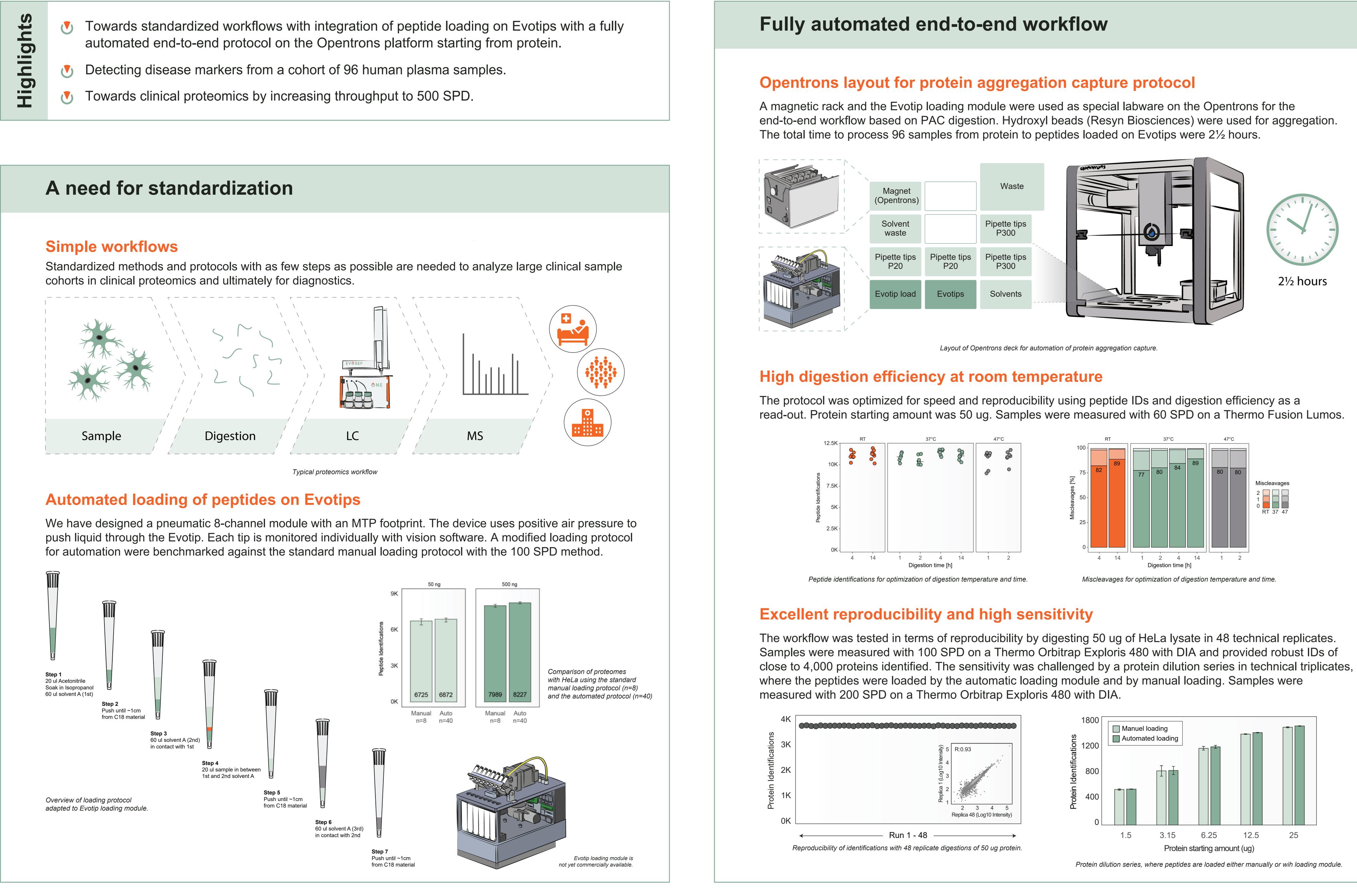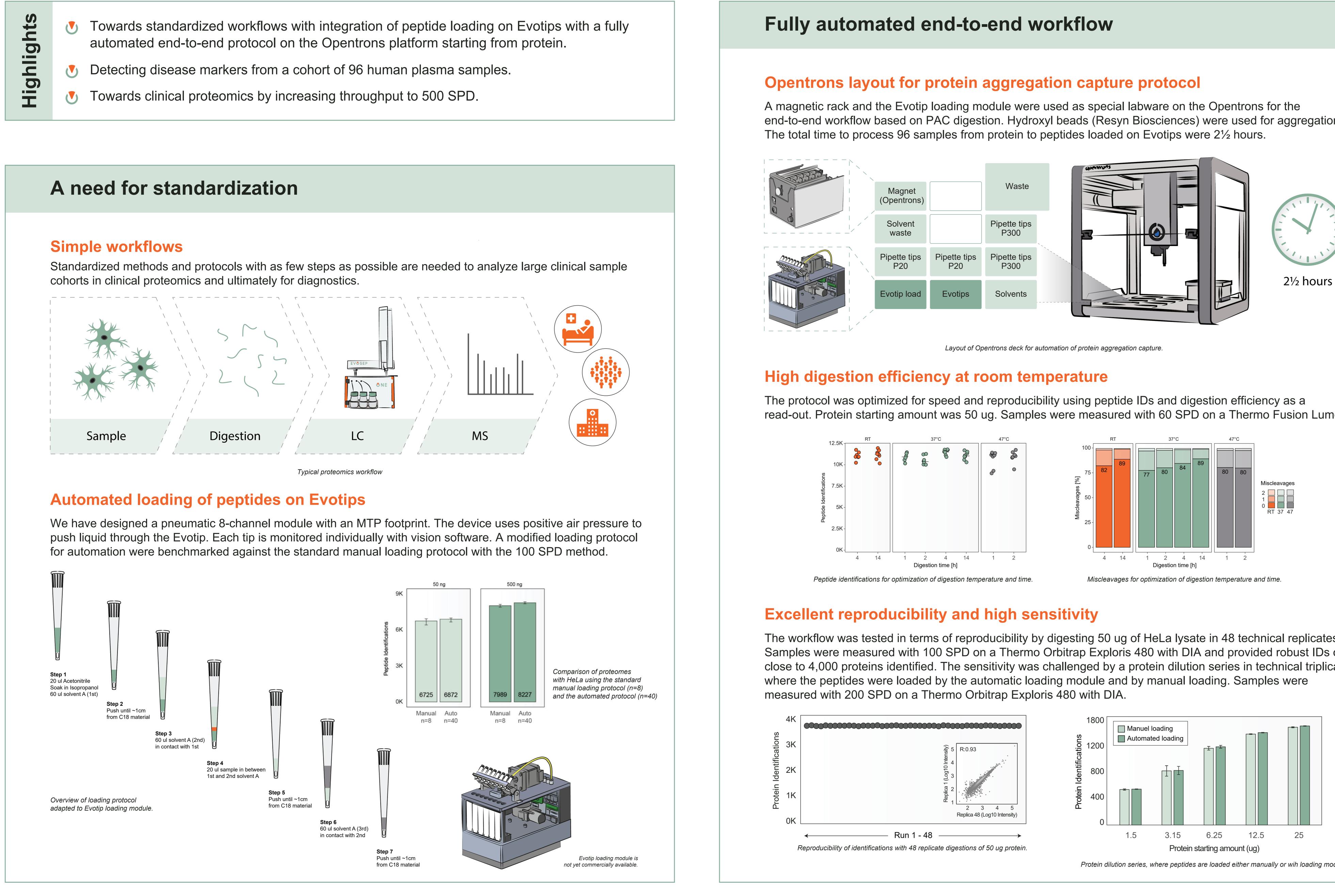