

Screening for Inhibitors of BRD4 Bromodomain 1 using a Homogeneous Proximity Assay



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Abstract

Bromodomains recognize acetylated lysine residues of histones and facilitate recruitment of protein complexes as part of the epigenetic regulation of gene transcription. The BET family of proteins, which contain tandem repeats of Bromodomains plus an Extra Terminal domain, play a role in bridging acetylation and transcriptional regulation of diverse cellular processes such as inflammatory gene expression, mitosis and viral/host interactions. Several small molecule inhibitors have been identified that disrupt bromodomain/acetylated lysine interactions. Here we demonstrate the automation of a homogeneous, TR-FRET (Time-Resolved Fluorescence Resonance Energy Transfer) assay method suitable for high throughput screening and rapid characterization of inhibitors of bromodomain/acetylated peptide interactions. Demonstration of the assay includes a screen of a small library of natural products under primary screening conditions against the BRD4 bromodomain 1 (human amino acids 49-170).

Introduction

The post-translational modification of histones have proven to be critical for modulation of chromatin structure by both direct interaction with DNA and indirectly via molecular interactions with recruited nuclear proteins. Modulation of chromatin structure has been shown to be necessary for such processes as gene regulation, repair and cell cycle progression (Figure 1).

Acetylated lysine residues of histone proteins are targets for binding of small nuclear proteins known as bromodomains¹. Once bound, these bromodomains recruit protein complexes involved in regulation of chromatin structure and thus gene expression. The BET family of proteins include BRD2, BRD3, BRD4 and BRDT, all of which play key roles in a diverse range of cellular processes such as inflammatory gene expression, mitosis, and viral/host interactions by linking acetylation marks to transcriptional regulation at promoters²⁻⁵. Small molecule inhibitors have been shown to successfully disrupt bromodomain/histone interactions holding promise as potential therapeutics for human disease⁶⁻⁸.

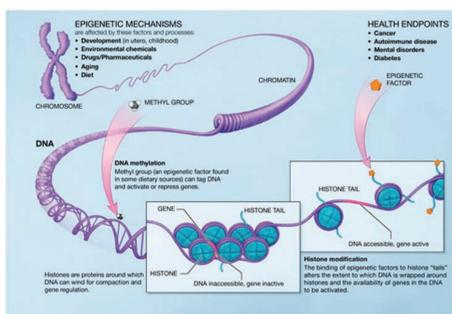


Figure 1 – Histones play a central role in chromosomal structure affecting the regulation of gene expression. Epigenetic factors act as modulators of histones in turn controlling epigenetic mechanisms such as development and aging.

Here we demonstrate the combination of a fluorescence-based assay with liquid handling and dispensing instrumentation and a high-throughput screening (HTS) multi-mode reader which can be used to monitor the biological activity of the BET bromodomain, BRD4 bromodomain 1, a model system. The assay method uses a time-resolved fluorescent energy transfer (TR-FRET) assay method amenable to rapid characterization of small molecule inhibitors of bromodomain/acetylated peptide interactions in a high throughput format. The 'donor' fluorophore in this assay consists of BRD4 bromodomain 1 (human amino acids 49-170) directly labeled with a europium (Eu³⁺) chelate. A biotinylated peptide containing target acetylated lysine serves as a ligand for BRD4 bromodomain 1. Allophycocyanin (APC)-labeled avidin binds with high affinity to the peptide substrate via the biotin moiety and serves as the 'acceptor' fluorophore in the assay. Inhibition of the interaction by an inhibitor results in a loss of TR-FRET signal.

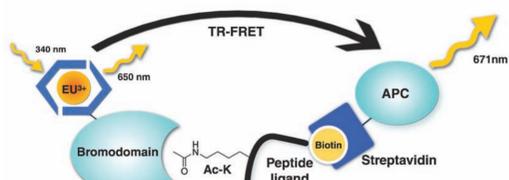


Figure 2 – Assay schematic for the bromodomain TR-FRET Assay Kit. Upon excitation, the europium chelate can fluoresce or transfer its energy to an APC molecule, provided the APC is in close proximity to the europium fluorophore. Inhibition of the BRD/peptide interaction displaces BRD-Eu³⁺ from the APC/avidin, resulting in loss of TR-FRET signal.

