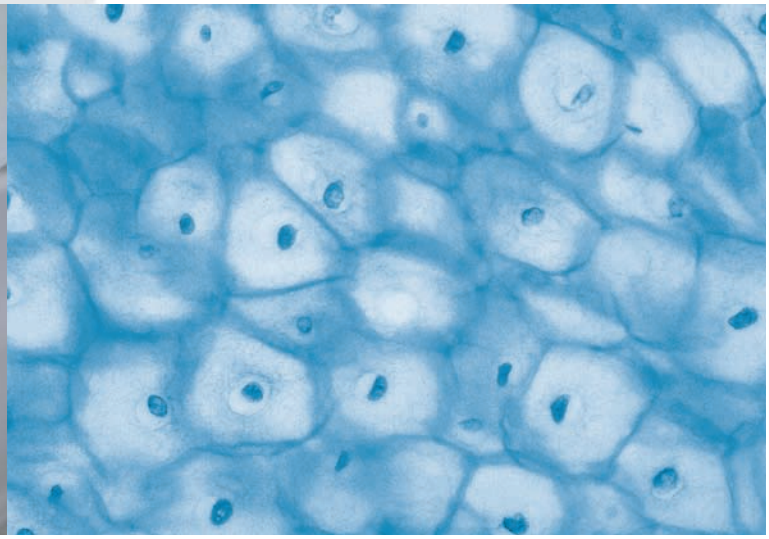
Application Note



EPIgeneous™ H3K27Me3 Cellular Assay for measuring epigenetic methylation

Easy automation of complex, two-plate protocol on the Fluent™ laboratory automation solution

Introduction

Epigenetics has played an important role in drug discovery for many years, but its importance has grown significantly recently due to the role epigenetic modifications can play in cancer and stem cell differentiation. Methylation is the principal epigenetic modification of DNA and of proteins known as histones, which package DNA into chromatin inside a cell. Epigenetic modifications define how genetic information is read and used by cells and, through cell divisions, these epigenetic changes may last for the duration of the cell's life. They may also last for multiple generations, even though they do not involve changes in the underlying DNA sequence of the organism. Epigenetic modifications can therefore be inherited, and are influenced by environmental factors, some of which can induce epigenetic signaling that may contribute to biological processes such as aging.
The Cisbio EPIgeneous cell-based assays enable direct measurement of methylation, demethylation, and total proteins on both adherent and suspension cells.

Tecan has re-invented automation with Fluent, a unique instrumentation concept built around the application-specific needs of your lab. Fluent breaks new ground, delivering more capacity and increased speed.

The platform provides superior throughput and walkaway time, making it easier to get more done, with a high level of confidence in the results.

Tecan and Cisbio – renowned for their HTRF® technology – have developed protocols that demonstrate the power of their combined expertise in automated liquid handling, detection and cell-based assays, bringing a new dimension to performing fast, reliable and completely automated assays without the need for expert automation personnel.

Materials and methods

Figure 1: The Fluent cell-based assay workstation. A Fluent 780 is shown, equipped with an eight-channel Flexible Channel Arm, a Multiple Channel Arm 384 and a Robotic Gripper Arm. A Carousel is integrated onto the right hand side of the instrument, along with the latest generation of CO2 incubator. An Infinite M1000 PRO microplate reader is located below the Dynamic Deck. The instrument was also equipped with a HEPA hood (not shown).

Fluent laboratory automation solution

Fluent (Figure 1) is the latest in Tecan's successful family of liquid handling automation platforms. The Fluent cell-based assay solution offers rapid high definition pipetting for both the eight-channel Flexible Channel Arm (FCA) and the Multiple Channel Arm 384 (MCA384) its patented Dynamic Deck™ increases the worktable capacity and boosts productivity by allowing integration of a wide range of Tecan modules – including a Carousel™, for storage of various consumables; a HydroSpeed™ plate washer (not required for this assay); an Infinite® M1000 PRO plate reader; and carriers for troughs, stacked disposable tips and microplates (up to six deep on the worktable, Figure 2). The Multiple Channel Arm 384 uses adapters, which can be automatically exchanged during a run, allowing it to act as either a 384- or a 96-channel arm within the same protocol. Fully independent, task-specific arms allow parallel processing and coordinated scheduling, ensuring the runs are completed faster and more efficiently. Each Fluent cell-based assay solution is equipped with:

- Flexible Channel Arm (FCA) – fitted with eight pipetting channels using disposable tips that can individually access any well or tube, perfect for explicit sample and control distribution or serial dilutions.
- Multi-Channel Arm (MCA) – instantly swaps between 96- and 384- channel adapters during a run, offering outstanding capabilities for reagent distribution or plate replication.
- Robotic Gripper Arm (RGA) – quickly and smoothly transfers plates and consumables between storage modules, integrated devices and the worktable without interrupting pipetting.

