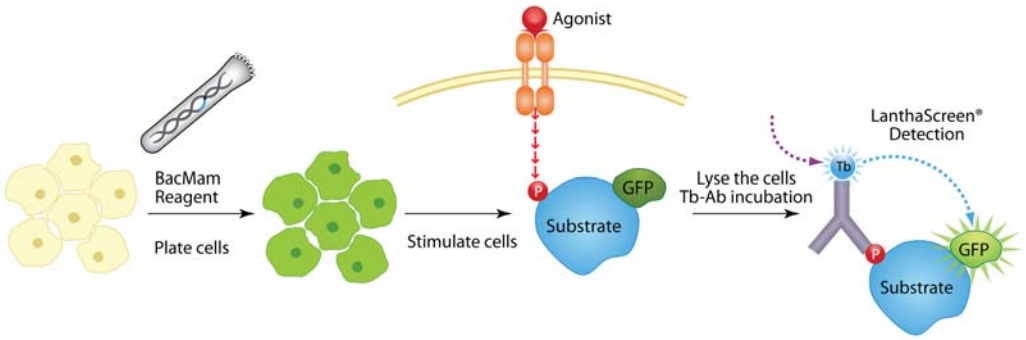
BacMam Histone H3 [pSer10] Cellular Assays

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 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 Histone H3 Assay Using LanthaScreen® Technology. The eukaryotic nucleosome, composed of histones H2A, H2B, H3, and H4, regulates the structure of chromatin and consequently modulates gene transcription profiles in a concerted manner. Nucleosome function is directly regulated by a multitude of posttranslational modifications on amino-terminal tails of core histones, including acetylation, phosphorylation, methylation and ubiquitination. The combination of baculovirus-mediated gene delivery (BacMam) with LanthaScreen® Cellular Assay technology enables a platform for the analysis of specific posttranslational modifications of histones. BacMam provides a convenient genetic delivery tool for a GFP-H3 fusion protein in the cell line of interest. This kit describes an HTS-compatible cellular immunoassay measuring phosphorylation of GFP-Histone H3 at Ser10.

Validation Table of Contents

Validation experiments of BacMam Histone H3 [pSer10] Cellular Assay were performed in a 384-well format using U-2 OS as a host cell line. Following treatment with Calyculin A, the phosphorylation status of GFP-Histone H3 was then measured using LanthaScreen® Tb-anti-H3 [pSer10] 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 Histone H3 pSer10 assay

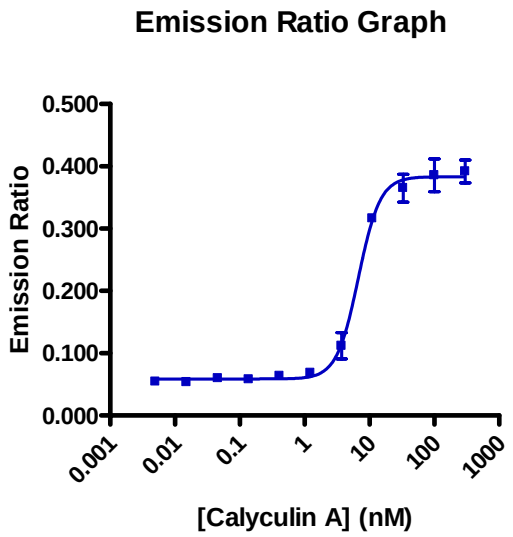
- a. Comparison of emission ratio and response ratio graphs
- b. Optimization of BacMam Histone H3 transduction
- c. Optimization of cell number per well
- d. Optimization of stimulation time
- e. Testing effect of carrier flask confluence
- f. Lysis buffer equilibration time
- g. Aurora Kinase Inhibitor Testing
- h. Cell-cycle dependent Histone H3 Ser10 phosphorylation
- i. Localization of GFP-Histone H3

