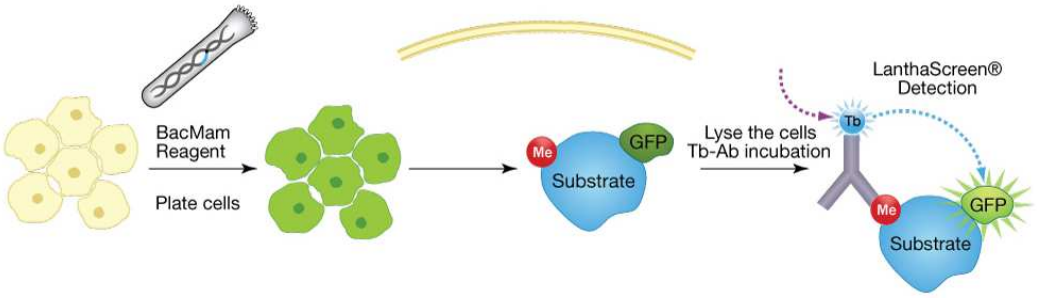
BacMam Histone H3K4me3 Cellular Assay

Overview

BacMam-enabled Cellular Histone H3 Assay Using LanthaScreen® Technology. BacMam-enabled Histone H3 assay is an HTS-compatible cellular immunoassay measuring Histone H3 post-translational modifications. 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 post-translational modifications of histones. BacMam provides a convenient genetic delivery tool for a GFP-Histone H3 fusion protein in the cell line of interest. GFP-Histone H3 is shown to localize in the nucleus and incorporate into chromatin (See reference Machleidt, et al)



Representative Assay Workflow. Cells are mixed with BacMam Reagent encoding GFP-tagged Histone H3 protein and plated in a 384-well assay plate. Cells are left untreated or treated with compound for 20 to 24 hours. Cells are lysed in the presence of a terbium-anti-Histone H3K4me2 antibody and TR-FRET is detected using a fluorescence microplate reader with standard TR-FRET settings.

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Validation experiments of BacMam Histone H3K4me3 Cellular Assay were performed in a 384-well format using U-2 OS as a host cell line. The methylation status of GFP-Histone H3 was measured using LanthaScreen® Tb-anti-Histone H3K4me3 antibody as a detection reagent. Included here are representative results, including an example of the assay optimization process, which can be applied to your cell line of choice.

1. Antibody Specificity Profile

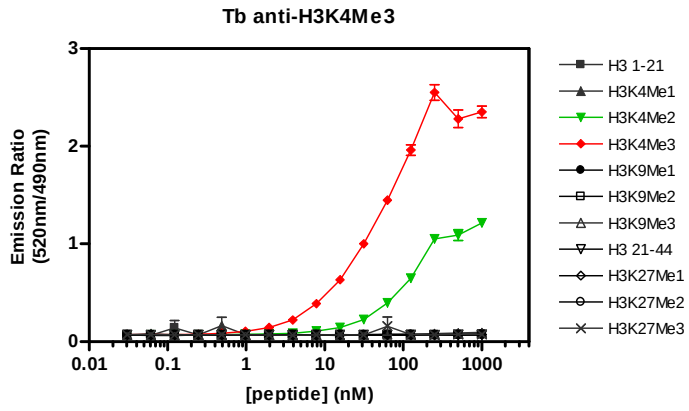
2. Representative assay optimization and validation for Histone H3K4me3 assay using U-2 OS cells

- a. Optimization of BacMam Histone H3 transduction
- b. Optimization of cell plating density
- c. Lysis buffer equilibration time

3. Alternate Cell Backgrounds Tested (HeLa, MM231, MCF7, T47D, SkBr3, HuMEC) and siRNA analysis

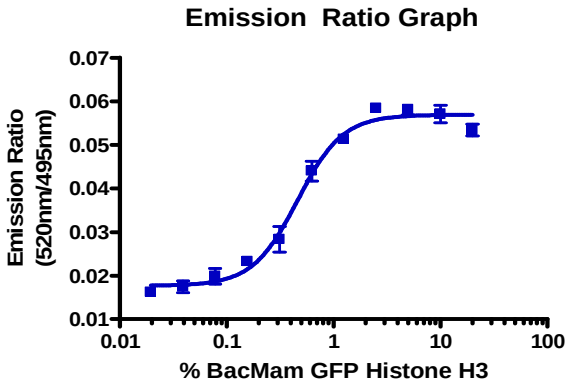
Representative Data for BacMam Histone H3K4me3 Cellular Assay

