TECHNICAL NOTE

LANCE TR-FRET

Development of a Simple Bromodomain Binding Assay Using LANCE *Ultra* (TR-FRET) Technology

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Introduction

Bromodomains are a class of epigenetic reader proteins that translate the acetylation state of histones into downstream cell-signaling events. They are important regulators of gene transcription, modulating the recruitment of different molecular partners to the nucleosome. Bromodomains are believed to be involved in many disease states, including cancer, inflammation, and cardiovascular disease.[1] In recent years, there has been increasing interest in developing small molecules that can target specific bromodomains.

The Bromodomain and Extra-Terminal Domain (BET) family of bromodomains include BRD2, BRD3, BRD4, and BRDT. Inhibition of BET-histone interactions has been shown to downregulate transcription of several oncogenes, providing a novel pharmacological strategy for the treatment of cancer.[2,3]

Here we demonstrate a biochemical bromodomain binding assay utilizing LANCE® *Ultra* TR-FRET technology. LANCE *Ultra* TR-FRET assays are simple mix-and-read assays that are amenable to low- and high-throughput screening and can be measured on a variety of plate readers. A LANCE *Ultra* assay was developed for BRD4 (BD1)/Histone H4 (K5,8,12,16Ac) peptide binding (Fig. 1). The assay was optimized for performance in experiments used to study and screen inhibitors of bromodomain binding. Using the optimized LANCE *Ultra* TR-FRET assay, we compared rank order potency of known BET inhibitors. Our results show the power and versatility of LANCE *Ultra* (TR-FRET) technology as a tool in drug discovery programs for the identification of specific inhibitors of bromodomain activity *in vitro*.

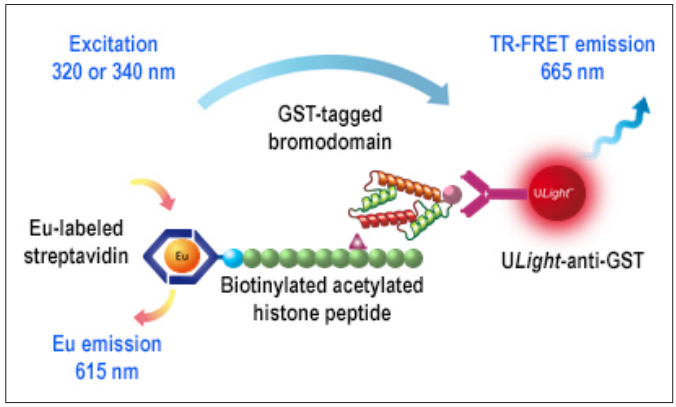

Figure 1. Detection of epigenetic marks uses Europium labeled streptavidin donor (Eu-SA) and U*Light*™ labeled Anti-GST acceptor (U*Light*-anti-GST) to capture the GST-tagged bromodomains and biotinylated peptides. Excitation of the Europium chelate in the complex generates signal at 615 nm and after the energy transfer, signal is generated at 665 nm.

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Materials and Methods

Reagents

LANCE *Ultra* Eu-W1024 Streptavidin (Cat #AD0063) and U*Light* anti-GST (Cat #TRF0104) were from PerkinElmer. GST-tagged Bromodomain-containing protein 4, Bromodomain 1 [BRD4 (BD1) – Cat #15-0012] and biotinylated tetra-acetylated histone H4 (H4 K5,8,12,16Ac) peptide (Cat #12-0034) were from EpiCypher. BET inhibitors were sourced from Tocris [(+)JQ-1 – Cat #4499, (-)JQ-1 – Cat #5630, CPI 203 – Cat #5331, XD 14 – Cat #5489, I-BET 151 – Cat #4650, PFI 1 – Cat #4445, LY303511 – Cat #2418, PFI 3 – Cat #5072]. Reagents were diluted in 1X LANCE Detection Buffer (PerkinElmer, Cat #CR97-100) or 1X AlphaLISA Epigenetics Buffer 1 (PerkinElmer, Cat #AL008) as indicated in each figure caption. Inhibitors were serially diluted in 100% DMSO, then diluted 1:10 into 1X AlphaLISA Epigenetics Buffer 1 and added to the plate at 10X concentration, for a final assay DMSO concentration of 1%.