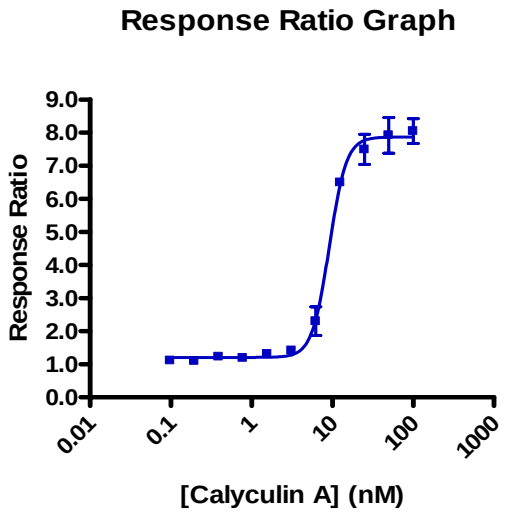
2. Assay Portability

- a. Alternate cell backgrounds tested: HeLa, NIH-3T3

Representative Data for BacMam Histone H3 [pSer10] Cellular Assays

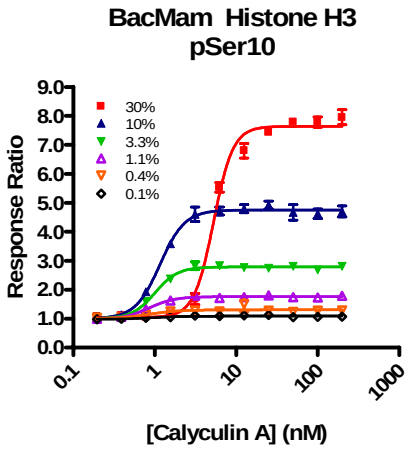
Figure 1a — Comparison of emission ratio and response ratio graphs





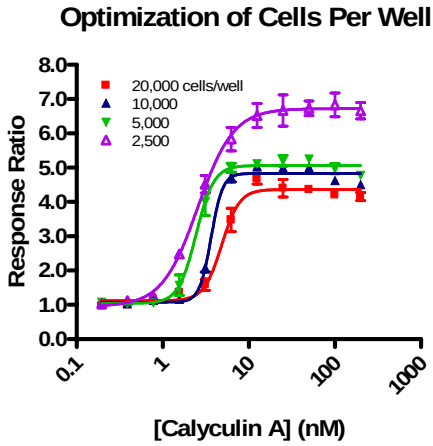
Measurement of Histone H3 Phosphorylation using the BacMam Histone H3 [pSer10] Cellular Assay. U-2 OS cells were harvested, resuspended in assay medium (Opti-MEM I + 0.1% charcoal-stripped FBS) and mixed with BacMam Histone H3 Reagent to achieve 10% virus (v/v) concentration. The mix was applied to wells of a 384-well plate, yielding a cell density of ~5,000 cells per well. Following a 24 hour incubation, cells were treated for 1 hour with a serial dilution of Calyculin A. Cells were lysed by adding fully-supplemented 6X lysis buffer containing Tb-anti-Histone H3 [pSer10] 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 Histone H3 transduction



Measurement of Histone H3 Phosphorylation using the BacMam Histone H3 [pSer10] Cellular Assay. U-2 OS cells were harvested, resuspended in assay medium and mixed with BacMam Histone H3 Reagent to achieve the indicated % virus (v/v) concentrations. The mixes were applied to designated wells of a 384-well plate, yielding a cell density of ~5,000 cells per well. Following a 24 hour incubation, cells were treated for 1 hour with a serial dilution of Calyculin A. Cells were lysed by adding fully-supplemented 6X lysis buffer containing Tb-anti-Histone H3 [pSer10] 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 Histone H3 Phosphorylation using the BacMam Histone H3 [pSer10] Cellular Assay. U-2 OS cells were harvested, resuspended in assay medium and mixed with BacMam Histone H3 Reagent to achieve 10% virus (v/v) concentration. The mix was applied to wells of a 384-well plate, yielding varying cell densities per well. Following a 24 hour incubation, cells were treated for 1 hour with a serial dilution of Calyculin A. Cells were lysed by adding fully-supplemented 6X lysis buffer containing Tb-anti-Histone H3 [pSer10] Antibody and equilibrated for 3 hours at room temperature before TR-FRET was measured on a BMG Labtech Pherastar plate reader.

