A comparison of detection technologies for the epigenetic enzyme JMJD2C histone H3 demethylase

Kathy Dodgson, and Gary Allenby
Aurelia Bioscience Ltd, BioCity, Pennyfoot Street, Nottingham, NG1 1GF, UK

Introduction

The field of epigenetics is a dynamic and rapidly growing area of science. While epigenetic changes are required for normal development and health, they can also be responsible for some disease states. Disrupting any of the three systems (DNA-methylation, histone modification and small regulatory RNA's) that contribute to epigenetic alterations can cause abnormal activation or silencing of genes. Such disruptions have been associated with cancer, inflammation and mental disorders.

JMJD2C histone H3 demethylase belongs to a class of enzymes that modify histone protein at specific lysine residues removing a methyl group. These enzymes are alpha-ketoglutarate "dependent" and also require Iron2+ (FeII) as a co-factor in the demethylation chemical reaction. The biological impact of histone demethylases in normal cellular function and in the pathogenesis of human disease is not well understood. This is partly because these enzymes have been discovered only recently but also because of a general lack of chemical tools to enable characterization of the role and function of these enzymes.

In this poster we compare and contrast three assay technologies for the measurement of JMJD2C histone H3 demethylase. The three technologies were HTRF (Cisbio), LANCE *Ultra* (PerkinElmer) and AlphaLISA (PerkinElmer). The JMJD2C enzyme demethylates the substrate [Lys(Me3)9]- Histone H3 (1-21)-GGK biotin to produce the di-methylated peptide (H3K9me2) that can be detected using a specific antibody labelled with an appropriate tag, relevant to the chosen detection technology. These assays are all homogeneous and measure the specific product rather than relying on coupled assays to indirectly measure a by-product of the reaction e.g. formaldehyde.

The poster will highlight the key steps we undertook to compare the three technologies. We will demonstrate the utility of each technology for screening in a drug discovery setting.

Methods

The assay protocol for both the HTRF and LANCE *Ultra* assays were available from the respective suppliers and used for the initial time course experiments. There was no recommended assay protocol available for the AlphaLISA detection reagents in combination with the JMJD2C enzyme. Therefore the LANCE *Ultra* assay conditions were also used for AlphaLISA.

All assays were conducted in 384 well, low volume, white plates (Greiner #784075). The AlphaLISA detection volumes were reduced in order to achieve a 20µl assay volume rather than the 25µl assay volume in the product literature.

JMJD2C enzyme was supplied by BPS Bioscience and the substrate [Lys(Me3)9]- Histone H3 (1-21)-GGK biotin was supplied by AnaSpec.

Co-factors and inhibitors were purchased from Sigma.

All assays were conducted in the same base buffer: 50mM HEPES pH 7.5 supplemented with 0.01% Tween-20 and 0.01% BSA.

AlphaLISA assays were measured on the EnSpire whilst HTRF and LANCE *Ultra* were measured on the EnVision.

Data for HTRF was calculated as a ratio of the Em 665/Em 620 x10^4

Results – Time course studies

Time course studies were conducted for all three technologies over 60 minutes at room temperature. Enzyme concentrations ranging between 30 and 0.3nM were investigated.

Initial experiments demonstrated that the JMJD2C enzyme was both pH and buffer sensitive. Activity was lost in TRIS buffer at pH 7.5 (data not shown). The technology comparison studies were therefore conducted in HEPES buffer at pH 7.5 with 0.01% Tween-20 and 0.01% BSA. The biotinylated H3K9Me3 peptide substrate concentration was constant for all technologies at 300nM. The JMJD2C enzyme also requires FeII and ascorbate as co-factors, these were included at 5µM and 1mM respectively.

For all three technologies the optimal enzyme concentration was found to be between 1 and 3nM. Subsequent experiments used 3nM JMJD2C enzyme and a 30 minute reaction time.

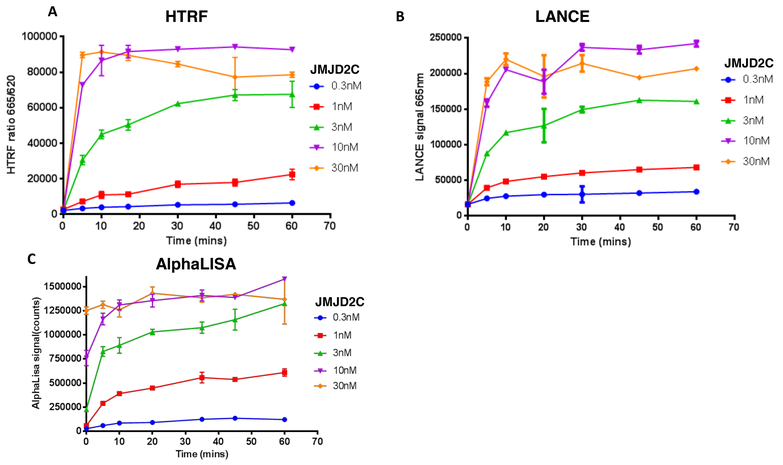

Fig. 1. JMJD2C was serially diluted to give 30, 10,3,1 and 0.3nM concentrations in the assay well. The assay was initiated with the addition of 300nM biotinylated H3K9 me3 peptide and 50µm alpha-ketoglutarate . The assay was stopped at the indicated time points by the addition of the appropriate detection reagents A) HTRF, B) LANCE and C) AlphaLISA

