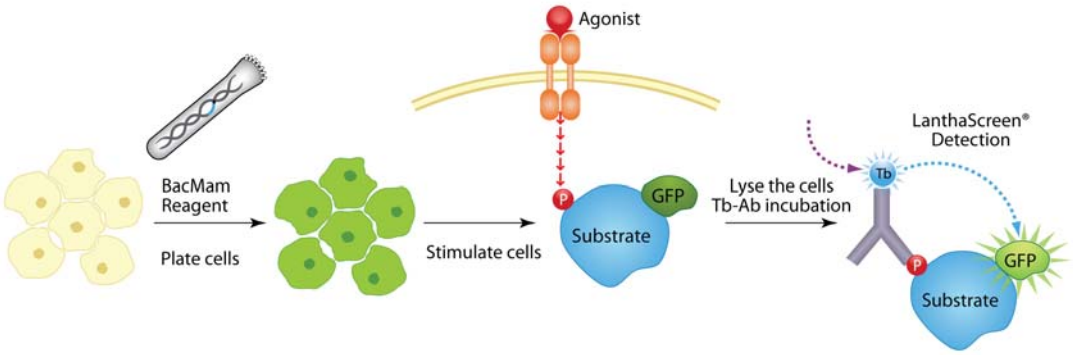
BacMam p53 [AcLys382] Cellular Assay

Overview

Introduction to BacMam. LanthaScreen® Cellular Assays can now be used together with a gene delivery method known as BacMam for the transient expression of the desired GFP-substrate fusion in many cell lines of interest. The BacMam delivery system utilizes modified baculovirus as a vehicle to deliver and express target genes in mammalian cells. This technology has several advantages over commonly used transient methods for heterologous gene expression, including: 1) high transduction efficiency across a broad range of cell types, including primary and stem cells; 2) little-to-no observable cytopathic effects; 3) reproducible and titratable target gene expression; 4) virus is non-replicating in mammalian cells, therefore it is designated as Biosafety Level 1 (BSL1); and 5) compatible with simultaneous delivery of multiple genes. Thus, combination of the LanthaScreen® Cellular Assay technology and BacMam-mediated gene delivery provides a fast, convenient, and robust method for interrogating specific signal transduction events in a cell background of choice. Please see Kost, et al for more information pertaining to BacMam gene expression in cells.



Schematic Illustration of BacMam-enabled Cellular Assay Using LanthaScreen® Technology Workflow. Cells
Schematic Illustration of the workflow of BacMam-enabled Cellular Assay Using LanthaScreen® Technology. Cells are treated with BacMam virus encoding a GFP-fusion protein and plated in a 384-well assay plate. 24-48 hours post-transduction, the cells are stimulated to induce the specific post-translational modifications (such as phosphorylation as shown) of the GFP-substrate fusion. Cells are then lysed in the presence of terbium-labeled anti-modification specific antibody and TR-FRET from terbium to GFP can be detected.

***BacMam-enabled Cellular p53 Assay Using LanthaScreen® Technology*.** p53 is a tumor suppressor that functions as a transcription factor to regulate genes involved in cell cycle arrest, DNA repair, and apoptosis. In unstressed cells, p53 is degraded via an MDM2-ubiquitin-proteasome pathway. In response to DNA damage, p53 becomes phosphorylated at Ser15 and also acetylated by the acetyltransferases Tip60, p300, and PCAF at Lys382. Together these p53 modifications are believed to enhance its stability and/or transcriptional activity. The combination of baculovirus-mediated gene delivery (BacMam) with LanthaScreen® Cellular Assay technology enables a platform for the analysis of specific posttranslational modifications of p53. BacMam provides a convenient genetic delivery tool for a GFP-p53 fusion protein in a cell line of interest. BacMam-enable p53 assay is an HTS-compatible cellular immunoassay measuring acetylation of GFP-p53 at Lys382. This assay is validated for detecting modulators for SIRT1 and/or type I and II HDACs.

Validation Table of Contents

Validation of BacMam p53 [AcLys382] Cellular Assay was performed in a 384-well format using U-2 OS as a host cell line. Following stimulation with EX537 and Trichostatin A (TSA), the acetylation status of GFP-p53 was then measured using LanthaScreen® Tb-anti-p53 [AcLys382] antibody as a detection reagent. Included are representative results using this assay, including an example of the assay optimization process, which can be applied to your cell line of choice.

