BRD4 Bromodomain 1 Inhibitor Screening using a Homogeneous Proximity Assay

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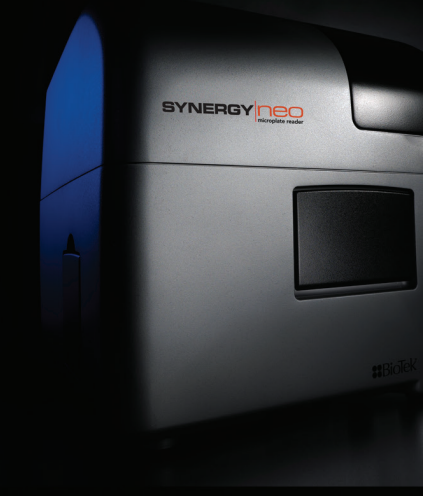

Key Words:

Epigenetics

Bromodomain

TR-FRET

Acetylation

Histone Modification

Introduction

Post-translational histone modifications are critical for modulation of chromatin structure by direct DNA interaction and indirect molecular interactions with recruited nuclear proteins. Chromatin structure modulation is proven necessary for gene regulation, repair and cell cycle progression (Figure 1) and other processes. Bromodomains are small nuclear proteins that selectively bind to acetylated lysine residues of histone proteins[1]. Once bound, these bromodomains recruit protein complexes involved in chromatin structure regulation, and thus play a role in gene expression. The bromodomain and extra-terminal (BET) protein family includes BRD2, BRD3, BRD4 and BRDT. These proteins all play key roles in diverse cellular processes, such as inflammatory gene expression, mitosis and viral/host interactions, by linking acetylation marks to transcriptional regulation at promoters[2-5]. Small molecule inhibitors disrupt bromodomain/histone interactions, holding promise as potential therapeutics for human disease[6-8].

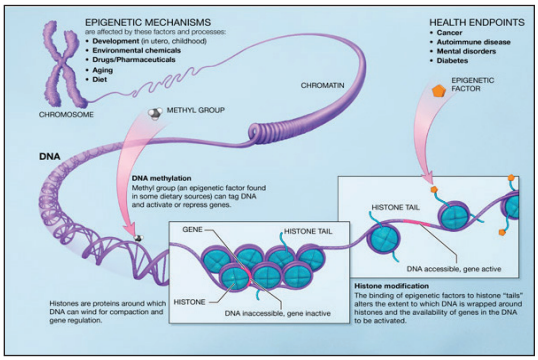

Figure 1. Histones play a central role in chromosomal structure affecting the regulation of gene expression. Epigenetic factors act as histone modulators, in turn controlling epigenetic mechanisms such as development and aging.

Here we demonstrate the combination of a homogeneous, time-resolved fluorescence resonance energy transfer (TR-FRET) assay with automated instrumentation and a high-throughput screening (HTS) multi-mode microplate reader that monitors BRD4 bromodomain 1 activity. This system provides rapid characterization in a high-throughput format as demonstrated using a small natural product library under primary screening conditions against BRD4 bromodomain 1 (human amino acids 49-170).

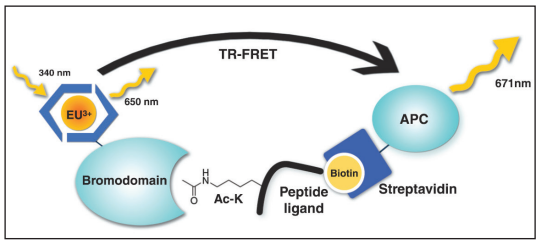

Figure 2. Bromodomain TR-FRET assay schematic.

In this assay (Figure 2), BRD4 bromodomain 1 is labeled with a europium (Eu^{3+}) chelate as the donor fluorophore. A biotinylated peptide-containing an acetylated lysine serves as the BRD4 bromodomain 1 ligand. Allophycocyanin (APC)-labeled avidin binds with high affinity to the peptide substrate via the biotin moiety, serving as the acceptor fluorophore. When a bromodomain/histone interaction occurs, the close proximity allows energy to be transferred from the europium donor to the APC acceptor. The emitted fluorescence signal can be read on a multi-mode microplate reader. Small molecule inhibitors can be screened for by monitoring the loss of TR-FRET signal via the commonly used FRET ratio analysis.

Materials and Methods

Materials

Assay Components

BRD4 bromodomain 1 TR-FRET Assay Kit (Catalog No. 600520) was a gift of Cayman Chemical Co., (Ann Arbor, MI). Screen-Well® Natural Product Library, v. 7.2 (Catalog No. BML-2865) was a gift of Enzo Life Science (Farmingdale, NY).

Labware

384-well, black, low-volume, plates (Catalog No. 4363MTX) used in all experiments were supplied by Thermo Scientific (Pittsburgh, PA). 96-well polypropylene microplates were used for reagent preparation (Catalog No. 3359) were procured from Corning Inc, (Lowell, MA).

