European Commission
Horizon 2020
European Union funding
for Research & Innovation

TopSpec - 829157

WP7 - Signal detection and data processing

Deliverable: D7.3 - Optimized FTMS Boosters test protocols

Task: Protocols of installation and testing of two optimized data acquisition systems FTMS Booster TD at KI and IP for protein top-down analysis on the Orbitrap mass spectrometers (SPS: the lead, performs the task using SPS infrastructure and personnel)

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Deadline: 10/31/2022

1. Executive summary

- Previously (D7.1.), we reported on the development and installation of the two prototype high-performance data acquisition (DAQ) systems, named FTMS Booster TD. The prototype systems were installed on a Q Exactive Orbitrap instrument equipped with the Omnitrap at FasmaTech (Greece) in January 2021 and on a Q Exactive HF Orbitrap at the Institute Pasteur (France) in September 2021;
- The FTMS Booster TD Installation and Acceptance Protocols were compiled and their content was verified during the two installations. These protocols were fulfilled at both installation sites;
- Presently, we report on the development of the optimized installation and acceptance protocols based on the experience obtained during the two installations described above and on the additional installation of the FTMS Booster TD;
- **Importantly, we now report on a first successful implementation of the FTMS Booster TD on the state-of-the-art Orbitrap platform, namely Exploris 480, that was selected as the new-generation Orbitrap-Omnitrap platform for a complete TopSpec system;**
- The complete TopSpec system installations, comprising an Orbitrap, Omnitrap, pI Trap, and FTMS Booster TD, are now active at the two TopSpec partner sites: in Karolinska Institute (Sweden) and Institute Pasteur (France);
- **The up-to-date installation and acceptance protocols have been generated and validated for the optimized software tools for data acquisition, data processing, and data analysis.**

Milestone M11 "Two FTMS Booster prototypes are designed, implemented, and evaluated":

This milestone has been reached and over-exceeded by implementation of the FTMS Booster TD prototypes on three TopSpec systems, each of which would be build around different model of an Orbitrap mass spectrometer: Q Exactive Plus, Q Exactive HF, and Exploris 480.

2. Description of the action

To reach the objectives of WP7' D7.3 deliverable according to the updated TopSpec plan (addition of one more model of an Orbitrap mass spectrometer), the unreduced data in the form of time-domain transients are to be acquired from the current state-of-the-art Orbitrap platform – the Exploris 480 - interfaced with an Omnitrap. The Exploris platform is detailed in the WP6 deliverables report of TopSpec by Alexander Makarov and Kyle Fort (Thermo Fisher Scientific). Due to the principally different architecture and electronics layout, the previously established interfacing of the FTMS Booster TD to the Q Exactive Orbitrap platforms reported in D7.1 does not work on the Exploris platforms. To enable this implementation, the Spectroswiss team visited TopSpec partner, Karolinska Institute (Roman Zubarev laboratories) and performed, for the first time, a successful installation of the FTMS Booster TD on the Exploris 480 platform, **Figure 1**.

