## HARVARD MEDICAL SCHOOL **TEACHING HOSPITAL**

## Building a universal proteomics sample preparation platform using low-cost liquid handling robotics and 3D-printing

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Reservoir

Reagent

20 μL

Tips

Addition of denaturation /

Reduction with dithiothreitol

(DTT): 95°C for 10 min and 56 °C

Alkylation with iodoacetamide

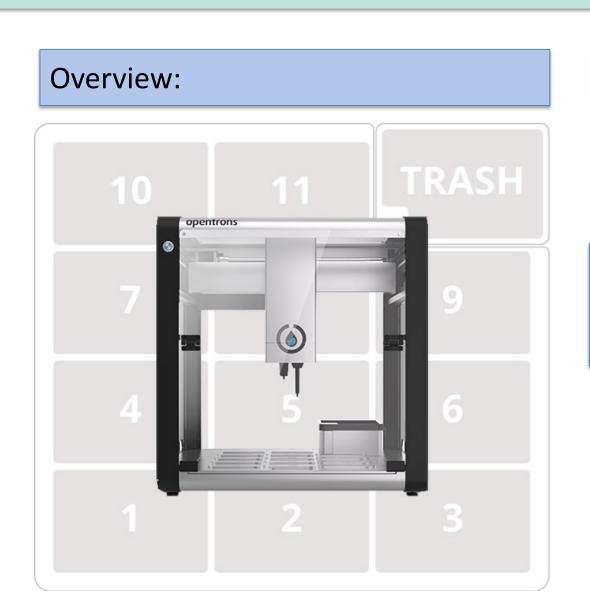
(IAA): 22 °C for 20 min at dark.

Quench with DTT: 22 °C for 20 min

dilution buffer

for 30 min.

at dark.



## Input Plasma, CSF, Cell Lysate, Tissue

Main steps of the single-pot, solidphase-enhanced sample-preparation (SP3)-Pipeline:

Pipettes

- Reduction and Alkylation
- . Protein Binding
- Ethanol Washes x3 On-Bead Proteolytic Digest
- 5. BCA Quantification
- 6. TMT Labeling

# Modules



Module

Shaking Module

8-Channel Pipettes: P300 and P20

Single-Channel Pipettes: P300 and P20

#### Denaturation

#### Unfolding of proteins to make them accessible for proteolytic digest.

#### Eliminating contaminants through repeated washing steps.

300 μL

300 μL

Tips

Manually adding magnetic

Addition of 100% ethanol to

3 washes with 80% ethanol to

Addition of Lys-C digestion buffer

carboxylated beads.

- Incubation while mixing.

remove contaminants.

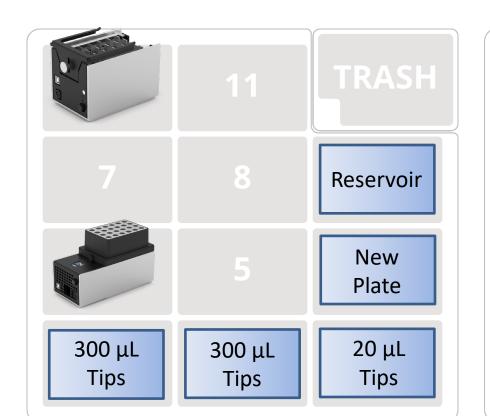
initiate binding.

and trypsin.

SP3 Washes

## After proteolytic digest the specimens are transferred to a new

Stop Digest



- Addition of 50% formic acid (FA) to stop enzymatic activity.

- Transfer sample to new plate.

- Wash magnetic beads with elution buffer and transfer supernatant to new plate.

#### **BCA** Assay

#### The bicinchoninic acid (BCA) assay allows protein quantification of 96 well plates to inject equal amounts of protein into the mass analyzer.

300 μL

BCA

Plate 1

Plate 2

20 μL

Plating out a series of protein

standards (BSA) into four rows of

Plating out sample specimen in

duplicates in BCA plates 1-3.

Addition of 200 μL working

reagent to each well.