Assay Protocol

The LANCE *Ultra* TR-FRET bromodomain binding assay was performed as indicated in the workflow in Figure 2. Briefly, 4 µL of GST-tagged BRD4 (BD1) protein and 4 µL of biotinylated H4 (K5,8,12,16Ac) were added to 2 µL buffer, DMSO, or inhibitor in a white, 384-well OptiPlate™ (PerkinElmer, Cat #6007290). Next, 10 µL of 2X Eu-SA/U*Light*-anti-GST mix was added to the wells. Plates were incubated for one hour at 23 °C prior to reading in an EnVision® multimode plate reader (PerkinElmer).

Instrumentation

All LANCE *Ultra* TR-FRET measurements were performed on the PerkinElmer EnVision multimode plate reader (Fig. 3), equipped with a laser using default values for TR-FRET detection for laser excitation. In addition to fast, sensitive LANCE technology detection, the EnVision multimode microplate reader provides fluorescence intensity, luminescence, absorbance, fluorescence

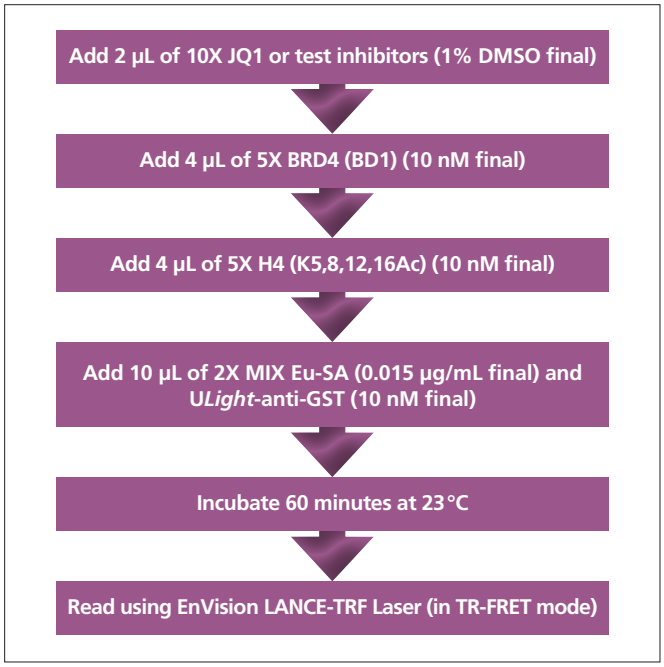


Figure 2. Workflow for LANCE *Ultra* TR-FRET bromodomain binding assay.

Figure 3. EnVision multimode plate reader.

polarization, and AlphaLISA® and AlphaPlex™ assay technologies. The system is based on hybrid technology, combining filters and a monochromator for enhanced flexibility. It incorporates proprietary Direct Double Optics™ technology for high speed and sensitivity in simultaneous filter-based readouts, such as FRET assays, and for TRF, lamp-based excitation is standard, with the option of a high energy laser for higher speed and sensitivity.

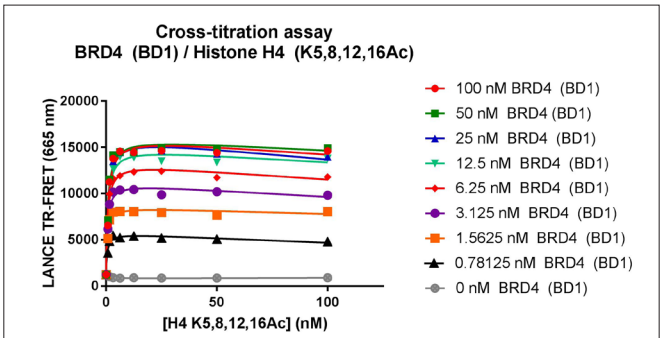
Data Analysis

Data are shown as either the raw 665 nm TR-FRET signal (from U*Light* acceptor dye) or the ratio of 665 nm signal (TR-FRET signal) to 615 nm signal (Europium donor emission). Data were plotted in GraphPad Prism version 7.0. For cross-titration and saturation experiments, curves were fit using a one-site fit saturation binding curve. Inhibition curves were generated using a four-parameter (variable slope) inhibition dose-response curve fit.

