Enabling epigenetics studies from HTS to SAR: a novel HTRF® platform to identify and characterize reader domain inhibitors

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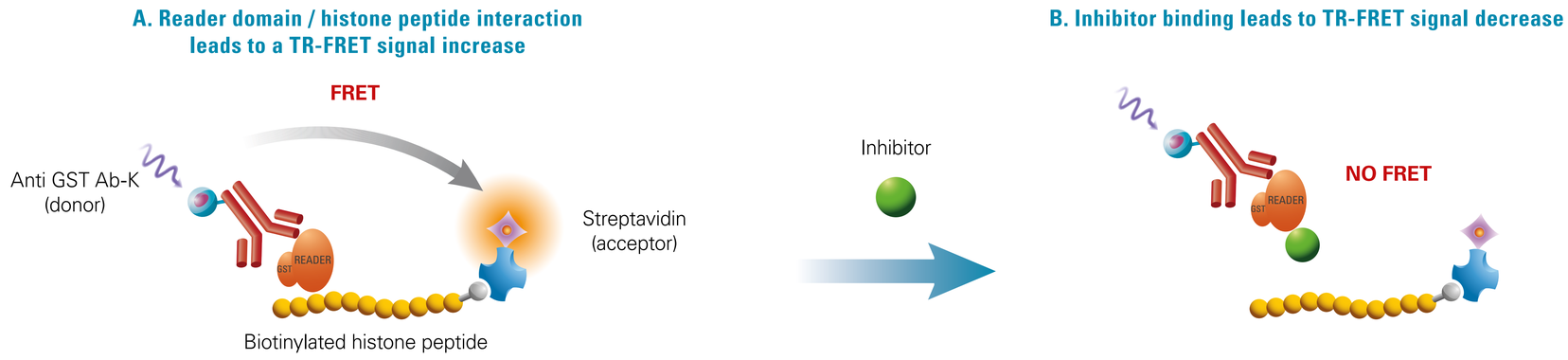
INTRODUCTION

Over recent years, significant drug discovery efforts have been made to identify potent and selective inhibitors of epigenetic targets. Proteins of this target class are classified into readers, writers and erasers of marks on histones or other nuclear proteins and DNA. By regulating a combination of posttranslational marks, they tightly control gene expression. Their deregulation has been linked to the development of various diseases, particularly in oncology.

Here we describe a novel assay platform based on the HTRF technology which enables the discovery and the characterization of novel reader domain inhibitors. More than 20 different assays have been built up to monitor the interaction of Bromodomain, Tudor domains, MBT domains and Chromodomains with histone peptides.

DESIGN OF AN HTRF PLATFORM FOR READER DOMAIN INHIBITOR STUDY



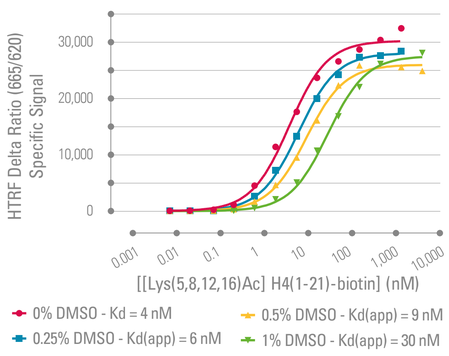
The assays are designed to measure the interaction between reader domain proteins and modified lysine residues of the N–terminal tails of histones and thus enables rapid characterization of interaction inhibitors in a high throughput format.

As shown in Panel A, the interaction between a GST-tagged reader and a biotinylated histone peptide (acetylated or methylated) is detected using an anti-GST antibody coupled to Eu^{3+} or Lumi4®-Tb cryptates (donor) and a red acceptor-labeled streptavidin (SA). When the protein and peptide interact, a TR-FRET signal can be measured. However when a compound binds to the reader domain and inhibits the protein/peptide interaction, the TR-FRET signal decreases (Panel B).