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Instrumentation

MultiFlo™ Microplate Dispenser

BioTek's MultiFlo™ Microplate Dispenser (Figure 3) offers up to four reagents dispensed in parallel within one compact instrument. The MultiFlo offers a choice of either peristaltic pump or microprocessor-controlled syringe drive technologies. A wide array of plate types is accommodated, from 6- to 1536-well formats, and MultiFlo also offers a broad dispensing volume range from 500 nL to 3 mL. BioTek's proprietary angled dispensing ensures compatibility with all dispense protocols, including media exchanges with loosely adherent cell monolayers. The instrument was used to dispense BRD4 bromodomain 1 Assay Kit reagents to the 384-well assay plates.

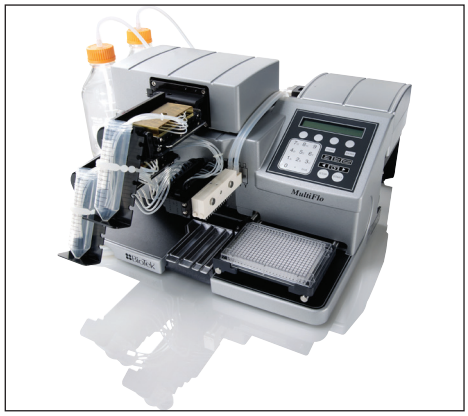

Figure 3. MultiFlo™ Microplate Dispenser.

Precision™ Microplate Pipetting System

The Precision™ Microplate Pipetting System (Figure 4) from BioTek is an affordable, walk-away solution for automated 96- or 384-well microplate liquid handling. The unique XY transport design provides effortless 96- to 384-well plate transfers with the same pipette mechanism. Additionally, a user-configurable deck and four liquid handling transfer tools provide flexible experimental design, and the instrument's small footprint allows for operation in laminar flow hoods. The instrument was used to serially titrate the inhibitor (+)-JQ1 across a 96-well microplate, and to transfer compounds to the 384-well assay plates.

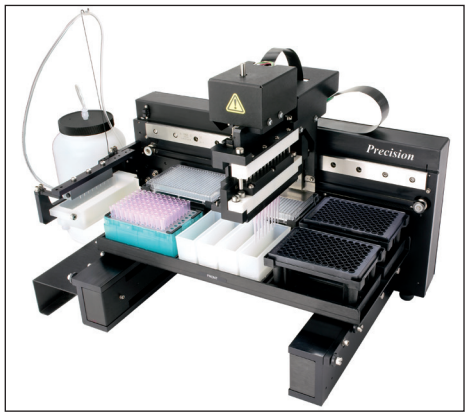

Figure 4. Precision™ Microplate Pipetting System.

Synergy™ Neo HTS Multi-Mode Microplate Reader

The Synergy™ Neo HTS Multi-Mode Microplate Reader (Figure 5) from BioTek is specifically designed for today's screening and core laboratories. Synergy Neo has multiple parallel detectors for ultrafast measurements, laser-based excitation, plate stacker and high sensitivity on low volume assays, and dedicated filter-based optics for live cell assays. The Synergy Neo also incorporates BioTek's unique patented Hybrid Technology™, a unique combination of filter- and monochromator-based fluorescence optics, for endless assay flexibility and high performance. Integrated Gen5™ Data Analysis Software controls every aspect of Synergy Neo, with an intuitive workflow interface, powerful data analysis, and flexible export options.

Figure 5. Synergy™ Neo HTS Multi-Mode Microplate Reader.

Methods

Reagent Preparation

Reagents were prepared in accordance with the manufacturers recommendations. Briefly, TR-FRET assay buffer was diluted to 1X with Milli-Q water, sterile filtered and stored at 4 °C until use. All samples, standards and assay-specific reagents were prepared in 1X TR-FRET assay buffer as directed. Samples and standards were diluted to 4X the desired final assay concentration with concentration normalization of any organic solvent present.

Compound Library Dilution

The Screen-Well Natural Product Library from Enzo Life Sciences are supplied as 100% DMSO stocks at variable concentrations. Each 1000X compound concentration was initially diluted 1:25 using the Precision by transferring 5 µL stock concentration into 120 µL 1X assay buffer. The compounds were further diluted 1:10 by transferring 10 µL of intermediate dilution into 90 µL of 1X assay buffer for a final 4X concentration.

(+)-JQ1 Titration

An 11-point 1:2 serial dilution of the known inhibitor (+)-JQl, including a zero point, was performed in a 96-well assay plate starting at 40 µM.

Synergy Neo Read Parameters	
Filter Sets	
Mode	Dual PMT
Excitation	330 nm
Dual Emmision	620/665
Gain (Side/Top PMTs)	Auto
Read Speed	
Read speed	Normal
Delay after plate movement	0
Measurement per data point	120
TRF Parameters	
Delay	150 µsec
Data collection time	500 µsec
Read Height	
Auto-Adjust determined	8.75 mm
Light Source	
Souce	Xenon Flash
Lamp energy	low

Table 1. Synergy™ Neo TRF Reading Parameters.