Results

Cross-titration of BRD4 (BD1) GST-Tagged Protein and Biotinylated-H4 (K5,8,12,16Ac) Peptide

The first experiment performed was a two-dimensional cross-titration experiment to determine optimal concentrations of GST-BRD4 (BD1) and biotinylated-H4 (K5,8,12,16Ac) peptide to use in the assay. Higher concentrations of protein and peptide can bind more Eu-streptavidin and U*Light*-anti-GST, enabling more FRET. Adding higher concentrations of one binding partner can also push the binding equilibrium to favor the protein-peptide complex. However, at some point the protein or peptide will saturate the Eu-streptavidin or U*Light*-anti-GST in the well and eventually create a hooking effect. In this experiment, the concentrations of Eu-streptavidin and U*Light*-anti-GST were kept constant (0.015 µg/mL Eu-SA and 10 nM U*Light*-anti-GST final concentration), while GST-BRD4 (BD1) protein and biotinylated tetra-acetylated peptide were titrated from 0 nM to 200 nM final concentration. Data are shown in Figure 4. Based on these data, 10 nM BRD4 (BD1) and 10 nM biotinylated H4 (K5,8,12,16Ac) peptide final concentrations were chosen for the next optimization experiment. This pair of concentrations provided best signal-to-background while conserving protein and peptide.



[BRD4 (BD1)] (nM)

[Biotin-H4 (K5,8,12,16Ac)] (nM)	S/B	100	50	25	12.5	6.25	3.125	1.5625	0.7812
	100	17	17	16	16	13	11	9	5
	50	17	17	17	16	14	12	9	6
	25	17	17	17	16	14	11	9	6
	12.5	16	16	16	16	14	12	9	6
	6.25	17	17	17	16	14	12	10	6
	3.125	15	16	15	14	13	11	9	6
	1.5625	11	11	11	11	10	9	7	5
	0.78125	6	6	6	6	6	5	4	3
	0	1	1	1	1	1	1	1	1

Figure 4. GST-BRD4 (BD1)/Biotinylated histone H4 (K5,8,12,16Ac) peptide cross-titration assay. Multiple concentrations of bromodomain protein and tetracetylated peptide were tested for optimal signal ratio. For this experiment, 1X AlphaLISA Epigenetics Buffer 1 was used to dilute the GST-BRD4 (BD1) protein and biotinylated H4 (K5,8,12,16Ac) peptide. 1X LANCE Detection Buffer was used to dilute the Eu-SA and U*Light*-anti-GST. In the table below the graph, signal-to-background is indicated for each pair of protein-peptide concentrations.

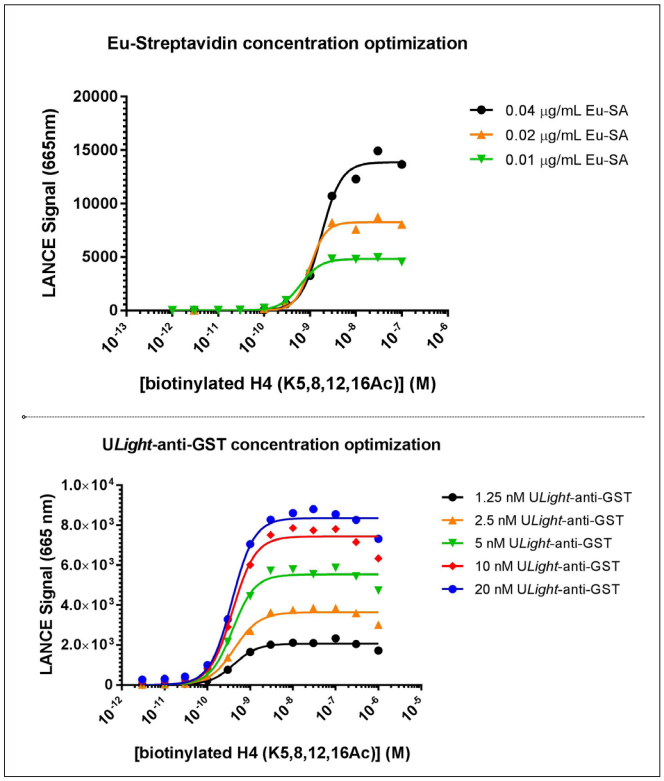


Figure 5. (A) Eu-streptavidin (donor) and (B) U*Light*-anti-GST (acceptor) titration experiments to determine optimal concentration of donor and acceptor in the assay. For this experiment, 1X AlphaLISA Epigenetics Buffer 1 was used to dilute the GST-BRD4 (BD1) protein and biotinylated H4 (K5,8,12,16Ac) peptide. 1X LANCE Detection Buffer was used to dilute the Eu-SA and U*Light*-anti-GST.