BioTek Instrumentation



Figure 3 – MultiFlo™ Microplate Dispenser.

MultiFlo™ Microplate Dispenser 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 are accommodated from 6- to 1536-well formats as well as a broad 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.

BioTek Instrumentation (Continued)



Figure 4 – Precision™ Microplate Pipetting System.

Precision™ is an affordable, innovative 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. The instrument was used to serially titrate agonists and antagonists across a 96-well plate, as well as transfer the compounds to the 384-well assay plates.



Figure 5 – Synergy™ NEO HTS Multi-Mode Microplate Reader.

The new Synergy™ NEO is an HTS Multi-Mode Microplate Reader designed for screening and core laboratories. Synergy NEO has multiple parallel detectors for ultra-fast measurements, laser-based excitation, plate stacker and high sensitivity on low volume assays. The Synergy NEO also incorporates BioTek's unique patented Hybrid Technology™ for wavelength flexibility.

Materials

Assay Components

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

Instrument Components

- Fluorescent signals were read on the Synergy NEO using 330/80 nm excitation and 620/10nm and 665/8nm emission filters, along with a 650 nm cutoff dichroic mirror with matched dual PMT technology.

Assay Plates

- 384-well, black, low-volume, plate (P/N 4363MTX, Thermo Scientific, Pittsburgh, PA) were used in all experiments.

Data Reduction

- TR-FRET ratio values are reported as the ratio of acceptor fluorophore intensity/donor fluorophore intensity
- Data was analyzed using Gen5™ Data Analysis Software (BioTek Instruments, Inc. Winooski, VT), Microsoft® Excel® (Microsoft, Redmond, WA), and GraphPad Prism (GraphPad Software, LaJolla, CA)

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: Compounds in the Natural Product Library come as 100% DMSO stocks at variable concentrations. Each 1000X compound concentration was initially diluted 1:25 using the Precision by transferring 5 µL of the stock concentration into 120 µL 1X assay buffer. The compounds were further diluted 1:10, using the Precision, by transferring 10 µL of the intermediate dilution into 90 µL of 1X assay buffer. Five microliters of the 4X stock was then transferred in triplicate to the appropriate assay plate, again using the Precision resulting in a 0.1% DMSO, 1X concentration during the assay procedure.

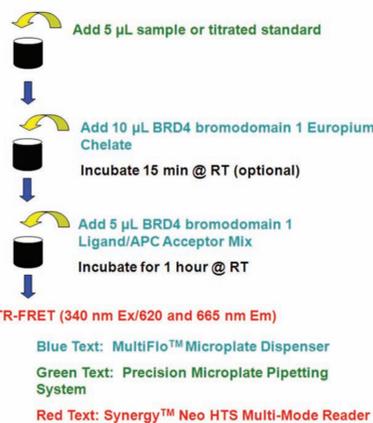


Figure 6 – Automated Assay Procedure. Dilution and transfer of compounds accomplished by Precision. Assay specific reagents dispensed using MultiFlo. Detection of 620 and 665 nm signals accomplished using the Synergy NEO.

BRD4 Bromodomain 1 Inhibition Titration

- An 11-point 1:2 serial dilution was performed, with a zero point, in a 96-well assay plate starting with an initial concentration of (+)-JQ1 at 40 µM.
- Four replicates of each dilution were transferred to a 384-well assay plate.
- The assay plate was incubated for 15 minutes at room temperature with shaking following the addition of the BRD4 bromodomain 1 Europium chelate.
- After addition of the BRD4 bromodomain 1 ligand/APC acceptor mix the plate was incubated for 1 hour at room temperature with shaking.
- Following the 1 hour incubation the plate was read on the Synergy NEO using the parameters shown in Table 1.
- The TR-FRET ratio was plotted versus inhibitor concentration for determination of the IC₅₀ concentration.

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

Table 1 – Synergy NEO TRF Read Parameters.

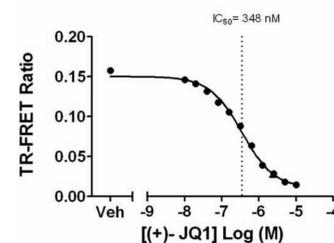


Figure 7 – BRD4 Bromodomain 1 Inhibition Curve. A dose response curve was generated for the displacement of the acetylated peptide from BRD4 bromodomain 1 by the known inhibitor (+)-JQ1. The IC₅₀ value was determined to be ~348 nM.