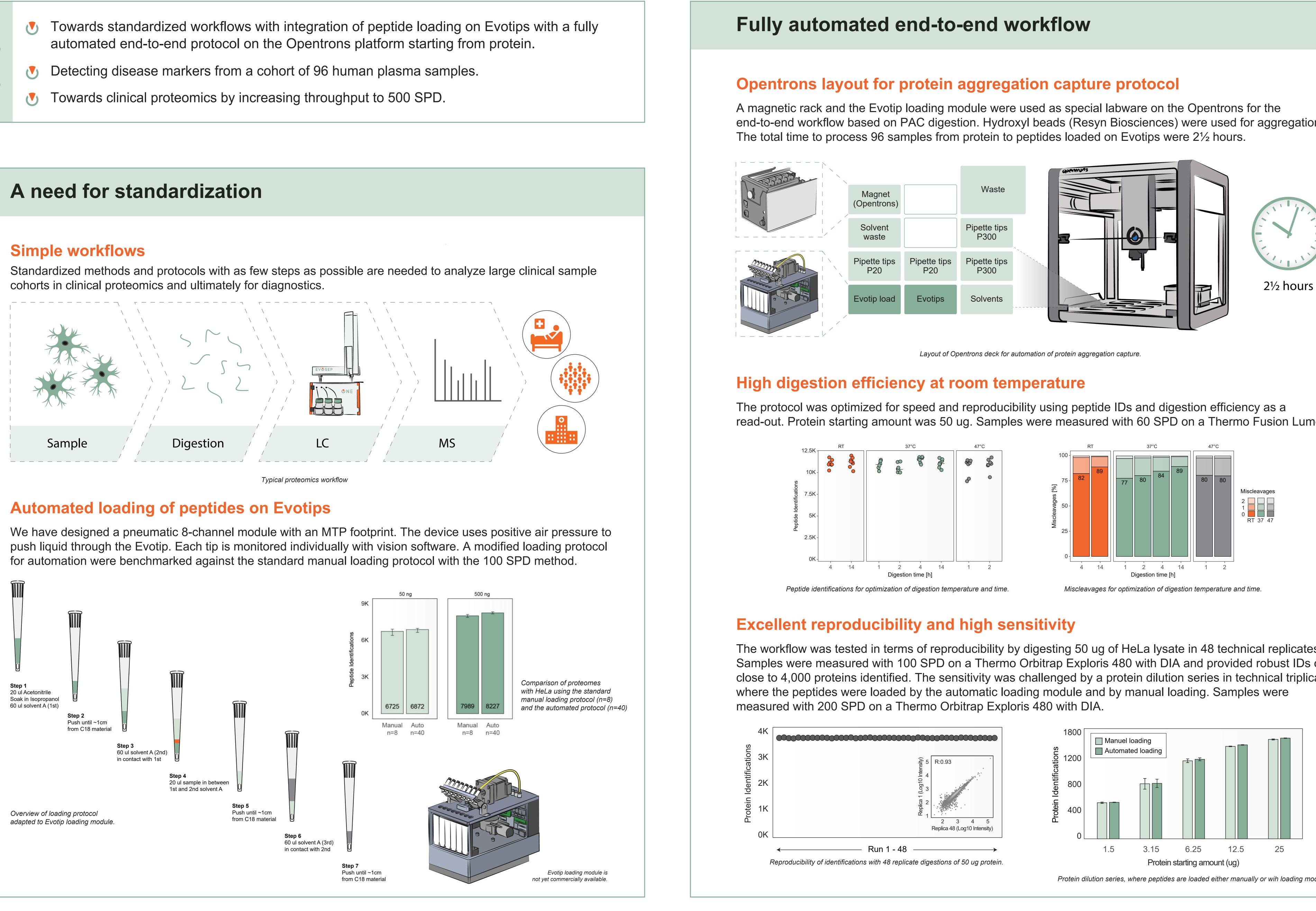# A complete and automated sample preparation strategy for high-throughput and standardized proteomics, applied to a clinical cohort of patient plasma samples