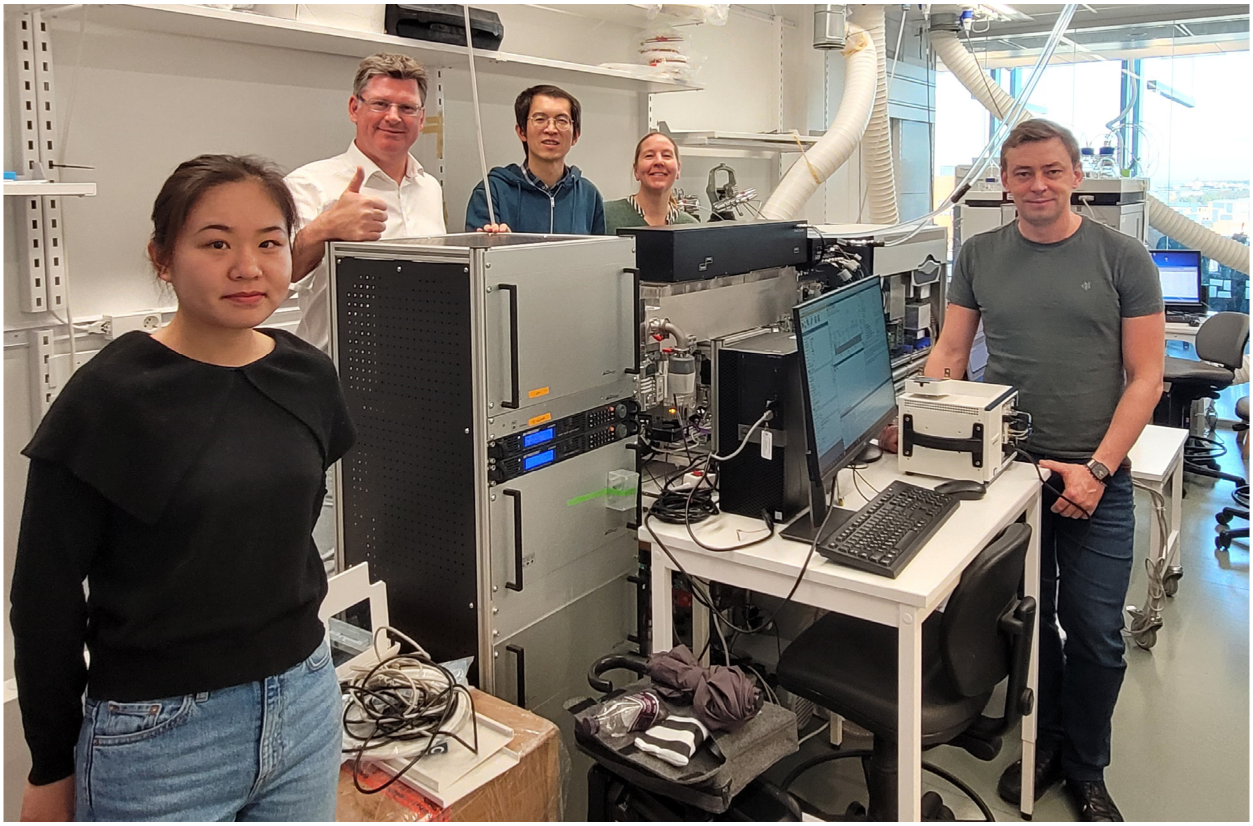

Figure 1. Work in progress – installation of the FTMS Booster TD on the Omnitrap-enabled Exploris 480 Orbitrap Zubarev at Karolinska Institute (KI) in Stockholm, Sweden (November 29 – December 1, 2022). In the picture, Hezheng Lyu (KI), Yury Tsybin (Spectroswiss), Zhaowei Meng (KI), Susanna Lundström (KI), and Konstantin Nagornov (Spectroswiss).

The interfacing was performed with a support of other TopSpec partners, Thermo Fisher Scientific and FasmaTech. The main difference and challenge for the installation was to evaluate possible triggers for the appropriate coupling of the Exploris and FTMS Booster TD electronics. The careful examination of the Exploris electronics system revealed a possible solution and the following work was focused on the evaluation and implementation of the thus found triggering solution. As a result, the connection between the FTMS Booster TD (as a guest system) and an Exploris 480 (as a host system), was established and stable performance regarding the recording of the time-domain transients was achieved, **Figure 2**. The FTMS Booster TD firmware was updated accordingly.

