

A high-throughput MALDI MS assay for multiplexed read-out of histone demethylase inhibition

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Aim

To describe technical aspects of a robust and high-throughput MALDI MS assay with multiplex capability for defining histone methylation status suitable for screening of inhibitors of histone demethylase function.

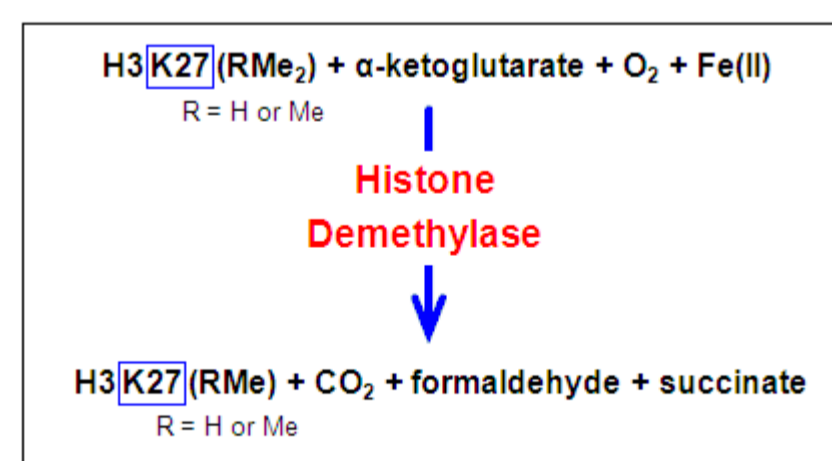
Introduction

Histone methylation and histone acetylation are dynamic post-translational modifications that are important in the epigenetic regulation of gene expression. Histone demethylases are implicated in a number of diseases including different types of cancer such as leukemia and prostate cancer. Inhibition of histone demethylases has significant potential for addressing unmet medical need.

Enzyme activity can be measured directly via mass spectrometry in a label free environment with native substrates. This allows for fast assay development as it eliminates the need to develop antibodies or fluorescent labels. Here we have optimised the measurement of peak areas for methylated histone peptides and use the peak area ratio of tri-methylated to di-methylated peptides as a measure of demethylase function to determine the potency of demethylase inhibitors.

Assay Method

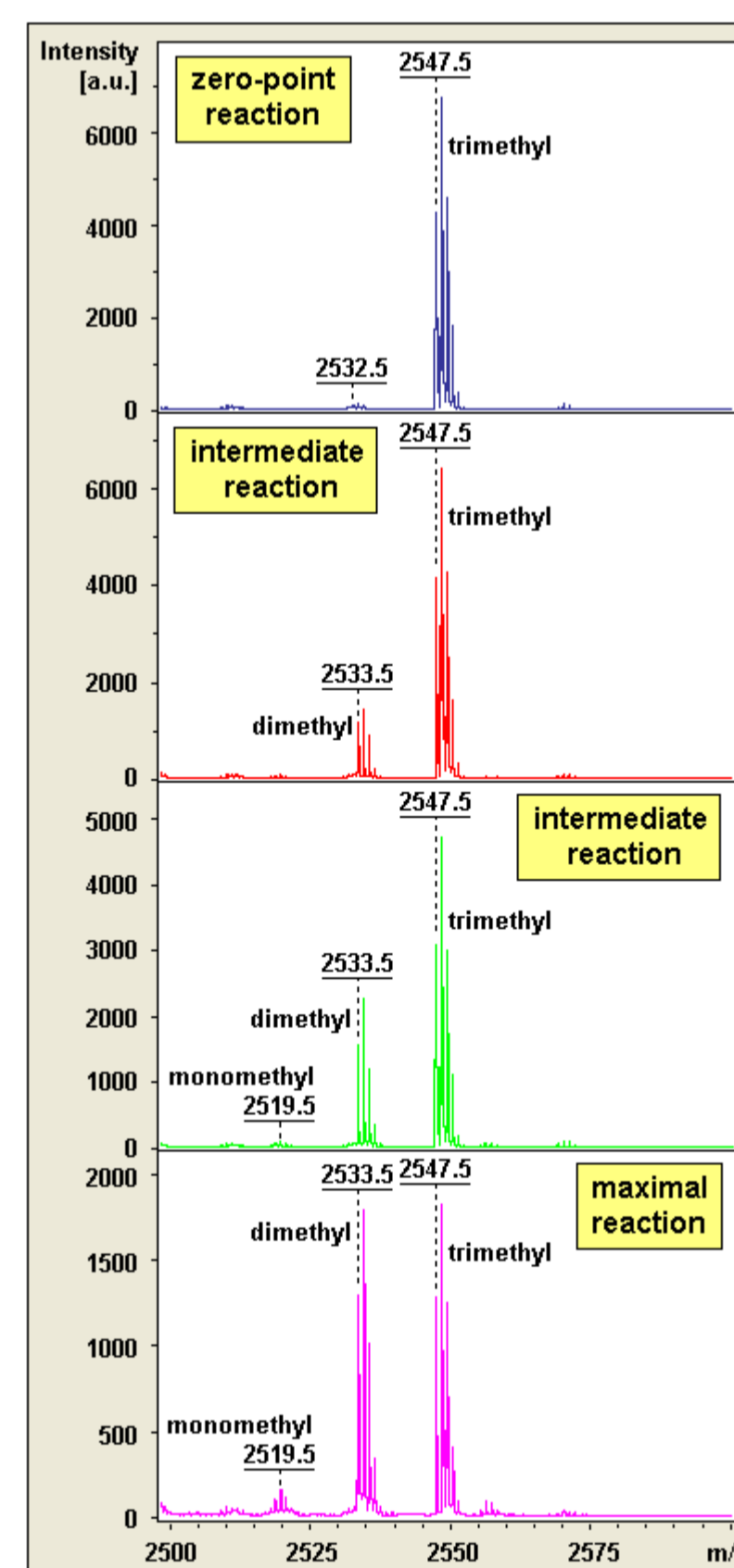
The enzymatic assay monitors demethylation of histone H3 peptides containing tri-methylated K27 (at 15 μM) by recombinant histone demethylases in the presence of α-ketoglutarate, Fe(II), and ascorbic acid at pH7.5/ room temperature. The reaction is stopped with 1% TFA, diluted 1:100 in 5% acetonitrile and analysed by MALDI-TOF MS (Ultraflex III) on a 384-well PAC target (Bruker Daltonics).



