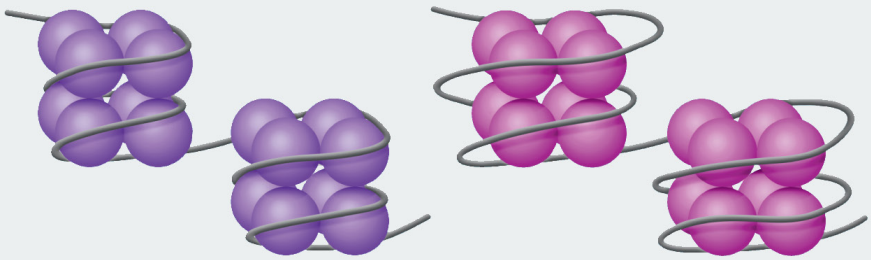
APPLICATION NOTE

Cellular Imaging and Analysis



HDAC inhibitor

Key Features

- Identify candidate compounds using high throughput biochemical AlphaLISA® assays
- Validate candidates in a high content imaging approach

Screening HDAC Inhibitors – A Workflow Comprising High Throughput and High Content Screening

Introduction

An epigenetic trait is a stably inherited phenotype resulting from changes in a chromosome without alterations in the DNA sequence [Berger et al., 2009]. Key mechanisms of epigenetic gene regulation are pathways which affect the packaging of DNA into chromatin, thereby determining the accessibility of DNA to transcription. These pathways include DNA methylation, chromatin remodeling and histone modifications. Aberrations in epigenetic mechanisms are well known to be associated with the biology of cancer, which suggests that epigenetic enzymes may be promising cancer drug targets [Ducasse and Brown, 2006].

In comparison to normal cells, cancer cell genomes are characterized by, among other things, a decrease in histone modifications such as acetylation [Ropero and Esteller, 2007]. Histone acetylation by histone acetyltransferases leads to loosening of DNA-histone interactions, allowing gene expression. In contrast, histone deacetylation by histone deacetylases (HDACs) increases the interaction between DNA and histones and leads to a decrease in gene expression (Figure 1).

PerkinElmer®
For the Better

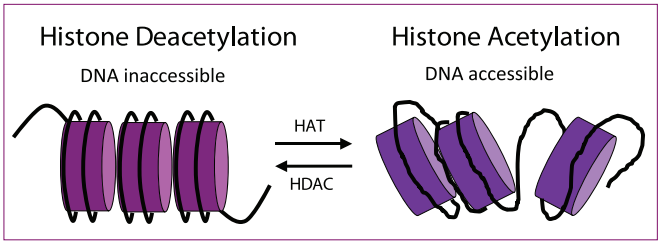


Figure 1: DNA accessibility is regulated by acetylation of core histones. In the non-acetylated state (left side), DNA and histones form a dense nucleosomal structure, so that DNA is not accessible for transcription. Histone acetylation allows loosening of the nucleosomal structure (right side) and is mediated by enzymes with histone acetyltransferase (HAT) activity. This state allows gene expression. Gene repression in return is induced by histone deacetylases (HDACs), which reverse acetylation and allow repackaging of the nucleosomes [Adapted from Adcock et al., 2004].

HDAC inhibitors block histone deacetylation resulting in hyperacetylation of core histones [Yoshida et al., 2001]. Previous studies have shown that HDAC inhibitors can induce tumor cell differentiation, apoptosis, or growth arrest, and can enhance the *in vitro* sensitivity of tumor cells to radiation [Chavez-Blanco et al., 2005]. In addition, the use of HDAC inhibitors as part of combinatorial therapies for the treatment of various cancers has shown to be promising [Pollock and Richon, 2009].

To allow efficient screening of large compound libraries, intelligent screening workflows are needed that combine different assay formats and multiplex various readouts, including phenotypic information, to provide a more holistic view of compound effects. This application note, developed in collaboration with the European ScreeningPort, describes a workflow for HDAC inhibitor screening and demonstrates the combination of a high throughput AlphaLISA® assay and a high content assay using the Operetta® High Content Imaging System.