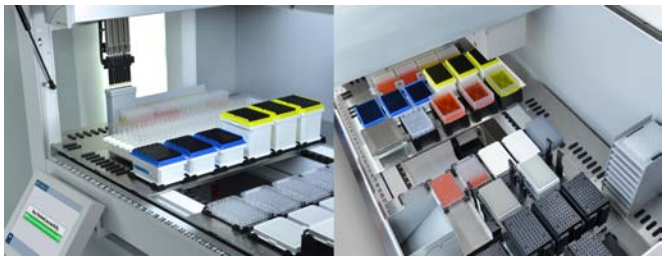


Figure 2: The Dynamic Deck can accommodate ANSI/SLAS-format carriers six deep on the worktable. (Left) Instrument equipped with six boxes of disposable tips for the Flexible Channel Arm (blue = 200 µl, yellow = 1,000 µl). (Right) Top view of the Dynamic Deck showing positions for nested disposable tips, troughs, the active carrier for the MCA 384 and a hotel at the rear.

EPIgeneous H3K27Me3 Cellular Assay Kit

Overview

Cisbio's cellular assay has been developed with optimized reagents and protocols for the direct detection of endogenous levels of H3K27Me3. HTRF is a flexible assay format that enables one kit to be used for a variety of applications, without having to compromise on sensitivity. The H3K27Me3 assay can be used for adherent or suspension cells, primary or secondary screening and inhibitor studies.

Assay technology principle

The assay is designed for simple, rapid, direct detection of endogenous levels of the H3K27Me3 epigenetic mark. The trimethylation of lysine 27 on histone H3 is detected using a sandwich assay incorporating two different specific antibodies; a donor labeled with Eu3+-cryptate and an acceptor labeled with d2. When the dyes are in close proximity, excitation of the donor with a light source (laser or flash lamp) triggers fluorescence resonance energy transfer (FRET) towards the acceptor, which the fluoresces at a specific wavelength (665 nm). One conjugate binds to histone H3 and the other binds to K27Me3, thereby generating FRET. The specific signal modulates positively in proportion to trimethylation on lysine 27 (Figure 3).

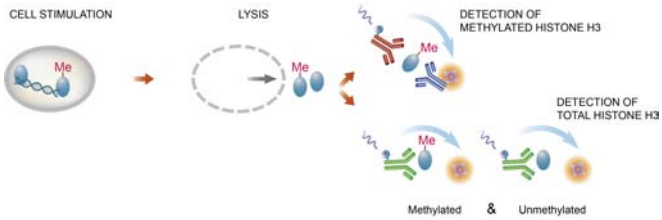


Figure 3: EPIgeneous H3K27Me3 Cellular Assay principle. The sandwich assay directly detects endogenous levels of methylation in cells. The site-specific methylation on histone H3 is detected using two different specific antibodies, one labeled with Eu3+ cryptate (donor), the other with d2 (acceptor). The signal is proportional to the level of methylation.

Automation process

Overview

The epigenetic H3K27Me3 Cellular Assay was run using both 96- and 384-well plates, testing the effect of the compounds in a dose-response experiment. A serial dilution plate was set up in 96-well format using the Fluent Flexible Channel Arm, and the contents were then transferred into a 96-well plate containing the cells using the Multiple Channel Arm with a 96-channel adapter. In order to determine the optimal assay window, three different cell lines were seeded in different cell densities, generating nine assay plates. The plates were incubated for 24, 48 or 72 hours respectively, before the cells were lysed. After lysis, the contents of the 96-well plate were divided into two 384-well plates, following a two-plate protocol to detect total H3 and H3K27Me3 independently.

Detailed Automation assay

The cells were plated at 100 µl/well in a lidded 96-well tissue culture-treated plate in an appropriate growth medium and incubated for 3 to 4h at 37°C in a CO_2 atmosphere to allow cell adhesion. This step could also be automated if required, however, offline cell plating is frequently preferred. The 96-well compound plates containing 30 µl of the test compounds, for example GSK 126 in column 10, were loaded into the Carousel together with the disposable tips (15 µl) for the Multiple Channel Arm 384. The tip boxes with disposable tips for the Flexible Channel Arm were loaded onto the Dynamic Deck ahead of the automation run. The reagents – Lysis Buffer A parts 1 and 2, mixed Lysis Buffer A and premixed HTRF conjugates – are added to the Dynamic Deck before starting the respective part of the assay.

When the run was initiated on the Tecan Fluent cell-based assay laboratory automation solution, the Robotic Gripper Arm transferred the compound plate from the Carousel to the Dynamic Deck of the system and the lid was automatically removed. A serial dilution of GSK 126 in cell culture medium was carried out using the Flexible Channel Arm with disposable tips to create a 10-point dilution gradient (Figure 4, top). When complete, the Robotic Gripper Arm replaced the plate lid.

Once serial dilution of all the compound plates was completed, the Robotic Gripper Arm collected a cell plate and automatically removed the lid. 20 µl aliquots of the serially diluted compound was transferred into the cell plates using the Multiple Channel Arm with a 96-channel adapter (Figure 4, center). The lid was replaced on the assay plate, and offline incubation was started in a CO_2 incubator.

A two-step cell lysis protocol was started after 24, 48 or 72 h. Using the Multiple Channel Arm with the 96-channel adapter, 40 µl of Lysis Buffer A part 1 was added to each well (Figure 4, bottom). The Robotic Gripper Arm automatically handled removal and replacement of the lid, transferring the plate afterwards to the MIO™ (Tecan) on-deck incubator for 45 minute shaking incubation at room temperature. Subsequently, the plate was taken from the MIO using the Robotic Gripper Arm and, after removing the lid, Lysis Buffer A part 2 was added using the Multiple Channel Arm with the 96-channel adapter and disposable tips, followed by a thorough mixing step.

