

Next-Level Performance in Label-Free High-Throughput Screening of Protein-Ligand Interactions with timsTOF MALDI PharmaPulse

We describe here for the first time the application of timsTOF MALDI PharmaPulse® (timsTOF MPP) to high-throughput screening (HTS) of protein-ligand interactions by label-free affinity-selection mass spectrometry (AS-MS).

Abstract

In an AS-MS test experiment, thrombin served as a protein target, and screening was performed against a chemical compound library comprising 1263 small molecules. Using this test setup, we demonstrate the unique capabilities timsTOF MPP provides for enhanced readout of AS-MS based ligand-binding HTS assays. These are:

- AS-MS reading rates of less than a second per well enabled by fast MALDI acquisition speed with 10 kHz smartbeam 3D laser technology.
- Minimized risk of false positive/negative AS-MS hits ensured by accurate-mass readout (routine sub-ppm accuracy) at high resolving power ($R=50,000$) and quantitative feature extraction at narrow tolerance width (typically 1 mDa).
- Fast separation of isobars and isomers by Trapped Ion Mobility Spectrometry (TIMS) reducing assay background interferences, opening up new possibilities for multiplexed AS-MS assays, and yielding collisional cross-section (CCS) values as an additional criterion in compound library validation and for verification of AS-MS hits.

Presented timsTOF MPP AS-MS screening data confirm strong thrombin-specific binding of argatroban and chlorhexidine, two compounds known for their activity as direct thrombin inhibitors.

Setup and execution of the AS-MS test screen was performed using MPP 2023, a newly developed HTS software facilitating large-scale AS-MS screening campaigns on the timsTOF MPP integrated in fully automated HTS environments.

Keywords:
timsTOF MALDI
PharmaPulse®, MPP, MALDI,
timsTOF, protein-ligand
interaction, affinity-selection
mass spectrometry, AS-MS,
binding assay, high-throughput
screening, HTS, pharma,
drug discovery

Introduction

In-vitro measurement of small-molecule ligand binding to a protein target represents an important but challenging step in early drug discovery. Matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS), due to its analysis speed, robustness and seamless integration in fully automated environments has proven itself a powerful reader in label-free high-throughput screening (HTS) [1,2]. Recently, readout by MALDI-MS has been successfully utilized in an in-vitro binding assay platform enabling HTS for small molecule ligands [3]. This novel approach, referred to as affinity-selection mass spectrometry (AS-MS), is based on incubation of compounds with a protein target, separation of resulting protein-ligand complexes by MTP formatted size-exclusion chromatography (SEC), subsequent denaturation and final detection of small-molecule ligands by MALDI-MS.

The new timsTOF MALDI PharmaPulse® (timsTOF MPP) label-free HTS solution takes advantage of MALDI speed and robustness in combination with Bruker's innovative timsTOF technology. In this application note, we describe the first-time application of timsTOF MPP to AS-MS based HTS of protein-ligand interactions. Using an AS-MS test setup with thrombin as a protein target and screening against a compound library comprising 1263 small molecules, we demonstrate the unique capabilities of timsTOF MPP for enhanced readout of AS-MS based ligand-binding HTS assays. These are:

- Fast MALDI acquisition speed with 10 kHz smartbeam 3D laser technology resulting in AS-MS reading rates of less than a second per well.
- Accurate mass (routine sub-ppm accuracy) and high resolving power ($R=50,000$) provided by orthogonal time-of-flight (TOF) mass analysis allowing for quantitative feature extraction at narrow tolerance width (typically 1 mDa) minimizing the risk of false positive/negative AS-MS hits.
- Trapped Ion Mobility Spectrometry (TIMS) enabling
 - fast separation of AS-MS assay background in case of isobaric interferences
 - discrimination between isobaric and even isomeric compounds providing new possibilities for multiplexed AS-MS assays
 - reproducible measurement of collisional cross-section (CCS) values that can serve as an additional criterion in compound library validation and for verification of AS-MS derived ligand hits.

Furthermore, we introduce MALDI PharmaPulse 2023, a newly developed HTS software providing comprehensive AS-MS workflow support on timsTOF MPP and allowing for automated processing of large-scale AS-MS HTS campaigns.

Experimental

Figure 1 displays a scheme describing the AS-MS based HTS workflow. In this study, thrombin was selected as a protein target and screening was performed against 1263 small molecules from a commercial compound library (Prestwick Chemical Libraries, Orléans, France). Argatroban, a known thrombin inhibitor, was selected as a reference compound for strong thrombin-specific binding. Bovine gamma globulin (BGG) was used as a negative-control protein for measurement of non-specific binding.

