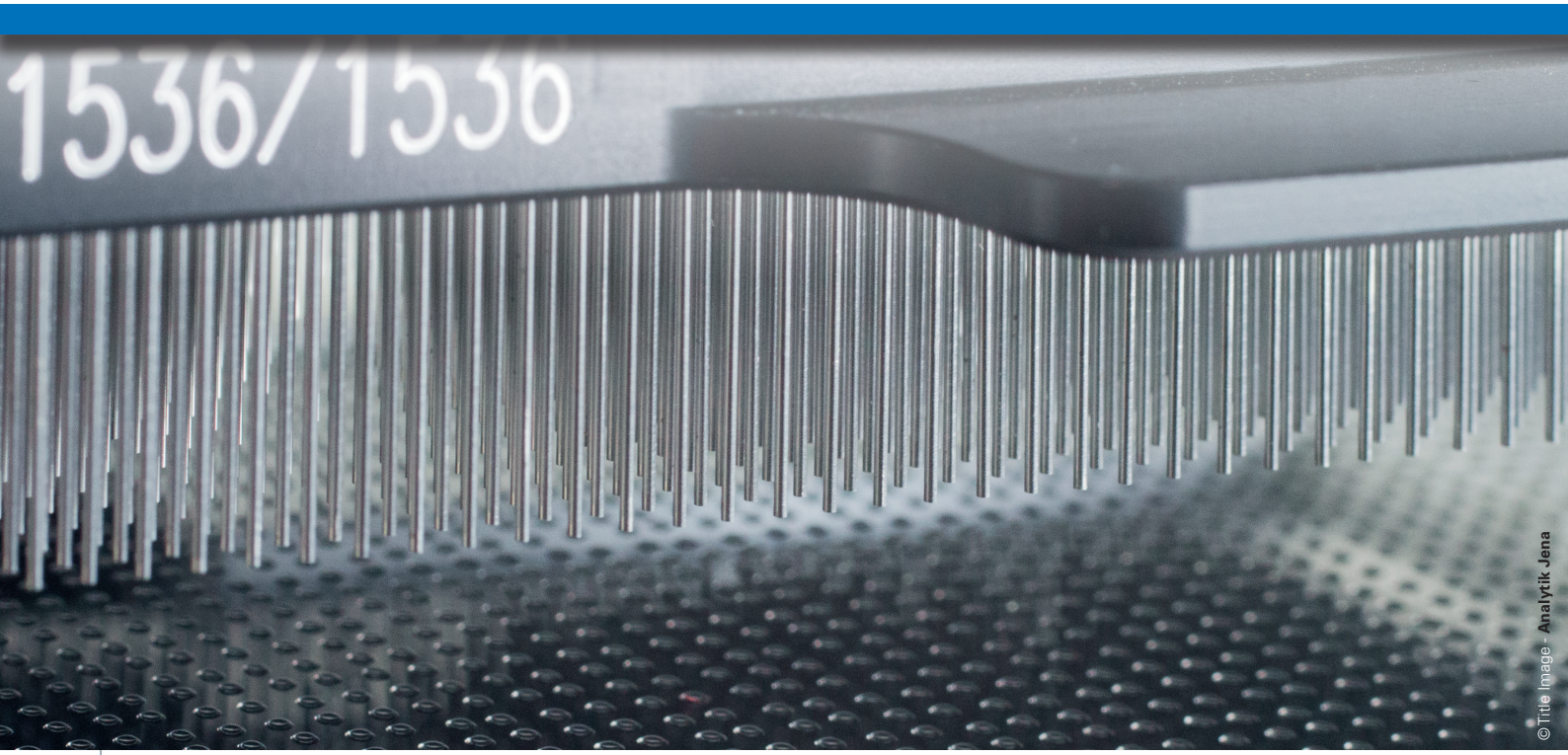

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Kinase inhibition screening by label-free mass spectrometry employing the MALDI PharmaPulse HTS solution

Bruker´s MALDI PharmaPulse solution, integrating the rapifleX high-speed MALDI-TOF instrument with the dedicated MALDI PharmaPulse software suite, represents an emerging technology for label-free high-throughput screening in drug discovery, offering high analysis speed and cost-efficiency as well as low false discovery rates.