1. Representative assay optimization and validation for p53 AcLys382 assay using U-2 OS cells

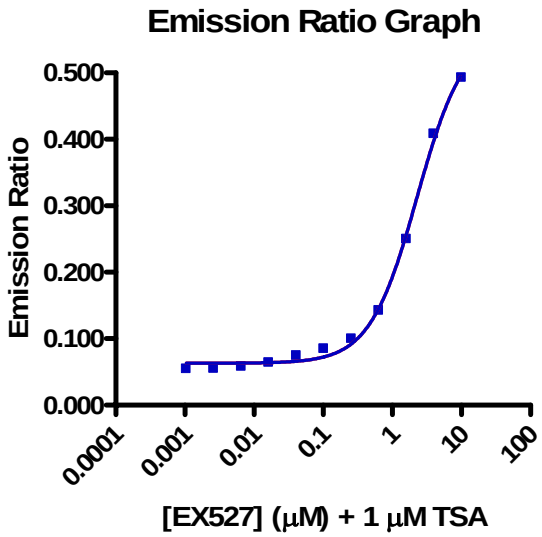
a. Comparison of response ratio and emission ratio graphs
b. Optimization of BacMam p53 transduction
c. Optimization of cell number per well
d. Optimization of cell treatment time
e. Lysis buffer equilibration time
f. Testing Trichostatin A concentrations
g. DNA-damage induced responses
h. SIRT1 siRNA testing

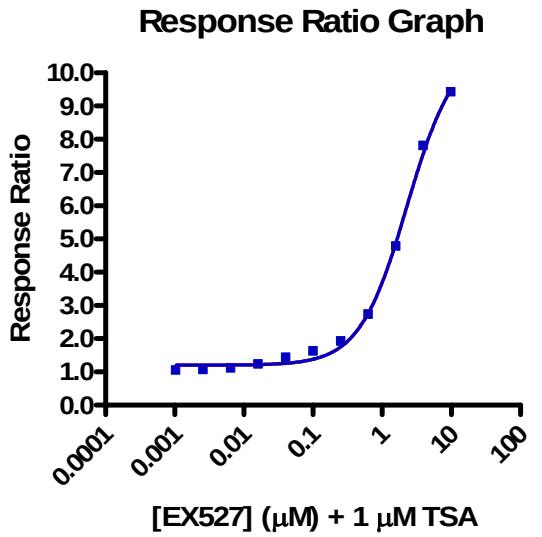
2. Assay Portability: Alternate cell backgrounds tested

a. HCT-116
b. PC-3
c. Human mammary epithelial cells,
d. Human astrocytes

Representative Data for BacMam p53 [AcLys382] Cellular Assay

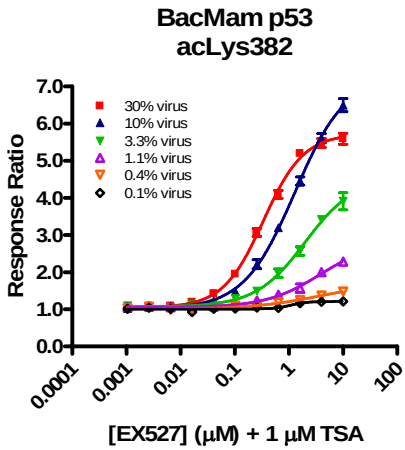
Figure 1a — Comparison of emission ratio and response ratio graphs





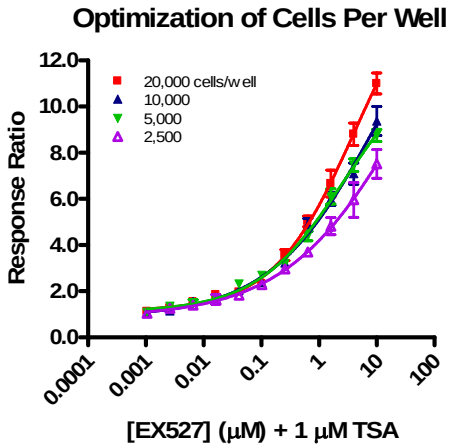
Measurement of p53 Acetylation using the BacMam p53 [AcLys382] Cellular Assay. A serial dilution of EX537 + 1µM Trichostatin A was pre-spotted onto a 384-well plate. U-2 OS cells were harvested, resuspended in low serum assay medium and then mixed with BacMam p53 Reagent to achieve 10% virus (v/v) concentration. The mix was applied to the compound wells, yielding a cell density of ~10,000 cells per well. Following a 20 hour incubation, cells were lysed by adding fully-supplemented 6X lysis buffer containing Tb-anti-p53 [acLys382] Antibody and equilibrated for 3 hours at room temperature before TR-FRET was measured on a BMG Labtech Pherastar plate reader. Raw TR-FRET Emission Ratios (520nm/490nm) and Response Ratios (measure of assay window) normalized to untreated controls are shown.