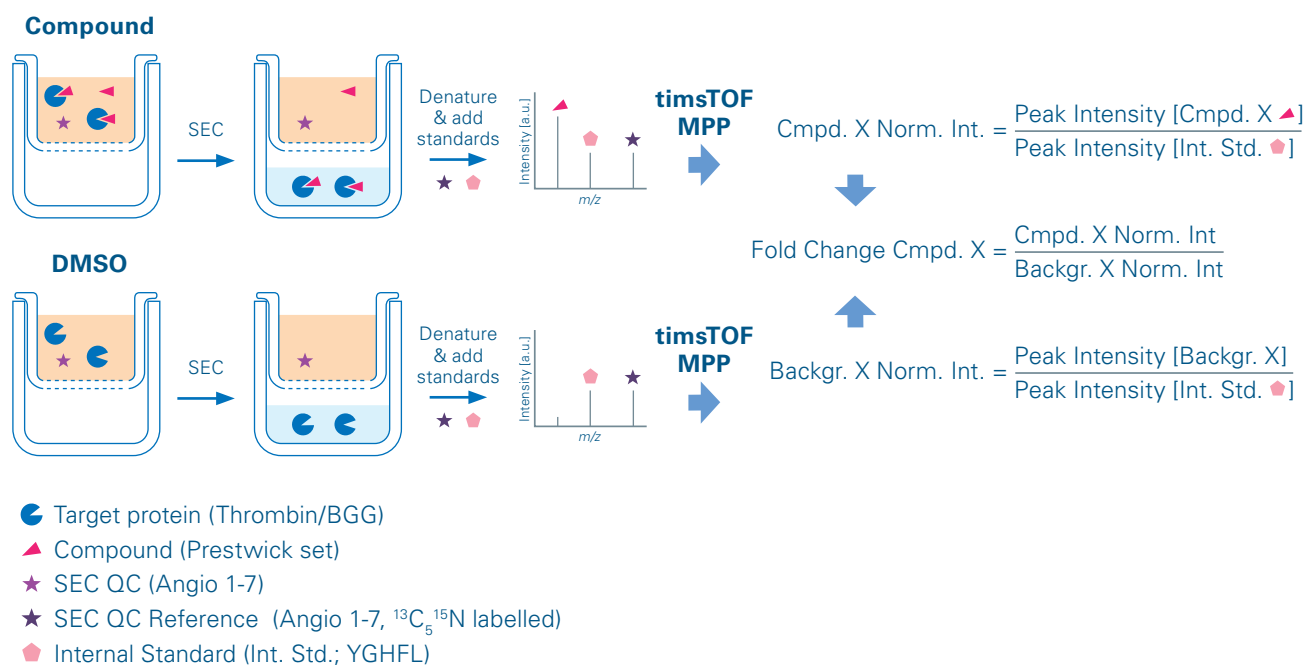


Figure 1
AS-MS workflow for label-free HTS of protein-ligand interactions adapted from Simon et al. [3]

Affinity-Selection (AS) based protein-ligand interaction screening

Single-compound incubation of library compounds (100 μM) with the protein target thrombin (0.3 mg/mL) was performed in 384-well plates. The assay plate accommodated additional background wells (supplied with DMSO instead of compound) and reference wells incubating argatroban (10 μM) with thrombin (0.3 mg/mL; strong specific binding) and bovine gamma globulin (0.3 mg/mL; minimum specific binding), respectively.

Separation of resulting protein-ligand complexes was performed by size-exclusion chromatography (SEC) in the format of 384-well filter plates (Multiscreen HTS 384-well filter plates, polyvinylidene fluoride (PVDF) membrane, pore size 0.45 μm , product no. MZHVN0W10, Merck-Millipore) using angiotensin 1-7 peptide as a SEC quality control (QC) for detection of SEC leakages. SEC eluates from four 384 elution plates were transferred to a 1536-well plate, and stop solution, SEC QC reference ($^{13}\text{C}_5^{15}\text{N}$ angiotensin 1-7) and internal standard (YGHFL peptide) were added. Further details of the affinity-selection based assay workflow are described in [3].

In a primary screening experiment, all 1263 compounds from the Prestwick chemical compound library were screened. Active compounds from the primary screen (i.e. compounds detected at a fold change ≥ 2.5 on both replicate plates) were included in a second round confirmatory screen investigating the level of specificity of thrombin binding. For this purpose, the confirmatory screen was set up as a differential assay comprising two experiments: A) Incubation with thrombin, B) Incubation with bovine gamma globulin (the latter as a measure of non-specific binding). Concentrations were applied as described above for the primary screen.

MALDI preparation

200 nL aliquotes of the denatured SEC eluates were spotted on disposable HTS MALDI plates (product no. 1847006) mounted in dedicated HTS MALDI adapters (product no. 1847571, both Bruker Scientific LLC, Billerica, MA, USA) using a CyBio Well Vario high-performance liquid handler equipped with 1536 capillary head (Analytik Jena GmbH, Jena, Germany). A double-layer matrix preparation protocol was applied using α -cyano-4-hydroxycinnamic acid (HCCA) as a MALDI matrix.

For an initial investigation of the MALDI ionization behaviour of the Prestwick library compounds, ionization test plates were prepared by spotting 200 nL aliquotes of 10 μ M compound solutions applying the HCCA preparation protocol described above.

MALDI plates for the ionization test and from the primary screen were prepared in duplicate. All samples from the second-round confirmatory screen were spotted on the MALDI plate in duplicate, and 3 plate replicates were prepared.

AS-MS readout by timsTOF MPP

AS-MS data were acquired on a timsTOF MALDI PharmaPulse[®] instrument (Bruker Daltonics) equipped with dual ESI/MALDI ion source, 10 kHz smartbeam 3D laser and MALDI plate autoloader. The instrument was operated in either positive or negative ion polarity MALDI-MS mode.