Abstract

Here, we demonstrate the unique capabilities of the MALDI PharmaPulse solution for the application field of in-vitro kinase inhibition screening, selecting human tyrosine kinase ABL as a show case. Data acquisition when performed on a rapifleX instrument enables analysis times of less than one second per sample. Resulting MALDI-TOF data are of high quality as indicated by Z´ and RSD values obtained in a proof-of-concept study described here. Bruker´s dedicated MALDI PharmaPulse software supports all steps of the MALDI-HTS workflow from campaign setup to final export of results to LIMS and data analysis software, e.g. Genedata Screener, providing significant improvements to the efficiency of large scale MS analyses in an HTS environment.

Keywords: MALDI PharmaPulse, MPP, rapifleX, MALDI-TOF, high-throughput screening, HTS, label-free, biochemical assays, kinase inhibition screening, drug discovery

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Introduction

With the dramatically increased speed and throughput provided by latest MALDI-TOF instrumentation, namely Bruker´s rapifleX MALDI PharmaPulse platform, MALDI-TOF mass spectrometry (MS) has become a highly attractive method for label-free high-throughput screening (HTS) analyses in drug discovery. Compared to traditional label-based screening methods, MALDI-MS based screening of even large libraries comprising millions of compounds can be accomplished faster, at reduced costs as well as significantly lowered false discovery rates, the latter reducing the effort required for further hit validation.

In addition to that, Bruker´s newly developed MALDI PharmaPulse (MPP) software contributes significantly to the elevation of MALDI-TOF based high-throughput screening to a previously unseen level of operational routine and efficiency. The software supports all steps of the MALDI-HTS workflow from setup and execution of acquisition campaigns to final export of results to LIMS and downstream data analysis software.

In this application note we demonstrate the unique capabilities of Bruker´s MALDI PharmaPulse HTS solution when employed for *in-vitro* kinase inhibition screening selecting human tyrosine kinase ABL (ABL(H)) as a show case. Kinases represent a family of highly relevant drug targets that are involved in a broad variety of diseases. Due to the high MALDI ionization efficiency of peptides and their respective phosphorylates, kinase activity can be assayed by quantitative monitoring of the mass change between a suitable substrate peptide and its product of enzyme catalyzed phosphorylation, making MALDI-TOF a particularly promising readout method in kinase (or phosphatase) inhibition screening. [1-4] Furthermore, this application note is highlighting key features of the MALDI PharmaPulse software illustrating the great benefits it provides with regard to the efficiency of MALDI-MS based workflows when integrated in an HTS lab environment.

Experimental

Sample preparation

The biochemical assay described here analyzes the enzymatic activity of human tyrosine kinase ABL (ABL(H)) when catalyzing tyrosine phosphorylation of substrate peptide EAIYAAPFAKKK (MW=1336Da) yielding EAIpYAAPFAKKK (MW=1416Da) as a product. Assay samples, including positive and negative controls, were obtained from an external source. Buffer conditions as well as concentrations of substrate (µM range), co-factor adenosine triphosphate (ATP) and enzyme were identical to those used in the respective ^{33}P radiolabeling reference method. For assay quenching, however, trifluoroacetic acid was applied at a final assay concentration of 1%.

Prior to MALDI preparation, assay samples were diluted by factor 100 in MALDI compatible buffer (10 mM $NH_4H_2PO_4$, 1% TFA in water).