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Highlights

- Towards standardized workflows with integration of peptide loading on Evotips with a fully automated end-to-end protocol on the Opentrons platform starting from protein.
- Detecting disease markers from a cohort of 96 human plasma samples.
- Towards clinical proteomics by increasing throughput to 500 SPD.

A need for standardization

Simple workflows

Standardized methods and protocols with as few steps as possible are needed to analyze large clinical sample cohorts in clinical proteomics and ultimately for diagnostics.

Sample — Digestion — LC — MS

Typical proteomics workflow

Automated loading of peptides on Evotips

We have designed a pneumatic 8-channel module with an MTP footprint. The device uses positive air pressure to push liquid through the Evotip. Each tip is monitored individually with vision software. A modified loading protocol for automation were benchmarked against the standard manual loading protocol with the 100 SPD method.

Step 1 20 ul of Acetonitrile Soak in Isopropanol 60 ul solvent A (1st)
Step 2 Push until ~1cm from C18 material
Step 3 60 ul solvent A (2nd) in contact with 1st
Step 4 20 ul sample in between 1st and 2nd solvent A
Step 5 Push ~1cm from C18 material
Step 6 60 ul solvent A (3rd) in contact with 2nd
Step 7 Push ~1cm from C18 material

Overview of loading protocol adapted to Evotip loading module.

Evotip loading module is not yet commercially available.

50 ng: Manual n=8: 6725, Auto n=40: 6872
500 ng: Manual n=8: 7989, Auto n=40: 8227

Comparison of proteomes with HeLa using the standard manual loading protocol (n=8) and the automated protocol (n=40)

Fully automated end-to-end workflow

Opentrons layout for protein aggregation capture protocol

A magnetic rack and the Evotip loading module were used as special labware on the Opentrons for the end-to-end workflow based on PAC digestion. Hydroxyl beads (Resyn Biosciences) were used for aggregation. The total time to process 96 samples from protein to peptides loaded on Evotips were 2½ hours.

Magnet (Opentrons) | Waste
Solvent waste | Pipette tips P300
Pipette tips P20 | Pipette tips P20 | Pipette tips P300
Evotip load | Evotips | Solvents

2½ hours

Layout of Opentrons deck for automation of protein aggregation capture.

High digestion efficiency at room temperature

The protocol was optimized for speed and reproducibility using peptide IDs and digestion efficiency as a read-out. Protein starting amount was 50 ug. Samples were measured with 60 SPD on a Thermo Fusion Lumos.

Peptide identifications for optimization of digestion temperature and time.

Miscleavages for optimization of digestion temperature and time.

Excellent reproducibility and high sensitivity

The workflow was tested in terms of reproducibility by digesting 50 ug of HeLa lysate in 48 technical replicates. Samples were measured with 100 SPD on a Thermo Orbitrap Exploris 480 with DIA and provided robust IDs of close to 4,000 proteins identified. The sensitivity was challenged by a protein dilution series in technical triplicates, where the peptides were loaded by the automatic loading module and by manual loading. Samples were measured with 200 SPD on a Thermo Orbitrap Exploris 480 with DIA.

Reproducibility of identifications with 48 replicate digestions of 50 ug protein.

