Validation & Assay Performance Summary

CellSensor® DBE-*bla* MDA-MB-468 Cell Line

Cat. no. K1814

Pathway Description

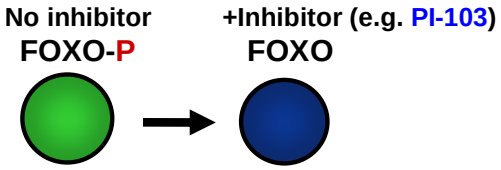
The phosphatidylinositol-3-kinase (PI3K) signaling cascade is essential for cell growth and survival. A series of recent studies indicate that the various PI3K pathway components are targeted by amplification, mutation, and translocation more frequently than any other pathway in cancer patients. The mammalian FOXO subfamily of Forkhead transcription factors is among the best characterized targets of PI3K signaling and consists of three members: FOXO1 (FKHR), FOXO3 (FKHRL1), and FOXO4 (AFX). Growth factor-induced activation of PI3K results in increased downstream activity of the serine/threonine kinase AKT, which in turn phosphorylates and inactivates FOXO family of transcription factors as well as other targets to promote cell survival and oppose apoptosis. Under stress conditions that activate FOXOs (e.g. serum-starvation), they translocate from the cytoplasm to the nucleus where they induce or repress transcription of sets of target genes.

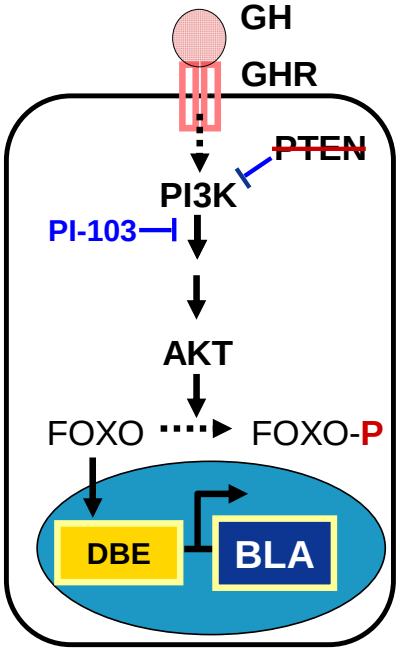
Cell Line Description

To interrogate endogenous PI3K/AKT signaling, MDA-MB-468 breast cancer cells were engineered with a FOXO responsive element driving beta-lactamase reporter gene expression (DBE-*bla*). In MDA-MB-468 cells the tumor suppressor PTEN, which normally functions as a negative regulator of PI3K in the absence of growth factor stimulation, is mutationally inactivated resulting in constitutive PI3K/AKT signaling. CellSensor® DBE-*bla* MDA-MB-468 is a clonal population isolated by flow cytometry and tested for assay responsiveness to PI3K inhibitor, PI-103. PI-103 blocks inactivation of downstream FOXO transcription factors by the endogenous constitutive PI3K/AKT signaling, thereby allowing for transcriptional upregulation of FOXO-driven beta-lactamase reporter gene expression.

CellSensor® DBE-*bla* MDA-MB-468 features:

- Constitutive PI3K/AKT signaling (PTEN mutant)
- FOXO3 response element
- Beta-lactamase reporter gene readout





Validation Summary

Testing and validation of this assay was evaluated in 384-well format using LiveBLAzer™-FRET B/G Substrate.