In the proof-of-concept study described here, diluted assay samples were spotted on prestructured Bruker MTP Anchorchip 384 BC target plates using HCCA as a MALDI matrix. 0.5 µl aliquots of diluted assay samples were spotted first and were dried under light vacuum. 0.5 µl HCCA matrix solution (1.4 mg/ml in 90% ACN, 0.1% TFA, 1 mM $NH_4H_2PO_4$) was added per

Table 1: MPP Kinase inhibition screening: MALDI-TOF acquisition parameters

Instrument operation mode	Reflector positive
Laser repetition rate	10kHz
Laser application profile	MS dried droplet
No. of laser shots accumulated per spectrum	2000
Sample carrier movement	RandomWalk (100 laser shots per raster position)
Detection range	m/z 1300 - 1500
Detector sampling rate	2.5GS/s
Calibration	External method precalibration with Bruker Peptide Calibration Standard II (Cubic Enhanced fit); Internal recalibration of MS spectra using substrate peptide as a calibrant (Linear Correction fit)

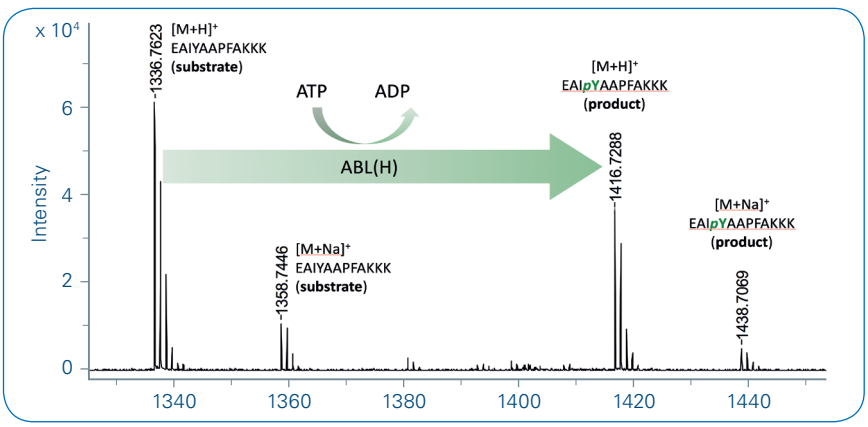

Figure 1: Annotated MALDI-TOF mass spectrum obtained from a typical assay sample. Activity of tyrosine kinase ABL(H) was monitored by quantifying the 80Da mass change from substrate peptide EAIYAAPFAKKK to its phosphorylation product EAIpYAAPFAKKK.

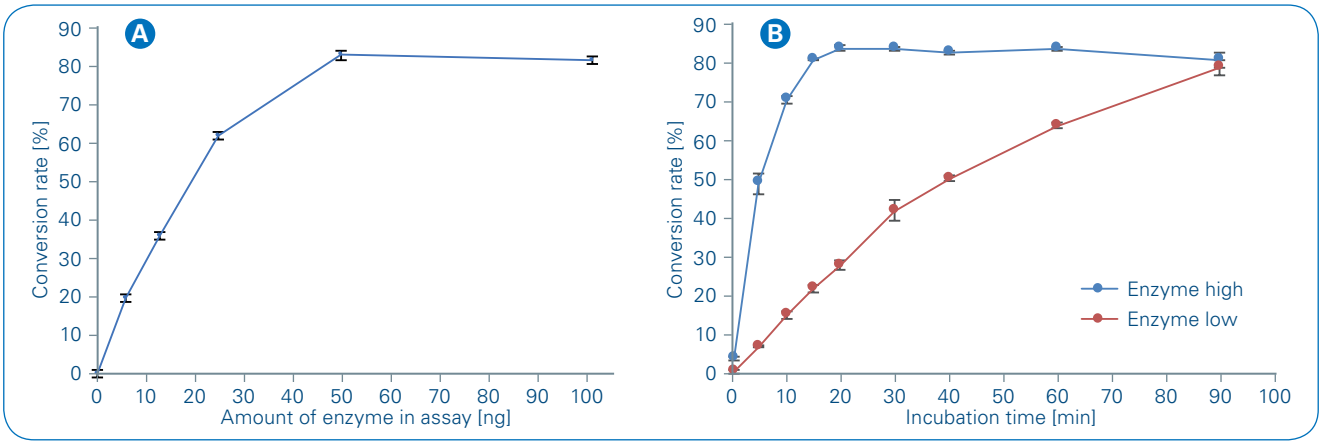

Figure 2: Enzyme titration experiment performed at 50 µM substrate concentration (A); Time course experiment for two enzyme concentrations (enzyme high = 5 ng/µl; enzyme low = 0.5 ng/µl) performed at 50 µM substrate concentration. Error bars indicate standard deviations (n=4 MALDI spots per datapoint) (B)

spot to the dry pre-spotted samples and was allowed to dry down under room conditions.