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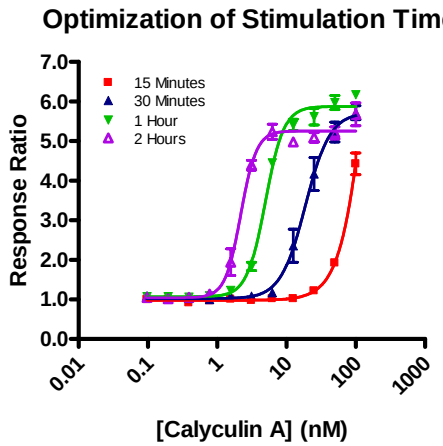
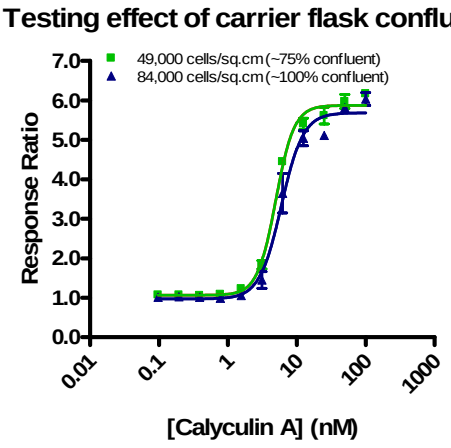
Figure 1d — Optimization of cell stimulation time



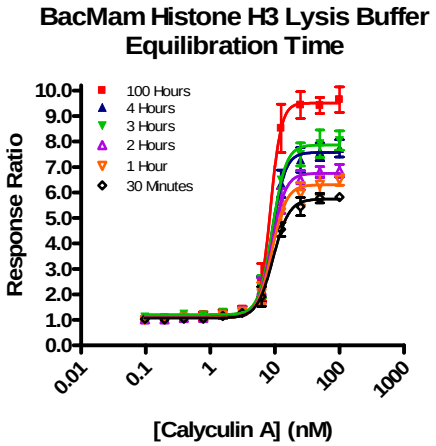
Measurement of Histone H3 Phosphorylation using the BacMam Histone H3 [pSer10] Cellular Assay. U-2 OS cells were harvested, resuspended in assay medium and mixed with BacMam Histone H3 Reagent to achieve 10% virus (v/v) concentration. The mix was applied to wells of a 384-well plate, yielding a cell density of ~5,000 cells per well. Following a 24 hour incubation, cells were treated for the indicated times with a serial dilution of Calyculin A. Cells were lysed by adding fully-supplemented 6X lysis buffer containing Tb-anti-Histone H3 [pSer10] Antibody and equilibrated for 3 hours at room temperature before TR-FRET was measured on a BMG Labtech Pherastar plate reader.

Figure 1e — Testing effect of carrier flask confluence



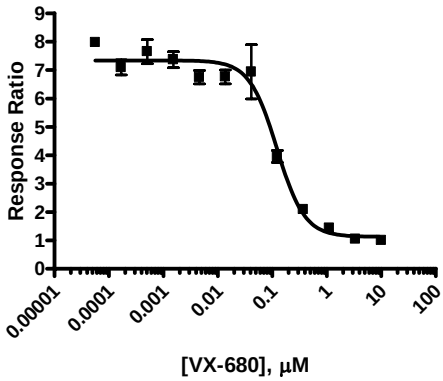
Measurement of Histone H3 Phosphorylation using the BacMam Histone H3 [pSer10] Cellular Assay. Two T225 flasks of U-2 OS cells at varying confluence were harvested, resuspended in assay medium and mixed with BacMam Histone H3 Reagent to achieve 10% virus (v/v) concentration. The mix was applied to wells of a 384-well plate, yielding a cell density of ~5,000 cells per well. Following a 24 hour incubation, cells were treated for 1 hour with a serial dilution of Calyculin A. Cells were lysed by adding fully-supplemented 6X lysis buffer containing Tb-anti-Histone H3 [pSer10] Antibody and equilibrated for 3 hours at room temperature before TR-FRET was measured on a BMG Labtech Pherastar plate reader.