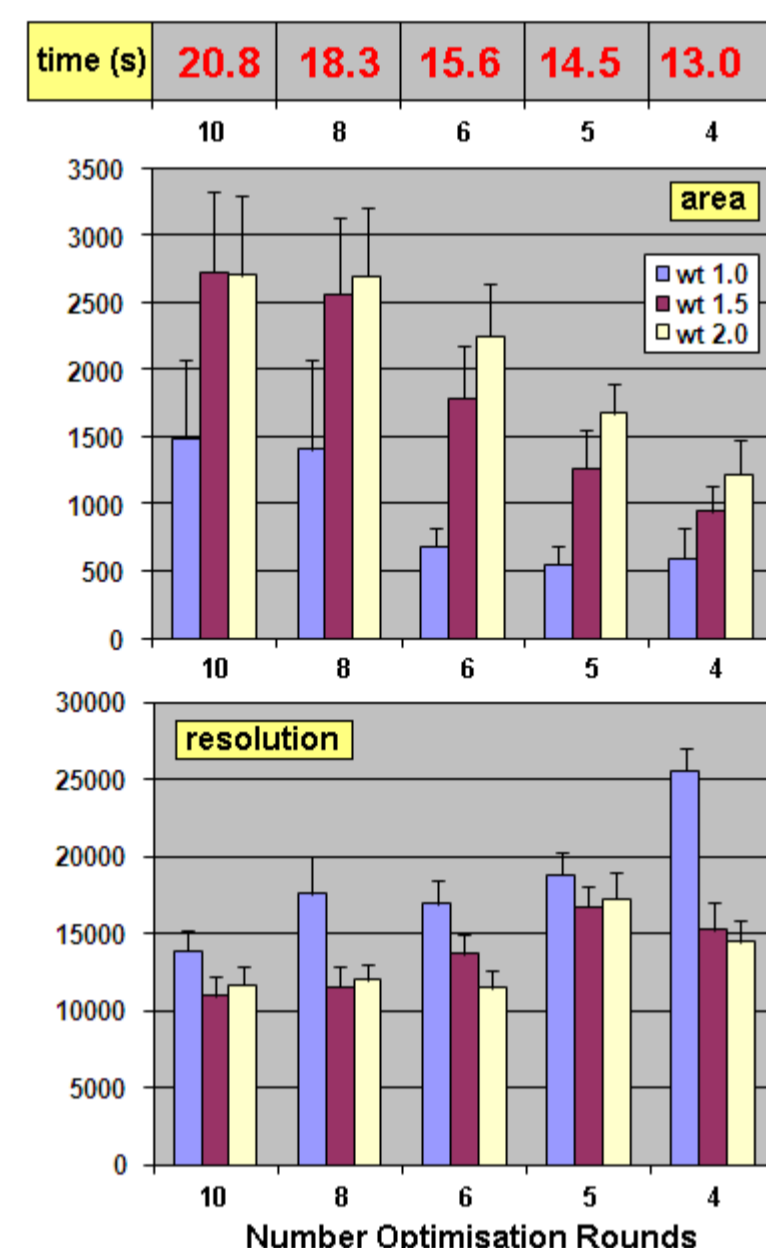
Data acquisition has been optimised using a random-walk routine under fuzzy logic control so that 600 laser shots are acquired per sample. All experiments are performed in 384-well format with automated dilution and spotting to the MALDI target (Biomek FX), data acquisition, data processing and result extraction using a report script. For dose-response curves, compound dilution series are added to the enzymatic assay and peak area ratio of tri-/di-methylated peptide compared to a control assay in absence of compound. Calculated Z prime values are 0.6 – 0.8 for a typical analysis.