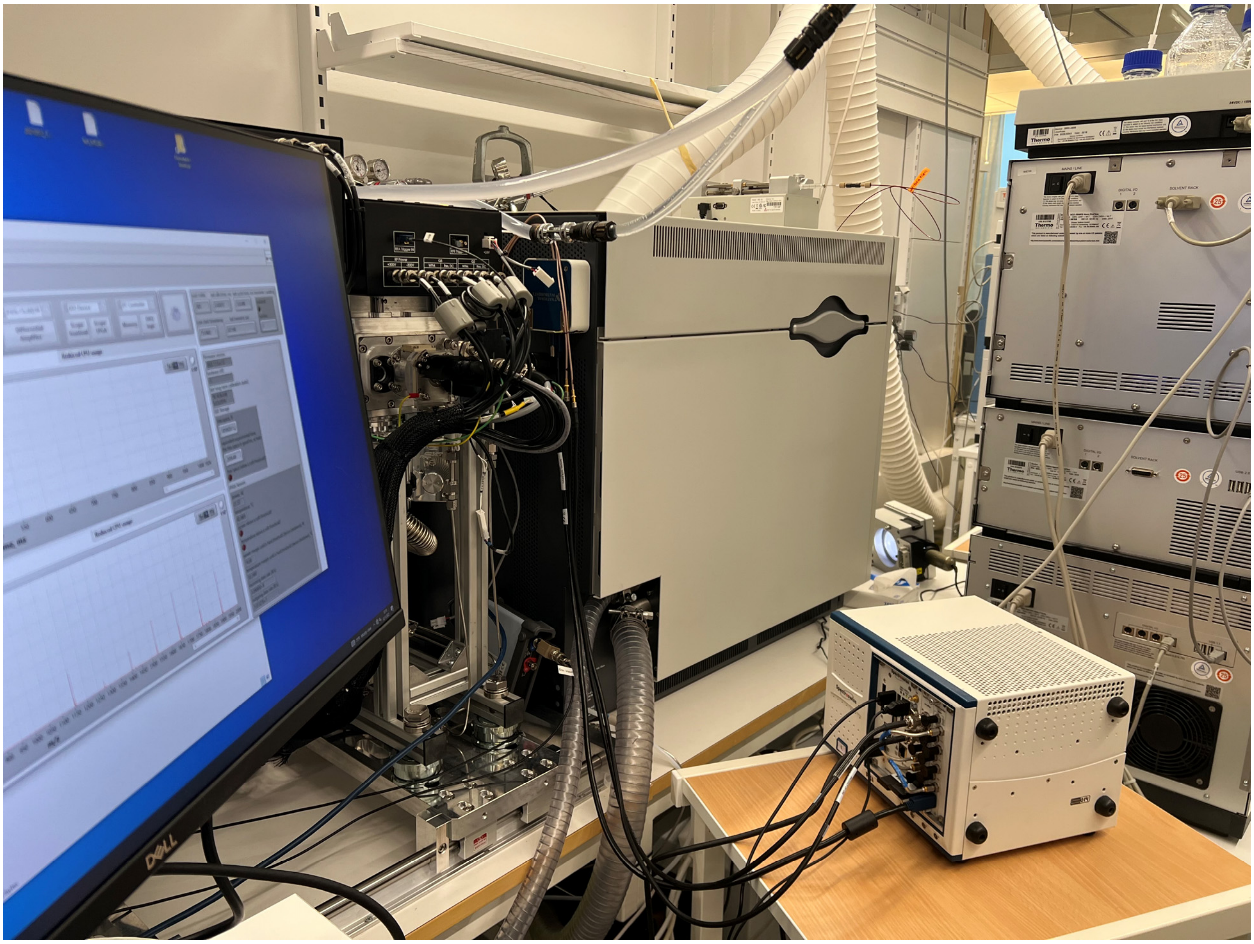

Figure 2. A prototype of a high-performance data acquisition system, FTMS Booster TD (shown to the right, bottom), is connected to the preamplifier output and the trigger of the Exploris 480 Orbitrap in parallel to the original built-in data acquisition system (Thermo Fisher Scientific). The unreduced FTMS data (time-domain transients) are received from the pre-amplifier in an analogue mode by the FTMS Booster TD, and then amplified and digitized. The software for data acquisition and FTMS Booster TD manipulation is shown on the screen to the left. The demonstrated installation is in the Karolinska Institute, Stockholm, Sweden (December 2022).

As a result, the prototype external high-performance DAQ systems designed specifically for top-down mass spectrometry applications have been interfaced to three different Orbitrap platforms

(i) Q Exactive Plus (FasmaTech, Greece), Q Exactive HF (Institute Pasteur, France), and Exploris 480 (Karolinska Institute, Sweden). The final outcome of the TopSpec project, a TopSpec system that includes all four major modules: (i) Omnitrap, (ii) Orbitrap, (iii) pI Trap, and (iv) FTMS Booster TD, has been developed and installed in two laboratories – at Institute Pasteur (France) and Karolinska Institute (Sweden), **Figure 3**.