For larger sized screens, in which MALDI preparation is routinely performed by liquid handlers, Bruker´s dedicated disposable Plain HTS MALDI plates (Bruker part number 1847006), mounted on the respective light weight Bruker HTS MALDI Adapter (Bruker part number 1847571), would be the MALDI sample plate of choice.

MALDI-TOF data acquisition

Mass spectra were recorded on a Bruker rapifleX MALDI PharmaPulse MALDI-TOF mass spectrometer equipped with 10 kHz smartbeam 3D laser and an autoloader enabling automated probe exchange by a lab robot. MS acquisition parameters applied in this study are given in Table 1. Automated MALDI-TOF data acquisition was run under control of MALDI PharmaPulse (MPP) 2.2 and flexControl 4.0 software.

MS data processing

Processing of raw MS spectra was performed automatically in flexAnalysis 4.0 applying the default flexAnalysis processing method MPP2_2_methdev_screen_peptides.FAMSMethod provided with the MPP 2.2 software installation. The method performed the following processing steps:

- Baseline correction
- Peak finding throughout narrow m/z tolerance windows defined in accordance with the substrate and product related m/z values specified in the Measurement Tasks table upon MPP campaign setup
- Internal recalibration employing $[M+H]^+$ of the substrate peptide as a calibrant
- Extraction of peak attributes (*m/z*, peak intensity, peak area, etc.) for MS signals representing substrate and product, respectively

The following target m/z values were taken into account for quantitative monitoring of ABL(H) activity:

- m/z 1336.7623 (substrate peptide EAIYAAPFAKKK; $[M+H]^+$)
- m/z 1416.7287 (phosphorylation product EAIpYAAPFAKKK; $[M+H]^+$)

Conversion rates [%] were calculated by the MPP software based on MS peak areas. Mean values and standard deviations of conversion rates determined for positive and negative controls were used by the software for automated calculation of Z´ as a measure of assay robustness.

MS peak attributes, resulting conversion rates and further information was compiled by the MPP software in a *.csv formatted result output file. This output file was imported in Genedata Screener (Genedata AG, Basel, Switzerland) for further data analysis, i.e. determination of IC50 values.

Results and discussion

Figure 1 displays an annotated MALDI-TOF mass spectrum obtained from a typical assay sample after accumulation of 2000 laser shots. Data acquisition took less than 1 s per spectrum (including spot-to-spot moving time) illustrating the high-throughput capabilities of the rapifleX instrument.

Figure 2 shows MALDI-TOF results obtained from enzyme titration and time course experiments performed as part of the assay development process. Error bars in the figure represent standard deviations derived from replicate analyses (n=4 MALDI spots per assay sample). Relative standard deviations (RSD) throughout these two experiments were in the range between 0.5 and 7% indicating a high level of reproducibility of the MALDI-TOF method.

In a subsequent experiment, the MALDI-TOF method was applied to generate an inhibitor profile of ABL(H) for a panel of 40 reference inhibitor compounds. Figure 3 displays the