Optimisation of analysis

To develop a robust and reproducible readout for peptide peak areas, we optimised MALDI preparation parameters: dilution factor from assay well, type of dilution buffers and type of plates. We found that a 1:100 dilution into 5% aqueous acetonitrile provided the best response. Application of sample solution to plates that are precoated with thin layer matrix allowed for an additional stringent washing step which increased reproducibility of the method.



Example mass spectra of demethylation of K-27 trimethylated histone H3 peptide. The top panel is the acid-stopped substrate control, the middle panels intermediate reactions and the bottom panel is the DMSO maximal reaction control that gives maximal dimethyl product generation in the fixed time point assay.

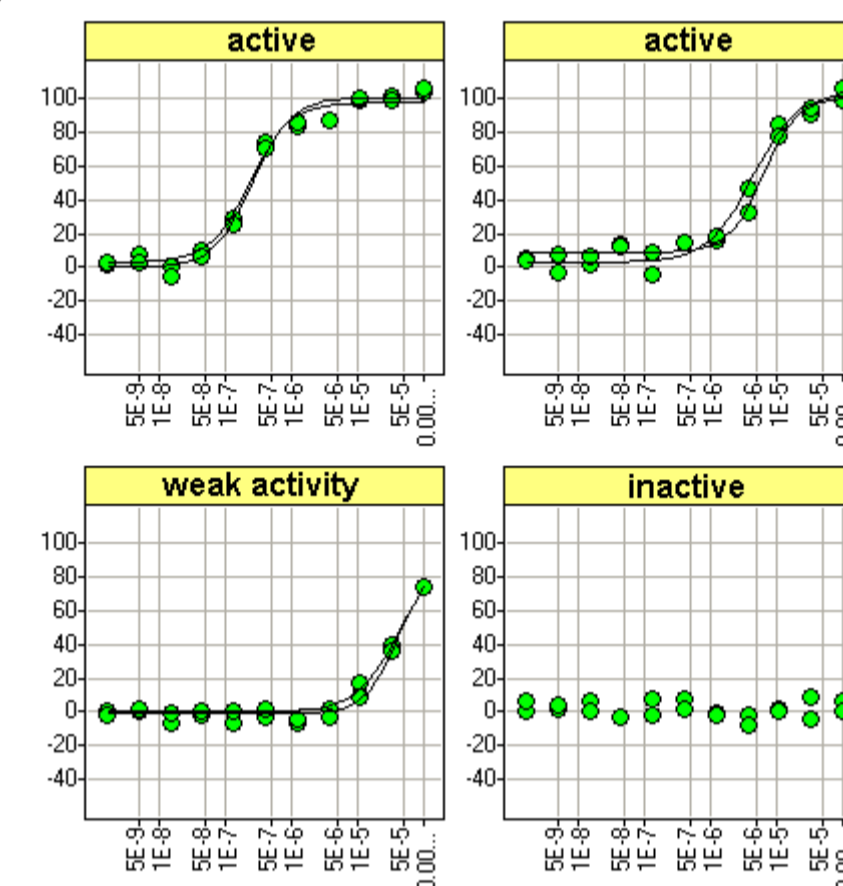


Effect of laser power weighting and number of rounds of fuzzy optimisation (total 600 shots of increasing batch size from 60 to 150 shots) on analysis time, peak area and resolution.