1. Primary inhibitor dose response under optimized conditions (n=3)

Average PI-103	EC_{50}	= 1.2 μM
Average Z'-Factor	(EC_{100})	= 0.55
Average Response Ratio		= 2.0
Recommended cell no.		= 8,000 cells/well
Recommended [DMSO]		= up to 0.25 %
Stimulation Time		= 16 hours
Max. [Stimulation]		= 5 uM PI-103

2. Inhibitor Panel

Wortmannin (PI3K)	EC_{50} = 24 nM
LY294002 (PI3K)	EC_{50} = 2.2 μM
LY303511 (control)	EC_{50} >50 μM
PI-103 (mTor/PI3K)	EC_{50} = 310 nM
PIK-90 (PI3Kα)	EC_{50} = 750 nM
PI3Ka inhib IV (PI3Kα/β)	EC_{50} = 770 nM
TGX-221 (PI3Kβ)	EC_{50} = 140 nM
PI3Kg inhib (PI3Kγ)	$EC_{50} \geq$ 1 μM*
PI3Kg inhib II (PI3Kγ)	EC_{50} >10 μM
AKT inhib II (AKT)	EC_{50} >10 μM
AKT inhib IV (AKT)	$EC_{50} \geq$ 2 μM*
AKT inhib X (AKT)	EC_{50} = 920 nM
AKTi 1,2 (AKT1/2)	EC_{50} = 63 nM
Triciribine (AKT1/2/3)	$EC_{50} \geq$ 5 μM*

*top of dose response curve not well-defined

3. Stealth™ RNAi Testing

4. Cell culture and maintenance

See Cell Culture and Maintenance Section and Table 1

Assay Testing Summary

5. Assay performance with variable cell number

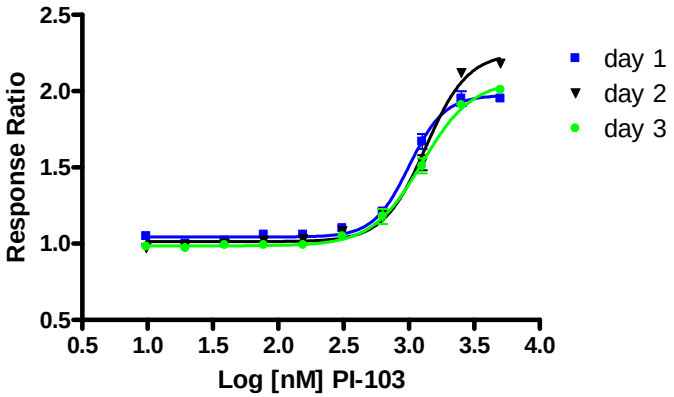
6. Assay performance with variable DMSO concentration

7. Assay performance with variable substrate loading time

8. Assay performance with variable stimulation time

Primary Inhibitor Dose Response

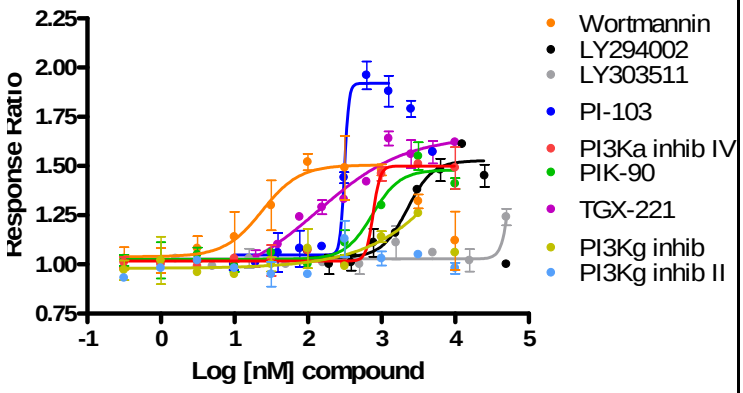
Figure 1 — PI-103 dose response under optimized conditions



DBE-*bla* MDA-MB-468 cells were assayed on three separate days in 384-well assay format in Assay Medium at 8,000 cells/well. Serial dilutions of dual PI3K/mTOR inhibitor PI-103 were applied to the wells (0.1 % final DMSO). Following overnight inhibitor incubation, cells were loaded with LiveBLAzer™-FRET B/G Substrate plus Solution D for 8 h. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and response ratios calculated by dividing the 460/530 ratios of the PI-103 treated wells from the untreated wells (n = 16 for each data point).