As shown in Figure 5, following the two-step lysis procedure a two-plate protocol began by transferring 10 µl from each well into a 384-well plate. Four transfers were made into a 384-well plate – potentially this could be four transfers from the same 96-well plate to establish a quadruplet data set (Figure 5B), or from four different 96-well plates, to increase the number of compounds tested. After adding the HTRF conjugate premix using the Multiple Channel Arm with the 384-channel adapter, the plate was used for detection of H3K27Me3. Fully automated exchange from a 96- to a 384-channel adapter took less than 5 seconds.

A second 384-well plate was prepared for the detection of total H3 by transferring 3 µl from the assay plate (Figure 5D). After automatic exchange of the 96-channel adapter with the 384-channel adapter, two subsequent liquid handling steps were carried out using the 15 µl diposable tips as shown in Figure 5E and 5F, in preparation for the read-out.

All assay plates were sealed to reduce evaporation during overnight incubation. Although in this case sealing was carried out offline, the procedure could also be automated. After an overnight incubation, the plates were read using the Infinite M1000 PRO plate reader.

Detailed assay information

Three different cell lines (SU-DHL-6, MDA-MB-231, OCI-LY-19) were used. In order to discover the optimal assay conditions, four different cell densities were deployed, in duplicate, per 96-well plate.

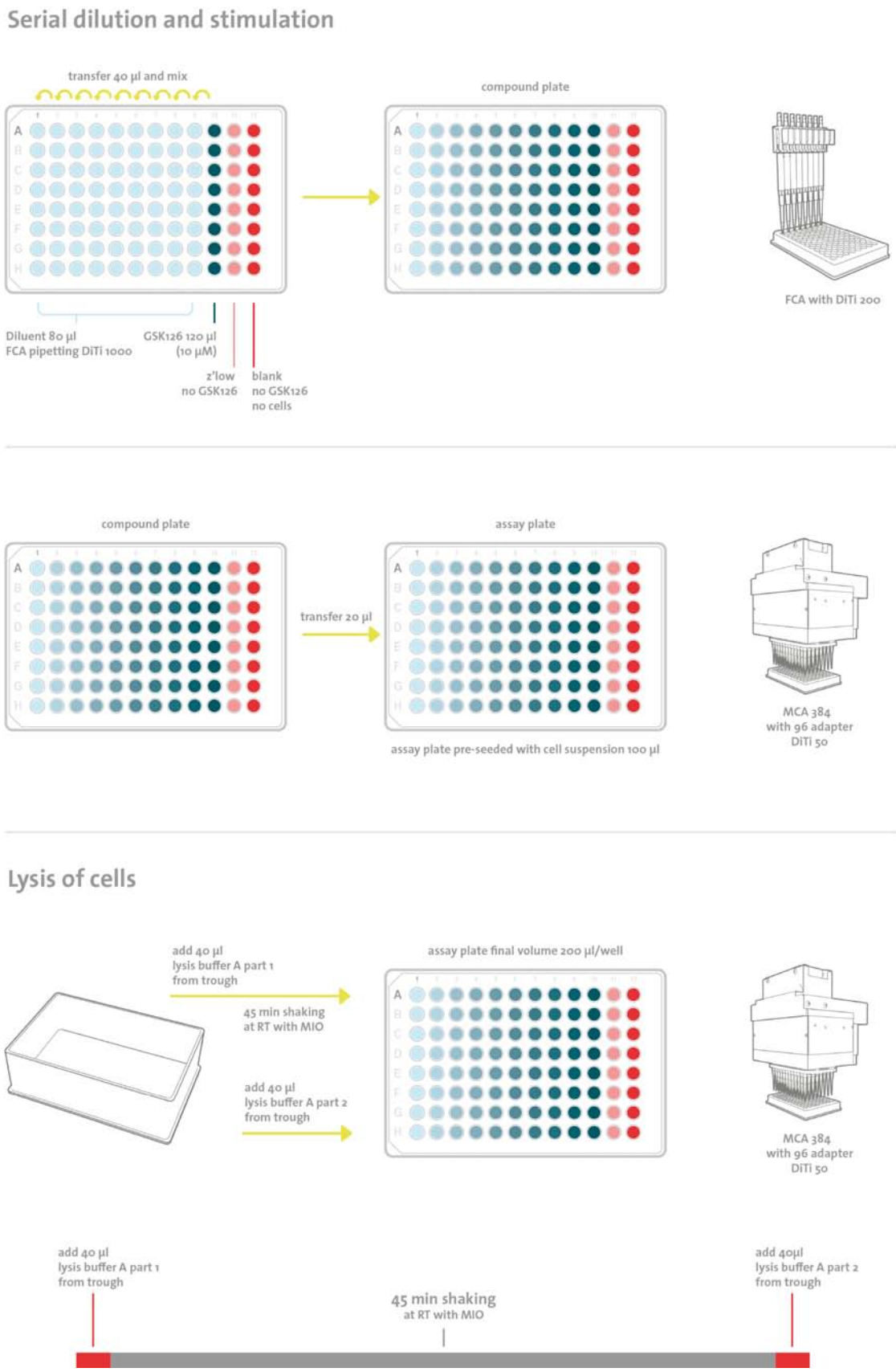


Figure 4: Plate layouts and pipetting schemes for serial dilution, stimulation and lysis of cells. The experiments began with a serial dilution using the Flexible Channel Arm, followed by stimulation of the pre-seeded cells, deploying the Multiple Channel Arm with the 96-chanell adapter. From this point onwards, the cell plate is referred to as the assay plate. Lysis of the cells takes place in two stages, with a 45 min incubation time in between. Lysis buffer is added with the Multiple Channel Arm and 96-channel adapter as indicated, followed by an additional mixing step after dispensing.

