Validation & Assay Performance Summary

CellSensor® p53RE-*bla* HCT-116 Cell Line

Cat. no. K1640

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

p53 is a transcription factor and tumor suppressor. At the molecular level, DNA damage induces the interaction of p53 with the transcriptional activator p300 as well as with the transcriptional co-repressor mSin3A. The ATM protein kinase detects DNA damage and in response activates DNA repair factors and inhibits cell cycle progression. Two of the proteins that ATM phosphorylates in response to DNA damage are the tumor suppressor p53 and the checkpoint kinase chk1. In turn, the tumor suppressor p53 interacts with p21 to block the activity of cdk2 (cyclin dependent kinase 2) preventing passage from G1 to S phase and harmful replication of damaged DNA. Activated p53 binds to a specific site in the promoter region and leads to the transcriptional activation of downstream genes involved in DNA repair, cell cycle arrest and in some cases, apoptosis.

Cell Line Description

The CellSensor® p53RE-*bla* HCT-116 cell line contains a beta-lactamase reporter gene under control of the p53RE Response Element (p53RE) stably integrated into HCT-116 cells. HCT-116 is a human colon carcinoma cell line. This cell line has been tested for assay performance under variable conditions, including DMSO concentration, stimulation time, and validated for Z' and EC_{50} concentrations of Mitomycin. Additional information using alternate ligands and Stealth™ RNAi 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)**

Mitomycin EC_{50}	= 0.8 µg/ml
Z'-Factor (EC_{100})	= 0.74
Response Ratio	= 5
Recommended cell no.	= 30K cells/well
Recommended [DMSO]	= up to 1%
Recommended Stim. Time	= 16 hours
Max. [Stimulation]	= ~ 9µg/ml

2. **Alternate Agonist Dose Response**
   Nutlin EC_{50} =2.5µM

3. **Ligand Panel**
   See Ligand panel Section

4. **Stealth™ RNAi Testing**
   See Stealth™ RNAi Testing section

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

Assay Testing Summary

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

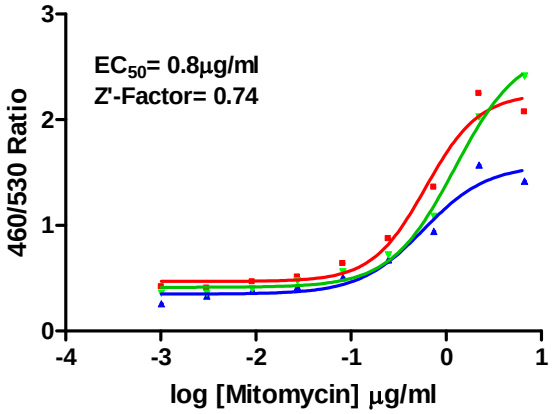
7. **Assay performance with variable stimulation time**

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

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

Primary Agonist Dose Response

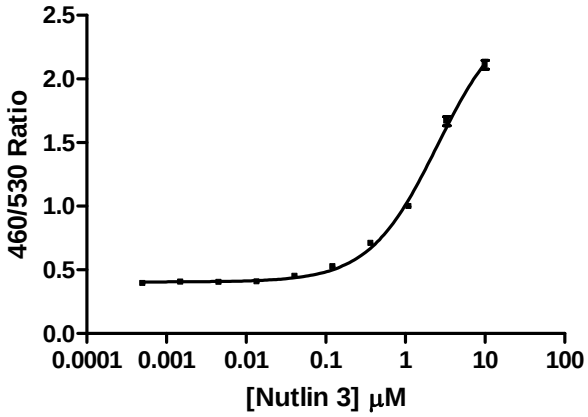
Figure 1 — p53RE-*bla* HCT-116 dose response to Mitomycin under optimized conditions



p53RE-*bla* HCT-116 cells (30,000 cells/well) were plated the day of the assay in a 384-well format. Cells were stimulated with Mitomycin (Calbiochem #475820) over the indicated concentration range in the presence of 0.5% DMSO for 16 hours. Cells were then loaded with LiveBLAzer™-FRET B/G Substrate for 2.5 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 against the indicated concentrations of Mitomycin (n=16 for each data point).