MALDI-MS spectra were accumulated from 8000 laser shots resulting in an acquisition time of less than 1 sec per well.

AS-MS HTS measurements were performed under control of MALDI PharmaPulse 2023 software. All experiment input information (i.e. MALDI plate ID, well positions and corresponding compound sum formula / neutral mass, internal standards in use) was imported as a *.csv file.

Additional MALDI-TIMS-MS analyses were performed for selected compound wells in positive ion mode. A TIMS ramp of 300 ms was applied covering the $1/K_0$ range 0.1 – 1.41. 400 laser shots were fired per TIMS frame, multiple shot bursts were averaged per well.

Data analysis

Quantitative feature extraction from raw MS data was performed by MPP 2023 software automatically in near-real time applying a customized ion profile. An extraction tolerance width of 1 mDa was applied.

Upon completed data acquisition of a MALDI plate, a *.csv formatted result file was automatically generated by MPP 2023 reporting normalized peak intensities, i.e. the ratio ([peak intensity (compound)] / [peak intensity (internal standard)]) for all wells.

KNIME Analytics Platform (KNIME AG, Zurich, Switzerland) was used for downstream analysis of MPP result files obtained from the ionization test and the primary AS-MS screen, i.e. to calculate fold changes (FC) as described in Figure 1.

Binding specificities were calculated as [%] values by comparing the fold change values obtained from compound wells after incubation with thrombin (representing specific + non-specific binding) and bovine gamma globulin (representing non-specific binding), respectively.

Prediction of CCS values was performed using the CCS Predict Pro algorithm featured in Bruker's MetaboScape[®] software.

Results

MALDI ionization of Prestwick library compounds

The vast majority of library compounds showed preferred ionization in positive ion mode. Almost 75% of all compounds were detected as protonated molecular ions $[M+H]^+$. A minority of compounds yielded dominating ion species other than $[M+H]^+$, i.e. $[M.]^+$, $[M+Na]^+$, $[M-H_2O+H]^+$, or ionized in negative mode $(M-H)^-$. Overall, more than 80% of the library compounds were successfully detected in MALDI-MS.

Because of the strong dominance of compounds detected as protonated ions in positive mode, all data shown in the following are solely based on quantitative feature extraction from $[M+H]^+$ ion signals. Figure 2 provides a summary of results obtained from the ionization test.

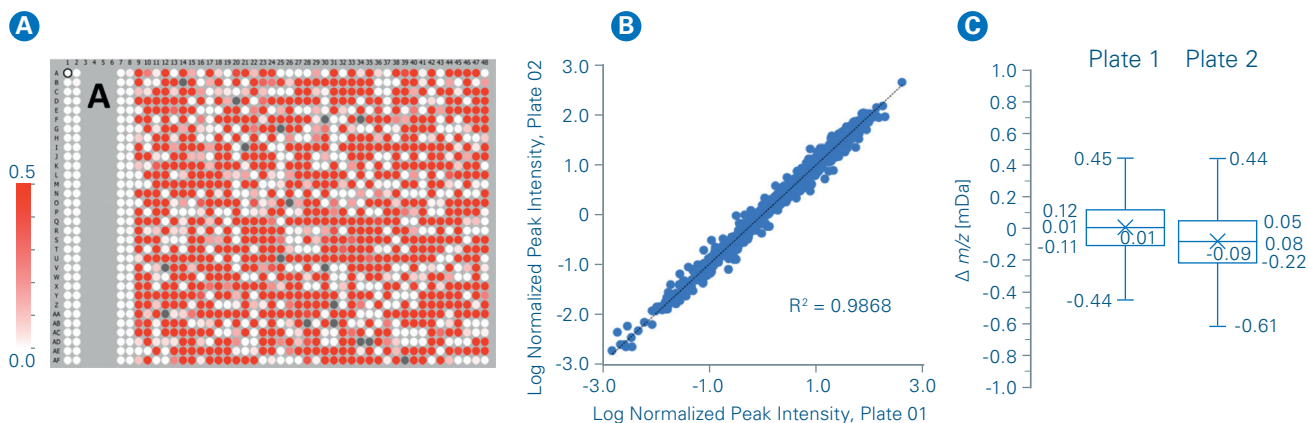


Figure 2

Results obtained from (+)MALDI-MS analysis of ionization test plates

(A) Plate view of one out of two replicate MALDI plates. Color gradient indicates compound $[M+H]^+$ peak intensities normalized against internal standard peptide YGHFL. Columns 9-48 accommodate the Prestwick library compounds. Grey colored wells indicate empty spot positions. Columns 1-2 and 7-8 served as background wells (DMSO). **(B)** Correlation plot evaluating plate-to-plate reproducibility of quantitative compound detection by MALDI-MS after normalization against the internal standard. Correlation coefficient close to 0.99 indicates excellent reproducibility. **(C)** Mass accuracy of the internal standard signal plotted for all wells on ionization test plates 1 and 2. On all wells, the internal standard signal was detected well within a tolerance of ± 1 mDa. Accordingly, 1 mDa was set as tolerance width for quantitative feature extraction in the subsequent AS-MS screening experiment.