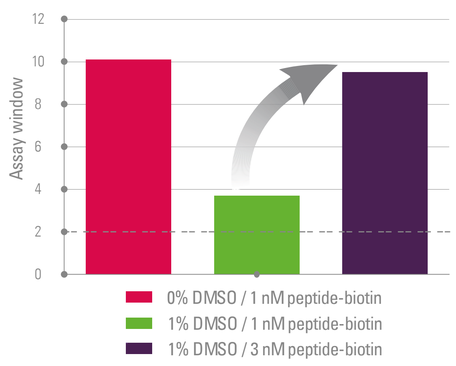
- The TR-FRET signal is treated as HTRF Ratio = Acceptor signal (665nm) / Donor Signal (620nm) x 10^4
- HTRF Delta Ratio = Ratio (Positive) – Ratio (Negative) where Negative control is performed without reader-protein to determine background signal.
- Assay window = S/B = Ratio (Positive) / Ratio (Negative)

CASE STUDY: BRD4(1) BROMODOMAIN

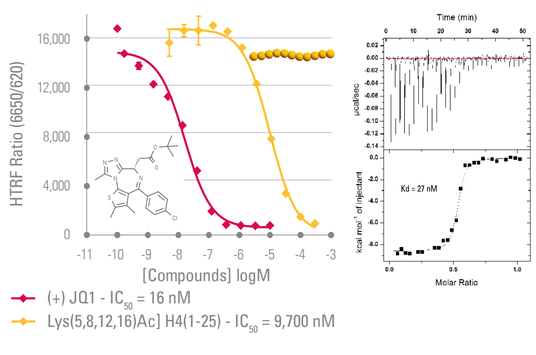
1. Histone Peptide titration



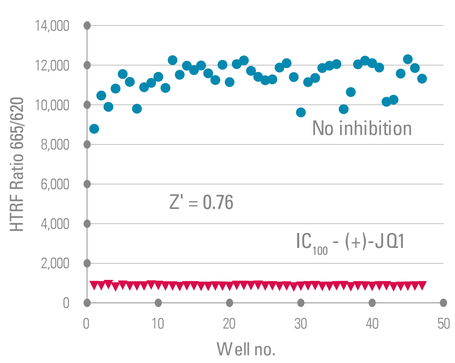
2. DMSO effect on assay window



3. Inhibitor titration



4. Z' factor



Measurement of BRD4(1) / histone H4 peptide interaction and DMSO effect.

The GST-BRD4(1) concentration was fixed at 5 nM while the peptide-biotin was serially diluted. The HTRF Delta Ratio was proportional to the specific interaction measured between GST-BRD4(1) and [Lys(5,8,12,16)Ac]-H4(1-21)-biotin peptide. The 4nM Kd value was determined from this experiment using a one site specific binding regression.

A shift of apparent Kd was observed while DMSO% increases. This is due to the competitive inhibitor nature of the DMSO on the BRD4(1)/H4 peptide interaction (already described (1)).

Selection of optimal peptide-biotin concentration depending on DMSO % used

Due to the competitive nature of DMSO, the assay window decreases as the DMSO percentage increases. The assay can then be recovered by increasing the peptide-biotin concentration. The optimal peptide-biotin concentration is selected (between real Kd and EC_{100} obtained on the titration without DMSO) with a compromise between assay window and assay sensitivity for inhibitor studies. For further study of inhibitors, 1% DMSO and 3nM peptide-biotin conditions were selected.

BRD4(1) HTRF inhibition assay was validated using reference inhibitors

The HTRF assay was performed using 3 nM peptide-biotin, 5 nM GST-BRD4(1) and 1% DMSO set constant all through the inhibitor titration. The IC_{50} of (+)-JQ1 and H4 tetra-acetylated peptide (panel on the left) are in good agreement with published data (1, 2) and with ITC reference experiment (panel on the right).

Assay robustness demonstrated through Z' factor determination.

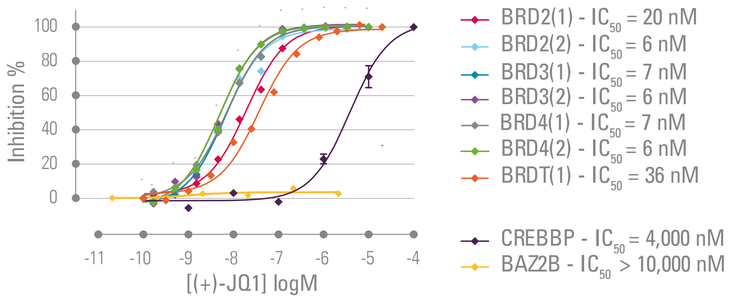
The assay was performed using 3 nM peptide-biotin, 5 nM GST-BRD4(1) and 1% DMSO.