Biochemical Approach

PerkinElmer AlphaLISA epigenetic assays are highly sensitive, no-wash immunoassays that allow the measurement of epigenetic enzyme activities. Critical components of the assay are a peptide substrate containing an epigenetic modification site, the epigenetic enzyme, Alpha Streptavidin Donor beads and AlphaLISA Acceptor beads.

Firstly, the effect of the reference compound Trichostatin A (TSA) on the HDAC-1 enzyme was measured using an AlphaLISA HDAC1 Histone H3-Lysine 9 Deacetylase Assay. This assay uses a biotinylated peptide derived from histone H3, acetylated at lysine 9, as a substrate (H3K9ac). Upon deacetylation of the substrate by HDAC-1, the AlphaLISA Acceptor beads bind to the substrate through a specific antibody reaction. This reaction brings the Donor bead, which is bound to the substrate by its Streptavidin moiety, and the Acceptor bead into close proximity. Upon laser irradiation at 680 nm, short-lived singlet oxygen molecules are produced by the Donor beads. If the Acceptor bead is in close proximity to the Donor bead, an AlphaLISA signal is generated at 615 nm.

To perform the AlphaLISA assay, all steps were performed as previously described [Rodriguez-Suarez et al., 2011]. Briefly, HDAC-1 was pre-incubated with different concentrations of the HDAC inhibitor TSA, or Assay Buffer alone, for 5 min at room temperature, before addition of the H3K9ac peptide (AnaSpec, 64361). After 1h of enzymatic reaction time, AlphaLISA Acceptor Beads (PerkinElmer, AL138C) were added and the reaction mix was incubated for 1h, followed by addition of Streptavidin Donor Beads (PerkinElmer, 6760002). After a final incubation of 30 min the AlphaLISA signal was measured using the EnVision® Multilabel Plate Reader, with the standard instrument settings for Alpha. TSA inhibits the HDAC-1 enzyme in a dose-dependent manner (IC50 = 2.3 nM) (Figure 3). With the validated assay, a compound library from the Centre for Agriculture and Biosciences International (CABI), based on extracts of the fungus *Beauveria bassiana*, was screened for potential HDAC-1 inhibitors (data not shown). Biochemically active HDAC-1 inhibitors were submitted for further investigation in a cellular imaging assay.

Cellular Imaging Approach

To compare the inhibitory effect of TSA on histone acetylation in the biochemical and the cellular setting, an imaging assay using U2OS w.t. cells was developed. In addition to histone H3K9 acetylation, the acetylation of lysine 27 and the expression of p21 were analyzed. P21 is known to be affected by HDAC inhibition and plays a crucial role in controlling cell growth at the G1/S cell cycle check point [Sambucetti, 1999]. To perform the assay, cells were seeded at a density of 7,500 cells per well into a 384-well CellCarrier™ microtiter plate (PerkinElmer, 6007558).

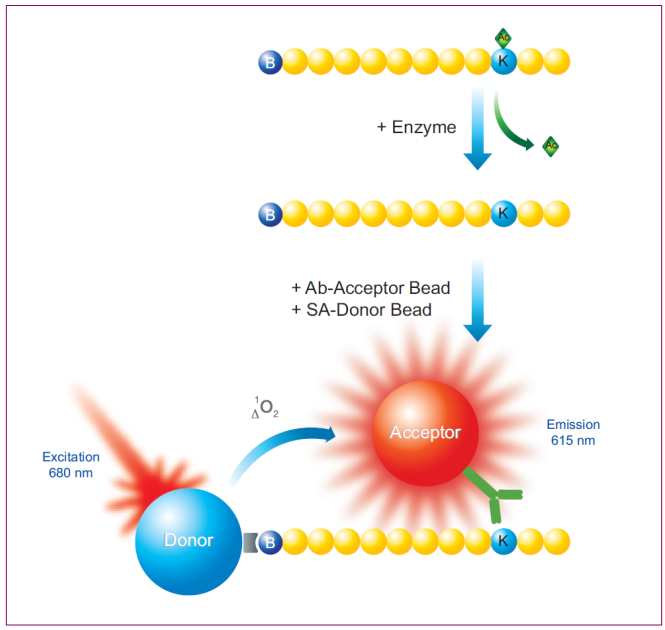


Figure 2: Working principle of the AlphaLISA assay. Acceptor beads bind to the target peptide by a specific antibody reaction. Upon laser irradiation at 680 nm, short-lived singlet oxygen molecules are produced by the Donor beads. If the Acceptor bead is in close proximity to the donor bead, an AlphaLISA signal is generated at 615 nm.