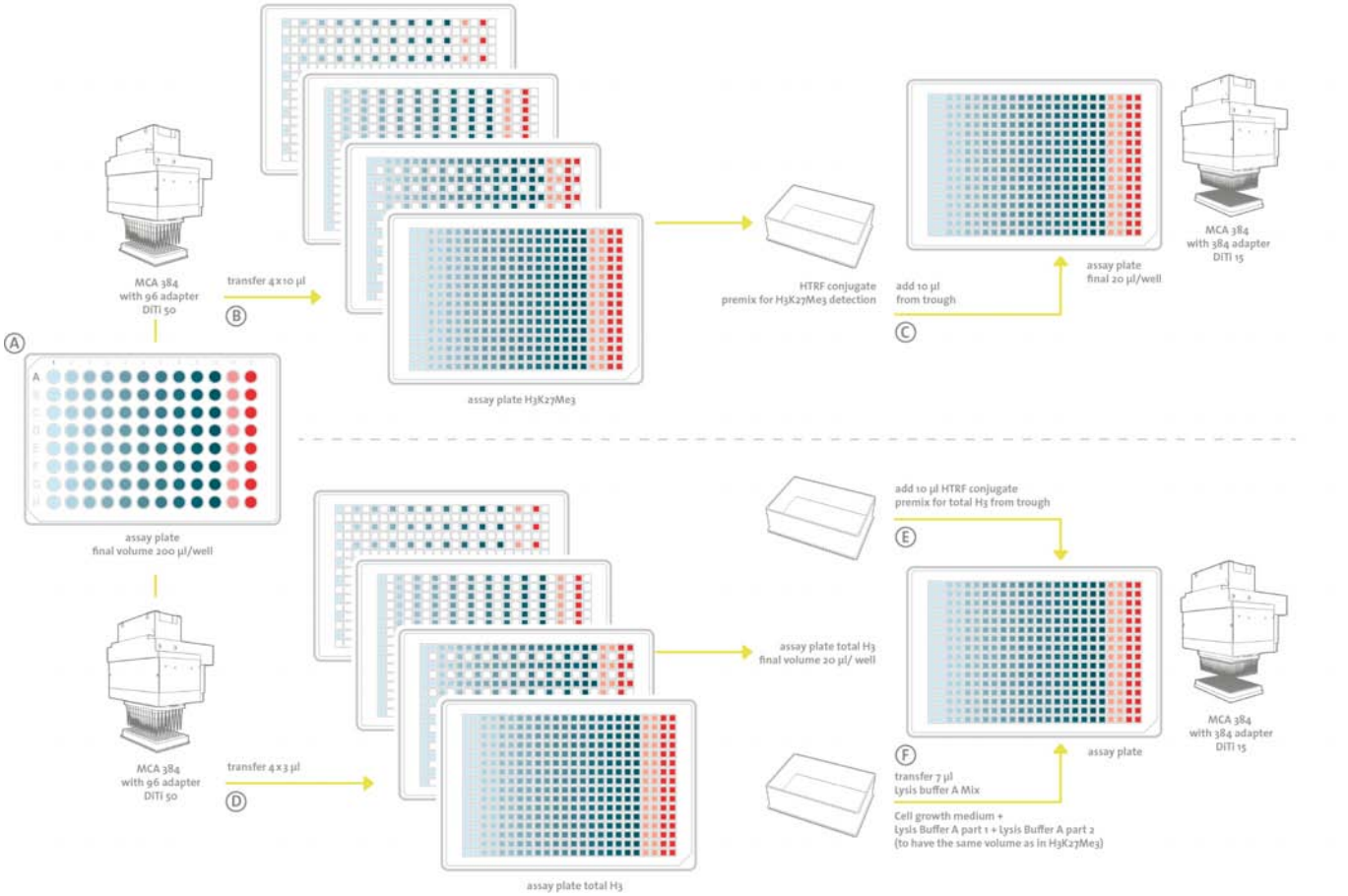


Figure 5: Two-plate protocol following cell lysis. Assay plate with lysed cells (A) is divided into the plates for H3K27Me3 (B,C) and total H3 (D, E, F) detection as indidcated.

Results

The overexpression of the well-known EZH2 histone methyltransferase is implicated in tumorgenesis, and generally correlates with poor prognosis in several tumor types. In addition, in diffuse large B cell lymphoma, somatic heterozygous mutations can occur on specific residues within the catalytic SET domain of EZH2. This allows the mutated enzyme to be very efficient at catalyzing the reaction from di- to tri-methylation. Cell lines such as the SU-DHL-6 used here co-express both the mutant and wild type (WT) EZH2, which leads to hypertrimethylation of lysine 27 on histone H3. However, cell lines such as OCI-LY-19 (B cell lymphoma without mutation) and MDA-MB-231 (breast cancer cell line) only express the WT version of the enzyme. These cell lines mostly accumulate H3K27Me2, and have a low level of H3K27Me3.