Results – Enzyme Inhibition

The effect of two known inhibitors of JMJD2C; 2,4-pyridinedicarboxylic acid (2,4-PDCA) and N-oxalylglycine (NOG) was investigated and the IC_{50} was compared across the three technologies. Similar IC_{50} values were obtained in HTRF and LANCE *Ultra* whilst the AlphaLISA technology generated lower IC50 values for both inhibitors.

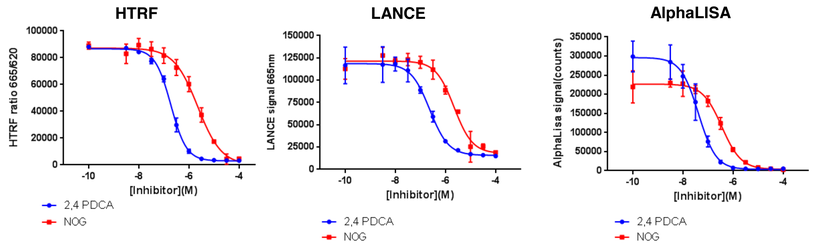

Fig. 2. Serial dilutions of inhibitors were pre-incubated with 3nM JMJD2C for 10 minutes prior to the addition of 300nM biotinylated substrate with 5µM alpha ketoglutarate (1.5µM alpha ketoglutarate for AlphaLISA due to lower Km)

	HTRF	LANCE	AlphaLISA
IC_{50} for 2,4-PDCA	187nM	234nM	41nM
IC_{50} for NOG	2.15µM	2.2µM	0.36µM

Z' and reproducibility studies

The reproducibility of the three technologies was compared using at least 40 wells each of uninhibited, IC_{80} and IC_{100} concentrations of 2,4-PDCA. The variability of each group of wells were compared and the Z' value calculated for each technology, using the uninhibited wells and the IC_{100} wells. The results of this comparison are shown in Fig.3. and Table 1.

The AlphaLISA technology had the largest signal window but was the most variable and therefore had the lowest Z' value.

Both HTRF and LANCE *Ultra* were highly reproducible and gave Z' values greater than 0.7

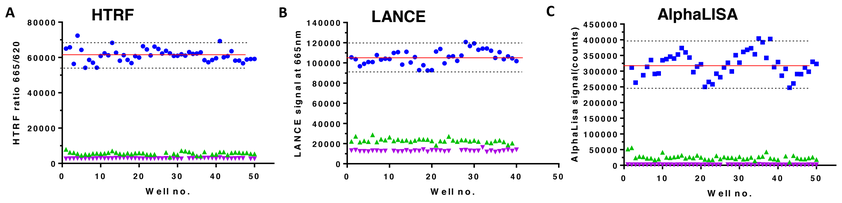

Fig. 3. 3nM JMJD2C was pre-incubated with either ● DMSO, uninhibited control; ▲ IC_{80} [2,4-PDCA]; ▼ IC_{100} [2,4-PDCA]. The red line represents the mean signal and dotted lines represent the mean +/- 2SD. **A**) HTRF; **B**) LANCE; **C**) AlphaLISA

	HTRF (665/620)	LANCE	AlphaLISA
CV at IC_{80}	14	9	23
CV at IC_{100}	6	6	13
Z' at IC_{80}	0.76	0.67	0.55
Z' at IC_{100}	0.8	0.75	0.63
S:B at IC_{100}	21	8	162

Table. 1. Comparison of coefficient of variation (CV); Z' values and the signal to background (S:B) for HTRF, LANCE and AlphaLISA

Summary and Conclusions

- Initial studies demonstrated that the enzyme was both pH and buffer sensitive. HEPES pH 7.5 was used for these studies.
- All three technologies gave similar time course profiles and enzyme consumption. 3nM JMJD2C was subsequently used for all three technologies.
- The performance of the HTRF and LANCE *Ultra* technologies was similar both in terms of enzyme consumption, incubation time and IC_{50} values for standards.
- The AlphaLISA assay had the largest signal window but was the least reproducible in this study with Z' values of 0.63. Whilst this is an acceptable Z' value the other two technologies were more suitable for screening due to lower variability.
- Overall the HTRF technology gave the best Z' values and had a better signal window than the LANCE *Ultra* technology.
- In this study we have demonstrated that all three technologies are fit for purpose for screening in a drug discovery setting dependent upon the availability of a suitable reader system.

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