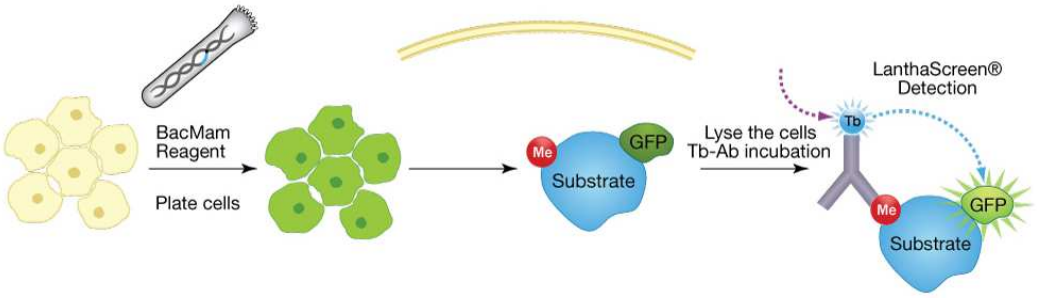
BacMam Histone H3K27me3 Cellular Assay

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 6-well format. 20–24 hours post-transduction, cells are harvested and plated onto a 384-well assay plate. Cells are left untreated or treated with compound for additional 20 to 24 hours. Cells are lysed in the presence of a terbium-anti-Histone H3K27me3 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 H3K27me3 Cellular Assay were performed in a 384-well format using HeLa as a host cell line. The methylation status of GFP-Histone H3 was measured using LanthaScreen® Tb-anti-Histone H3K27me3 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

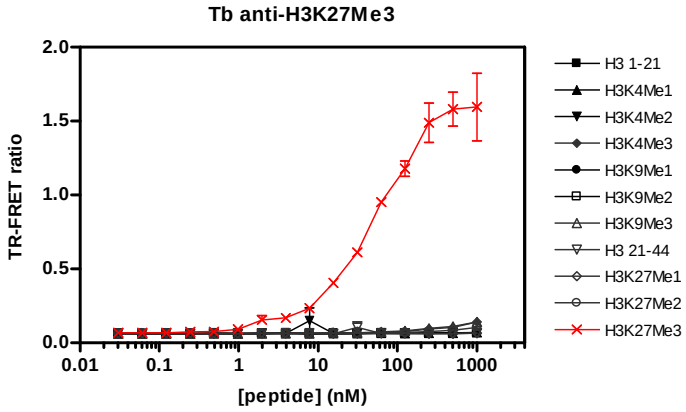
2. Representative assay optimization and validation for Histone H3K27me3 assay using Hela cells

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

3. Alternate Cell Backgrounds Tested (MM231, MCF7, T47D, SkBr3, HuMEC) and RNAi Testing

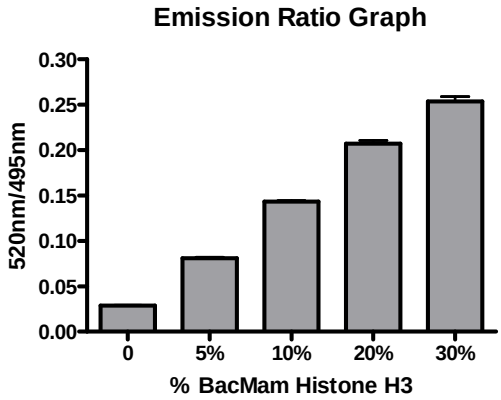
Representative Data for BacMam Histone H3K27me3 Cellular Assay

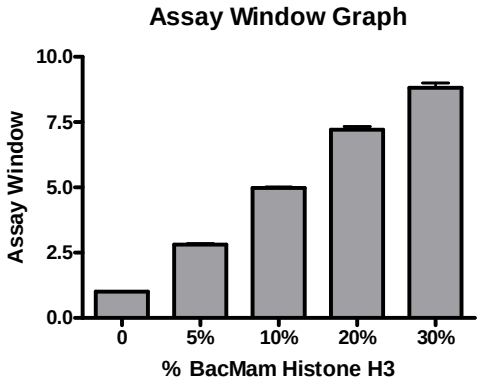
Figure 1 Antibody Specificity Profile



LanthaScreen® Tb-anti-Histone H3K27me3 antibody specifically binds to Histone H3K27me3 peptide. Tb-anti-Histone H3K27me3 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)

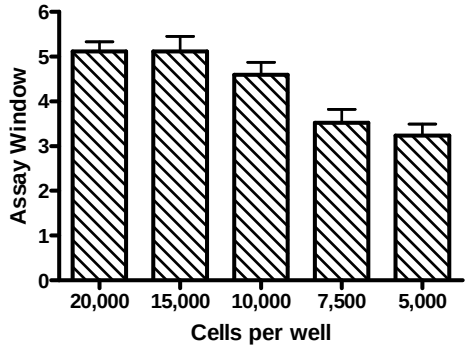




BacMam Histone H3 reagent titration. HeLa cells were harvested, resuspended in growth medium, and seeded at ~1,000,000 cell per well of a 6-well plate. BacMam Histone H3 Reagent was added to achieve the desired virus concentration (v/v) in the presence of 0.5 X BacMam Enhancer Solution (Invitrogen, PV5835). After 24 hours transduction, cells were harvested, resuspended in growth medium, and then plated onto a 384-well plate, to a 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 H3K27me3 Antibody and equilibrated for 3 hours at room temperature before TR-FRET was measured on a PerkinElmer EnVision™ 2104 plate reader. Emission ratios (520nm/490nm) are plotted against the concentration of BacMam reagent (Top graph). Assay Window is calculated by dividing each emission ratio value by the value from the non-transduced wells (cells not expressing GFP-Histone H3) and plotted in the bottom graph. In this case, 30% BacMam Histone H3 generated the highest assay window.