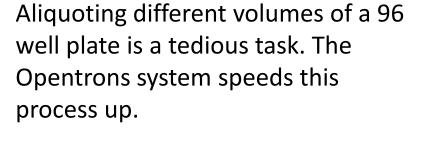
absorption (562nm).

Read-out of wavelength

BSA

Tips

each BCA plate.



Aliquoting

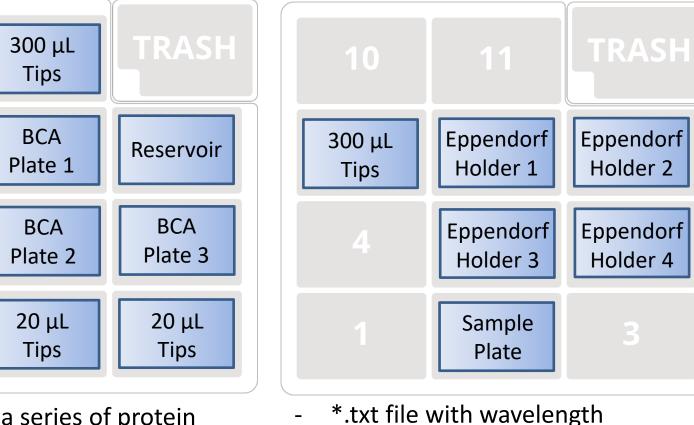
Manually

- TMT-labelling (SPS-MS3)

Offline fractionation

Single shot (DIA)

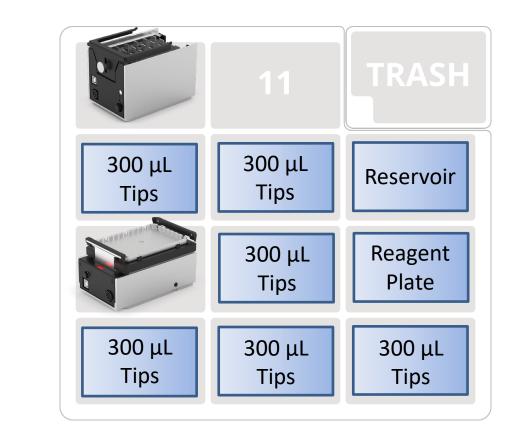
- Etc.



- absorption is used to calculate protein concentration for each well respectively. A determined amount of
- protein (e.g., 25 μg for plasma) is aliquoted into Eppendorf tubes.

#### **Bonus: Corona Formation Protocol**

The CF-Protocol was developed to optimize binding efficiencies for low abundance plasma proteins. The protocol allows the depletion of high-abundant proteins such as immunoglobulins (Igs).



The core features distinguishing this protocol from the SP3-protocol is that protein denaturation is initiated only after the depletion process together with intensive washing steps and increased incubation time.

Note: This protocol is not yet optimized for a full 96 well plate and has only been tested for validation experiments.