Alternate Agonist Dose Response

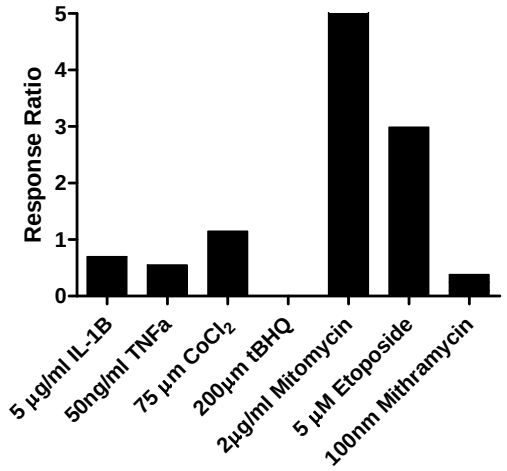
Figure 2 — p53RE-*bla* HCT-116 dose response to Nutlin



p53RE-*bla* HCT-116 cells (25,000 cells/well) were plated the day of the assay in a 384-well format. Cells were stimulated with Nutlin 3 (EMD #444143) over the indicated concentration range 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 were obtained using a standard fluorescence plate reader and the 460/530 Emission Ratios plotted against the indicated concentrations of Nutlin (n=8 for each data point).

Ligand Panel

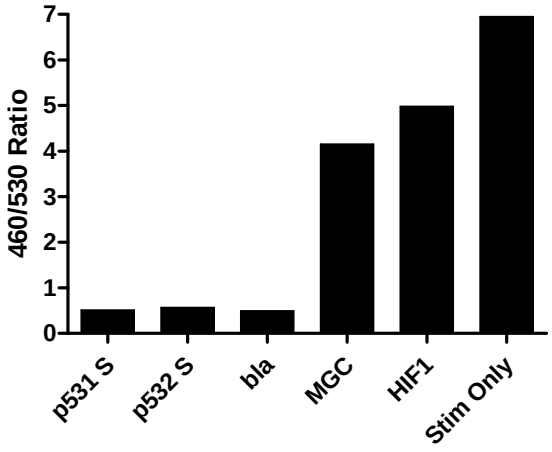
Figure 3 — p53RE-*bla* HCT-116 dose response to various ligands



p53RE-*bla* HCT-116cells (40,000 cells/well) were plated the day of the assay in a 96-well format. Cells were stimulated with listed ligands in 0.5% DMSO for 16 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 Response Ratios plotted.

Stealth™ RNAi Testing

Figure 4 — p53RE-*bla* HCT-116 response to various Stealth™ RNAi oligos.



p53RE-*bla* HCT-116 cells were plated the day of the assay at 6,000 cells per well in a 96-well format. Lipofectamine™ 2000 mixtures containing RNAi oligos for p53RE, beta-lactamase (*bla*), Medium GC control or HIF1 were added to the plate and incubated for 60 hours. 2µg/ml Mitomycin (Calbiochem #475820) was then added to the plate with 0.5% DMSO and cells were incubated at 37ºC & 5% CO_2 for 16 hrs. Cells were 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 RNAi.

Cell Culture and Maintenance

Thaw cells in Growth Medium without Blasticidin and culture them in Growth Medium with Blasticidin. 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 60% confluency. Do not allow cells to reach confluence.

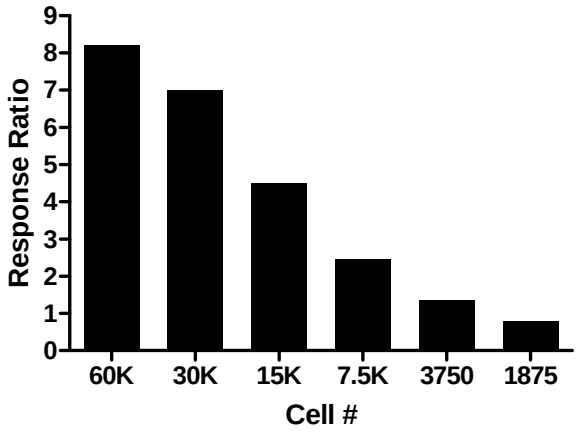
Note: We recommend passing cells for three passages after thawing before using them in the beta-lactamase assay. Freeze cells at $2x10^6$ cells/ml in Freezing Medium. For optimal cell line performance, use dialyzed FBS (Invitrogen #26400-010).

Table 1 – Cell Culture and Maintenance

Component	Growth Medium	Assay Medium	Freezing Medium
McCoy's 5A Medium	90%	-	85%
Opti- MEM®	-	99.5%	-
Dialyzed FBS **Do Not Substitute!**	10%	0.5%	10%
NEAA	-	0.1 mM	0.1 mM
Sodium pyruvate	-	1 mM	1 mM
Penicillin	100 U/ml	100 U/ml	100 U/ml
Streptomycin	100 µg/ml	100 µg/ml	100 µg/ml
Blasticidin	5 µg/ml	-	-
DMSO	-	-	5%