Figure 1b — Optimization of BacMam p53 transduction



Titration of BacMam p53 Reagent. A serial dilution of EX537 + 1µM Trichostatin A was pre-spotted onto a 384-well plate. U-2 OS cells were harvested, resuspended in low-serum assay medium and then mixed with serial dilutions of BacMam p53 Reagent to achieve the indicated % virus (v/v) concentrations. The mixes were applied to the compound wells, yielding a cell density of ~10,000 cells per well. Following 20 hour incubation, cells were lysed by adding fully-supplemented 6X lysis buffer containing Tb-anti-p53 [acLys382] Antibody and equilibrated for 3 hours at room temperature before TR-FRET was measured on a BMG Labtech Pherastar plate reader.

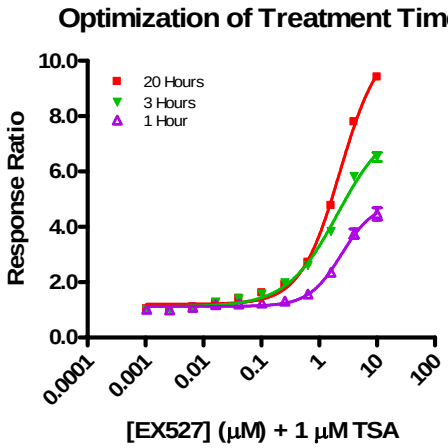
Figure 1c – Optimization of cell number



Measurement of p53 Acetylation using the BacMam p53 [acLys382] Cellular Assay. A serial dilution of EX537 + 1µM Trichostatin A was pre-spotted onto a 384-well plate. U-2 OS cells were harvested, resuspended in low serum assay medium in a serial dilution, and then mixed with BacMam p53 Reagent to achieve 10% virus (v/v) concentration. The mixes were applied to the compound wells, yielding various cell densities per well. Following a 20 hour incubation, cells were lysed by adding fully-supplemented 6X lysis buffer containing Tb-anti-p53 [acLys382] Antibody and equilibrated for 3 hours at room temperature before TR-FRET was measured on a BMG Labtech Pherastar plate reader.

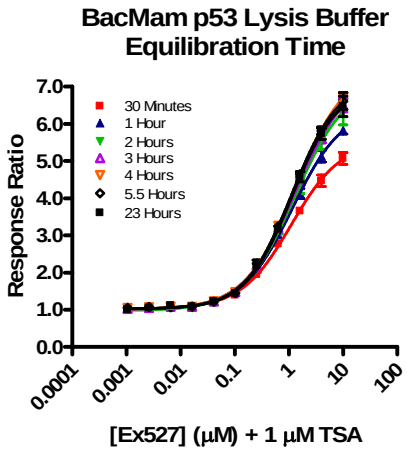
Representative Data for BacMam p53 [AcLys382] Cellular Assay

Figure 1d — Optimization of cell treatment time



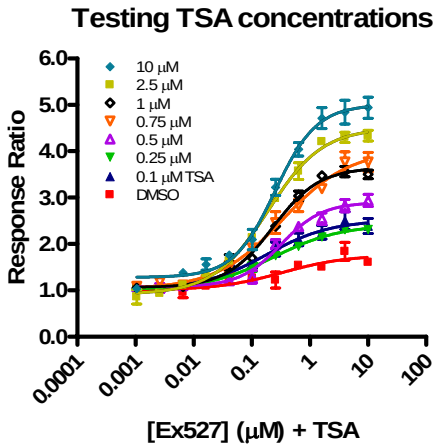
Measurement of p53 Acetylation using the BacMam p53 [acLys382] Cellular Assay. A serial dilution of EX537 + 1µM Trichostatin A was pre-spotted onto a 384-well plate (for the 20 hour time) or added onto the cells the next day (for the 1 and 3 hour times). U-2 OS cells were harvested, resuspended in low serum assay medium, and then mixed with BacMam p53 Reagent to achieve 10% virus (v/v) concentration. The mixes were applied to the plate, yielding a cell density of ~10,000 cells per well. Following a 20 hour incubation, cells were lysed by adding fully-supplemented 6X lysis buffer containing Tb-anti-p53 [acLys382] Antibody and equilibrated for 3 hours at room temperature before TR-FRET was measured on a BMG Labtech Pherastar plate reader.

