Use of PASEF for Accelerated Protein Sequence Confirmation and *de novo* Sequencing with High Data Quality

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Overview

- Biopharmaceutical sequences can be well confirmed or established by multiple protease digests - e.g., trypsin, elastase and chymotrypsin – followed by LC-MS/MS data analysis.
- **PASEF** (Parallel Accumulation and Serial Fragmentation, **1**) on the timsTOF instrument (***Fig 1***) allowed to generate high-quality peptide maps suitable for sequence confirmation and *de novo* sequencing as well.
- Sequences were established by combination of results from 3 enzyme digests using a short LC gradient.
- Nivolumab and Dulaglutide sequences were confirmed and Nivolumab sequenced *de novo*.

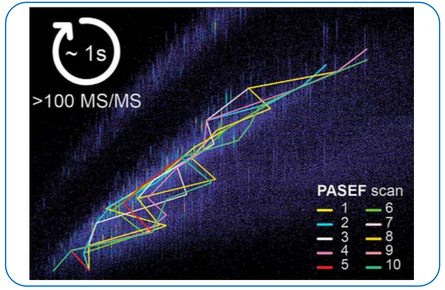

Fig. 1 **PASEF** Spectra were obtained with a standard 1.1 sec PASEF acquisition cycle. In this method, **P**arallel **A**ccumulation and **SE**rial **F**ragmentation results in increased sensitivity due to ion mobility focusing of the ions, which are sequentially fragmented at > 100 Hz. This increased sensitivity and analysis depth results in more peptides, fragment ions and a high sequence coverage even in antibody *de novo* sequencing analyses.

Methods

Chemistry Nivolumab (IgG4κ) and dulaglutide (IgG4 based Fc-fusion peptide) were reduced and carbamidomethylated prior to proteolytic digest by trypsin, elastase or chymotrypsin (**2**).

LC-separation Acquity 2.1x150 mm UPLC C18 column, 45 min gradient length, 60 min cycle time.

PASEF Peptide maps were obtained on the timsTOF (Bruker) with standard proteomics parameters.

Data Analysis PASEF datasets were processed directly either in BioPharma Compass 2021 (Bruker) where spectra were matched with the reference sequence (5 ppm MS tolerance, no enzyme) for confirmation of provided sequences or to Supernovo (Protein Metrics) to establish sequences *de novo*.

Dulaglutide

For dulaglutide 96/100/90% SC were obtained and 92/90/83 % FC; combined, 100 % SC and 100 % FC were obtained (***Fig. 2***). The merged peptide map from the 3 digests resulted in 221 peptides; enough to safely confirm the full dulaglutide.

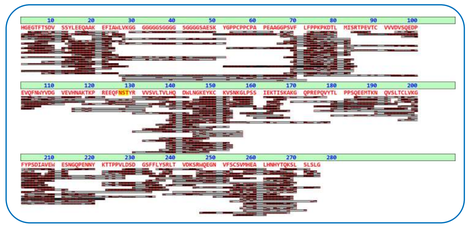

Fig. 2 Sequence coverage map of dulaglutide generated by combination of the results from the individual 3 digests. .

Sequence confirmation:

Nivolumab

Proteins were digested by 3 enzymes individually. For nivolumab 94/94/90% sequence coverage (**SC**) and 86/84/85% fragment coverage (**FC**) were obtained from the individual digest analyses with Trypsin/Chymotrypsin/Elastase, respectively.

The combined data (***Fig. 3***) resulted in 305 HC and 148 LC peptides and 100% SC and 99% FC; enough to safely confirm the full sequence – including the sequences of all 6 CDRs - and to determine the nivolumab sequence *de novo* (***Fig. 4***).

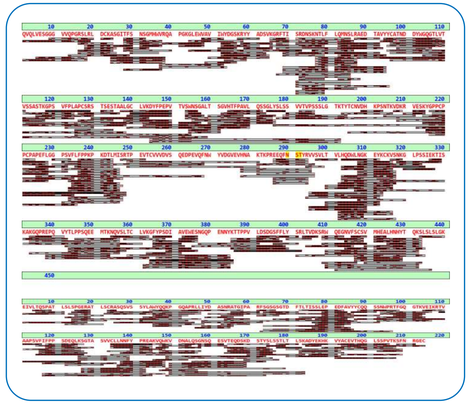

Fig. 3 Sequence coverage maps of the nivolumab HC (top) and LC (bottom) generated by combination of the results from the individual 3 digests. Red sequence text indicates the presence of identified peptides covering the particular sequence stretch, black: not covered by any peptide - this is not observed here. Grey bars: matching peptides contributing to cover the sequence. Red bricks: matching fragment ions; upper row of bricks: matching N-terminal b-ion fragments, lower row of bricks: matching C-terminal y-ion fragments.

De novo sequencing

The combined nivolumab peptide maps were analyzed using Supernovo. The *de novo* determined sequence had 99.5% identity to known sequence of Nivolumab (Drugbank). The differences were localized at heavy chain residue 27, where the determined was Leucine instead of Isoleucine, and at 99-100 where the determined motif was DN rather than ND.

The metrics/visualizations, which include the confidence of each deduced residue, an aggregate fragmentation map, differences from germline, and a peptide inspection dashboard, helped to spot and fix the errors.

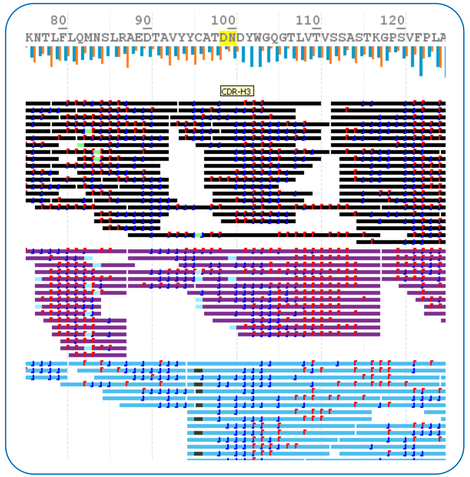

Fig. 4 Sample Supernovo output. Three samples' peptides are shown. Yellow highlight on the protein sequence indicates low confidence in the determined sequence, and vertical blue bars below the sequence show aggregated fragment coverage in these residues.

References

Meier F, Beck S, Grassl N, Lubeck M, Park MA, Raether O, Mann M. Parallel Accumulation-Serial Fragmentation (PASEF): Multiplying Sequencing Speed and Sensitivity by Synchronized Scans in a Trapped Ion Mobility Device. J Proteome Res. 2015;14(12):5378-87.

Suckau D, Evers W, Belau E, Pengelley S , Resemann A , Tang W, Sen KI, Wagner E, Colas O, Beck A. Use of PASEF for accelerated protein sequence confirmation and *de novo* sequencing with high data quality. Methods in Molecular Biology 2020, Mass spectrometric sequencing of therapeutic antibodies, Chapter X Editor: Gunnar Houen; in press.

Conclusions

- Sequence conformation and *de novo* analysis was based on only 3 enzyme digests and 1 hr LC cycle time using PASEF
- Nivolumab and the fusion protein Dulaglutid sequences were fully confirmed
- Nivolumab sequence was fully established *de novo* including the 6 CDR sequences
- PASEF shortened the typical time for sequence analysis of biopharmaceuticals significantly and improved data quality concomitantly
- It can be expected that the method will reduce costs for full biologics sequence analysis and make such analysis more readily available

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