Titration of Europium-streptavidin and U*Light*-anti-GST

To determine optimal concentrations of Eu-SA and U*Light*-anti-GST, titration experiments were performed. Peptide saturation curves were created using increasing concentrations of biotinylated H4 (K5,8,12,16Ac) peptide (1 pM – 100 nM final concentration), with a fixed concentration of GST-BRD4 (BD1) (10 nM). Three concentrations of Eu-SA (0.04 µg/mL, 0.02 µg/mL, and 0.01 µg/mL) were tested, keeping the concentration of U*Light*-anti-GST fixed at 10 nM (Fig. 5A). Five concentrations of U*Light*-anti-GST (20 nM, 10 nM, 5 nM, 2.5 nM, and 1.25 nM) were also tested in a separate peptide saturation experiment, keeping the concentration of Eu-SA fixed at 0.1 µg/mL (Fig. 5B). For the Eu-SA titration experiment, higher concentrations of Eu-SA gave higher signal, but with higher background (data not shown). For the U*Light*-anti-GST titration experiment, 10 nM and 20 nM U*Light* acceptor concentrations gave similar signal-to-background. Based on these data, final concentrations of 0.015 µg/mL Eu-SA and 10 nM U*Light*-anti-GST were chosen for the assay. This combination provided good signal-to-background while conserving reagents.

Buffer Optimization

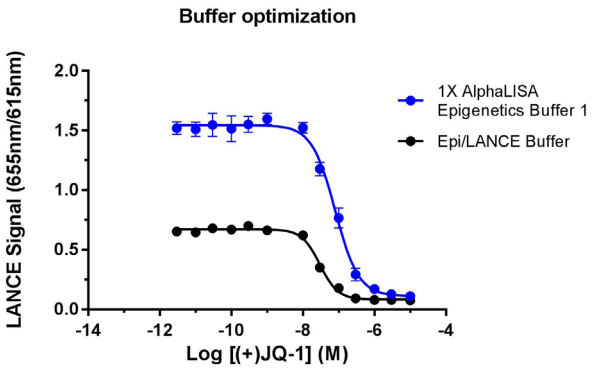
In the first few experiments, we optimized the concentrations of the BRD4 (BD1) and H4 peptide binding partners and the concentrations of Eu-SA and U*Light*-anti-GST donor and acceptors. Based on these data, we next decided to test inhibition of the BRD4 (BD1)/H4 (K5,8,12,16Ac) binding interaction at optimized reagent concentrations to verify that the current assay buffer is adequate. To do this, we ran an inhibition assay with (+)JQ-1 inhibitor. (+)JQ-1 is a known bromodomain inhibitor that binds competitively to acetyl-lysine binding motifs and displaces BRD4 from chromatin, producing anti-proliferative effects in xenograft models.[2]

In previous experiments, 1X AlphaLISA Epigenetics Buffer 1 (a buffer developed for histone peptide binding assays) was used to dilute the BRD4 (BD1) protein and H4 (K5,8,12,16Ac) peptide, while the Eu-SA and U*Light*-anti-GST reagents were diluted in 1X LANCE Detection Buffer. We compared these buffer conditions to conditions where all reagents were diluted in 1X AlphaLISA Epigenetics Buffer 1. As shown in Figure 6, 1X AlphaLISA Epigenetics Buffer 1 gave the best signal-to-background and was chosen as the assay buffer for the final, optimized assay. A nice dose-response effect was observed with the (+)JQ-1 inhibitor.