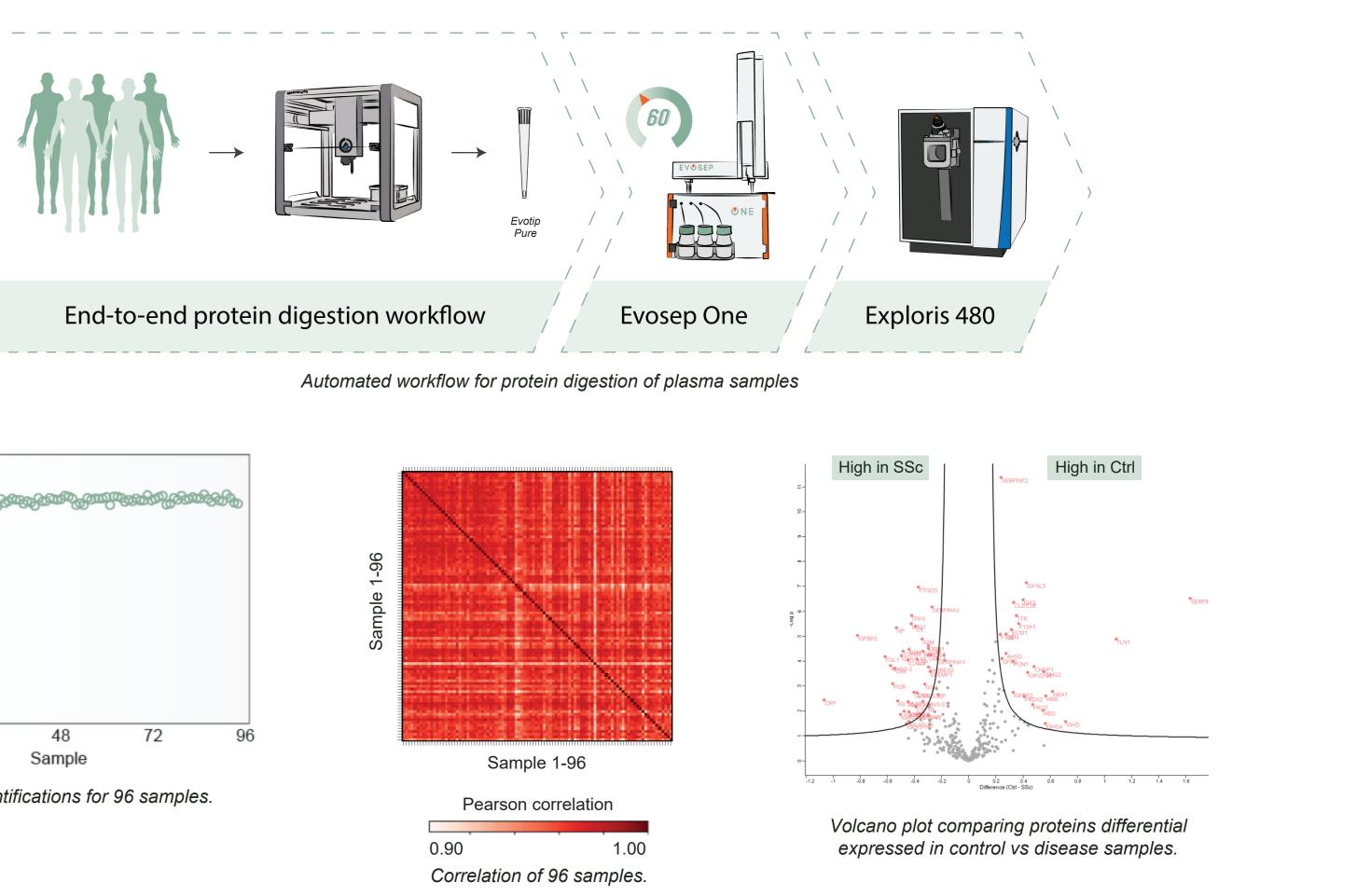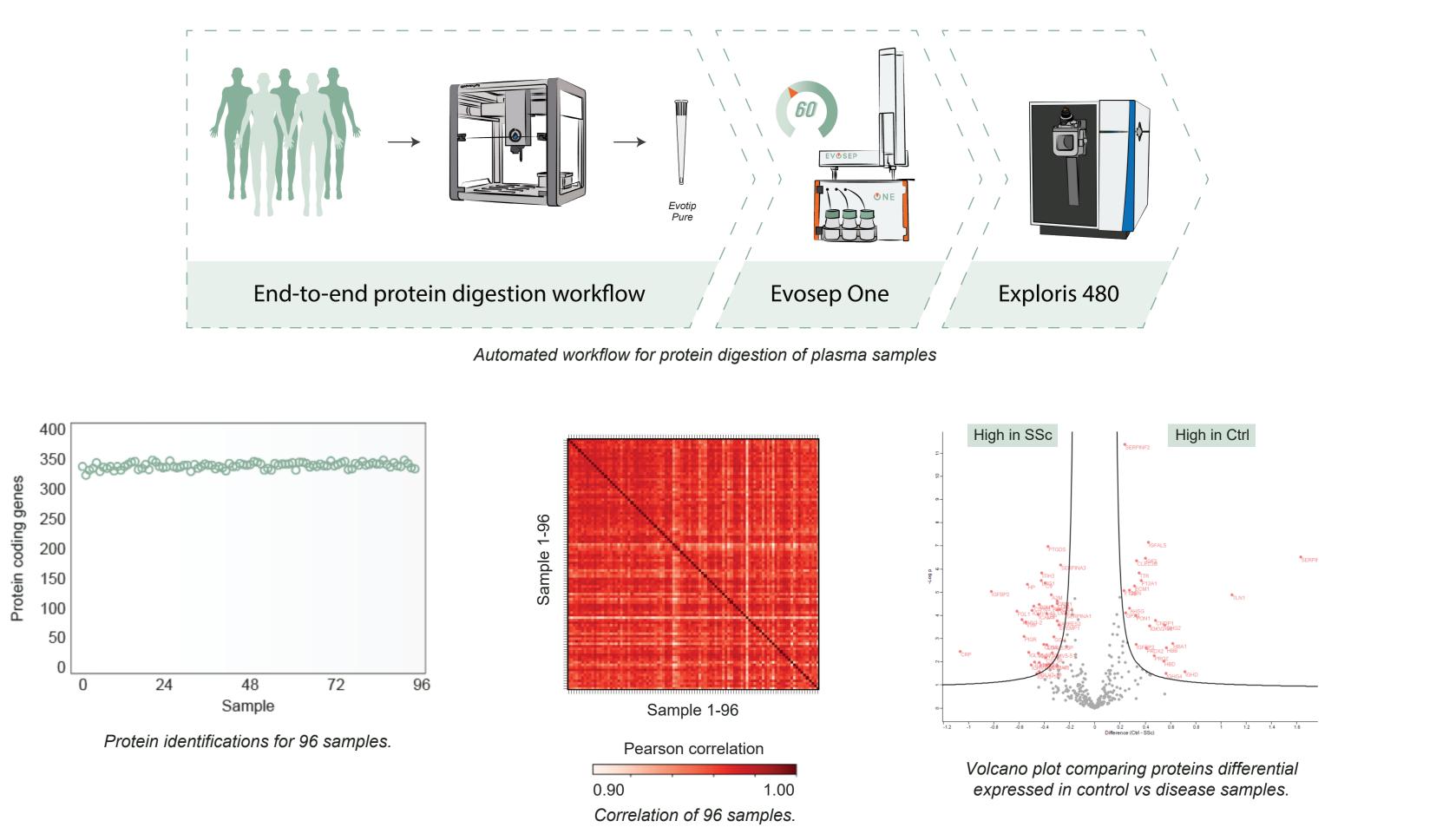*Protein dilution series, where peptides are loaded either manually or wih loading module.*

High-throughput plasma proteome profiling

Clinical applicability to patients with systemic sclerosis

To demonstrate clinical applicability, we tested a sample cohort of 96 plasma samples collected from 48 patients diagnosed with the incurable autoimmune disease, systemic sclerosis, where diagnosis in an early phase is still difficult due to lack of symptoms, and compared these to plasma samples from 48 healthy control individuals.

End-to-end protein digestion workflow — Evosep One — Exploris 480

Automated workflow for protein digestion of plasma samples.

Protein identifications for 96 samples.

Correlation of 96 samples.

Volcano plot comparing proteins differential expressed in control vs disease samples.

Faster chromatography with up to 500 samples analyzed per day

Automated sample preparation workflows require faster chromatography. We have developed a robust method with 500 samples analyzed per day with two minutes gradient time. For high throughput analysis of complex samples, highest peak capacity in the shortest time is a relevant measure, which is achieved by the fastest method.

Plasma control samples, n=48

Peptide identifications for 48 samples with different Evosep methods.

Protein identifications for 48 samples with different Evosep methods.

Peak capacity (total and per minute) for ultra fast methods.

CV distribution for all peptides identified across methods.

Dynamic range for proteins identified across all methods.