Several laser sampling modes were evaluated on the Ultraflex III mass spectrometer. Although using a fixed laser power is potentially the fastest mode it was found difficult to set an optimum power to accommodate spot-to-spot sample differences across the plate. This was overcome by use of a random walk method combined with a fuzzy logic algorithm to adjust laser power that allowed for fast analysis with peak resolution maintained close to defined target resolution of 12000.

It can be seen that varying the number of rounds of optimisation by varying the batch size of shots has a marked effect on analysis time, peak area recovery and resolution. The optimal conditions for collection were a total of 600 shots in 120 shot batches and laser frequency of 100 Hz and a power weighting factor of 1.5, with concurrent data processing and reporting that gave a total analysis time per spot of 15 second.

Reproducibility of data analysis was assessed by comparing peak area vs peak area ratio with the latter giving a coefficient of variation of 2% across a 384-well plate (compared to 40% for peak area). Peak area ratios were used for determining dose-response curves of small molecule inhibition of a histone demethylase.



Examples of dose-response curves (n=2) across a range of potencies demonstrating excellent reproducibility of assay read-out.

Increasing throughput

In order to further increase the assay throughput, assay plates were analysed on an autoflex speed MALDI-TOF by collecting 1000 shots using a fixed laser power at a frequency of 1 kHz. Because of the improved smartbeam II laser technology used on this instrument, it was found there was no difficulty in assigning a consistent fixed laser power that gave uniform performance across the plate.

Data acquisition was consequently reduced down to ~2 second per spot and gave equivalent quality of data to that obtained using the fuzzy logic method on the Ultraflex III, with comparative assay plates giving Z prime values of 0.8 on both instruments. This would reduce the analysis time per 384-well plate to 13 minute.

	Ultraflex III	autoflex speed
Laser Mode	Fuzzy Logic	Fixed
Laser Frequency	100 Hz	1 kHz
Shots	600 (5 x 120)	1000
Raster	Random Walk	Random Walk
Time per spot	14.5 sec	2 sec
Time per 384-well plate	93 min	13 min
Analysis Z prime	0.6 - 0.8	0.6 - 0.8