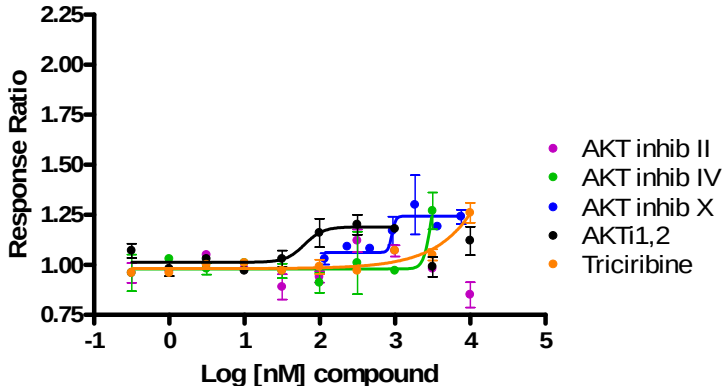
Inhibitor Panel

Figure 2A — PI3K inhibitor panel



DBE-*bla* MDA-MB-468 cells were assayed in 384-well assay format in Assay Medium at 8,000 cells/well. Serial dilutions of the indicated PI3K inhibitors or controls were applied to the wells (0.1 % final DMSO). Following overnight inhibitor incubation, cells were loaded with LiveBLAzer™-FRET B/G Substrate plus Solution D for 8 h. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and response ratios calculated by dividing the 460/530 ratios of the compound treated wells from the untreated wells (n = 2 for each data point).

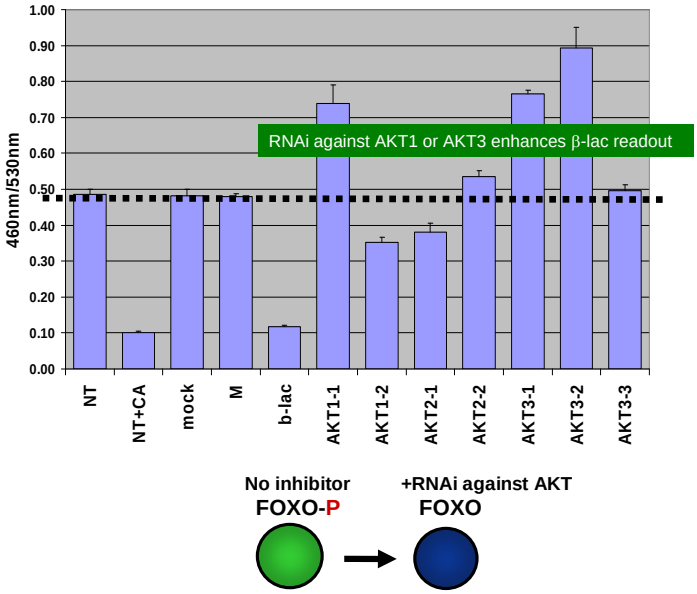
Figure 2B — AKT inhibitor panel



DBE-*bla* MDA-MB-468 cells were assayed in 384-well assay format in Assay Medium at 8,000 cells/well. Serial dilutions of the indicated AKT inhibitors were applied to the wells (0.1 % final DMSO). Following overnight inhibitor incubation, cells were loaded with LiveBLAzer™-FRET B/G Substrate plus Solution D for 8 h. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and response ratios calculated by dividing the 460/530 ratios of the compound treated wells from the untreated wells (n = 2 for each data point)

Stealth™ RNAi Testing

Figure 3 — RNAi panel



DBE-*bla* MDA-MB-468 cells were plated in 384-well assay format in Growth Medium at 8,000 cells/well and reverse transfected using using Lipofectamine™ RNAiMAX Transfection Reagent and 20 nM of Stealth™ RNAi duplexes against AKT1, AKT2, and AKT3. Controls were as follows: nontransfected cells (NT), nontransfected cells plus beta-lactamase inhibitor clavulonic acid (NT+CA), mock transfected (no RNAi duplex), Stealth™ RNAi Negative Control Med GC, and Beta-lactamase positive control RNAi duplex. At ~48 h post-transfection, the Growth Medium was removed from the wells and replaced with Assay Medium and the plate was incubated for an additional ~16 h prior to loading with LiveBLAzer™-FRET B/G Substrate plus Solution D for 8 h. Fluorescence emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and the 460/530 ratios were plotted (n = 4 for each data point).