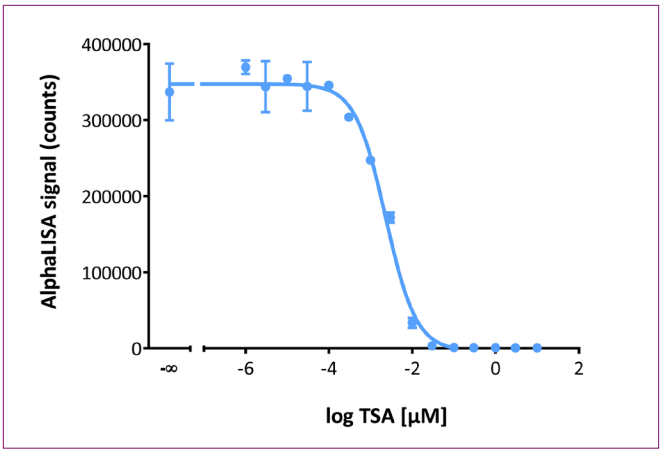


Figure3: Dose response curve representing the deacetylation of H3K9 by HDAC-1 in the presence of various Trichostatin A (TSA) concentrations (IC50 = 2.3 nM). TSA was pre-incubated for 5 min with 1.5 nM HDAC-1. Enzymatic reactions were initiated by the addition of 50 nM biotinylated H3K9ac peptide substrate.

After 8-10 h cultivation, cells were treated with TSA, the DMSO vehicle, or cultivation medium. After 24 h of incubation, cells were fixed with formaldehyde, permeabilized (0.2% Triton X-100) and incubated with 3% BSA to block all unspecific binding sites. The cells were labeled using an anti-p21 primary antibody (Santa Cruz, sc-397), followed by an AlexaFluor®555 secondary antibody conjugate (Invitrogen, A21428). Subsequently, histone H3 acetylation at lysine 9 or lysine 27 was visualized using FITC labeled primary monoclonal antibodies. The anti-H3K9ac or anti-H3K27ac antibodies were applied as a mixture with 22.7 µM Hoechst 33342 (Invitrogen, H-3570). Images were acquired using the Operetta® High Content Imaging System equipped with a 20X high NA objective in wide field fluorescence mode (Figure 4).

Quantitative evaluation of the imaging results was performed using Harmony® High Content Imaging and Analysis Software. To segment the nuclei, the Find Nuclei Building Block was applied to the Hoechst channel image. The total number of nuclei provided the first readout. Next, a ring region of 9 px width was generated around the nuclei and the Calculate Intensity Properties Building Block was used to determine the mean H3K9ac or H3K27ac intensity as well as the mean p21 intensity inside the nuclear region and inside the ring region. To correct for background intensity from the cytoplasm, the mean intensity in the ring region was subtracted from the mean intensity in the nuclear region. The resulting value was used as a readout parameter for histone H3 acetylation. The dose-response curve calculated to show the effect of TSA on H3K9 resulted in an IC50 value of 37.7 nM (Figure 5A, similar data for H3K27 is not shown). In parallel, the effect of TSA on the cell cycle was monitored. While the expression of the cell cycle regulatory protein p21 increased with increasing TSA concentrations (EC50 = 34.8 nM), the total number of cells decreased, indicating a p21-mediated inhibition of cell cycle progression (Figure 5B).

Using this imaging assay, the effect of a novel HDAC-inhibitor, BML-210, was also monitored. Due to the low solubility of BML-210 in aqueous solutions, 1.0 mM was the highest concentration that could be tested. Treatment of the cells with various concentrations of BML-210 resulted in a dose-dependent increase in H3K9ac fluorescence intensity with an IC50 value of 10.02 µM (Figure 5C). As observed with TSA, treatment with BML-210 also increased the expression of p21 (EC50 = 50.81 µM) and reduced the total number of cells per well (Figure 5D). Although higher concentrations were needed to induce a response, BML-210 treatment resulted in a stronger response on the p21 intensity readout and in a stronger effect on the total number of cells when compared to TSA treatment.