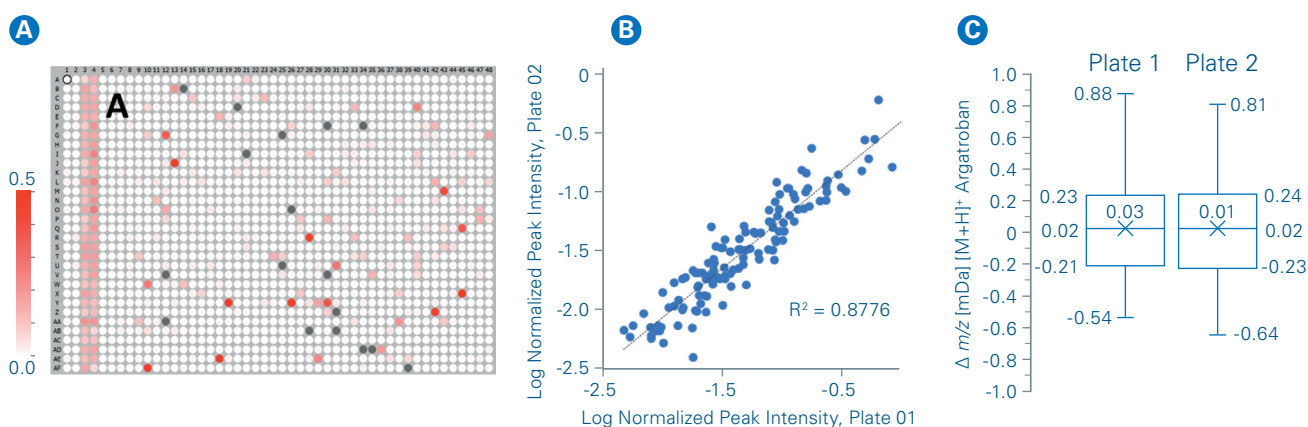


Figure 3

Results obtained from the primary AS-MS screening experiment

(A) Plate view reporting normalized peak intensities of Prestwick library compounds after incubation with thrombin (columns 9-48; grey colored spots indicate empty wells). Readout obtained from argatroban reference wells after incubation with thrombin (columns 3-4) and BGG (columns 5-6), respectively, confirm the expected strong thrombin-specific binding. Columns 1-2 and 7-8 represent background wells (DMSO incubated with thrombin). **(B)** Correlation plot comparing the results from two AS-MS MALDI plate replicates of 129 compounds for which thrombin binding was detected (fold change ≥ 2.5). Correlation coefficient close to 0.9 confirms high level of reproducibility of timsTOF MPP AS-MS readout. **(C)** Mass accuracy of reference compound argatroban $[M+H]^+$ signal acquired from 64 reference wells (columns 3-4) on two replicate AS-MS MALDI plates. All signals were well within the 1 mDa tolerance applied for quantitative feature extraction confirming outstanding quality of timsTOF MPP AS-MS data collected under real assay conditions.



Figure 4

Results obtained from the second-round confirmatory AS-MS screen investigating the level of specificity of thrombin binding for 129 compounds detected as binders in the primary screen.

(A) Selection of compounds, for which highly thrombin-specific binding was confirmed based on second-round confirmatory AS-MS data. **(B)** Box plot displaying normalized peak intensities obtained from argatroban reference wells after incubation with thrombin and BGG (64 wells each), respectively. Data confirm the expected strong specific binding of argatroban, a compound known as a direct thrombin inhibitor. High level of consistency of results throughout argatroban reference wells indicates good reproducibility of timsTOF MPP AS-MS readout within a MALDI plate as well as across replicate MALDI plates. **(C)** MALDI-MS spectra obtained from a well containing compound PRESTWICK-0143, chlorhexidine. Data suggest highly thrombin-specific binding which is in agreement with chlorhexidine's known activity as a direct thrombin inhibitor. Absence of signal of SEC QC peptide angio 1-7 rules out any potential interferences caused by SEC leakage.

AS-MS based HTS of protein-ligand interactions

In a primary screening experiment, two replicate MALDI plates holding the SEC eluates from the affinity-selection based protein-ligand binding assay were analyzed. Results are presented in Figure 3.

129 compounds detected as binders in the primary screen were further investigated in a second-round confirmatory AS-MS experiment regarding the level of specificity of thrombin binding. A selection of results is given in Figure 4.

Accurate mass and high resolving power are key to high-quality AS-MS results

Sub-ppm mass accuracy and 50,000 resolving power provided by timsTOF MPP allow for quantitative feature extraction from AS-MS raw data at a tolerance width as narrow as 1 mDa. Applying such narrow tolerance represents an efficient means to avoid signal false assignments, and, thus, minimize the risk of false positive/negative ligand hits. Two examples from the primary screen are given in Figure 5.