As expected, using EPIgeneous cellular assays we were able to detect a high level of H3K27Me3 in SU-DHL-6 compared to a low level in the OCI-LY-19 and MDA-MB-231 cell lines that only express WT EZH2. However, endogenous levels of total H3 were very close for all three cell lines. Moreover, as the grow over time in the well, the level of H3K27Me3 increases in parallel of total H3, until the signal reaches a plateau. The efficient integrated hood system ensures no contamination is observed, even after 72 h incubation. For a given time and cell line, this type of data illustrates perfectly that choosing a suitable cell concentration is essential to make sure that further compound study is in the linear range of the assay (Figure 6).

Figures 7, 8 and 9 show the time course for GSK 126 in a dose dependent manner for three different cell lines. Investigation of the mode of action of this compound showed that it is active on both EZH2 wild type and mutant cell lines, with close IC50 values. Analysis showed that although inhibition of H3K27Me3 began after 24 h, potency was maximal after two days. The 72 h incubation time establishes a good assay window that clearly demonstrates the compound effect. Moreover, the data perfectly illustrates the importance of selecting a suitable cell concentration, which may vary for different cell lines. For example, 72 h incubation of SU-DHL-6 (20,000 cells/well) results in a dose-response dependent inhibition with an acceptable modulation

assay window, and IC50 shows good correlation with the literature. However, at higher cell density (60,000 cells/well), this cell line is clearly outside the linear range of the assay, with a shift in the IC50 value, resulting in a reduced assay window.
In addition, a normalization study performed using the EPIgeneous Total H3 Cellular Assay clearly illustrates the toxicity of the compound above a concentration of 3.33 µM. Below this concentration, total histone H3 is not affected by the compound, indicating that the observed reduction of H3K27 methylation is specific and not due to cell death.

All results presented in this application note are calculated from octuplet data. The robustness of this assay on the Fluent laboratory automation solution has been investigated, and the z' value was calculated using two plates of SU-DHL-6 cells with and without GSK126 inhibitor at 1 µM (380 samples of each condition).The concentration has previously been shown to have no effect on total H3. The z' value calculated was 0.71 (Figure10), qualifying the assay as excellent.