## Introduction

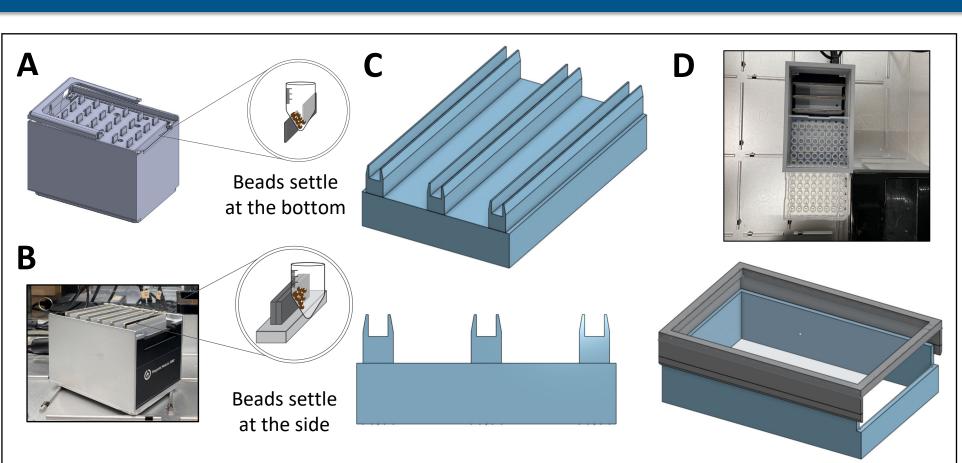
Multiplexed proteomics through isobaric labeling and the combination of data-independent acquisition (DIA) methods with artificial intelligence (AI)-driven data analysis approaches have — over the last years – substantially increased the throughput of mass spectrometry-based proteomics. The developments have reached a point where the major costs in analyzing proteome samples are shifted from the required instrument time to sample preparation. Addressing this situation requires improvements in sample preparation processing that match those in mass spectrometry technology. Key features for these improvements are sample preparation automation that is highly versatile to cover needs across different applications and research groups as well as cost-effectiveness regarding hardware and consumables to attract wide-spread usage. We present such a platform based on Opentrons liquid handler robotics.