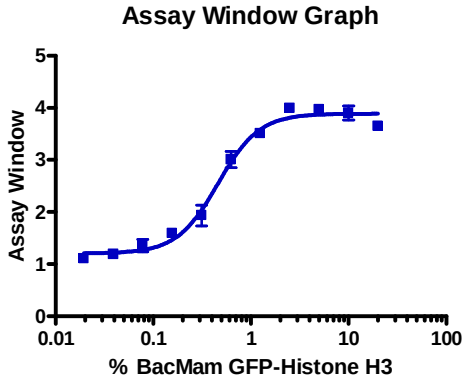
Figure 1 Antibody Specificity Profile



LanthaScreen® Tb-anti-Histone H3K4me3 antibody specifically binds to Histone H3K4me3 peptide and to a lesser degree H3K4me2 peptide. Tb-anti-Histone H3K4me3 antibody (1 nM) was incubated with Alexa Fluor® 488-Streptavidin (10 nM) and indicated amounts of biotinylated-Histone H3 peptides (Anaspec) for 30 min in a 384-well assay plate at room temperature. Following the incubation, TR-FRET signal was measured on a BMG Labtech PHERAstar Plus plate reader. Raw TR-FRET Emission Ratios (520nm/490nm) are shown.

Figure 2a — Optimization of BacMam Histone H3 Transduction (Titration of BacMam Reagent)

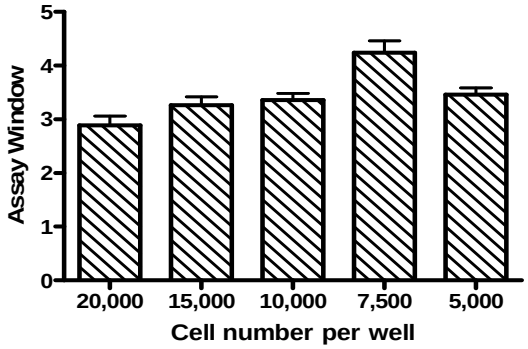




BacMam Histone H3 reagent titration. U-2 OS cells were harvested, resuspended in assay medium (McCoy's + 10% dialyzed FBS) and plated in a 384-well plate to a density of ~7500 cells per well. A serial dilution of BacMam Histone H3 Reagent (v/v) was applied to designated wells of a 384-well plate. Following a 20 hour incubation, cells were lysed by adding fully-supplemented 6X lysis buffer containing Tb-anti-Histone H3Kme3 antibody (6 nM) and equilibrated for 3 hours at room temperature before TR-FRET signal was measured on a PerkinElmer EnVison™ 2104 plate reader. Raw TR-FRET Emission Ratios (520nm/490nm) and Assay Window normalized to untransduced cell controls are shown. In this case, 2.5% BacMam Histone H3 generated the highest assay window.

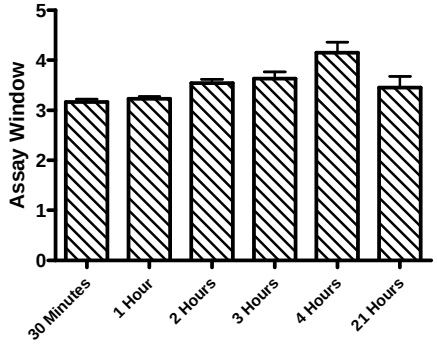
Representative Data for BacMam Histone H3K4me3 Cellular Assay

Figure 2b – Optimization of Cell Plating Density



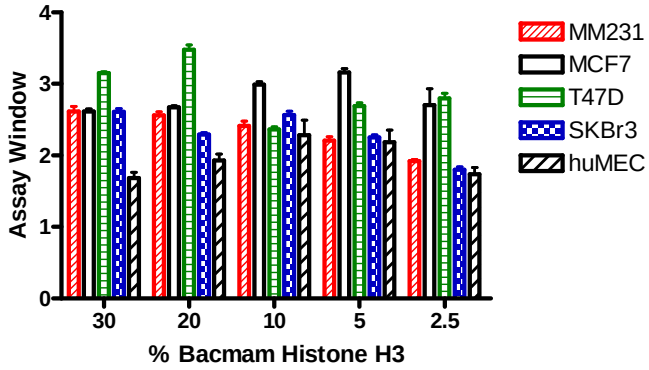
Optimization of cell plating density on an assay plate U-2 OS cells were harvested, resuspended in assay medium, and serial dilutions of the cells were mixed with BacMam Histone H3 Reagent to achieve 2.5% virus (v/v) concentration. The mixtures were applied to the designated wells of a 384-well plate, yielding varying cell densities per well. Following a 20 hour incubation, cells were lysed by adding fully-supplemented 6X lysis buffer containing Tb-anti-Histone H3K4me3 antibody and equilibrated for 3 hours at room temperature before TR-FRET was measured on a PerkinElmer EnvVson™ 2104 plate reader. In this case, 7500 cells/well generated the highest assay window. Assay window is calculated as the emission ratio of each sample divided by the emission ratio of untransduced samples (virus-free).