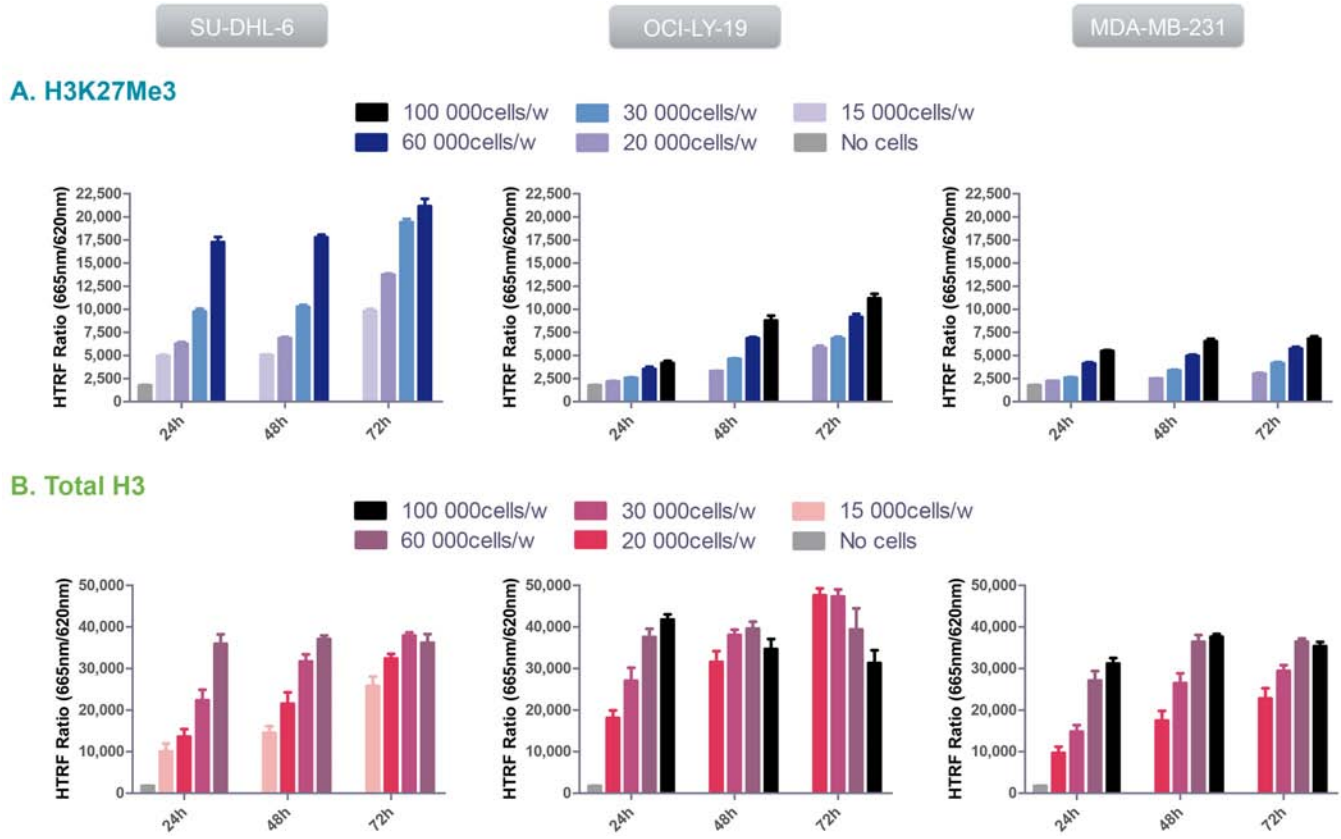


Figure 6: HTRF detection of H3K27Me3 mark basal level (Panel A) and associated total H3 (Panel B) in three cell lines: SU-DHL-6 (suspension cells, with heterozygous mutation of EZH2 histone methyltransferase (Y461N) affecting substrate selectivity, leading to high levels of H3K27Me3), OCI-LY-19 (suspension cells) and MDA-MB-231(adherent cells) with an associated weak level of H3K27Me3.

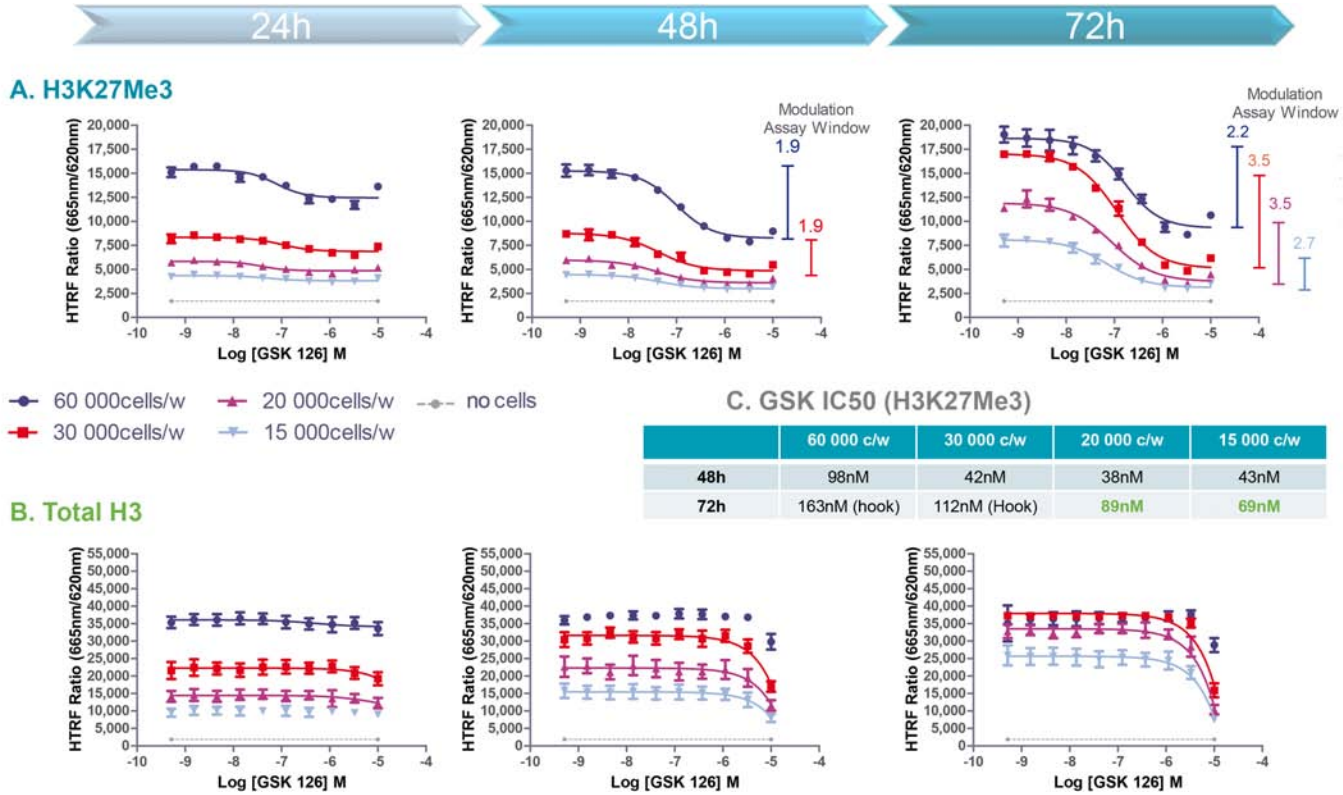


Figure 7: Detection of H3K27Me3 mark (Panel A) and associated total H3 used for normalization (Panel B) on SU-DHL-6. Cell line treated with GSK126 for 24, 48 and 72 h. Associated IC50 observed on H3K27Me3 summarized in Panel C. Green values highlighting the best conditions to allow good correlation with reference publications and HTRF technology (linear range of the assay). Only 10 µM of GSK 26 induced a decrease on total H3 (toxicity). Data from octuplicates with standard deviation.