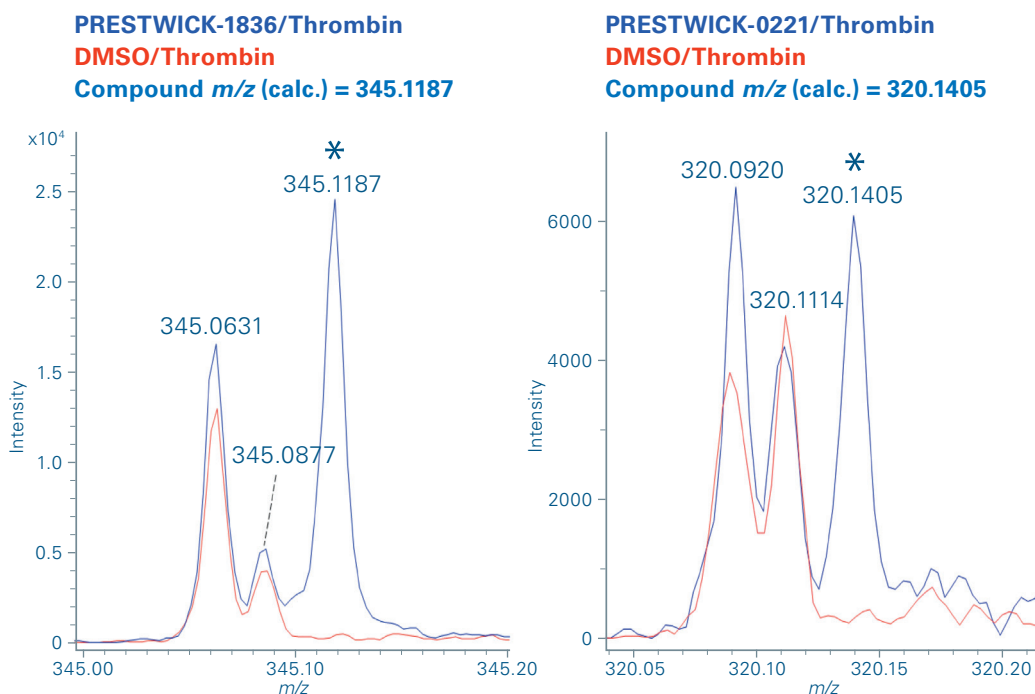


Figure 5

Zoomed view on MALDI-MS spectra obtained from two compound wells analyzed in the primary AS-MS screen.

Signals marked with asterisk represent the targeted compounds. Mass resolving power provided by timsTOF MPP enabled the separation of complex patterns of background signals appearing in a minimum distance to the target signal of only 20-30 mDa. At the same time, accurate-mass measurement at sub-ppm accuracy level ensured correct assignment of the target signals and, thus, avoided false negative results.

Expanding the scope of AS-MS with TIMS

TIMS adds an orthogonal dimension of fast separation and, thereby, has the potential to further enhance AS-MS in multiple regards by means of

- leveraging TIMS resolving power to eliminate interferences with isobaric assay background
- TIMS enabled discrimination between isobaric and isomeric compounds offering new possibilities for multiplexed AS-MS assays and isomer-specific AS-MS readout
- reproducible CCS values that can serve as an additional criterion in compound library validation and for verification of AS-MS hits.

Examples illustrating these TIMS capabilities are given in Figures 6-7 and Table 1.