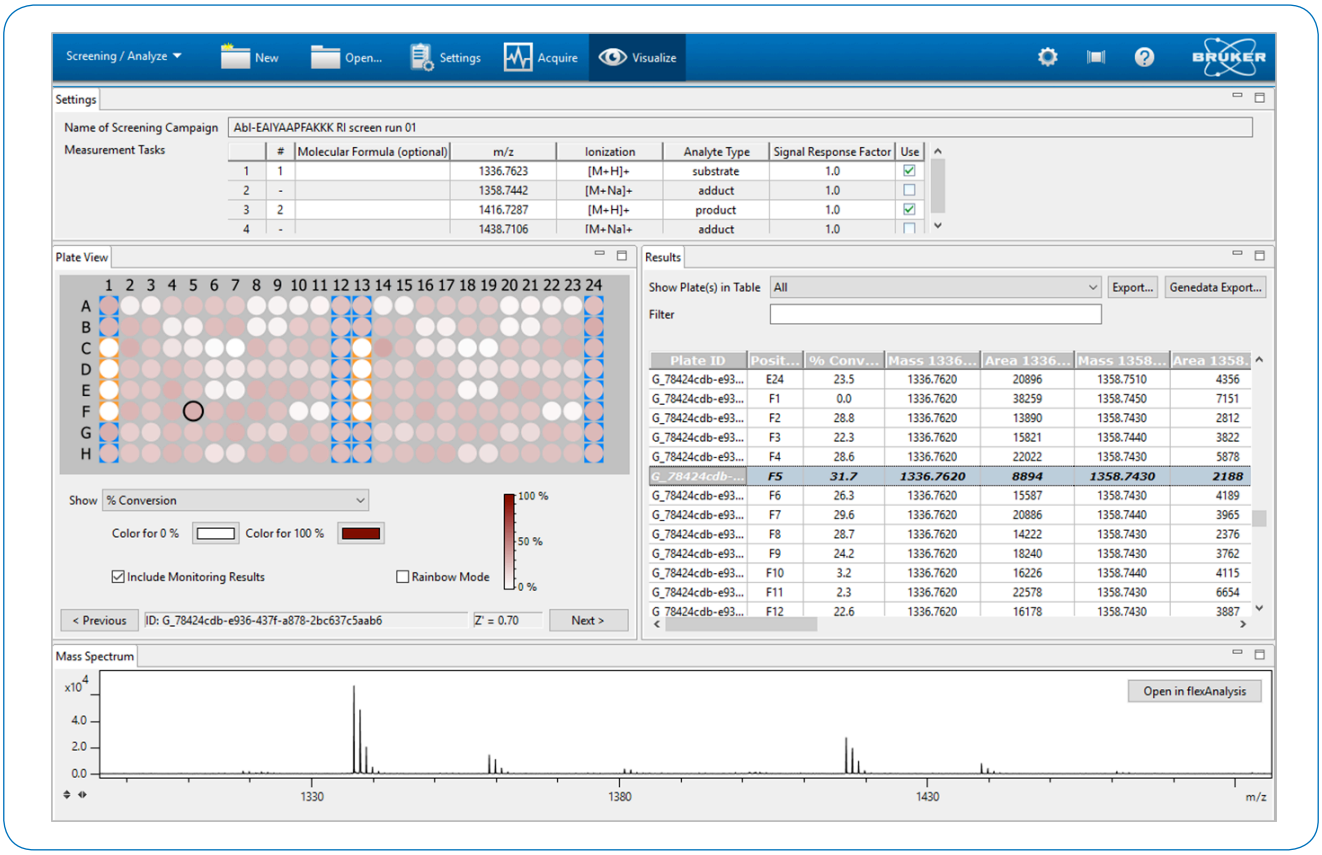

Figure 3: MALDI PharmaPulse 2.2 software user interface controlling the inhibitor profile experiment for ABL(H) against a panel of 40 reference inhibitor compounds