Figure 1e — Lysis buffer equilibration time



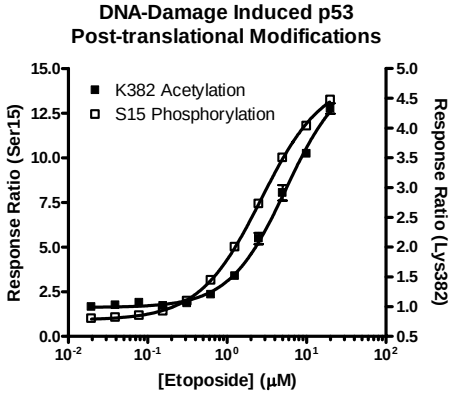
Measurement of p53 Acetylation using the BacMam p53 [acLys382] Cellular Assay. A serial dilution of EX537 + 1µM Trichostatin A was pre-spotted onto a 384-well plate. U-2 OS cells were harvested, resuspended in low serum assay medium and then mixed with BacMam p53 Reagent to achieve 10% virus (v/v) concentration. The mix was applied to all wells, yielding a cell density of ~10,000 cells per well. Following 20 hour incubation, cells were lysed by adding fully-supplemented 6X lysis buffer containing Tb-anti-p53 [acLys382] Antibody and equilibrated for the indicated times before TR-FRET was measured on a BMG Labtech Pherastar plate reader.

Figure 1f — Testing Trichostatin A concentrations



Measurement of p53 Acetylation using the BacMam p53 [acLys382] Cellular Assay. A serial dilution of EX537 + different concentrations of Trichostatin A were pre-spotted onto a 384-well plate. U-2 OS cells were harvested and resuspended in low serum assay medium. The mix was applied to all wells, yielding a cell density of ~10,000 cells per well. Then enough BacMam p53 Reagent was added to all wells to achieve ~10% virus (v/v) concentration. Following 17.5 hour incubation, cells were lysed by adding fully-supplemented 6X lysis buffer containing Tb-anti-p53 [acLys382] Antibody and equilibrated for 3 hours at room temperature before TR-FRET was measured on a BMG Labtech Pherastar plate reader.

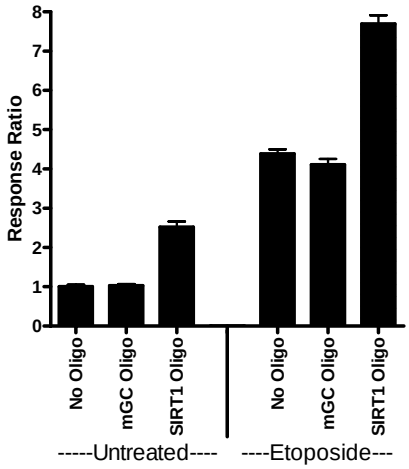
Figure 1g — DNA damage-induced responses



Measurement of p53 Acetylation and phosphorylation induced by DNA damaging agent etoposide. For measurement of p53 phoshporylation and acetylation using TR-FRET, U-2 OS cells were transduced with BacMam GFP-p53. Treatment with serially-diluted etoposide for 18 hours results in a dose-dependent increase in TR-FRET signal for both phosphorylated p53 at Ser15 (□) and acetylated p53 at Lys382 (■). Error bars represent the average of at least 4 data points ± S.E.

Representative Data for BacMam p53 [AcLys382] Cellular Assay

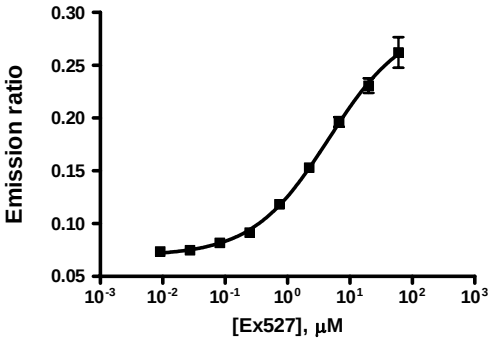
Figure 1h — SIRT1 siRNA testing



Knockdown of SIRT1 using siRNA results in increased acetyl GFP-p53 levels. Lipofectamine® RNAi Max transfection reagent, validated Stealth RNAi™ SIRT1 siRNA and negative control (Medium, Low, and High GC Control) duplexes were from Life Technologies. Cells were transfected by first preparing cell suspensions (as indicated above for the TR-FRET assays), and adding a 1/5 volume of RNAi:lipid complexes such that the final concentration of each duplex was 40 nM. For TR-FRET assays, immediately after mixing cells with RNAi:lipid complexes, Cell suspension (including RNAi oligos) were added to white 384-well plates (Corning). Cells were then incubated 24 hours prior to addition of BacMam GFP-p53 and etoposide to a final concentration of 40 μM. Cells were then incubated an additional 24 hours prior to cell lysis and TR-FRET measurement as described above.