Cell Culture and Maintenance

Thaw cells in Growth Medium without selection (Blasticidin) 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 20% and 80% confluence.

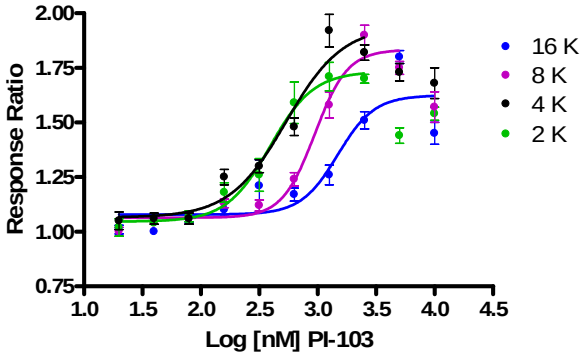
Note: We recommend passing cells for three passages after thawing before using them in the beta-lactamase assay. For more detailed cell growth and maintenance directions, please refer to protocol.

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	0.5 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	—	—
Recovery™ Cell Culture Freezing Medium	—	—	—	100%

Assay Performance with Variable Cell Number

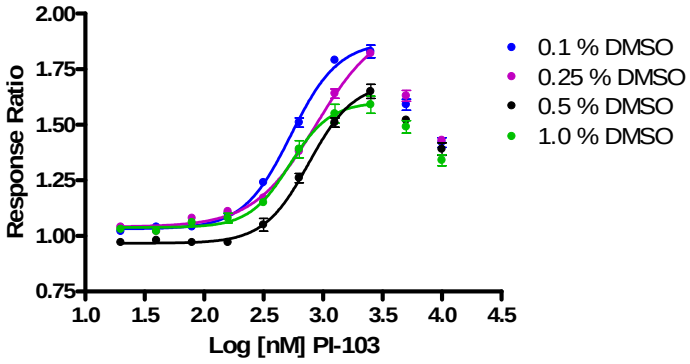
Figure 4 — PI-103 dose response with varying cell plating density



DBE-*bla* MDA-MB-468 cells were plated onto a poly-D-lysine coated 384-well assay plate in low serum medium (DMEM supplemented with 0.1% dFBS) at varying cell densities. Serial dilutions of dual PI3K/mTOR inhibitor PI-103 were applied to the wells (0.1 % final DMSO). Following overnight inhibitor incubation, cells were loaded with LiveBLAzer™-FRET B/G Substrate plus Solution D for 7 h. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and response ratios calculated by dividing the 460/530 ratios of the PI-103 treated wells from the untreated wells (n = 4 for each data point).

Assay Performance with variable DMSO concentration

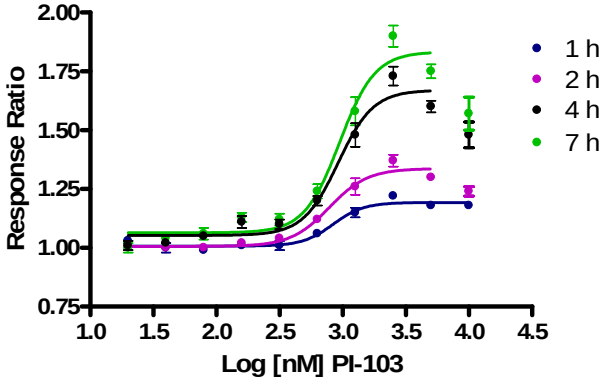
Figure 5 – PI-103 dose response with 0.1, 0.25, 0.5 and 1% DMSO.