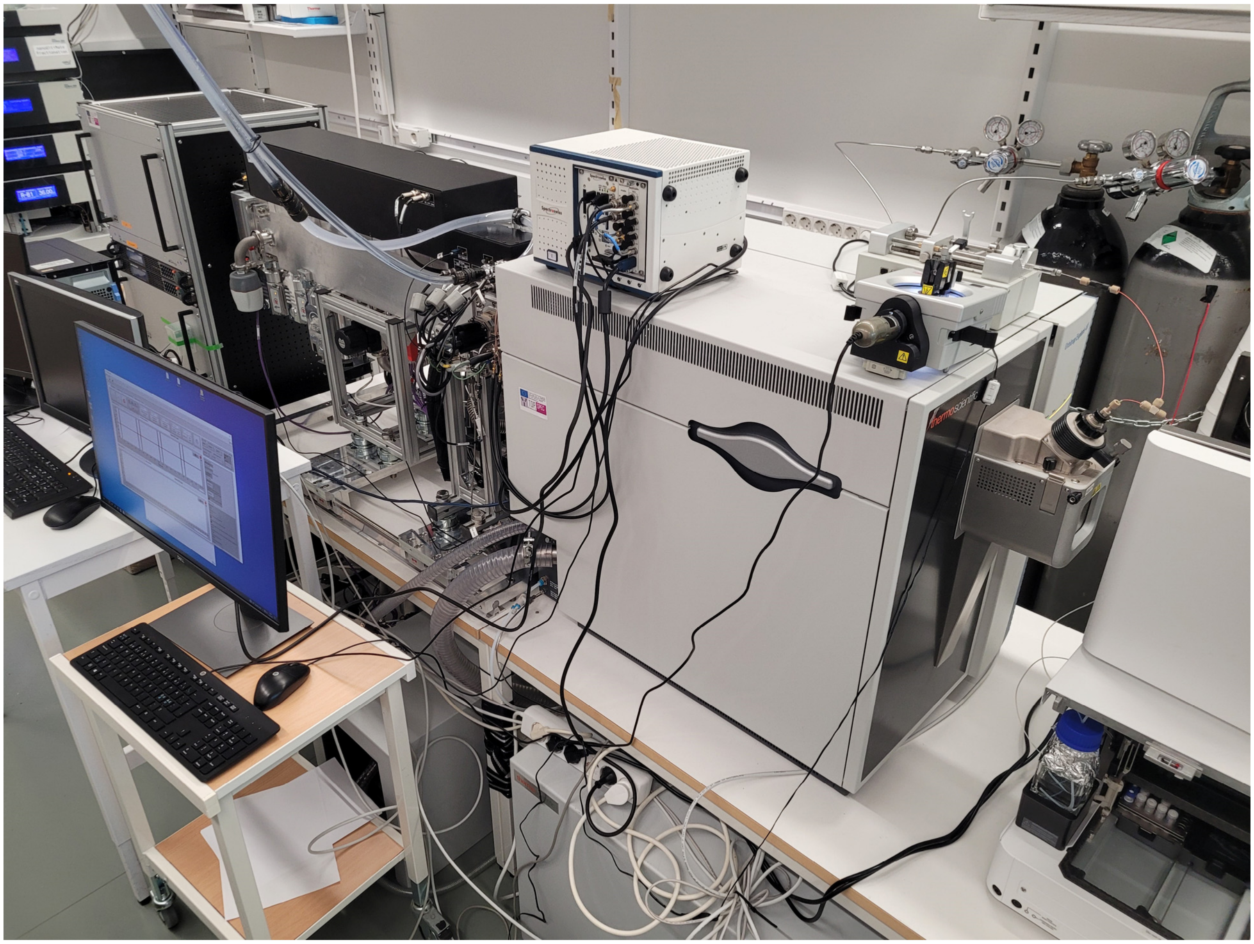

Figure 3. A complete TopSpec system that includes (i) Omnitrap, (ii) Orbitrap Exploris 480, (iii) pI Trap, and (iv) a high-performance data acquisition system FTMS Booster TD, as installed in Karolinska Institute (Sweden). In addition, the Omnitrap is outfitted with the ion mobility capability.

In addition to the FTMS Booster TD installation on Exploris 480, Spectroswiss team continued to (remotely) work with the teams in Institute Pasteur and FasmaTech to evaluate and optimize the installation and acceptance protocols, as well as to facilitate the use of the FTMS Booster TD and of the embedded firmware and of the different software tools – from data acquisition to reporting.

3. Performance

The general objective of acquiring and processing the unreduced data (time-domain transients) is to maximize information output from Orbitraps for the monoclonal antibodies (mAbs) analysis: to deliver increased confidence and productivity, including sensitivity and throughput. In this regard, we continued to evaluate the performance of the TopSpec systems equipped with access to the unreduced data (time-domain transients).

Figure 4 shows an example of such evaluation – aimed for improved antibody analysis when a low-resolution setting is selected for mass spectra acquisition on a Q Exactive Orbitrap HF.

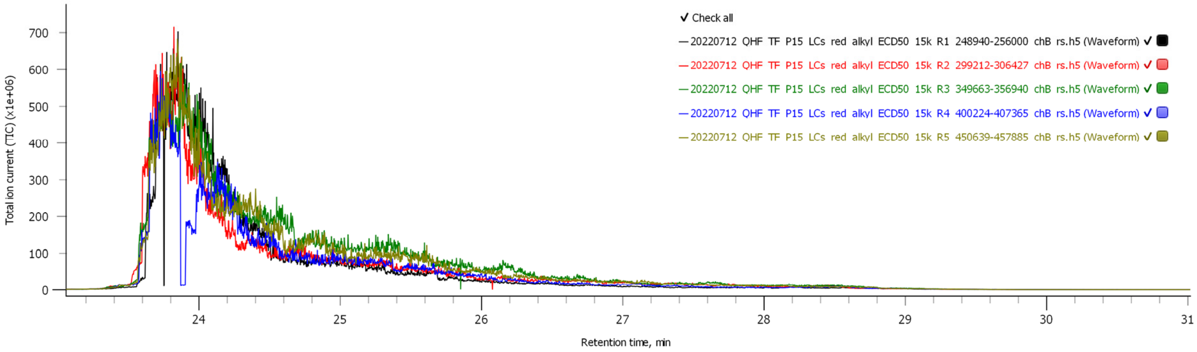

Figure 4. Analysis of mAbs' light chains with electron capture dissociation (ECD) on a Q Exactive HF equipped with an Omnitrap and FTMS Booster TD (Institute Pasteur, France). Orbitrap settings: the resolution of 15'000 at *m/z* 200, AGC of 5e5, and ITmax of 200 ms. Shown are the total ion current (TIC) chromatograms for five technical replicates. Data were processed and analyzed with Peak-by-Peak BioPharma software (Spectroswiss).

Data processing and analysis of the LC-MS/MS datasets shown in Figure 4 revealed a substantial overhead that is introduced by performing ECD in an Omnitrap, **Figure 5**. Due to the overhead introduced by ion-electron dissociation (ECD) in an Omnitrap, the time-domain transient lengths acquired with the FTMS Booster TD is proportionally higher than the one acquired with the original Orbitrap DAQ system (150 ms versus 32 ms). As a result, the obtainable resolution with the longer time-domain transient is proportionally higher and isotopically resolved mass spectra can be obtained (Figure 5 insets). When mass spectra of similar time-domain transient periods are compared, the resolution and the peak shapes are almost coinciding (Figure 5 insets).