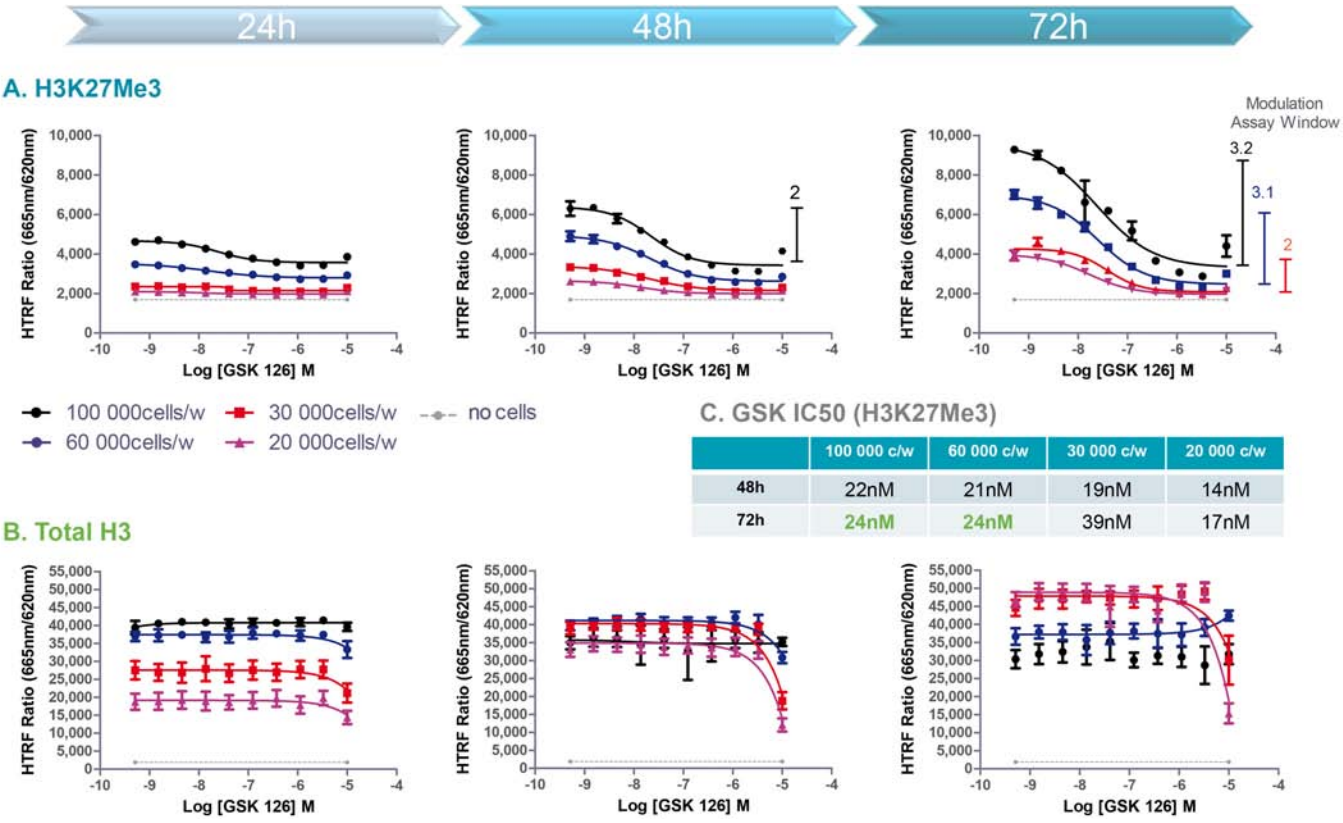


Figure 8: Detection of H3K27Me3 mark (Panel A) and associated total H3 used for normalization (Panel B) on OCI-LY-19. Cell line treated with GSK126 for 24, 48 and 72 h. Associated IC50 observed on H3K27Me3 summarized in Panel C. Only 10 µM of GSK126 induced a decrease in total H3 (toxicity). Data from octuplicates with standard deviation.