Z'-Factor

- Forty eight replicates of either 10 µM of (+)-JQ1 or negative control (DMSO/TR-FRET assay buffer) were assayed to determine the Z'-factor for the assay.

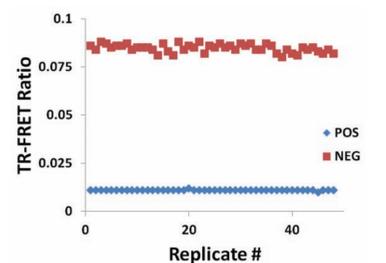


Figure 8 – Z' data for the BRD4 bromodomain 1 inhibitor Screening Assay. Data shown is from 48-replicate well of both positive and negative controls. The Z'-factor calculated from this experiment was determined to be 0.91.

- The determined Z'-factor of 0.91 is indicative of robust assay performance.

Natural Product Library Screen

- A total of 502 compounds from plates 1-6 of the Screen-well Natural Product Library were screened in triplicate.
- Compounds were diluted 1:1000 from the original 100% DMSO stocks as previously described.
- The known inhibitor of BRD4 bromodomain 1, (+)-JQ1, was also included in the library, as well as a negative control.

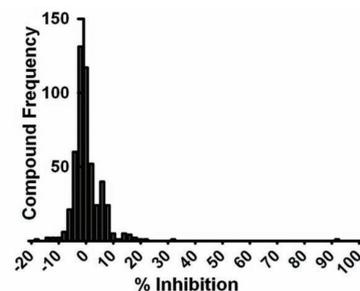


Figure 9 – Compound Library % Inhibition Distribution. Distribution of percent inhibition data from Natural Product Library compound screen.

- The inhibition data from the compounds tested exhibit a distribution pattern consistent with primary screening of larger compound libraries.
- The majority of compounds tested showed little or no inhibition.
- A few of the tested compounds showed low levels of inhibition (20-30%).
- One compound showing inhibition >30% (positive control can be seen showing inhibition >90%) and may warrant further investigation in dose response format.

Conclusions

- The BRD4 bromodomain TR-FRET Assay can be performed using automated methods and is amenable to HTS applications.
- The Synergy NEO HTS Multi-Mode Microplate Reader allows for dual-detection dramatically increasing throughput capabilities.
- The MultiFlo Microplate Dispenser for BRD4 bromodomain 1 europium chelate and Ligand/APC Acceptor Mixture reagent dispensing and Precision Microplate Pipetting System for serial dilution and compound transfer simplify assay processes and facilitates primary screening efforts.
- A robust Z'-factor determination when using automated methods indicates excellent assay performance as well as the additional benefit of increased throughput.

1. Mujtaba, S., Zeng, L., and Zhou, M.-M. Structure and acetyl-lysine recognition of the bromodomain. *Oncogene* 24, 5521-5527 (2011). 2. Florence, B. and Falter, D.V. You Bet-cha: A novel family of transcriptional regulators. *Front. Biosci.* 6, D1008-D1018 (2011). 3. Hargreaves, D.D., Horng, T., and Medhritov, R. Control of inducible gene expression by signal-dependent transcriptional elongation. *Cell* 138(1), 129-45 (2009). 4. LeRoy, G., Rickards, B., and Flint, S.J. The double bromodomain proteins Brd2 and Brd3 couple histone acetylation to transcription. *Mol. Cell* 30(1), 51-60 (2008). 5. Weidner-Glunde, M., Ottlinger, M., and Schulz, T.F. WHAT do viruses BET on? *Front. Biosci.* 15, 537-549 (2010). 6. Filippakopoulos, P., Qi, J., Picaud, S., et al. Selective inhibition of BET bromodomains. *Nature* 468(7327), 1067-73 (2011). 7. Hewings, D.S., Wang, M., Philpott, M., et al. 3,5-Dimethylisoxazoles act as acetyl-lysine-mimetic bromodomain ligands. *J. Med. Chem.* 54(19), 6761-70 (2011). 8. Chung, C.W., Coste, H., White, J.H., et al. Discovery and characterization of small molecule inhibitors of the BET family bromodomains. *J. Med. Chem.* 54(11), 3827-3838 (2011).