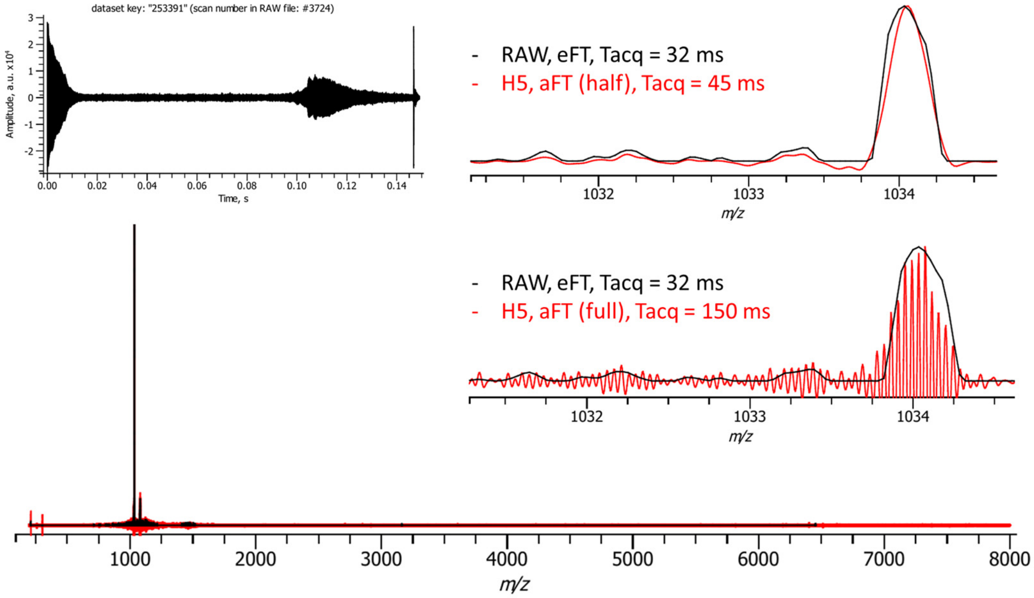

Figure 5. Mass spectra of a light chain of a monoclonal antibody as measured in parallel with a Q Exactive HF (data shown in black) and FTMS Booster TD (data shown in red). Analysis of mAbs' light chains with ECD on a Q Exactive HF equipped with an Omnitrap and FTMS Booster TD (Institute Pasteur, France). Orbitrap settings: the resolution of 15'000 at *m/z* 200, AGC of 5e5, and ITmax of 200 ms. Data were processed and analyzed with Peak-by-Peak BioPharma software (Spectroswiss).

Results reported in Figure 5 confirm the overall appropriate performance of the modified Orbitrap and of the FTMS Booster TD, including its ability to generate phased transients and to output absorption mode mass spectra. In addition, the FTMS Booster TD shows its capability to record time-domain transients during all the time ions are generating useful signals in the Orbitraps and to maximize the duty cycle of the measurements. Overall, the obtained result is important in regard to understanding the performance capabilities and principles of the Orbitrap-Omnitrap coupling. Results reported in Figure 5 suggest that the resolution setting for ion detection with the built-in DAQ system could be increased to match the available useful time-domain signal. **Figure 6** shows results of such experiments, measured with the setting of 120'000 at *m/z* 200. In this case, the ion detection period that corresponds to 120'000 resolution (256 ms) exceeds the overhead due to the ECD in the Omnitrap, **Figure 7**. Therefore, both time-domain transients recorded with the built-in data acquisition system and the FTMS Booster TD are of an equal period. As a result, the obtainable resolutions are comparable for both datasets (Figure 7 insets). Importantly, for these datasets we see another confirmation of an appropriate and expected performance.

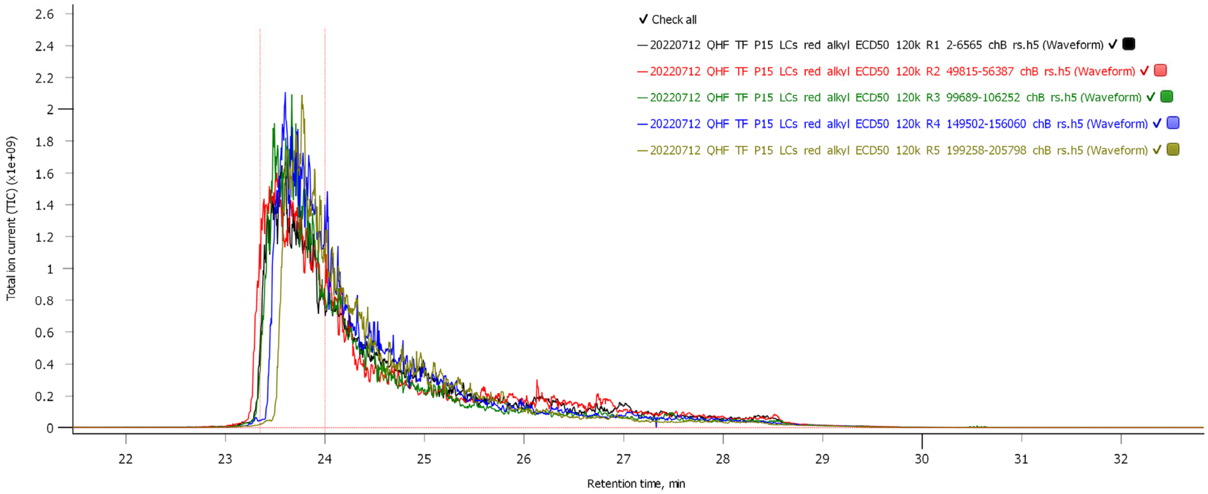

Figure 6. Analysis of mAbs' light chains with electron capture dissociation (ECD) on a Q Exactive HF equipped with an Omnitrap and FTMS Booster TD (Institute Pasteur, France). Orbitrap settings: the resolution of 120'000 at *m/z* 200, AGC of 5e5, and ITmax of 200 ms. Shown are the total ion current (TIC) chromatograms for five technical replicates. Data were processed and analyzed with Peak-by-Peak BioPharma software (Spectroswiss).