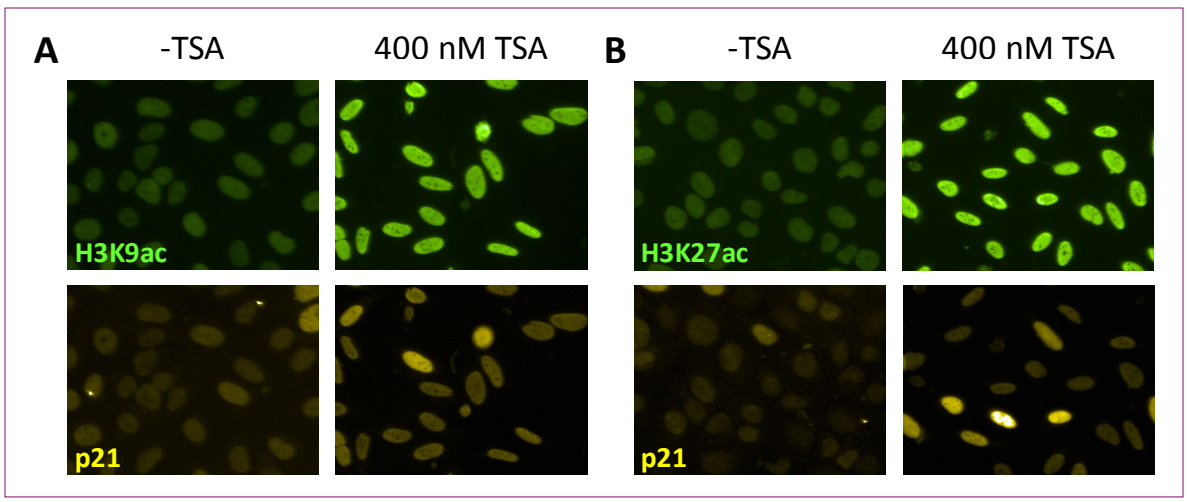


Figure 4: Trichostatin A (TSA) inhibits histone deacetylation and induces p21 expression in U2OS w.t. cells. By inhibiting HDAC enzymes with TSA, acetylated of histone H3 lysine 9 (H3K9ac) and lysine 27 (H3K27ac) sites are more abundant and the fluorescence intensity increases (upper images). TSA treatment also leads to higher p21 expression levels in the nuclei of individual cells (lower images). (A) Cells labeled with anti-H3K9ac and anti-p21 antibody. (B) Cells labeled with anti-H3K27ac and anti-p21 antibody.