## 3D Design

Reservoir

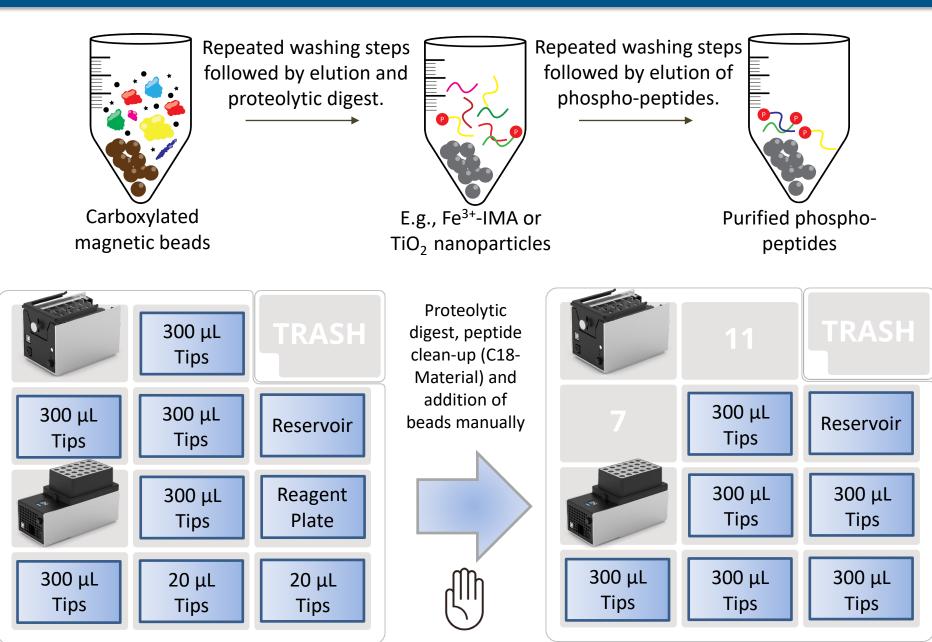
300 μL

Tips

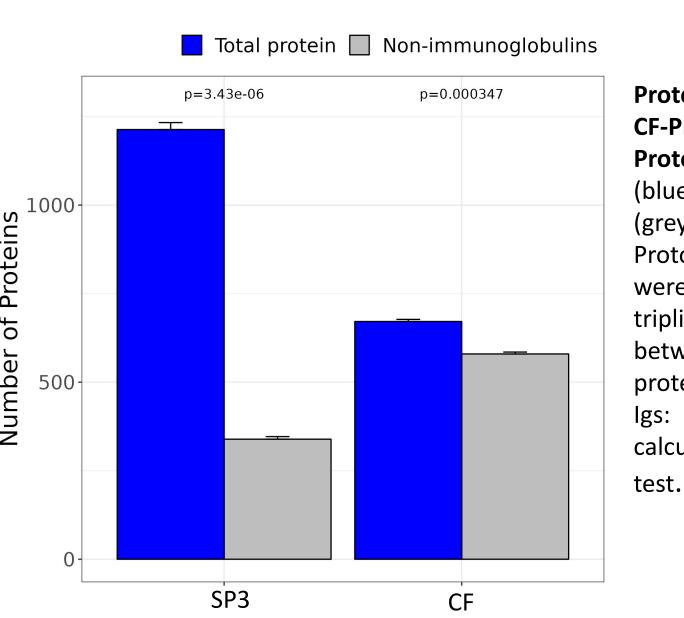


Upgrading the robotic platform with 3D-modules: (A) Bead settlement with the Opentrons Gen2 magnetic module. Magnetic beads settle at the bottom of the plate and are aspirated. (B) Custom 3D printed magnet holder. Magnetic beads settle at the side and no nanoparticles are aspirated during liquid handling. (C) 3D template of the magnetic holders. (D) 3D template to make the robotic platform compatible with frequently used labware.

## Set up for Phospho-Proteomics



## Corona-Formation Protocol



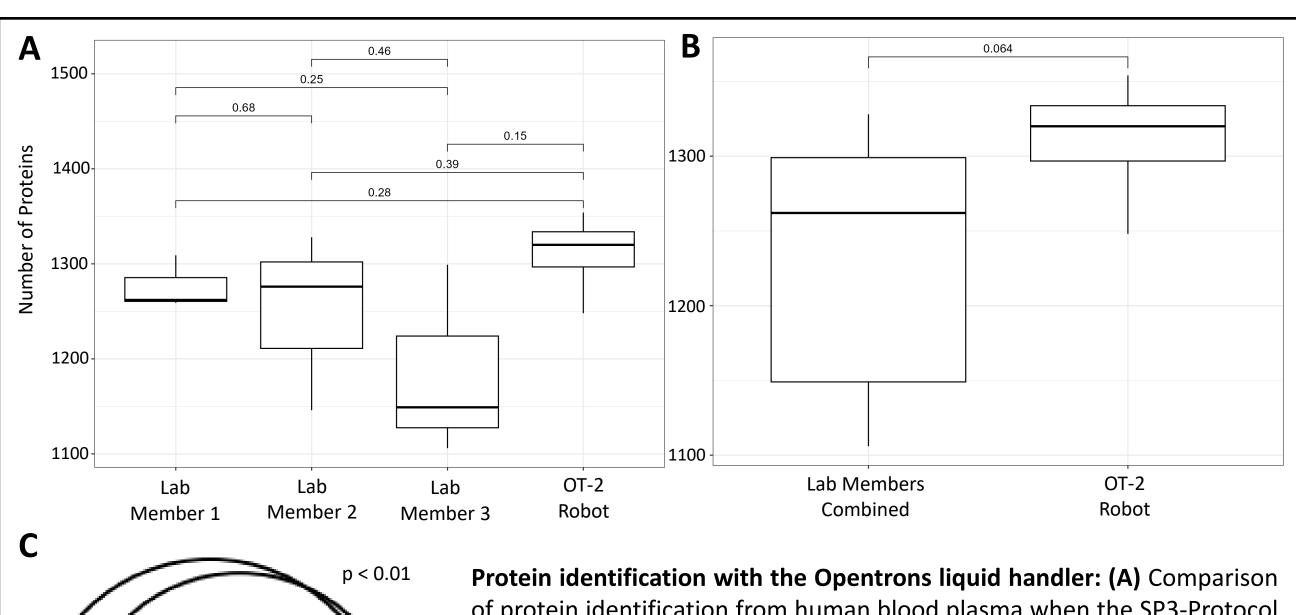
Lab technician operating the Opentrons

Hands-on time:

Protein recovery with the CF-Protocol and the SP3-**Protocol:** Protein recovery (blue) and non-lg recovery (grey) for the SP3 and CF-Both protocols conducted triplicates and significance between protocols (total proteins:  $p = 3.43x10^{-6}$ , non-Igs:  $p = 3.47x10^{-4}$ ) was calculated by an unpaired ttest.