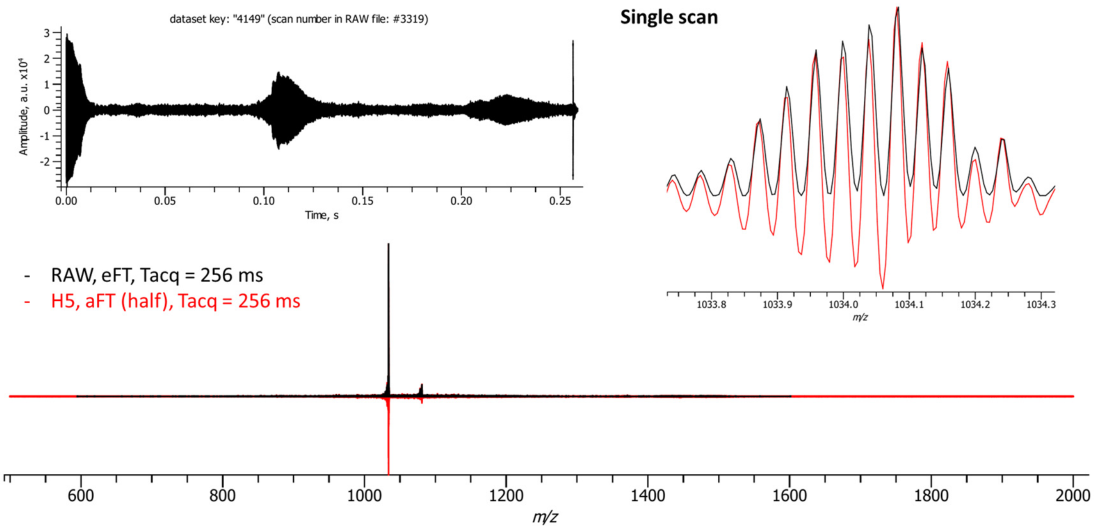

Figure 7. Mass spectra of a light chain of a monoclonal antibody as measured in parallel with a Q Exactive HF (data shown in black) and FTMS Booster TD (data shown in red). Analysis of mAbs' light chains with ECD on a Q Exactive HF equipped with an Omnitrap and FTMS Booster TD (Institute Pasteur, France). Orbitrap settings: the resolution of 120'000 at *m/z* 200, AGC of 5e5, and ITmax of 200 ms. Data were processed and analyzed with Peak-by-Peak BioPharma software (Spectroswiss).

The next step from confirming the performance of the Omnitrap-Orbitrap-Booster platform is in demonstrating the benefits of the unreduced data, **Figure 8** and **Figure 9**.

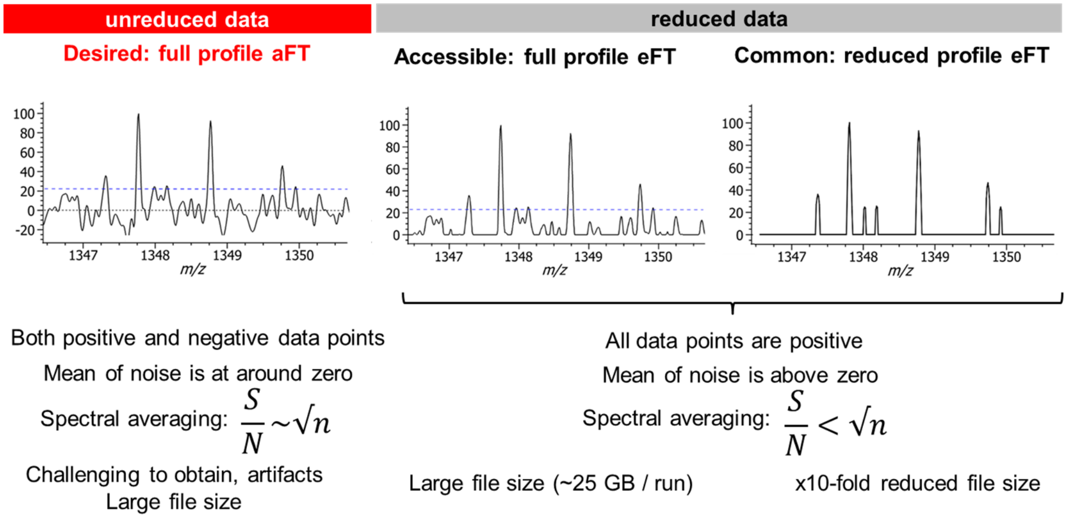

Figure 8. The types of mass spectra data in Orbitrap mass spectrometry. The FTMS Booster TD systems provide access to the truly unreduced data, represented as the absorption mode Fourier transform (aFT) mass spectra.

