf Serum (Invitrogen # 16010-159) in the presence of 0.5% DMSO for 15 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 460/530 Emission Ratios plotted for unstimulated and serum stimulated samples (n=16 for each data point).

Inhibitor Profile

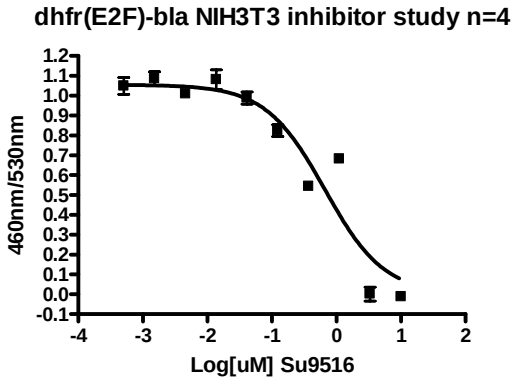


Figure 2 — dhfr(E2F)-*bla* NIH3T3 response to various known compound treatment.
Dhfr(E2F)-*bla* NIH3T3 cells (3750 cells/well) were plated in a 384-well format in assay medium. The cells were treated with Su9516 (EMD, #572650) at the indicated concentrations for 0.5 hours before 10% Newborn Calf Serum was added. Cells were stimulated with the serum for 16 hours and then loaded with LiveBLAzer™-FRET B/G Substrate for 3 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 treatment (n=4 for each data point).
IC50 of Su9516 = 0.66 μM

Cell Culture and Maintenance

Thaw cells in Growth Medium without Blasticidin and culture them in Growth Medium with Blasticidin. Pass or feed cells at least twice a week and maintain them in a 37°C/5% CO_2 incubator. Maintain cells between 10% and 85% confluency. Do not allow cells to reach confluence.

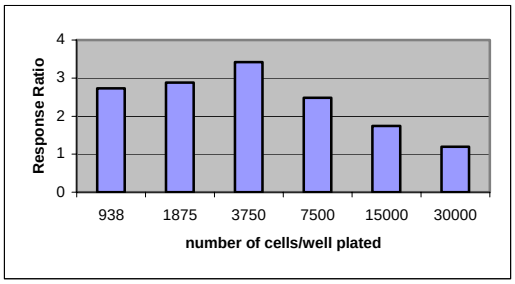
Note: We recommend passing cells for three passages after thawing before using them in the beta-lactamase assay. For more detailed cell growth and maintenance directions, please refer to protocol.

Table 1 – Cell Culture and Maintenance

Component	Growth Medium	Assay Medium	Freezing Medium
DMEM with GlutaMAX™	90%	—	—
DMEM without phenol-red	--	98%	
FCS **Do Not Substitute!**	10%	0.5%	—
NEAA	0.1 mM	0.1 mM	—
HEPES (pH 7.3)	25 mM	—	—
Na Pyruvate	—	1 mM	—
Penicillin (antibiotic)	100 U/ml	—	—
Streptomycin (antibiotic)	100 μg/ml	—	—
Blasticidin (antibiotic)	5 μg/ml	—	—
Recovery™ Cell Culture Freezing Medium	—	—	100%

Assay Performance with Variable Cell Number

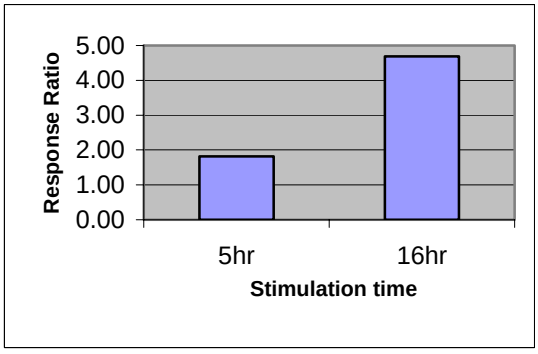
Figure5 — Serum response with different plating cell numbers / well



Dhfr(E2F)-*bla* NIH3T3 cells were plated in a 384-well format in assay medium with indicated number of cells/well and stimulated with Newborn Calf Serum (Invitrogen # 16010-159) in the presence of 0.5% DMSO for 16 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 each cell number.

Assay Performance with Variable Stimulation Time

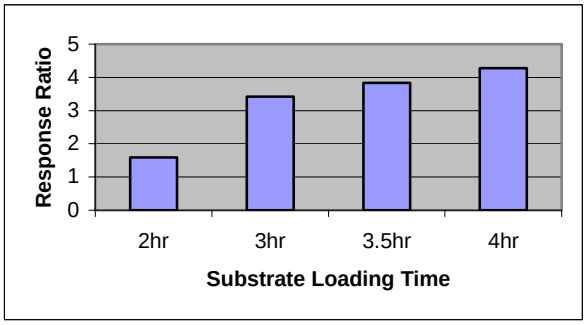
Figure 6 – Serum response with 5 and 16 hour stimulation times



Dhfr (E2F)-*bla* NIH3T3 cells were plated in a 384-well format in assay medium with 3750 cells/well and stimulated with Newborn Calf Serum (Invitrogen # 16010-159) in the presence for 5 or 16 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 is plotted for each stimulation time.

Assay Performance with Variable Substrate Loading Time

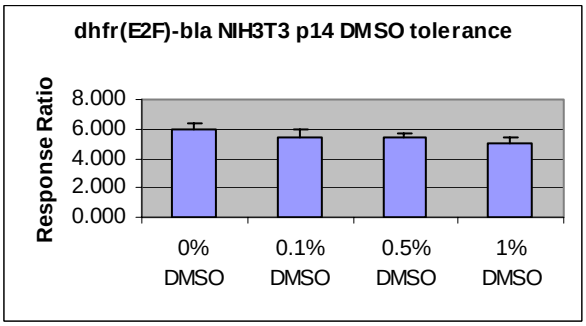
Figure 7 — Serum response with various substrate loading times



Dhfr (E2F)-*bla* NIH3T3 cells were plated in a 384-well format in assay medium with 3750 cells/well and stimulated with Newborn Calf Serum (Invitrogen # 16010-159) in the presence of 0.5% DMSO for 16 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for indicated hours. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the Response Ratios is plotted for each cell number.

Assay Performance with Variable DMSO Concentration

Figure 8 – Serum response with 0, 0.1, 0.5 and 1% DMSO



Dhfr (E2F)-*bla* NIH3T3 cells were plated in a 384-well format in assay medium with 3750 cells/well and stimulated with Newborn Calf Serum (Invitrogen # 16010-159) in the presence of indicated concentrations of DMSO for 16 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 is plotted for each DMSO concentration.