BioTek
Get a Better Reaction.

Application Note

# BacMam-Enabled Cellular Assays Measuring Histone Posttranslational Modifications

Roland Leathers, Kevin Lowitz, Coby Carlson, Thomas Machleidt, Kun Bi, Justin Wetter and Matthew Robers, Invitrogen, 501 Charmany Drive, Madison, WI, USA;
Peter Banks, BioTek Instruments, Inc., Winooski, VT, USA

*Enzymes that modulate histone function through posttranslational modifications are interesting drug targets for a variety of indications, including cancer. Here we demonstrate the utility of using transient transfection of GFP fusions of histones to develop LanthaScreen® Cellular assays to monitor histone posttranslational modifications in a homogeneous format suitable for high throughput screening.*

## Introduction

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. These modifications occur in a tightly regulated fashion, and affect numerous cellular processes including histone deposition, chromatin assembly, and chromosome condensation during both mitosis and meiosis. Therefore, a number of histone modifying enzymes have been identified as valuable targets for therapeutic intervention. The combination of Baculovirus-mediated gene delivery (BacMam) with LanthaScreen® cellular assay technology and measurements using the BioTek Synergy™ 4 Hybrid Multi-Mode Microplate Reader, enables a powerful platform for the analysis of target-specific posttranslational modifications of histones in the cell line of interest. Specifically, we have developed HTS-compatible cellular assays measuring acetylation of histone H3 at Lys9, phosphorylation of histone H3 at Ser10, and the ubiquitination status of histone H2B. These assays together with sensitive filter-based detection on the Synergy™ 4 represent a flexible method to measure the ability of compounds to affect histone posttranslational modifications in a HTS-compatible format.

## Role of HDACs on chromatin structure and transcription.

Histone deacetylases (HDACs) are enzymes that catalyze the removal of the acetyl modification on lysine residues of proteins, including the core nucleosomal histones H2A, H2B, H3, and H4. Together with histone acetylases (HATs), HDACs regulate the level of acetylation of the histones. The balance of acetylation of nucleosomal histones plays an important regulatory role in the transcription of many genes. Hypoacetylation of histones is associated with a condensed chromatin structure resulting in the repression of gene transcription, whereas acetylated histones are associated with a more open or relaxed chromatin structure and activation of transcription (see Figure 1).

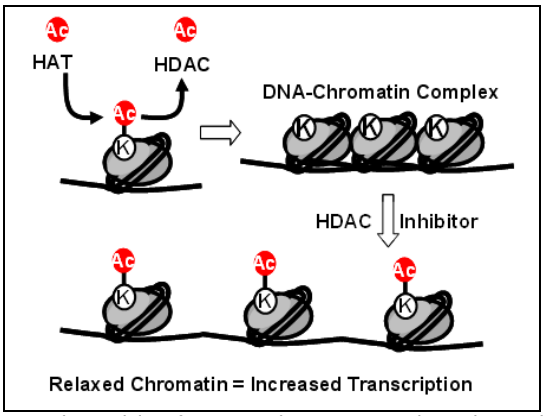



**Figure 1**. The Activity of HATs and HDACs Regulate Chromatin Structure and Transcription.

HDAC inhibitor drug discovery is an active area in oncology in general[1]. This activity has been validated by the recent FDA approval of the HDAC inhibitor vorinostat (SAHA) for treating lymphoma[2].

### LanthaScreen® Cellular Assay Principle

LanthaScreen® cellular assays utilize genetic fusions of GFP with substrates known to be modified in a signal transduction pathway. GFP serves as a FRET acceptor for modification-specific Tb-labeled antibodies in a lysate-based immunoassay. Specific post-translational modifications are measured on a microplate reader as an increase in TR-FRET.

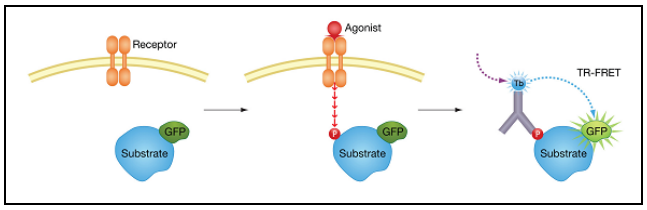



**Figure 2.** LanthaScreen® Cellular Assay Schematic. In this study, the GFP-substrate fusion was GFP-Histone H3 and GFP-Histone H2B.

### Baculovirus-mediated Gene Delivery (BacMam)

Baculoviruses are insect cell viruses that can be modified to express proteins in mammalian cells. The unmodified baculovirus is able to enter mammalian cells, however its genes are not expressed unless a mammalian recognizable promoter is incorporated upstream of a gene of interest. Both unmodified baculovirus and baculovirus modified with a mammalian promoter (BacMam) are unable to replicate in humans and are thus non infectious.