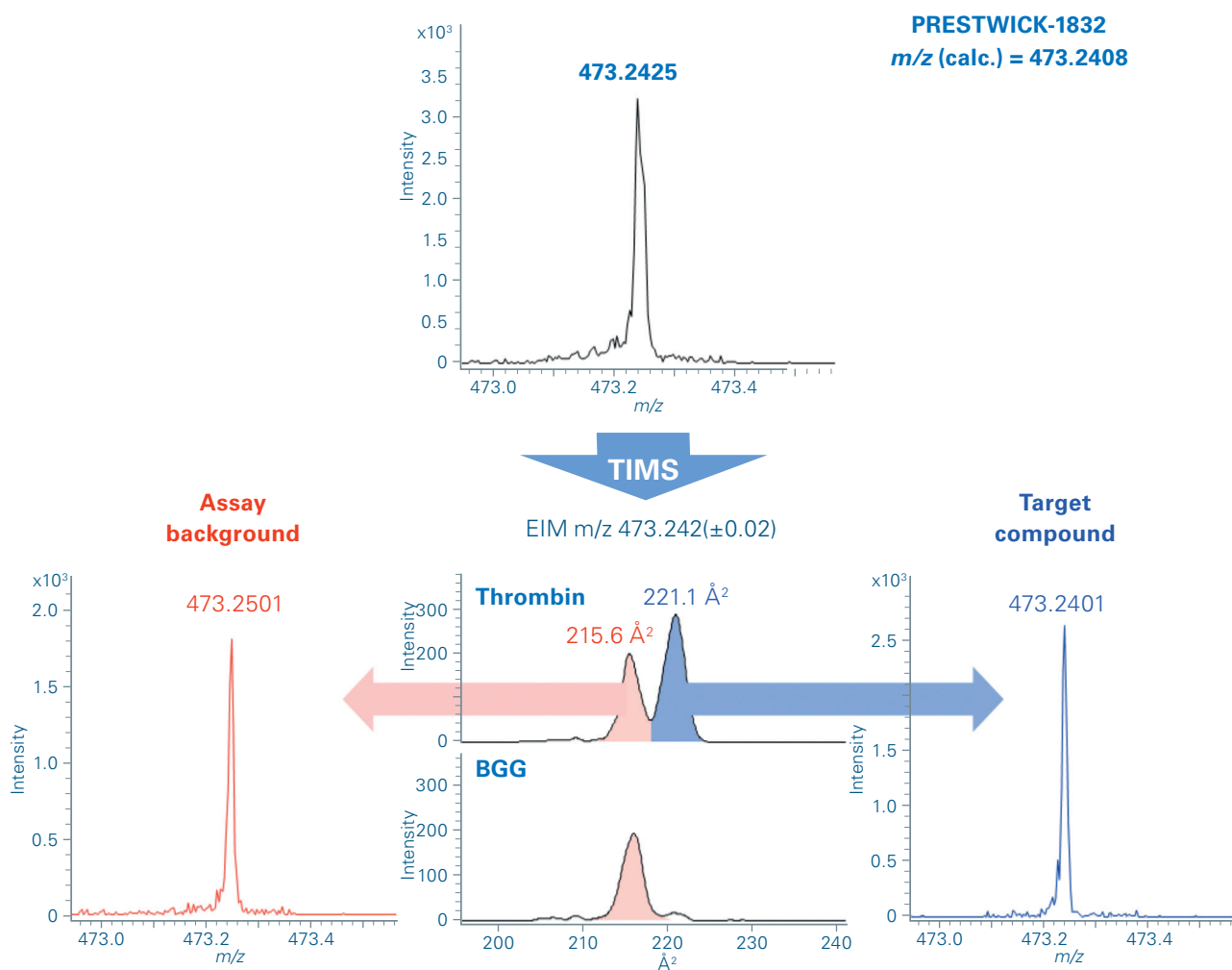


Figure 6
TIMS separation of isobaric assay background.

Top: MALDI-MS spectrum obtained from a compound well in the second-round confirmatory AS-MS screen showing a composite m/z signal merging the target compound with an isobaric background component of unknown identity (critical m/z distance approx. 0.01). **Bottom:** MALDI-TIMS-MS analysis resolved the background overlap in the TIMS dimension allowing for interference-free quantitative detection of the target compound.

PRESTWICK-0536
PRESTWICK-0332
 m/z (calc.) = 267.1703

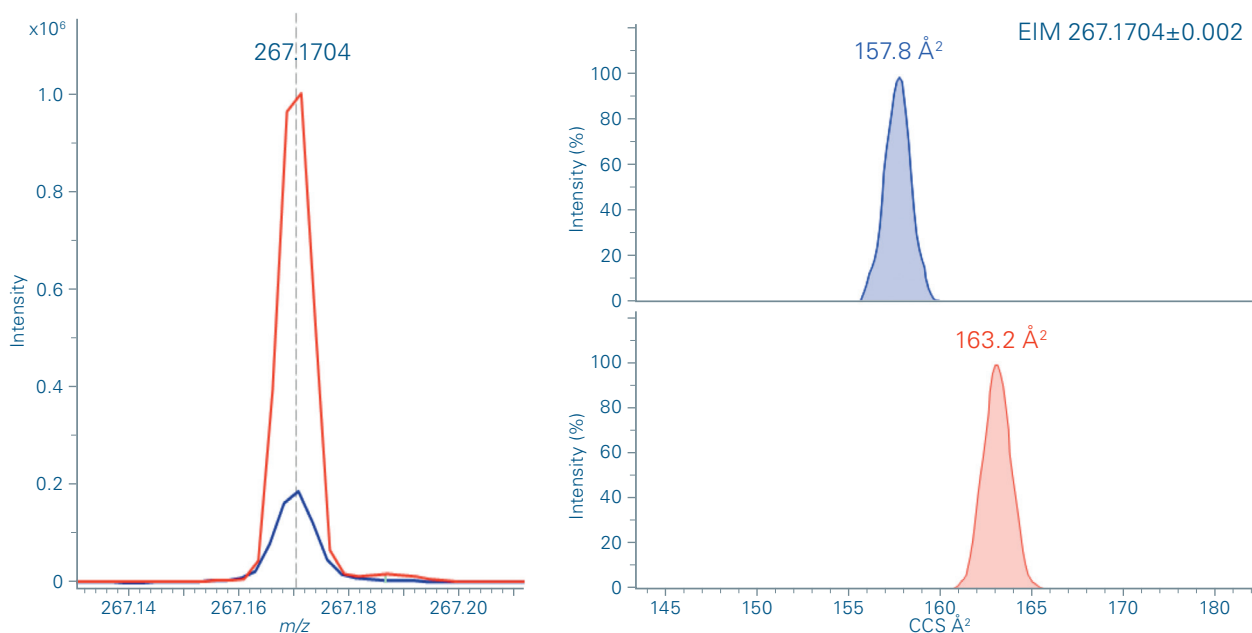


Figure 7

Discrimination between isomers by TIMS.

Left: Overlaid MALDI-MS spectra of two isomeric library compounds; **Right:** Extracted ion mobilograms obtained from MALDI-TIMS-MS analysis demonstrating separation of these isomeric compounds based on a distinct difference in their CCS values.