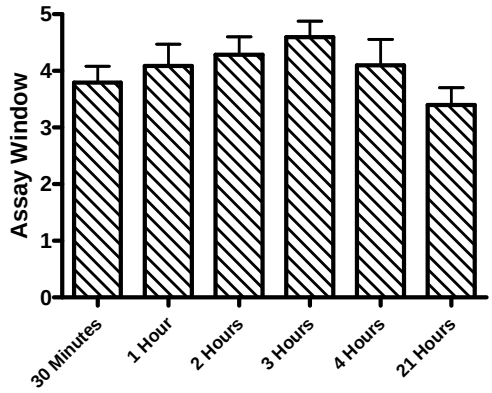
Representative Data for BacMam Histone H3K27me3 Cellular Assay

Figure 2b – Optimization of Cell Plating Density



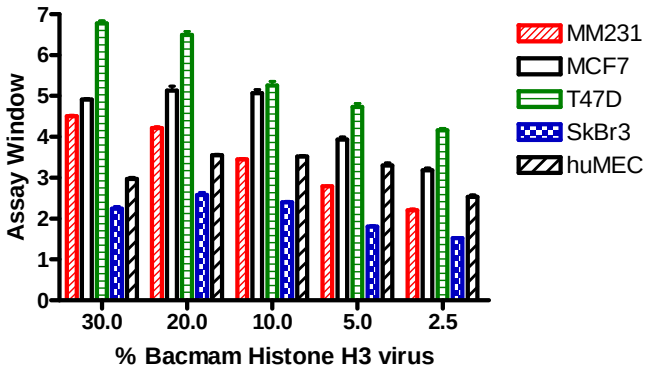
Optimization of cell plating density on an assay plate. HeLa cells were harvested, resuspended in growth medium, and seeded at ~1,000,000 cell per well of a 6-well plate. BacMam Histone H3 Reagent was added to achieve the 30% virus concentration (v/v) in the presence of 0.5 X BacMam Enhancer Solution (Invitrogen, PV5835). After 24 hours transduction, cells were harvested, resuspended in assay medium, and then applied to wells of a 384-well plate, yielding varying cell densities per well. Following an additional 20 hour incubation, cells were lysed by adding fully-supplemented 6X lysis buffer containing Tb-anti-Histone H3K27me3 Antibody and equilibrated for 3 hours at room temperature before TR-FRET was measured on a PerkinElmer EnVision™ 2104 plate reader.

Figure 2c — Optimization of Lysis Buffer Equilibration Time



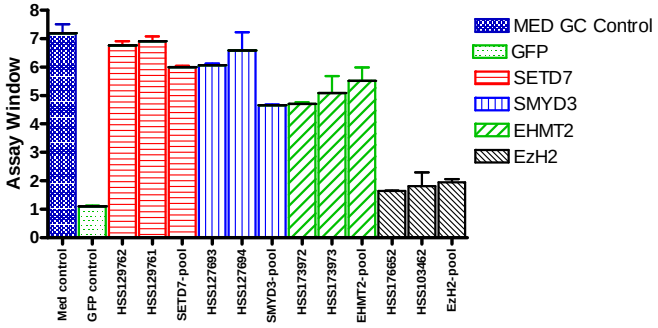
Optimization of lysis buffer (antibody) equilibration time HeLa cells were harvested, resuspended in growth medium, and seeded at ~1,000,000 cell per well of a 6-well plate. BacMam Histone H3 Reagent was added to achieve the 30% virus concentration (v/v) in the presence of 0.5 X BacMam Enhancer Solution (Invitrogen, PV5835). After 24 hours transduction, cells were harvested, resuspended in assay medium, and plated in designated wells of a 384-well plate to a 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 H3K27me3 antibody and equilibrated for the indicated times before TR-FRET was measured on a PerkinElmer EnVision™ 2104 plate reader. In this case, 3 hrs generated the highest assay window.

Figure 3a — Alternate Cell Backgrounds Tested



Measurement of Histone H3 methylation 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 24 hour incubation, cells were lysed by adding fully-supplemented 6X lysis buffer containing Tb-anti-Histone H3K27me3Antibody and equilibrated for 3 hours at room temperature before TR-FRET was measured on a PerkinElmer EnVision™ plate reader.

Figure 3b — siRNA Knockdown Analysis



siRNA knockdown analysis shows EZH2 RNAi decreases H3K27me3 level in HeLa 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. HeLa 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 40nM. Immediately after mixing the cells with RNA:lipid complexes, 2.5 mLs of cell suspension (including RNAi oligos) were added to wells of a 6-well plate, yielding a cell density of ~1,000,000 cell per well. Cells were incubated 24h prior to addition of 500 µl of 90% BacMam Histone H3 Reagent, yielding a final virus

concentration of 15%. After 24 hours transduction, cells were harvested, resuspended in assay medium, and plated in designated wells of a 384-well plate to a 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 H3K27me3 antibody and equilibrated for the indicated times 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*.
- 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.
- 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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