Figure 1f — Lysis buffer equilibration time



Measurement of Histone H3 Phosphorylation using the BacMam Histone H3 [pSer10] Cellular Assay. U-2 OS cells were harvested, resuspended in assay medium and mixed with BacMam Histone H3 Reagent to achieve 10% virus (v/v) concentration. The mix was applied to wells of a 384-well plate, yielding a cell density of ~5,000 cells per well. Following a 24 hour incubation, cells were treated for 1 hour with a serial dilution of Calyculin A. Cells were lysed by adding fully-supplemented 6X lysis buffer containing Tb-anti-Histone H3 [pSer10] Antibody and equilibrated for 3 hours at room temperature before TR-FRET was measured on a BMG Labtech Pherastar plate reader.

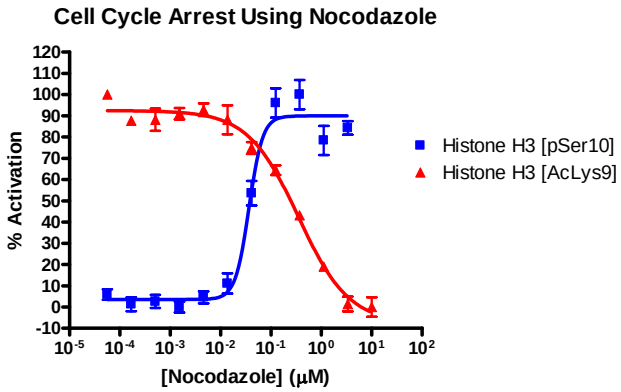
Figure 1g — Aurora Kinase Inhibitor Testing



Aurora Kinase Inhibitor VX-680 Profile. U-2 OS cells were transduced and plated onto a 384-well assay plate as described in Figure 1. Following a 24 hour incubation, cells were treated for 1 hour with a serial dilution of VX-680 followed by one hour incubation with 80 nM of Calyculin A. Cells were lysed by adding fully-supplemented 6X lysis buffer containing Tb-anti-Histone H3 [pSer10] Antibody and equilibrated for 3 hours at room temperature before TR-FRET was measured on a BMG Labtech Pherastar plate reader.

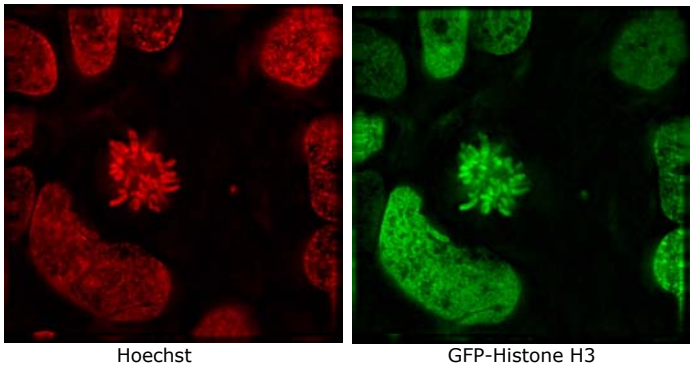
Representative Data for BacMam Histone H3 [pSer10] Cellular Assays

Figure 1h — Cell cycle-dependent phosphorylation



Cell-cycle arrest at M phase leading to increased Ser10 phosphorylation of Histone H3. U-2 OS cells were harvested, resuspended in assay medium and mixed with BacMam Histone H3 Reagent to achieve 10% virus (v/v) concentration. The mix was applied to wells of a 384-well plate, yielding a cell density of ~5,000 cells per well. Following a 24 hour incubation, cells were treated for 3 hours with serial dilutions of nocodazole. Cells were lysed by adding fully-supplemented 6X lysis buffer containing Tb-anti-Histone H3 [acLys9] antibody or [pSer10] antibody and equilibrated for 3 hours at room temperature before TR-FRET was measured on a BMG Labtech Pherastar plate reader.