Compound	Ion	CCS (measured) [Å ²]	CCS (predicted) [Å ²]	ΔCCS (meas.-predict.) [%]
PRESTWICK-0143	[M+H] ⁺	222,3	218,9	1.6
PRESTWICK-0204	[M+H] ⁺	152,7	157,2	-2.8
PRESTWICK-0221	[M+H] ⁺	173,5	170,9	1.5
PRESTWICK-0385	[M+H] ⁺	196,9	201,2	-2.1
PRESTWICK-0428	[M+H] ⁺	178,5	184,6	-3.3
PRESTWICK-0858	[M+H] ⁺	204,2	208,5	-2.0
PRESTWICK-0862	[M+H] ⁺	215,5	210,3	2.5
PRESTWICK-1120	[M+H] ⁺	184,2	184,6	-0.2
PRESTWICK-1432	[M+H] ⁺	188,7	189,6	-0.5
PRESTWICK-1446	[M+H] ⁺	194,1	194,3	-0.1
PRESTWICK-1464	[M+H] ⁺	188,6	190,2	-0.8
PRESTWICK-1825	[M+H] ⁺	192,1	189,1	1.6
PRESTWICK-1752	[M+H] ⁺	228,1	220,4	3.5
PRESTWICK-0777	[M+H] ⁺	238,7	233,1	2.4
PRESTWICK-0318	[M+H] ⁺	196,3	197,9	-0.8
PRESTWICK-1832	[M+H] ⁺	221,1	212,9	3.9

Table 1

CCS values derived from MALDI-TIMS-MS analysis of a selection of compound wells for which thrombin-specific binding had been detected in the confirmatory AS-MS screen. Measured CCS values are in good agreement with predicted CCS values and, thus, can serve as an additional criterion in compound library validation as well as AS-MS hit verification.

MPP 2023 software: Seamless AS-MS HTS support for timsTOF MPP

MPP 2023 provides the following AS-MS relevant key features:

- Intuitive graphical user interface for simplified HTS campaign setup and execution as well as result visualization
- Convenient *.csv import of input information required for experiment setup, i.e. plate IDs, compound well information and internal standards in use
- Automated calculation of target m/z values applying a customized ion profile
- Quantitative feature extraction in near-real time
- Generation of *.csv formatted result files for instant data transfer to external software, i.e. Genedata Screener or KNIME
- Well documented automation interface for timsTOF MPP system integration with external scheduling software and lab robotics for automated MALDI plate exchange