Automated Assay Procedure

Five microliters of each titrated standard or (+)-JQL were transferred to a 384-well assay plate. Ten µL of BRD4 bromodomain 1 europium chelate were added to each well, and the plate was incubated for 15 minutes at room temperature with shaking. Following incubation, 5 µL of BRD4 bromodomain 1 ligand/APC acceptor mix was added to each well, and the plate was again incubated for one hour at room temperature with shaking. Following the second incubation, the fluorescent signals were read on the Synergy Neo using 330/80 nm excitation and 620/10 nm emission filters, along with additional parameters listed in Table 1.

Results

BRD4 Bromodomain 1 Inhibition Titration

Using four replicates of each 11-point 1:2 serial dilution of the known inhibitor (+)-JQ1, a dose response curve was generated, with TR-FRET ratio values reported as the ratio of acceptor fluorophore intensity:donor fluorophore intensity. The TRF-FRET ratio was plotted versus (+)-JQ1 concentration (Figure 6) to calculate an IC_{50} value of approximately 348 nM.

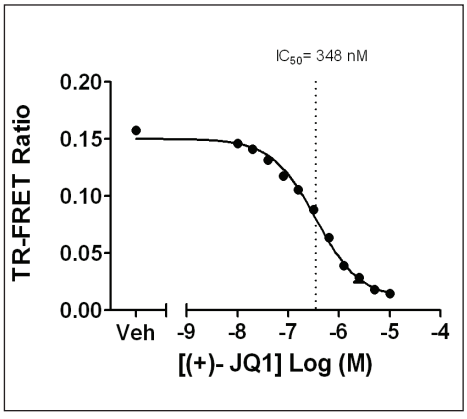

Figure 6. BRD4 Bromodomain 1 inhibition curve using (+)-JQ1.

Automated Assay Z'-Factor Validation

Forty-eight replicates of either 10 µM of (+)-JQ1 or negative control (DMSO/TR-FRET assay buffer) were assayed to determine the Z'-factor[9] for the assay. A Z'-factor value takes into account the difference in signal between a positive and negative control, as well as the signal variation amongst replicates, as an indicator of assay robustness. Per Figure 7 data, the Z'-factor was calculated to be 0.91, indicative of robust assay performance.

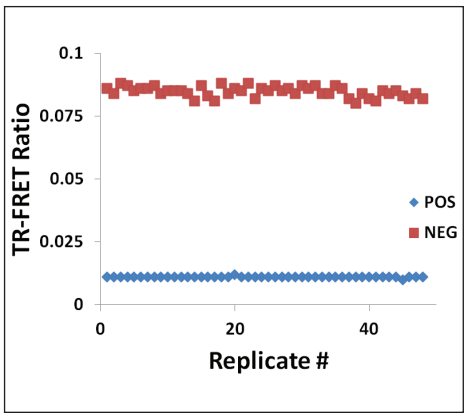

Figure 7. Z'-factor data for the BRD4 Bromodomain 1 inhibitor screening assay.

Natural Product Library Screen

A total of 502 compounds from plates 1-6 of the Screen-Well Natural Product Library were screened in triplicate using the aforementioned dilution method and automated assay procedure. The known inhibitor (+)-JQ1 was also included as a negative control. The compounds exhibited a distribution pattern consistent with primary screening using larger compound libraries. As demonstrated in Figure 8, the majority of compounds tested showed little to no inhibition, and some showed low inhibition levels of 20-30%. One compound showing greater than 30% inhibition may warrant further investigation in dose response format.

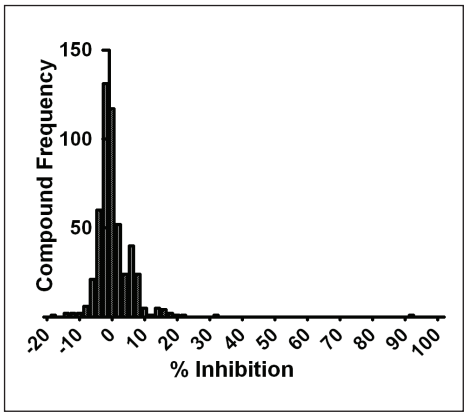

Figure 8. Compound Library Percent Inhibition Distribution, including positive and negative control.

Conclusions

We have demonstrated that the BRD4 Bromodomain TR-FRET assay may be automated for HTS applications. The automated assay workflow using MultiFlo™ and Precision™ for dispensing and diluting/transferring, respectively, simplifies the process and facilitates primary screening efforts, while Synergy ™ Neo increases throughput, thus adding to overall laboratory efficiency. The combination of assay and automated microplate-based instrumentation provides excellent performance and increased throughput as demonstrated through a robust Z'-factor determination.

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Acknowledgements

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