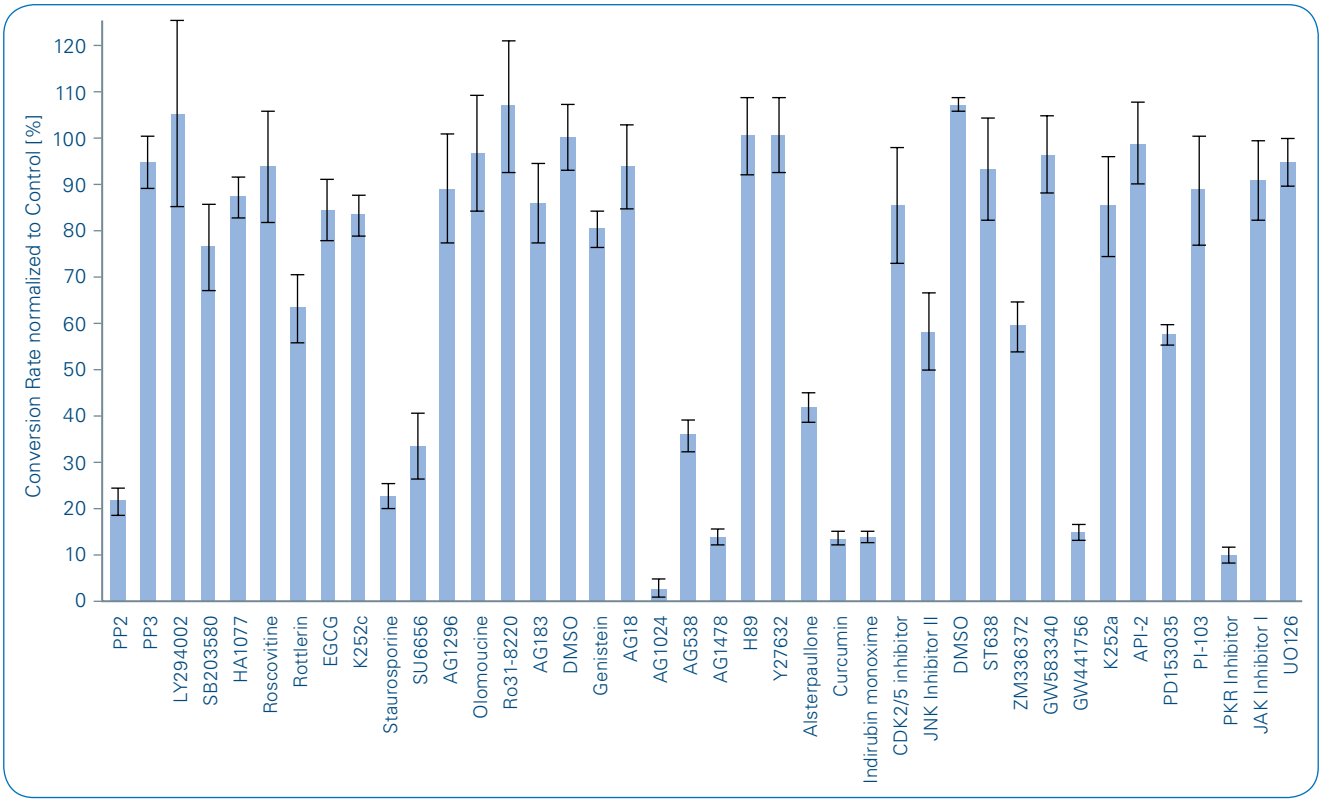

Figure 4: MALDI-TOF based inhibitor profile of ABL(H) against 40 reference inhibitor compounds (50 µm substrate concentration; inhibitor concentrations varied individually). Error bars indicate standard deviations from n=4 measurements (2 technical assay replicates, 2 MALDI spots each).

MPP 2.2 software user interface when controlling the inhibitor profile experiment described here. Reference inhibitor assays were run in duplicate in a 96 well plate together with 12 positive and 4 negative controls. For MALDI-TOF readout, the assay plate was transferred to the MALDI plate in duplicate resulting in 192 MALDI spots. The measurement tasks table, displayed in the upper part of the software GUI, accomodates the target m/z values representing the substrate peptide and its tyrosine phosphorylation product, respectively. This m/z information is being used by the MPP software for MS feature extraction and calculation of quantitative results (conversion rates).