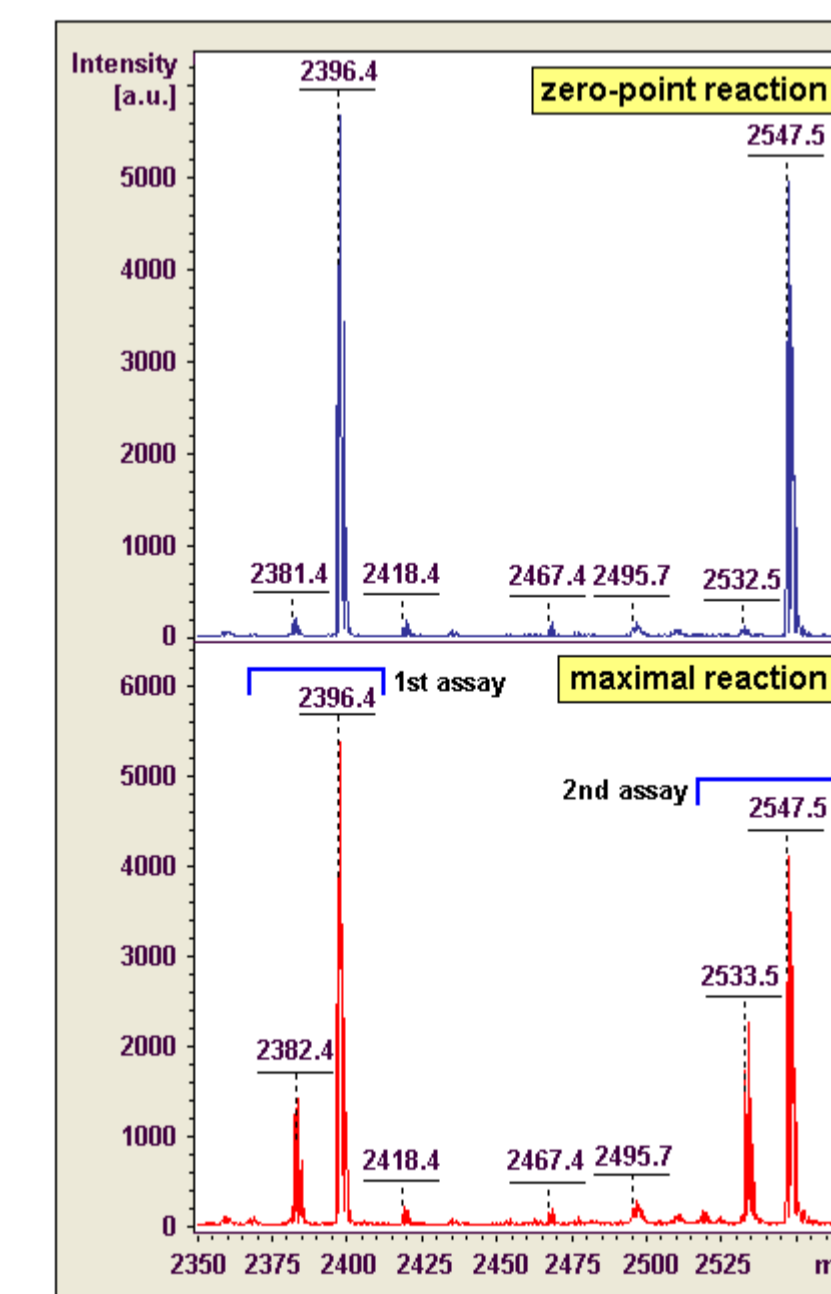
Multiplex capability for assay read-out

Provided substrate and product masses are distinct for multiple assay types, it is possible to mix samples prior to dilution and spotting such that a multiple readout can be obtained from a single plate analysis.

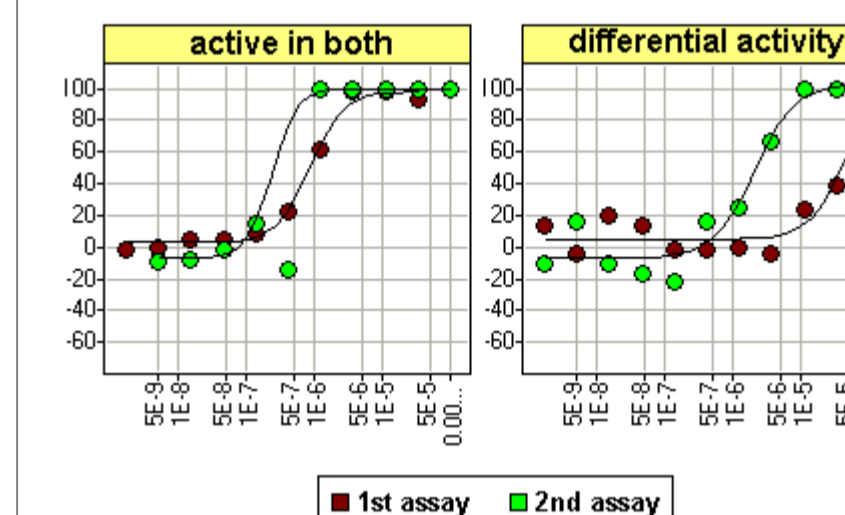
This allows a 384-well screening plate containing dilution ranges of target compounds and controls to be tested in several different related enzyme assays to assess selectivity profiles for each compound.

The analysis method is the same as for a single assay, but the analysis report script was modified to report multiple product and substrate pairs into a single report file for post-analysis processing.

Dose-response curves for two test compounds are shown that indicate different comparative potencies when tested in the pair of enzyme assays that used different histone H3 peptide substrates.



Duplex analysis of two assay reactions. The top panel is the acid-stopped zero-point reaction control and the bottom panel is the DMSO maximal reaction control in the fixed time point assay.



Examples of two comparative dose-response curves for compound tested in two separate demethylase assays that had been mixed after the reaction was stopped and spotted for duplex analysis.

Conclusions

- A robust and high-throughput MALDI-TOF MS assay has been developed for defining histone methylation status suitable for screening of inhibitors of histone demethylase function by direct detection of substrate and product.
- Automated protocol established for sample preparation from 384-well plate onto matrix pre-coated 384 spot target plates.
- Assay Z prime range of 0.6 - 0.8 for a typical analysis.
- Data collection time 2 second per spot using a fixed laser power at 1 kHz firing rate (smartbeam II laser) on the new autoflex speed MALDI-TOF allows rapid read-out of a 384-well target in 13 minute.
- Multiplexing proof-of-concept for the assay has been shown for the duplex case. Multiplexing will lead to further increased throughput of read-out and reduced cost-per-data point.
- The assay method is capable of high-throughput read-out of compound effect on demethylase function for driving lead optimisation in drug discovery.