The following attributes have led to its wide spread usage in life science applications for recombinant expression of proteins:

- Biosafety level 1
- Non-toxic to cells
- Easy to use – add BacMam to plated cells and run assay 24 hours later
- High, CMV-driven expression
- Titratable target expression
- Transduces “hard-to-transfect” cells
    - Primary cells
    - Stem cells
- Transient expression for 5 days
- Tolerates large gene inserts up to 38 kb

## Materials and Methods

### GFP Imaging – Verification of BacMam Transduction

HeLa cells were transduced with BacMam GFP-H3 and Invitrogen Organelle Lights™ ER-OFP. 24 hours post-transduction, cells were seeded on sterile coverslips and allowed to adhere overnight. Cells were then imaged on a DeltaVision fluorescence microscope using a 60X objective and a TRITC or GFP filter set.

### LanthaScreen® Cellular Assays

U-2 OS cells were used for all LanthaScreen® Cellular assays. Histone H3 acetylation and phosphorylation was monitored by the use of BacMam GFP-Histone H3 virus; histone H2B ubiquitination was monitored using BacMam GFP-H2B virus. In all assays, BacMam virus was added directly to plated cells in 384-well microplates.

### Histone H3 Acetylation

Following BacMam virus introduction, cells were incubated for 24 hours before being stimulated with serially diluted HDAC inhibitors trichostatin, HDAC inhibitor III, SAHA, CHANA and Na-butyrate. Plates were then incubated for 3 hours. Cells were then aspirated and lysed by addition of lysis buffer containing LanthaScreen® Tb-anti-H3 [acetyl-lysine-9] antibody. Following a 2 hour equilibration at room temperature, TR-FRET signals were measured.

### Histone H3 Phosphorylation

24 hours after BacMam virus introduction, cells were treated for 1 hour with serial diluted VX680 inhibitor prior to 1 hour treatment with calyculin A (phosphatase inhibitor). Cells were then aspirated and lysed by addition of lysis buffer containing LanthaScreen® Tb-anti-H3 [pS10] antibody. Following a 2 hour equilibration at room temperature, TR-FRET signals were measured.

### Histone H2B Ubiquitination

U-2 OS cells were transduced with a fixed concentration of BacMam GFP-H2B and immediately treated with E1 Inhibitor PYR-41, followed by 24 hour incubation. 24 hours posttransduction, cells were then aspirated and lysed by addition of lysis buffer containing LanthaScreen® Tb-anti-ubiquitin-FK2 antibody. Following a 2 hour equilibration at room temperature, TR-FRET signals were measured.

### Synergy 4 Hybrid Multi-Mode Microplate Reader Settings

TR-FRET signals were measured on a Synergy™ 4 Hybrid Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT USA). LanthaScreen® Tb TR-FRET instrument setup: 340/30nm excitation filter, 495/10nm and 520/25nm emission filters, 400nm dichroic mirror, 100µsec delay, 200µsec integration and auto sensitivity selected. Error bars in Figures 4, 5 and 6 represent the average of 3 data points +/- S.E.

## Results and Discussion

### GFP Imaging – Verification of BacMam Transduction

Figure 3 demonstrates the ability of BacMam to deliver GFP-histone fusion proteins into cell lines of interest. GFP-Histone H3 is distinctly localized to the nucleus, indicative of proper functionality.

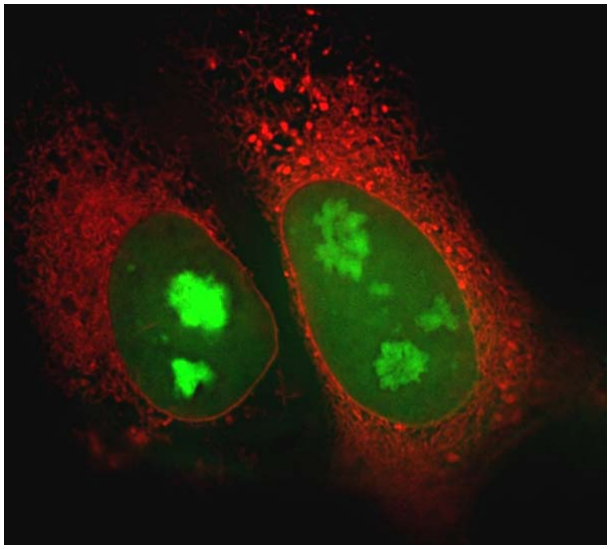
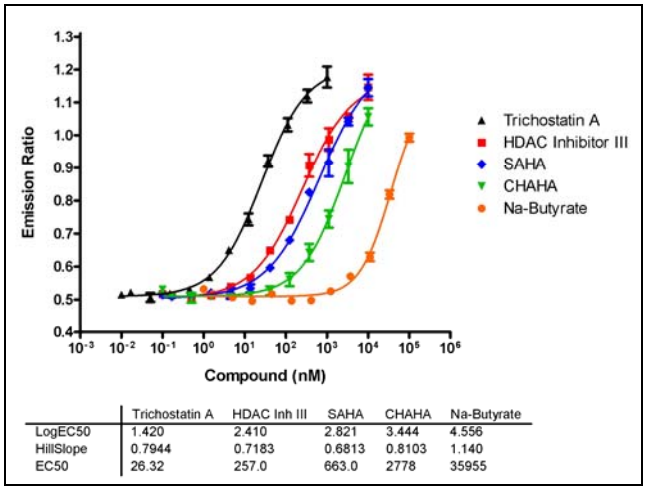