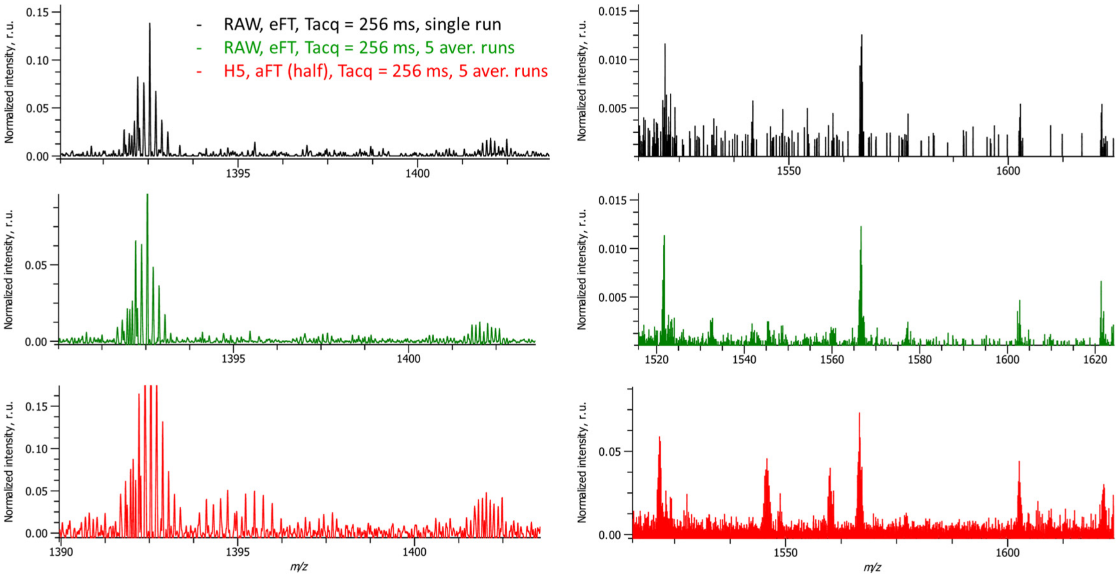

Figure 9. Sensitivity improvement demonstrated for averaging of the reduced data (eFT mass spectra, top and middle rows) and of the unreduced data (aFT mass spectra, bottom row). Mass spectra of a light chain of a monoclonal antibody as measured in parallel with a Q Exactive HF and FTMS Booster TD. Data analyzed with Peak-by-Peak BioPharma.

4. Protocols

The “FTMS Booster TD Acceptance Protocol” (see D7.1 report) included the following parts:

- General information (title page)
- Product setup (completeness of delivery, visual inspection, hardware interfaces)
- Product calibration (connections calibration, transient trigger decoder calibration, and LC decoder calibration procedures)
- Product basic performance (does standard FTMS calibration pass? Does standard FTMS noise test pass? Does standard reference experiment performance match that prior to the FTMS Booster installation?)
- Special agreements (special agreements are made? List them. If a special specification supersedes one of the standard specifications, the special specification will be demonstrated during product installation only if this has been agreed upon.)
- Signature page with Warranty information (The warranty period starts today)
- Appendices: product calibration routine description and contents of the delivery.

The described above protocol was applied for the installation of the FTMS Booster TD on an Exploris 480 Orbitrap in Karolinska Institute (Sweden) and its applicability and versatility were confirmed. It can now be extended to the new family of the Orbitrap systems – the Exploris.

However, practical usage of the original FTMS Booster TD, with its allied software for data acquisition, data processing, and data analysis, revealed several areas of concern. These concerns were in the direction of ease of use and usability rather than technical issues. The following aspects were considered, discussed with the TopSpec partners, and worked on:

- Use of **microscans** versus scans for data collection with the Orbitraps. It was revealed that using the microscan data collection option may jeopardize the indexing of the time-domain transients acquired with the external DAQ system. Therefore, data collection protocols were updated, and specific information was added in regard to the experimental settings of the Orbitrap method design. A proper solution was found. However, further technical improvements, including updates of the firmware of the FTMS Booster TD could be made in order to further simplify the user experience.

- The **file sizes** of the datasets with the time-domain data were prohibitively large and raised concerns of the users. To resolve this and add other suggested improvements, the data acquisition firmware was upgraded with the following features: (i) Selectable "low *m/z* limit" parameter was introduced to reduce the time-domain transient sizes: e.g., 2-fold size reduction for "low *m/z* limit" at 84 *m/z*, 3-fold for 206 *m/z*, 4-fold for 336 *m/z*, and so on, for Q Exactive instruments. This also translates to reduced data processing times in Peak-by-Peak software; (ii) On-the-fly correction of abnormal start/stop events from the FTMS instrument (a rare but possible case for certain FTMS instruments); (iii) Post-measurement automation: execution of the user's EXE file or BAT/PS1 script after a data acquisition experiment completes; (iv) Visualization of mass spectra and peak maxima, with a noise thresholding level; and other features. The protocols have been updated accordingly.