DBE-*bla* MDA-MB-468 cells were plated onto a poly-D-lysine coated 384-well assay plate in low serum medium (DMEM supplemented with 0.1% dFBS) at ~8,000 cells/well. Serial dilutions of dual PI3K/mTOR inhibitor PI-103 were applied to the wells along with increasing final DMSO concentrations. Following overnight inhibitor incubation, cells were loaded with LiveBLAzer™-FRET B/G Substrate plus Solution D for 7 h. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and response ratios calculated by dividing the 460/530 ratios of the PI-103 treated wells from the untreated wells (n = 8 for each data point).

Assay performance with Variable Substrate Loading Time

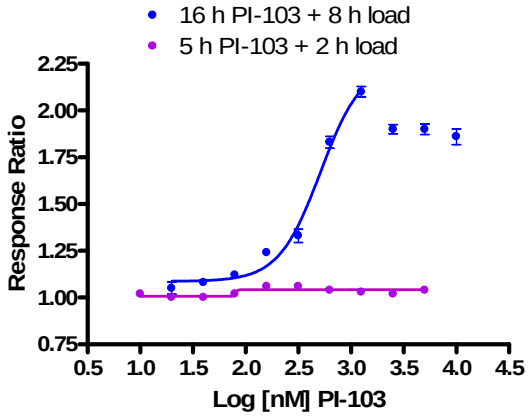
Figure 6 – PI-103 dose response with increasing loading times



DBE-*bla* MDA-MB-468 cells were plated onto a poly-D-lysine coated 384-well assay plate in low serum medium (DMEM supplemented with 0.1% dFBS) at ~8,000 cells/well. Serial dilutions of dual PI3K/mTOR inhibitor PI-103 were applied to the wells (0.1 % final DMSO). Following overnight inhibitor incubation, cells were loaded with LiveBLAzer™-FRET B/G Substrate plus Solution D and the plate was read at varying time points. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and response ratios calculated by dividing the 460/530 ratios of the PI-103 treated wells from the untreated wells (n = 4 for each data point).

Assay performance with Variable Stimulation Time

Figure 7 – PI-103 dose response with 5 and 16 hour stimulation times



DBE-*bla* MDA-MB-468 cells were plated onto poly-D-lysine coated 384-well assay plates in low serum medium (DMEM supplemented with 0.1% dFBS) at ~8,000 cells/well. Serial dilutions of dual PI3K/mTOR inhibitor PI-103 were applied to the wells (0.1 % final DMSO). Following either 5 h or 16 h inhibitor incubation times, cells were loaded with LiveBLAzer™-FRET B/G Substrate plus Solution D for 2 h or 8 h, respectively. Emission values at 460 nm and 530 nm were obtained using a standard fluorescence plate reader and response ratios calculated by dividing the 460/530 ratios of the PI-103 treated wells from the untreated wells (n = 8 for each data point).

References

1. Hennessy BT, Smith DL, Ram PT, Lu Y, and Mills GB. (2005) **Exploiting the PI3K/AKT pathway for cancer drug discovery**. *Nature Reviews Drug Discovery* 4:988-1004.

2. Birkenkamp KU and Coffer PJ. (2003) **Regulation of cell survival and proliferation by the FOXO (Forkhead box, class O) subfamily of Forkhead transcription factors**. *Biochemical Society Transactions* 31:292-297.

3. Accili D and Arden KC. (2004) **FoxOs at the crossroads of cellular metabolism, differentiation, and transformation**. *Cell* 117:421-426.

4. Fan Q-W, Knight ZA, Goldenberg DD, Yu W, Mostov KE, Stokoe D, Shokat KM, and Weiss WA. (2006) **A dual PI3 Kinase/mTOR inhibitor reveals emergent efficacy in glioma**. *Cancer Cell* 9:341-349.