**Figure 3.** 60x imaging of HeLa cells transiently transfected with BacMam GFP-Histone H3 (green) and Organelle Lights ER-OFP (red).

### LanthaScreen® Cellular Assays

Figures 4-6 demonstrates dose-response curves of various inhibitors to a variety of histone post-translational modifications. Assay performance in the form of assay window and precision (error bars of data points) and pharmacology are consistent with useful assays for high - throughput drug screening of inhibitors to deacetylation, phosphorylation and ubiquitination of histones.




| | Trichostatin A | HDAC Inh III | SAHA | CHAHA | Na-Butyrate |
|---|---|---|---|---|---|
| LogEC50 | 1.420 | 2.410 | 2.821 | 3.444 | 4.556 |
| HillSlope | 0.7944 | 0.7183 | 0.6813 | 0.8103 | 1.140 |
| EC50 | 26.32 | 257.0 | 663.0 | 2778 | 35955 |

**Figure 4.** BacMam-Enabled Assays Measuring Acetylation of Lysine 9 of GFP-Histone H3.

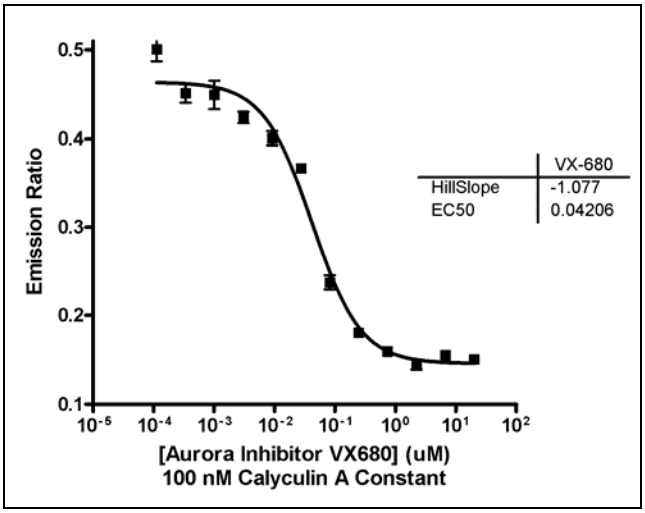



**Figure 5.** Analysis of GFP-H3 Phospho-serine-10 Mediated by Aurora Kinase.

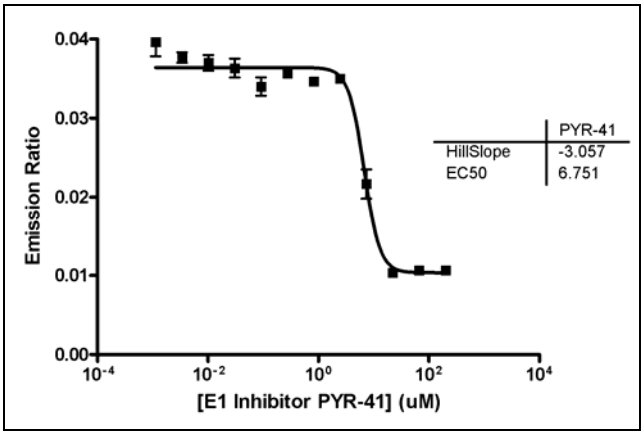



**Figure 6.** Inhibition of Histone H2B Ubiquitination.

## Conclusions

We have demonstrated the ease of use of BacMam technology to effectively transduce GFP-Histone fusion proteins into cell lines of interest and create high throughput screening assays based on LanthaScreen® Cellular assays suitable for the characterization of inhibitors to histone posttranslational modifications. The ability to directly introduce the BacMam into assay plates, greatly facilitates transfection and simplifies assay development.

## References


1. Milos Dokmanovic, Cathy Clarke and Paul Marks (2007). "Histone Deacetylase Inhibitors: Overview and Perspectives." *Mol. Cancer Res*, **5**(10), pp. 981-989.
2. Bhupinder Mann, John Johnson, Martin Cohen, Robert Justice and Richard Pazdur 2007). "FDA Approval Summary: Vorinostat for treatment of Advanced Primary Cutaneous T-Cell Lymphoma." *Oncologist*, **12**, pp. 1247-1252.