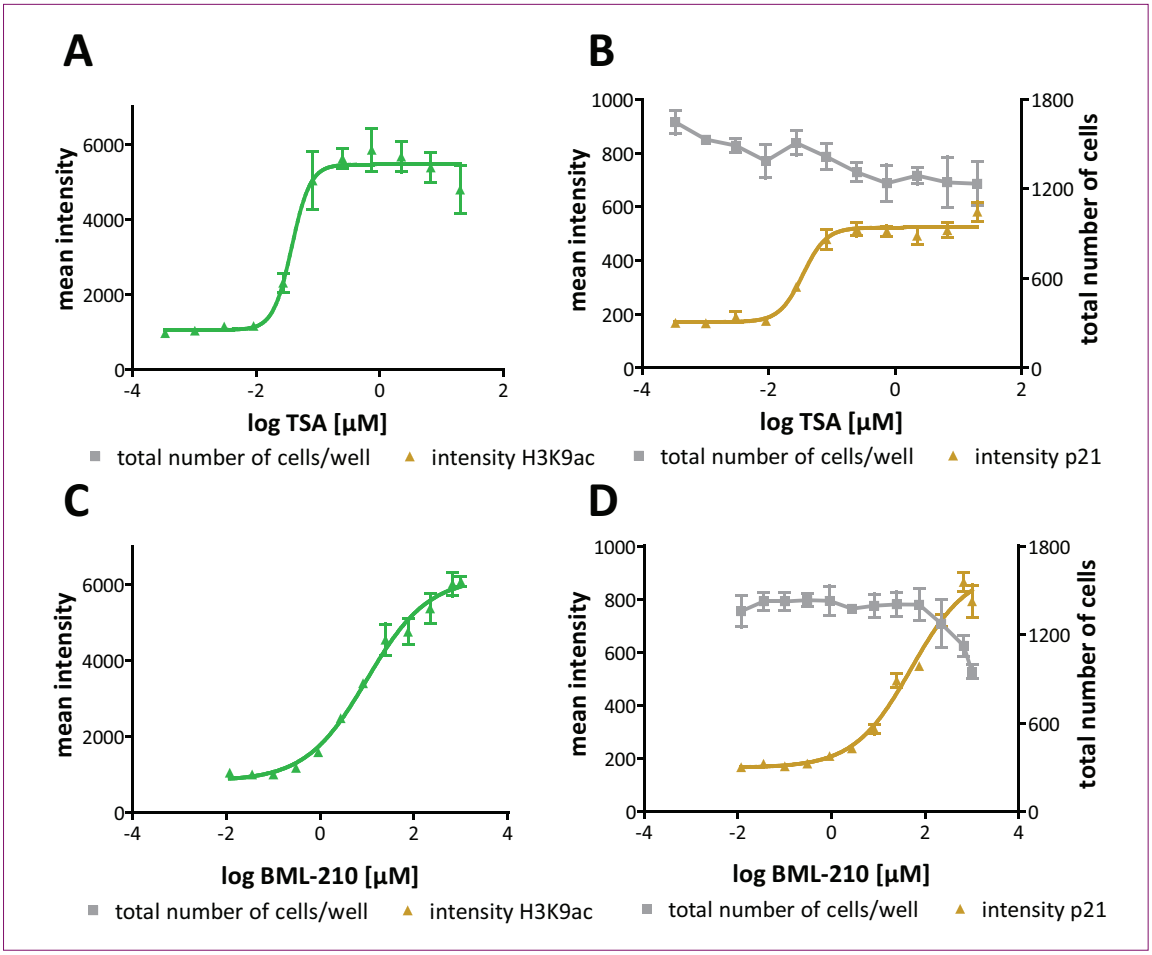


Figure 5: High Content Analysis of the effects of TSA and BML-210 on acetylation of H3K9 and the cell cycle in U2OS w.t. cells. (A and C) H3K9ac signal. Acetylation of H3K9 increases in a dose dependent manner (IC50 = 37.7 nM; Z' = 0.48 for TSA and IC50 = 10.02 µM; Z' = 0.86 for BML-210).
(B and D) P21 signal (yellow) combined with the total number of cells/well (gray). The expression of p21 increases (EC50 = 34.8 nM; Z' = 0.36 for TSA and EC50 = 50.81 µM; Z' = 0.41 for BML-210) and the total number of cells decreases dose-dependently. This suggests that both compounds induce a p21-mediated cell cycle arrest.

Conclusion

Biochemical assays often mark the early stage of target-based drug screening approaches and allow screening for active compounds in a sensitive and reliable high throughput manner. The cell-free approach provides a simple and controlled environment to test a wide spectrum of compound libraries. The AlphaLISA epigenetic toolbox reagents provide high sensitivity, a wide dynamic range, resistance to fluorescent interference, and a rather native peptide substrate compared to other biochemical epigenetic assays on the market. This makes them an attractive choice to screen compound libraries for effects on epigenetic targets. They are also easily scalable and automatable using the JANUS® Automated Workstation.

High content imaging assays provide a variety of information in addition to the direct effects of compounds on specific targets. These assays can also be multiplexed to address more, and perhaps indirect, targets. Additional information, for instance about cell cycle effects, phenotypic changes in cells or cell organelles, changes in staining textures or cytotoxic effects, can be also derived. Today, an increasing number of drug approvals are due to phenotypic based cellular screenings [Swinney and Anthony, 2011], which can be run on high content instruments such as the Operetta system. The high content imaging assay described in this application combines several readouts on just one plate – acetylation of histone H3, expression of p21 and cell growth arrest.