Figure 2c — Optimization of Lysis Buffer Equilibration Time



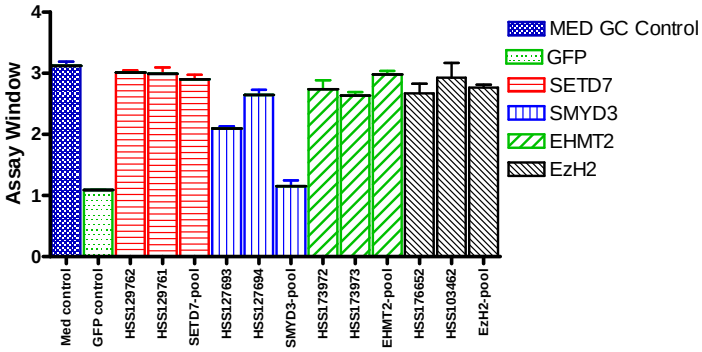
Optimization of lysis buffer (antibody) equilibration time U-2 OS cells were harvested, resuspended in assay medium and mixed with BacMam Histone H3 Reagent to achieve 2.5% virus (v/v) concentration. The mixture was applied to wells of a 384-well plate, yielding a cell density of ~7,500 cells per well. Following a 20 hour incubation, cells were lysed by adding fully-supplemented 6X lysis buffer containing Tb-anti-Histone H3K4me3 antibody and equilibrated for the indicated times before TR-FRET was measured on a PerkinElmer EnVison™ 2104 plate reader. In this case, 4-hour incubation generated the highest assay window.

Figure 3a — Alternate Cell Backgrounds Tested



Measurement of Histone H3K4me3 levels in various cell backgrounds Cells were harvested, resuspended in growth medium, and seeded at ~1,000,000 cell per well of a 6-well plate. BacMam Histone H3 Reagent was added to achieve the required virus concentration (v/v) in the presence of 0.5 X BacMam Enhancer Solution (Invitrogen, PV5835). 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 20 hour incubation, cells were lysed by adding fully-supplemented 6X lysis buffer containing Tb-anti-Histone H3K4me3 antibody and equilibrated for 3 hours at room temperature before TR-FRET was measured on a PerkinElmer EnVision™ 2104 plate reader.

Figure 3b — siRNA Knockdown Analysis



siRNA knockdown analysis shows SMYD3 RNAi decreases H3K4me3 level in U-2 OS cells Lipofectamine® RNAiMAX transfection reagent, validated Stealth RNAi™ Negative Universal Control Med #3 (Cat#46-5373, Cat#46-2001), GFP Reporter Control (Cat#46-5376), SETD7 (Cat#HSS129762, Cat#HSS129761), SMYD3 (Cat#HSS127693, Cat#HSS127694), EHMT2 (Cat#HSS173972, Cat#HSS173973), and EzH2 (Cat#HSS176652, Cat#HSS103462) RNAi oligos are from Life Technologies. Target specific oligo pools were incubated with RNAiMAX (volume relative to surface area of culture vessel) in 1/5 volume final plating volume in Opti-MEM® reduced serum media, and incubated for 20 minutes at room temperature. U-2 OS cells were harvested, resuspended in assay media (Opti-MEM® reduced serum media + 10% dialyzed FBS), and mixed with 1/5 volume of lipid:RNA complexes such that the final concentration for each duplex was 40 nM. Immediately after mixing the cells with RNA:lipid complexes, 26 µl of cell suspension (including RNAi oligos) were added to wells of a 384-well plate, yielding a cell density of

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~7,500 cell per well. Cells were incubated 24h prior to addition of 4 µl of 37.5% BacMam Histone H3 Reagent, yielding a final virus concentration of 5%. Following a 24 hour incubation, cells were lysed by adding fully-supplemented 6x lysis buffer containing Tb-anti-Histone H3K4me3 Antibody and equilibrated for 3 hours at room temperature before TR-FRET was measured on a PerkinElmer EnVision™ 2104 plate reader.

Histone methyltransferase	Histone H3 lysine modifications
SETD7 (SET7/9)	H3K4me1
SMYD3	H3K4me2 and H3K4me3
EHMT2 (G9a)	H3K9me1 and H3K9me2
EZH2	H3K27me1, K27me2, K27me3

General References:

- Machleidt, T.; Robers, M. B.; Hermanson, S. M.; Dudek, J. M.; Bi, K., TR-FRET Cellular Assays for Interrogating Post-Translational Modifications of Histone H3. *J. Biomol. Screen* **2011**, *in press*.
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- 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.
- Kost, T. A.; Condreay, J. P.; Ames, R. S.; Rees, S.; Romanos, M. A., Implementation of BacMam virus gene delivery technology in a drug discovery setting. *Drug Disc. Today* **2007**, *12*, 396-403.
- Robers, M. B.;Loh, C.; Carlson, C. B., Yang, H.; Frey, E. A.; Hermanson, S. A; Bi, K., Measurement of the cellular deacetylase activity of SIRT1 on p53 via LanthaScreen technology. *Mol. Biosyst.* **2011**, 7, 59-66.

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