- The ease of use of the Peak-by-Peak BioPharma software for data processing was questioned. Naturally, the required number of steps in the processing of top-down data is high. Therefore, to streamline the processing and to facilitate the user experience and improve the usability of the software, we introduced the step-by-step logic (as the top line in the software) and added further explanations into the embedded tutorial, **Figure 10**.

Status: Idle

Name	Type	Project folder
Proteins		
ADCs		
DIA-O-glyco	proteins (high resolution)	C:/Users/Konst...

N compounds/entries: 1266 / 10128
N detected comp./feat.: 0 / 0

		Name	Mass, Da	RT, min.	Sca
0	✓	RAEASIKPLVLASK	1482.767	36.6899681091...	FTMS
1	✓	AEASIKPLVLASK	1326.581	42.3603019714...	FTMS
2	✓	GAVIEPEQATELPYK	1644.82	49.4774436950...	FTMS
3	✓	GVTESLDPWKR	1287.421	44.4601707458...	FTMS
4	✓	DQVATLGPK	928.041	28.837100982666	FTMS
5	✓	LSASSVTPR	917.019	28.4330158233...	FTMS
6	✓	TTAPLTTR	859.967	24.098575592041	FTMS
7	✓	GPTLAPLLPMASPH	1401.673	66.7734527587...	FTMS
8	✓	ESVAPGVSPTSASMT...	1774.947	42.5776748657...	FTMS
9	✓	TPGPQRPPK	977.118	16.1386413574...	FTMS
10	✓	AADPGAYLSAAATR	1334.434	47.9840431213...	FTMS
11	✓	LLLPSTATSK	1030.216	41.1392555236...	FTMS
12	✓	SLNTVPITEYK	1264.424	46.6688270568...	FTMS
13	✓	SSTPSVTSGH	958.969	21.5446548461...	FTMS
14	✓	IETQTQEEVR	1232.298	24.3591785430...	FTMS
15	✓	SPKPGPHTLSPLPPGP...	2203.499	36.988956451416	FTMS
16	✓	AAAAGPLGPPTKPPS...	1744.99	43.4240303039...	FTMS
17	✓	TSAQPTAIPANGSP	1311.398	37.2015609741...	FTMS
18	✓	KGPEIGFLPGSDPDP...	3269.658	72.457878112793	FTMS

Processes / precision: 10 float32
Time range, min: from: 0.001 to: 90.049
Mass range, m/z: from: 400.000 to: 1200.000
Noise level: 0.00
☐ Truncate spectrum baseline:
Median size/Gauss npts: 99 1
Gauss sigma/Level: 1 1.0
MSF library: nstantin\Calculations\Seva\DIA-O-glyco\MSF_library.msf
☑ MSF hot link
Calculate MSF
Update MSF
Load MSF

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Ref.RT, min.: None

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Ref.RT, min.: None

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Ref.RT, min.: None

single scan at t=75.19 min:
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Ref. m/z: 1773.788813, 1774.791688, ..
Similarity: 15.84
Mass error: 2.566 ppm

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4	Group_adduct	(HexNAc)2(Hex)4(Fuc)2(NeuAc)3,+H
5	Monoisotopic (neutral), Da	1407.6492868261498
6	Average mass, Da	1408.5773200000003
7	Reference RT, min.	None
8	DBE	26.0
9	Polarity	positive
10	Instrument	Orbitrap QExHF™
11	Resolution:	R=120k@200m/z

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2. ☑ QEHF_201203_42.raw
3. ☑ QEHF_201203_43.raw
4. ☑ QEHF_201203_44.raw

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☑ (HexNAc)1(Hex)1,+H
☑ (HexNAc)1(Hex)2(NeuAc)1,+H
☑ (HexNAc)1(Hex)2(Fuc)1,+H
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☑ (HexNAc)2(Hex)1(NeuAc)1,+H
☑ (HexNAc)2(Hex)2,+H
☑ (HexNAc)2(Hex)2(NeuAc)1,+H

SIC(s) extraction: 100% (Elapsed time: 0:00:06)

Figure 10. A screenshot of Peak-by-Peak BioPharma software that boasts a selection of the steps required for data processing at the top panel, including: (i) Projects; (ii) Experimental data; (iii) Compounds; (iv) Ions and modifications; (v) Simulate profiles; (vi) Detect features; and (vii) Analyze results. Each step has its own tutorial guide that is embedded into the step description.

Furthermore, the following areas of improvement have been identified and either already implemented or under implementation (with the objectives going beyond the capabilities of the TopSpec project tasks): (i) improvement in the estimation of the space charge control in the Orbitrap mass analyzer, such as automatic gain control (AGC) function; (ii) speed-up of the real-time calculations for FT processing of large and very large time-domain transients; and (iii) automation of the data processing routines both for the real-time conversion of the time-domain transient files (.bin file format) into the Python file format (.H5 files) and for data analysis, such as targeted and untargeted deconvolution of the antibody mass spectra in low- and high-resolution.