The plate view, integrated in the MPP software´s Visualize tab, allows for instant evaluation of results. In Figure 3, the plate view displays conversion rate values calculated by the software from substrate and product related MS peak areas. Well positions holding negative controls (orange color label) and positive controls (blue color label) were defined upon MPP campaign setup to enable automatic Z´calculation. Analysis results obtained from these controls yielded a final Z´of 0.7 indicating good robustness of the MALDI-TOF assay.

In Figure 4, the MALDI-TOF based inhibitor profile of ABL(H) against 40 reference inhibitors is shown. Error bars indicate standard deviations of conversion rates normalized to controls for n=4 measurements (2 assay replicates, 2 MALDI spots each). Averaging over all 40 inhibitors, RSD was 12%.

Figure 5 presents MALDI-TOF results obtained from dose-response experiments performed for four compounds: PP2 and staurosporine were selected as potential ABL(H) inhibitors based on the results obtained from the screening experiment described before. Imatinib and dasatinib were chosen as additional references of known inhibitor activity. Measurements were done in duplicate (2 assay replicates, 1 MALDI spot each). MALDI-TOF results were exported to Genedata Screener software for further analysis, i.e. non-linear fitting with variable slope and calculation of IC50 values.

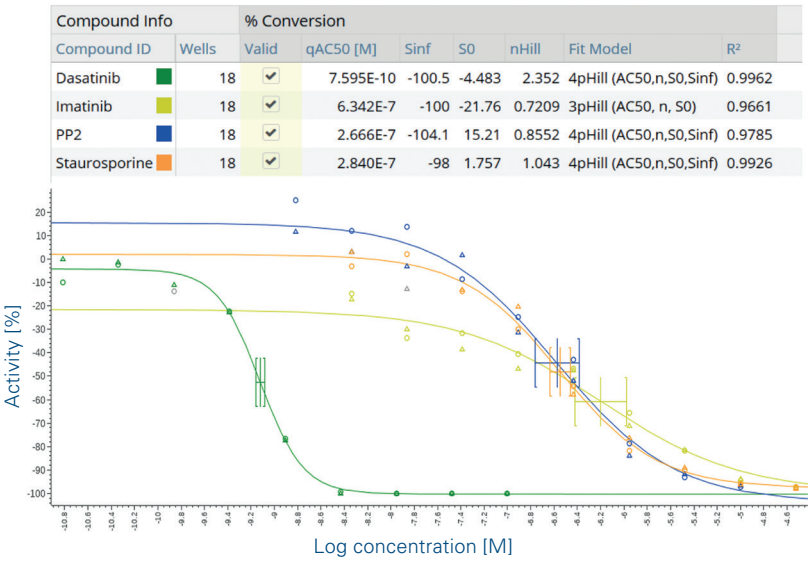

Figure 5: MALDI-TOF based dose-response curves and resulting IC50 values of PP2, Staurosporine, Imatinib and Dasatinib. Substrate concentration was 50 µM. Δ and o represent data points obtained from assay duplicates. Data analysis was performed in Genedata Screener.

For all experiments described above, comparison with ^{33}P labeling reference data (not shown here) indicated a striking level of consistency confirming the high quality of the MALDI-TOF data. This underlines the potential of MALDI-TOF to serve as a simple, fast and cost-efficient readout method in kinase inhibition screening.

Conclusion

- Bruker MALDI PharmaPulse solution represents an emerging technology for MS based label-free high-throughput screening offering time and cost efficiency as well as low false discovery rates.
- rapifleX MALDI-TOF instrument enables very high acquisition speed of up to 10 samples per second.
- MALDI PharmaPulse software provides a workflow-oriented user interface for highly convenient setup and execution of large MALDI-HTS campaigns, allows for instant evaluation of results including quality control based on Z´calculation and features an export interface for seamless data transfer to LIMS or data analysis software, e.g. Genedata Screener.
- MALDI PharmaPulse results obtained from a proof-of-concept kinase inhibition screening experiment are of high quality as indicated by RSD and Z´values and show a remarkable level of consistency with ^{33}P labeling reference data.

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