As a cellular system is more biologically relevant compared to the biochemical setting, it is much more likely that a compound identified in a cell-based screen can be translated into a therapeutic agent. For example, it is critical that compounds are able to permeate the cell membrane. Several putative HDAC inhibitors that were identified in the biochemical AlphaLISA screen proved ineffective in our cellular assay (data not shown), which might be due to lack of cell permeability.

In this application we illustrate the potential power of combinatorial screening campaigns. By running an AlphaLISA assay on the EnVision Multilabel Plate Reader as a primary screen, a number of active compounds can be selected which can then be further analyzed and verified in a cellular assay using the Operetta High Content Imaging System.

References

Adcock, I.M., Ito, K., and Barnes, P.J. (2004): Glucocorticoids: effects on gene transcription. Proceedings of the American Thoracic Society, 1 (3), 247–54.

Berger, S.L., Kouzarides, T., Shiekhattar, R., and Shilatifard, A. (2009): An operational definition of epigenetics. Genes & development, 23 (7), 781–3.

Bertrand, P. (2010): Inside HDAC with HDAC inhibitors. European journal of medicinal chemistry, 45 (6), 2095–116.

Chavez-Blanco, A., Segura-Pacheco, B., Perez-Cardenas, E., Taja-Chayeb, L., Cetina, L., Candelaria, M., Cantu, D., Gonzalez-Fierro, A., Garcia-Lopez, P., Zambrano, P., Perez-Plasencia, C., Cabrera, G., Trejo-Becerril, C., Angeles, E., and Duenas-Gonzalez, A. (2005): Histone acetylation and histone deacetylase activity of magnesium valproate in tumor and peripheral blood of patients with cervical cancer. A phase I study. Molecular cancer, 4 (1), 22.

Ducasse, M. and Brown, M.A. (2006): Epigenetic aberrations and cancer. Molecular cancer, 5 (1), 60.

Pollock, R.M. and Richon, V.M. (2009): Epigenetic approaches to cancer therapy. Drug Discovery Today: Therapeutic Strategies, 6 (2), 71–79.

Rodriguez-Suarez, R., Loiselle, M.-C., Gauthier, N., Roy, M., Pedro, L., and Beaudet, L. (2011): AlphaLISA HDAC1 Histone H3-Lysine 9 Deacetylase Assay. PerkinElmer Technical Note, Available at: http://www.perkinelmer.com/CMSResources/Images/44-131908TCH_AlphaLISA_HDAC1_Histone_H3-Lysine_9_Deacetylase_Assay.pdf

Ropero, S. and Esteller, M. (2007): The role of histone deacetylases (HDACs) in human cancer. Molecular oncology, 1 (1), 19–25.

Sambucetti, L.C. (1999): Histone Deacetylase Inhibition Selectively Alters the Activity and Expression of Cell Cycle Proteins Leading to Specific Chromatin Acetylation and Antiproliferative Effects. Journal of Biological Chemistry, 274 (49), 34940–34947.

Swinney, D.C. and Anthony, J. (2011): How were new medicines discovered? Nature reviews. Drug discovery, 10 (7), 507–19.

Yoshida, M., Furumai, R., Nishiyama, M., Komatsu, Y., Nishino, N., and Horinouchi, S. (2001): Histone deacetylase as a new target for cancer chemotherapy. Cancer chemotherapy and pharmacology, 48 Suppl 1 S20–6.

Authors

Nadine Fricke
Karin Boettcher

PerkinElmer
Cellular Technologies Germany GmbH
Cellular Imaging & Analysis
Hamburg, DE

Stephan Spindler
Sheraz Gul

European ScreeningPort GmbH
Hamburg, DE

PerkinElmer, Inc.
940 Winter Street
Waltham, MA 02451 USA
P: (800) 762-4000 or
(+1) 203-925-4602
www.perkinelmer.com

For a complete listing of our global offices, visit www.perkinelmer.com/ContactUs

Copyright ©2013, PerkinElmer, Inc. All rights reserved. PerkinElmer® is a registered trademark of PerkinElmer, Inc. All other trademarks are the property of their respective owners.

010974_01