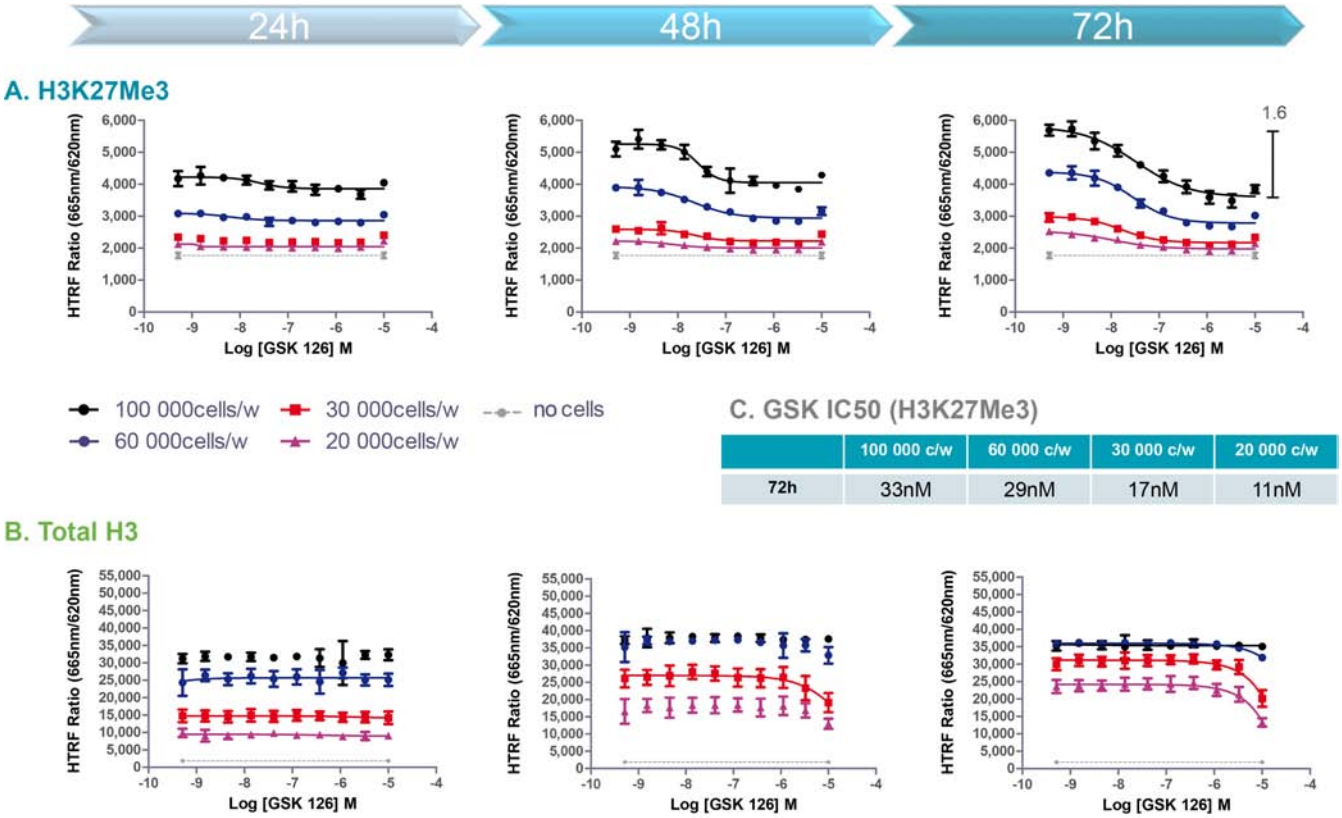


Figure 9: Detection of H3K27Me3 mark (Panel A) and associated total H3 used for normalization (Panel B) on MDA-MB-231. Cell line treated with GSK126 for 24, 48 and 72 h. Associated IC50 observed on H3K27Me3 summarized in Panel C. Only 10 µM of GSK126 induced a small decrease in total H3 (toxicity). Interestingly, GSK126 compound seems to be less active and toxic on this breast cancer cell line. Data from octuplicates with standard deviation.

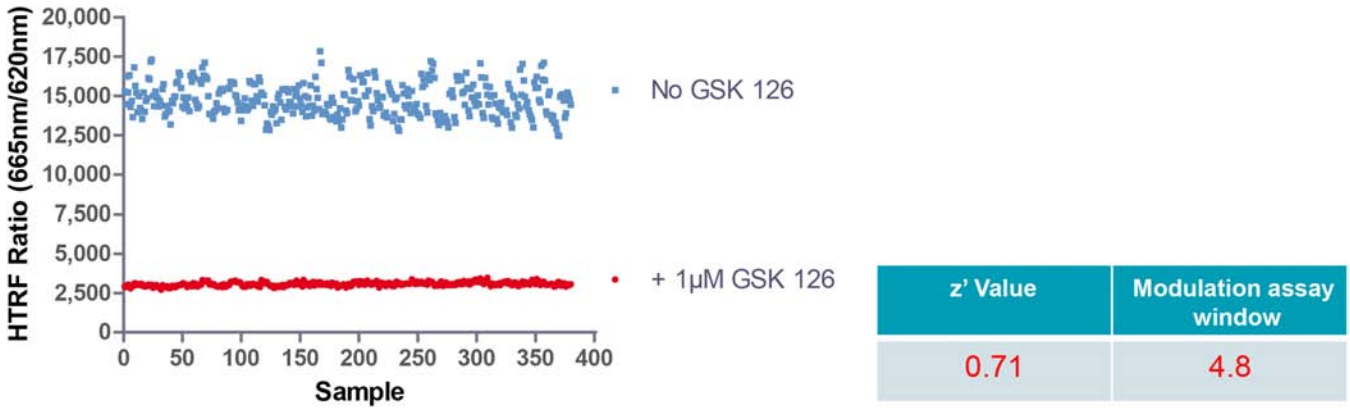


Figure 10: Assessment of robustness for EPIgeneous H3K27Me3 Cellular Assay using SU-DHL-6 cell line (20,000 cells/well) and 1 µM GSK126 (no effect on total H3 at this concentration) incubated with cells for 72 h. 95 wells of each condition were transferred in quadruplicate, leading to 380 samples for both basal (no GSK126) and inhibited conditions (+1 µM GSK126).

Summary

These cell-based assays were optimized to meet the format requirements of researchers screening epigenetic targets or using them as phenotypic read-out of upstream cell targets. For high throughput screening purposes, the no-wash streamlined assay protocol needs a robust liquid handling system in order to produce the most reliable output. From that perspective, this paper illustrates very clearly how HTRF assays can be easily implemented on the Fluent laboratory automation solution to offer an efficient and robust high throughput screening solution.

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