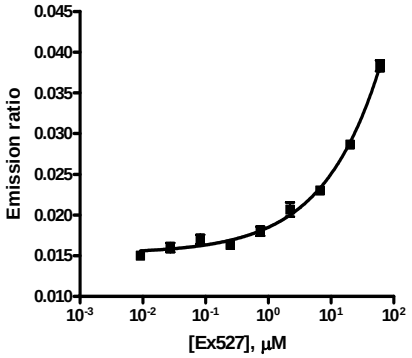
Figure 2 — Alternate Cell Backgrounds

a. HCT-116



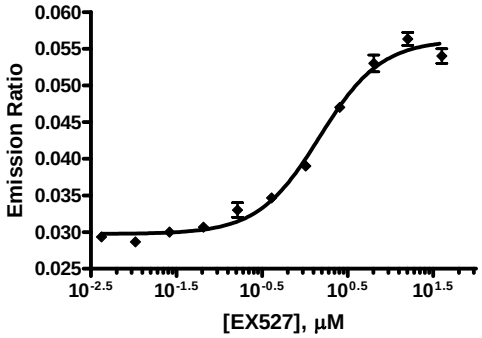
Measurement of p53 acetylation in HCT-116 cells. HCT-116 cells were seeded and simultaneously transduced overnight in a T75 flask using 10% (v/v) BacMam p53 Reagent. After 24 hours transduction, cells were harvested, resuspended in assay medium, A serial dilution of EX537 + 1 μM of Trichostatin A were pre-spotted onto a 384-well plate. Cells were plated yielding a cell density of ~10,000 cells per well. Following 16 hour incubation, cells were lysed by adding fully-supplemented 6X lysis buffer containing Tb-anti-p53 [acLys382] Antibody and equilibrated for 3 hours at room temperature before TR-FRET was measured on a BMG Labtech Pherastar plate reader.

b. PC-3



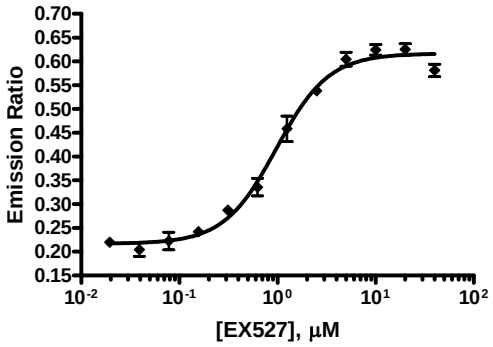
Measurement of p53 acetylation in PC-3 cells. PC-3 cells were seeded and simultaneously transduced overnight in a T75 flask using 10% (v/v) BacMam p53 Reagent. After 24 hours transduction, cells were harvested, resuspended in assay medium, A serial dilution of EX537 + 1 μM of Trichostatin A were pre-spotted onto a 384-well plate. Cells were plated yielding a cell density of ~10,000 cells per well. Following 16 hour incubation, cells were lysed by adding fully-supplemented 6X lysis buffer containing Tb-anti-p53 [acLys382] Antibody and equilibrated for 3 hours at room temperature before TR-FRET was measured on a BMG Labtech Pherastar plate reader.

c. Human mammary epithelial cells



Measurement of p53 acetylation in human mammary epithelial cells. Cryopreserved human mammary epithelial cells were thawed, resuspended in assay medium and mixed with BacMam p53 Reagent (10%, v/v). A serial dilution of EX537 + 1 μM of Trichostatin A were pre-spotted onto a 384-well plate. Cells were plated yielding a cell density of ~10,000 cells per well. Following 16 hour incubation, cells were lysed by adding fully-supplemented 6X lysis buffer containing Tb-anti-p53 [acLys382] Antibody and equilibrated for 3 hours at room temperature before TR-FRET was measured on a BMG Labtech Pherastar plate reader.

d. Human Astrocytes



Measurement of p53 acetylation in human astrocytes. Cryopreserved human astrocytes were thawed and plated into a 384 well assay plate pretreated with Geltrex at a cell density of ~10,000 cells per well. After 48 hours growth media was removed and assay media containing 10% v/v Bacmam p53 Reagent was added. After 2.5 hours media containing Bacmam p53 reagent was replaced with assay medium and a serial dilution of EX527 + 1 µM of Trichostatin A was added to the plate. Following 16 hour incubation, cells were lysed by adding fully-supplemented 6X lysis buffer containing Tb-anti-p53 [acLys382] Antibody and equilibrated for 3 hours at room temperature before TR-FRET was measured on a BMG Labtech Pherastar plate reader.

General References:

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