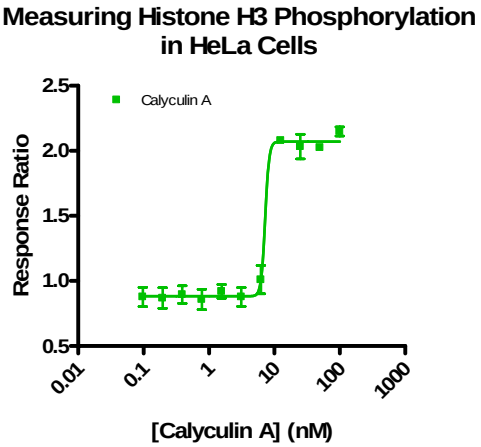
Figure 1i — Localization of GFP-Histone H3



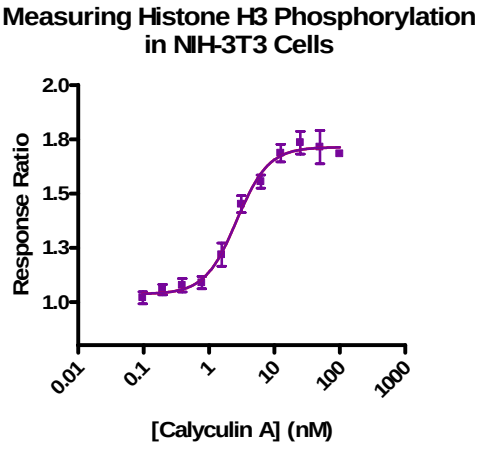
Hoechst GFP-Histone H3

Colocalization of GFP-Histone H3 with DNA in the nucleus. U-2 OS cells were transduced with 10% (v/v) BacMam GFP-Histone H3 reagent for 20 hours prior to Hoechst staining and imaging.

Figure 2a — Alternate Cell Backgrounds



Measurement of Histone H3 Phosphorylation using the BacMam Histone H3 [pSer10] Cellular Assay. HeLa cells were harvested, resuspended in assay medium and mixed with BacMam Histone H3 Reagent to achieve 30% virus (v/v) concentration. The mix was then applied to wells of a 384-well plate, yielding a cell density of ~10,000 cells per well. Following a 24 hour incubation, cells were treated for 1 hour with a serial dilution of Calyculin A. Cells were lysed by adding fully-supplemented 6X lysis buffer containing Tb-anti-Histone H3 [pSer10] Antibody and equilibrated for 3 hours at room temperature before TR-FRET was measured on a BMG Labtech Pherastar plate reader.



Measurement of Histone H3 Phosphorylation using the BacMam Histone H3 [pSer10] Cellular Assay. NIH-3T3 cells were seeded and simultaneously transduced overnight in a T75 flask using 25% (v/v) BacMam Histone H3 Reagent in combination with 0.5 µM Trichostatin A. After 24 hours transduction, cells were harvested, resuspended in assay medium, and then applied to wells of a 384-well plate at a cell density of ~10,000 cells per well. Following an additional 24 hour incubation, cells were treated for 1 hour with a serial dilution of Calyculin A. Cells were lysed by adding fully-supplemented 6X lysis buffer containing Tb-anti-Histone H3 [pSer10] Antibody and equilibrated for 3 hours at room temperature before TR-FRET was measured on a BMG Labtech Pherastar plate reader.

General References:

- Carlson, C. B.; Mashock, M.J.; Bi, K., BacMam-enabled LanthaScreen cellular assays for PI3K/Akt pathway compound profiling in disease-relevant cell backgrounds. *J. Biomol. Screen* **2010**, *15*, 327-334.
- Robers, M. B.; Horton, R. A.; Bercher, M. R.; Vogel, K. W.; Machleidt, T., High-throughput cellular assays for regulated post-translational modifications. *Anal. Biochem*. **2008**, *372*, 189–197.
- Huwiler, K.G.; Machleidt, T.; Chase, L.; Hanson, B.; Robers, M. B., Characterization of serotonin 5-hydroxytryptamine-1A receptor activation using a phosphor-extracellular-signal regulated kinase 2 sensor. *Anal. Biochem*. **2009**, *393*, 95-104.
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