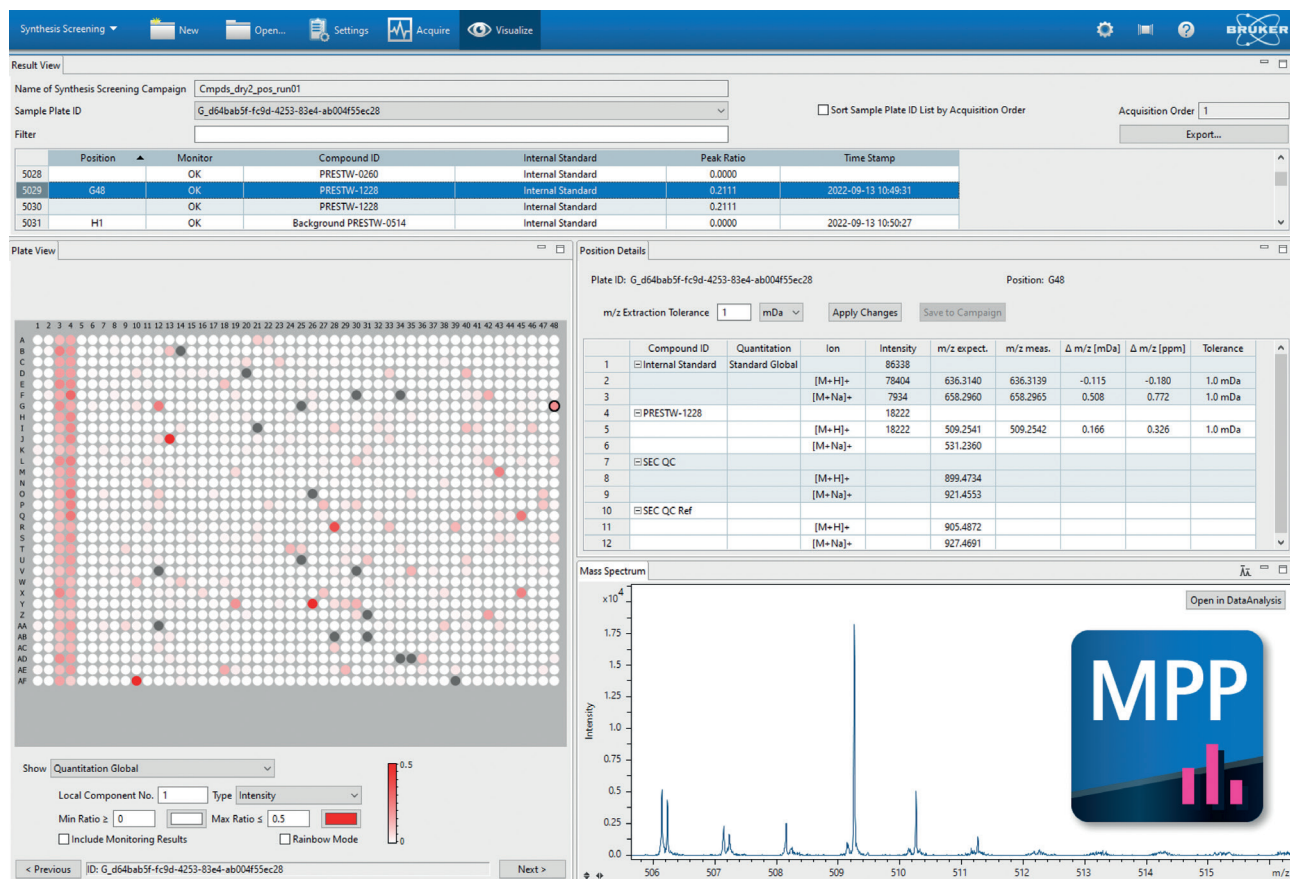


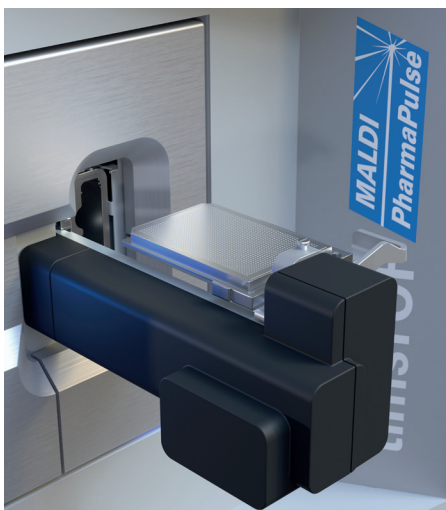
Figure 8

MPP 2023 graphical user interface allowing for intuitive visualization of AS-MS screening results.

Product overview

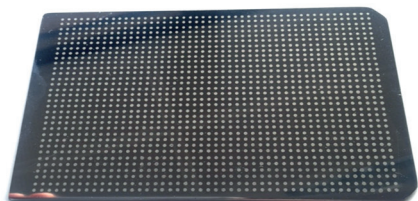
timsTOF MALDI PharmaPulse® instrument

- Dual ESI/MALDI ion source with 10 kHz smartbeam 3D laser
- Autoloader enabling MALDI plate exchange by robot
- High-capacity dual TIMS-XR analyzer



Disposable MALDI HTS sample plates

- Compatible with any HTS format (96, 384, 1536 and beyond)



Light-weight MALDI plate adapter

- Designed for safe handling by lab robot



MALDI PharmaPulse 2023

- Dedicated HTS software supporting timsTOF MALDI PharmaPulse®
- Seamless HTS campaign setup, execution and result visualization
- Supporting various HTS workflows and timsTOF operation modes
- Automation interface for system integration in automated HTS environments
- Export interface for instant data transfer to third-party software (i.e. Genedata Screener)



Conclusion

- timsTOF MALDI PharmaPulse® enables unmatched performance in AS-MS based label-free HTS of protein-ligand interactions by taking advantage of MALDI speed and robustness in combination with innovative timsTOF technology.
- timsTOF accurate-mass measurement at routine 50,000 resolving power allows for quantitative feature extraction at 1 mDa tolerance width, thereby minimizing the risk of false positive/negative AS-MS hits.
- TIMS as an added dimension of fast separation offers unique potential for further enhanced AS-MS by eliminating interferences with isobaric assay background, providing new possibilities for multiplexing even with isobaric and isomeric compounds, and using reproducible CCS values as a criterion in compound library validation and for verification of AS-MS hits.
- Newly developed HTS software MPP 2023 provides a workflow-oriented graphical user interface enabling large-scale timsTOF MPP AS-MS screening campaigns.

References

- [1] Haslam C, Hellicar J, Dunn A, Fuetterer A, Hardy N, Marshall P, Paape R, Pemberton M, Resemann A, Leveridge M (2016). *The Evolution of MALDI-TOF Mass Spectrometry toward Ultra-High-Throughput Screening: 1536-Well Format and Beyond*. *SLAS Discovery*. **21**(2) 176-186
- [2] Winter M, Ries R, Kleiner C, Bischoff D, Luippold AH, Bretschneider T, Buettner FH (2019). *Automated MALDI Target Preparation Concept: Providing Ultra-High-Throughput Mass Spectrometry-Based Screening for Drug Discovery*. *SLAS Technology*. **24**(2) 209-221
- [3] Simon RP, Winter M, Kleiner C, Wehrle L, Karnath M, Ries R, Zeeb M, Schnapp G, Fiegen D, Häbe TT, Runge F, Bretschneider T, Luippold AH, Bischoff D, Reindl W, Büttner FH (2021). *MALDI-TOF-Based Affinity Selection Mass Spectrometry for Automated Screening of Protein-Ligand Interactions at High Throughput*. *SLAS Discovery*. **26**(1) 44-57

For Research Use Only. Not for use in clinical diagnostic procedures.

Bruker Switzerland AG

Fällanden · Switzerland
Phone +41 44 825 91 11

Bruker Scientific LLC

Billerica, MA · USA
Phone +1 (978) 663-3660