Assay Performance with Variable Cell Numbers

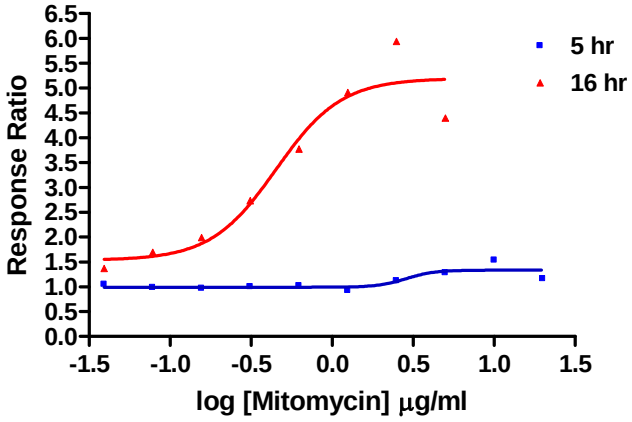
Figure 5 – p53RE-*bla* HCT-116 response to Mitomycin with variable cell number



p53RE-*bla* HCT-116 cells were plated the day of the assay at 60K, 30K, 15K, 7.5K, 3750 or 1875 cells/ well in a 384-well assay plate. 2µg/ml Mitomycin (Calbiochem #475820) was then added to the plate and cells were stimulated for 16hrs 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 Response Ratios for each cell number were plotted.

Assay Performance with Variable Stimulation Time

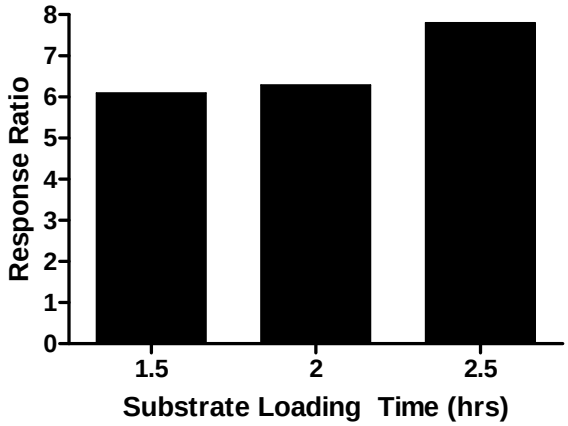
Figure 6 – p53RE-*bla* HCT-116 dose response to Mitomycin with 5 and 16 hour stimulation times



p53RE-*bla* HCT-116 cells (25,000 cells/well) were plated the day of the assay in a 384-well assay plate. Mitomycin (Calbiochem #475820) was then added to the plate over the indicated concentration range and cells were stimulated for 5 or 16 hrs 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 for each stimulation time were plotted against the indicated concentrations of Mitomycin.

Assay Performance with Variable Substrate Loading Time

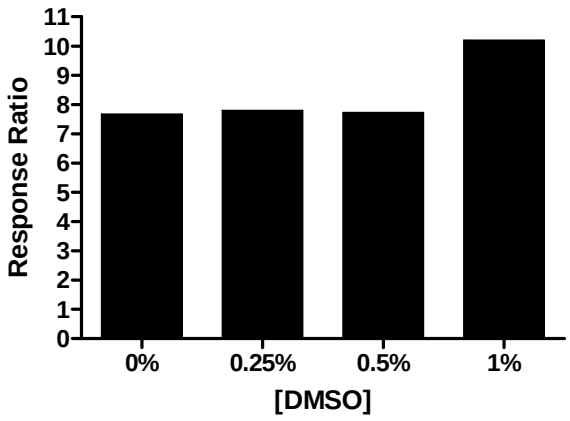
Figure 7 – p53RE-*bla* HCT-116 response to Mitomycin with 1.5, 2 and 2.5 hour substrate loading times



p53RE-*bla* HCT-116 cells (25,000 cells/well) were plated the day of the assay in a 384-well assay plate. 2µg/ml Mitomycin (Calbiochem #475820) was added to the plate and cells were stimulated for 16 hrs in 0.5% DMSO. Cells were then loaded for 1.5, 2 or 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 for each stimulation time were plotted.

Assay Performance with Variable DMSO Concentration

Figure 8 – p53RE-*bla* HCT-116 response to Mitomycin with 0, 0.25, 0.5 and 1% DMSO



p53RE-*bla* HCT-116 cells (30,000 cells/well) were plated the day of the assay in a 384-well black-walled tissue culture assay plate. 2µg/ml Mitomycin (Calbiochem #475820) was then added to the plate and DMSO was added to the assay at concentrations from 0% to 1%. Plates were stimulated for 16 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 florescence plate reader and the Response Ratios for each DMSO concentration were plotted (n=8 for each data point).