The 0.76 Z' factor value underlines the robustness of the assay and its suitability for High Throuput Screening.

COMPOUND PROFILING

Compound selectivity can be assessed over a broad range of validated reader domain assays.

(+)-JQ1 compound was profiled on the BET bromodomain family, CREBBP and BAZ2B bromodomains. As already described (3), (+)-JQ1 is a non selective inhibitor over the BET family but displays selectivity over non BET bromodomains (CREBBP and BAZ2B).



LIST OF VALIDATED READER DOMAINS

READER FAMILY	READER	DMSO TOLERANCE (UP TO)
Bromodomain – (I)	CERC2	0.25% DMSO
Bromodomain – (I)	FALZ (BPTF)	1% DMSO
Bromodomain – (II) (BET)	BRD2(1)	1% DMSO
Bromodomain – (II) (BET)	BRD2(2)	1% DMSO
Bromodomain – (II) (BET)	BRD2(1/2)	1% DMSO
Bromodomain – (II) (BET)	BRD3(1)	1% DMSO
Bromodomain – (II) (BET)	BRD3(2)	1% DMSO
Bromodomain – (II) (BET)	BRD3(1/2)	1% DMSO
Bromodomain – (II) (BET)	BRD4(1)	2% DMSO
Bromodomain – (II) (BET)	BRD4(2)	1% DMSO

READER FAMILY	READER	DMSO TOLERANCE (UP TO)
Bromodomain – (II) (BET)	BRD4(1/2)	1% DMSO
Bromodomain – (II) (BET)	BRDT(1)	1% DMSO
Bromodomain – (II) (BET)	BRDT(1/2)	1% DMSO
Bromodomain – (III)	CREBBP	0.1% DMSO
Bromodomain – (IV)	ATAD2A	1% DMSO
Bromodomain – (IV)	ATAD2B	1% DMSO
Bromodomain – (IV)	BRD1	1% DMSO
Bromodomain – (IV)	BRD9	1% DMSO
Bromodomain – (IV)	BRPF3	1% DMSO

READER FAMILY	READER	DMSO TOLERANCE (UP TO)
Bromodomain – (VI)	BAZ2B	2% DMSO
Bromodomain – (VII)	TAF1L(2)	0.25% DMSO
Bromodomain – (VII)	TAF1L(1/2)	0.5% DMSO
Bromodomain – (VII)	TAF1(2)	0.5% DMSO
Bromodomain – (VII)	TAF1(1/2)	0.25% DMSO
Bromodomain – (VIII)	SMARCA4 (BRG1)	1% DMSO
MBT domain	L3MBTL1	1% DMSO
Chromodomain	CBX1	1% DMSO
Tudordomain	UHRF1	1% DMSO

This binding domain HTRF assay format has been successfully applied to 28 reader domain proteins from different subfamilies of bromodomains, but also from chromo, MBT and tudor methyl binding domain families. The DMSO tolerance of each assay has been checked and is presented in the table above.

CONCLUSION

We have developed a novel HTRF platform to identify and characterize reader domain inhibitors, providing:

- A large validated offer. 28 reader domains from different families have been successfully validated. Moreover, the same assay principle can be applied to validate other proteins of interest by following the protocol provided .
- High robustness for HTS suitability as proven by good Z' factor.
- Good DMSO tolerance. Among the 28 proteins validated, 22 assays are compatible with at least up to 1% DMSO. The other 6 assays display lower DMSO tolerance due to higher affinity for the DMSO.
- Low consumption of substrates (protein and peptide). All the assays have been optimized to use only 5nM of reader domain proteins (384 low volume plate – 20µl).
- Selectivity profiling of compounds over the reader proteins.

REFERENCES

(1) Philpott et al. Mol. BioSyst., 2011, 7, 2899-2908
(2) Filippakopoulos and Knapp, FEBS Letters 586 (2012) 2692–2704
(3) Filippakopoulos et al. Nature 2010

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