Inhibition Experiments

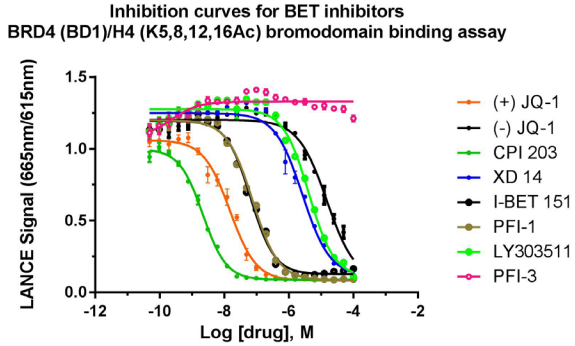
After optimizing the initial inhibition assay using the positive control (+)JQ-1, a variety of known BET inhibitors were then tested in an inhibition assay using the optimized BRD4 (BD1)/H4 (K5,8,12,16Ac) binding assay. (-)JQ-1 is an inactive stereoisomer of (+)JQ-1. CPI 203 is a BET bromodomain inhibitor that has shown synergistic activity with drugs that have already been clinically approved for cancer treatment.[4] XD 14 is a bromodomain inhibitor that was discovered in a high-throughput screening to screen for inhibitors of BRD4 (BD1) binding.[5] I-BET 151 (GSK1210151A) was reported in 2011 as a novel BET bromodomain inhibitor with efficacy in mixed-lineage leukemia (MLL) cell lines.[6] PFI-1 is a BET inhibitor for BRD2 and BRD4 that shows anti-proliferative efficiency in leukemic cell lines.[7] LY303511 has been shown to inhibit BET bromodomain proteins BRD2, BRD3, and BRD4.[8] PFI-3 is a bromodomain inhibitor with high selectivity for SMARCA2/4 and PB1 bromodomain-containing proteins.[9]

Data from compound inhibition experiments are shown in Figure 7. The compound rank order potency and IC_{50} values correlate with published data.



	1X AlphaLISA Epigenetics Buffer 1	1X AlphaLISA Epigenetics Buffer + 1X LANCE Detection Buffer
Bottom	0.1109	0.08316
Top	1.544	0.6711
IC_{50} (M)	8.05E-08	2.98E-08

Figure 6. Buffer optimization for LANCE *Ultra* TR-FRET bromodomain binding assay. A dose-response curve of (+)JQ-1 inhibitor diluted in 1% DMSO (final) was used for this experiment. Final reagent concentrations: 10 nM GST-BRD4 (BD1), 10 nM biotinylated -H4 (K5,8,12,16Ac) peptide, 0.015 µg/mL Eu-SA, and 10 nM U*Light*-anti-GST. Data using the raw 665 nm signal vs. the ratiometric 665 nm/615 nm signals were compared (data not shown). The ratiometric signal gave higher signal-to-background, so we chose to display ratiometric data in subsequent inhibition experiments. The table underneath the graph indicates maximum and minimum ratiometric signal and IC_{50} for (+)JQ-1.



Inhibitor	IC_{50} (M) in LANCE *Ultra* Assay	Published IC_{50} (M)
(+) JQ-1	1.483E-08	5.0E-08 (Tocris)
(-) JQ-1	1.514E-05	1.0E-05 (Tocris)
CPI 203	2.157E-09	9.1E-08 (Tocris)
XD 14	2.644E-06	2.37E-06[5]
I-Bet 151	6.486E-08	5.0E-08[1]
PFI 1	7.496E-08	2.2E-07[1]
LY303511	4.143E-06	9.05E-06[8]
PFI 3	n/a	n/a

Figure 7. Drug profiling inhibition curves obtained under optimized assay conditions for BRD4 (BD1)/H4 (K5,8,12,16Ac) binding using LANCE *Ultra* TR-FRET. The table below the graph shows the calculated IC_{50} for each inhibitor. Inhibitors were serially diluted in 100% DMSO, then diluted 1:10 in assay buffer for a final DMSO concentration of 1%. All other reagents were diluted in 1X AlphaLISA Epigenetics Buffer 1. Assays were run on three plates (duplicate in each plate). The average Z' was calculated to be 0.82.

Conclusion

In this technical note, we developed a no-wash LANCE *Ultra* TR-FRET bromodomain binding assay for the BRD4 (BD1)/Histone H4 (K5,8,12,16Ac) binding interaction. Performance indicates the assay is suitable for screening applications. The assay was used to measure inhibition of bromodomain binding with known BET inhibitors. BET inhibitor rank order of potency and IC_{50} values obtained were in agreement with published data. Excellent reproducibility was observed (Z' = 0.82). LANCE *Ultra* TR-FRET bromodomain binding assays can be used to study and screen BET inhibitors in a simple biochemical assay format.

References

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