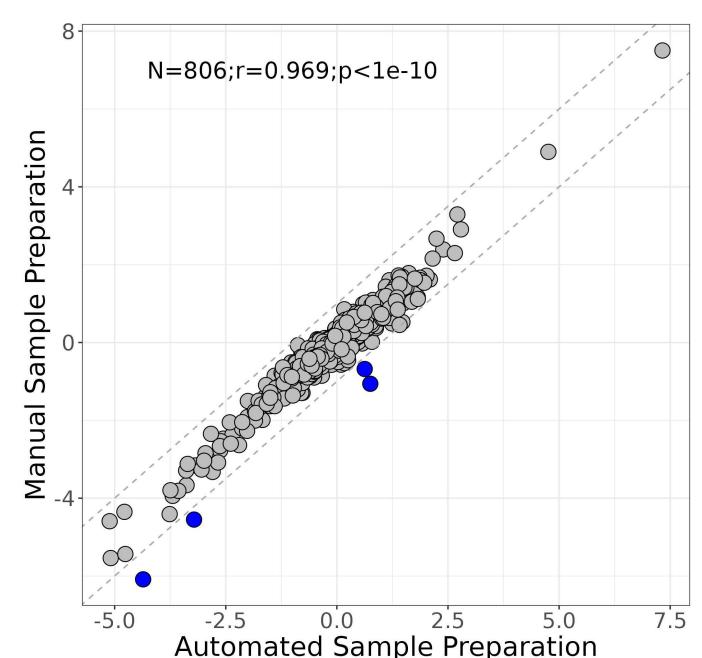
Lab technician alone

## Automated vs Manual Preparation



of protein identification from human blood plasma when the SP3-Protocol was performed in triplicates by three lab members and the OT-2 (four replicates). Statistical significance was determined by a two-sided t- test. (B) Data from the three lab members were collapsed into a single data set and the robotic platform was evaluated against manually processing. Statistical significance was determined by a two-sided t- test. Coefficient of variation in protein recovery when using the robotic platform was at 3.4% and 6.6% when processing samples manually. (C) Venn diagram showing the overlap of nonredundant proteins in the robotic/manually processed data sets with significant overlap (fisher's exact test, p < 0.01).

### Quantitative Evaluation



Quantitative evaluation of automated and manual sample preparation:

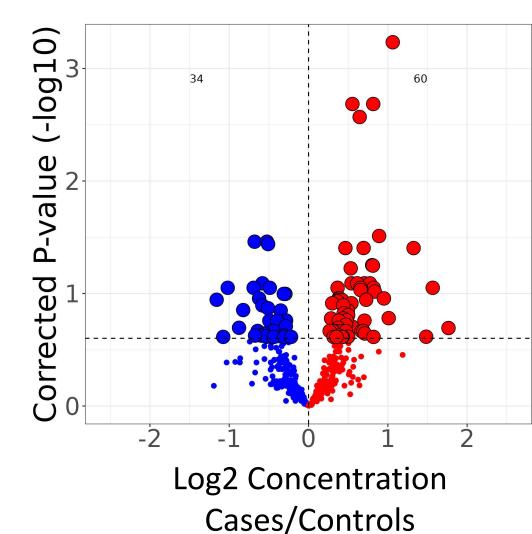
profiles were Proteome generated for M23 and M7192 KRAS mutant cell lines. The protein data was normalized and relative into protein intensities. The fold change between M23 and M7192 protein intensities was calculated to assess variability for both human and automated preparation. The plot shows a high similarity in the fold change ratios between both sample preparation methods. The

analysis demonstrates that the manual and automated sample preparation protocols exhibit comparable protein quantification results. Blue dots indicate proteins with a fold difference of more than 2 between the manual and automated protocol ratios, highlighting potential differences in protein abundance. The two gray lines represent the 2-fold threshold lines. A total of 806 proteins was assessed.

## >1,200 Blood Plasma Samples

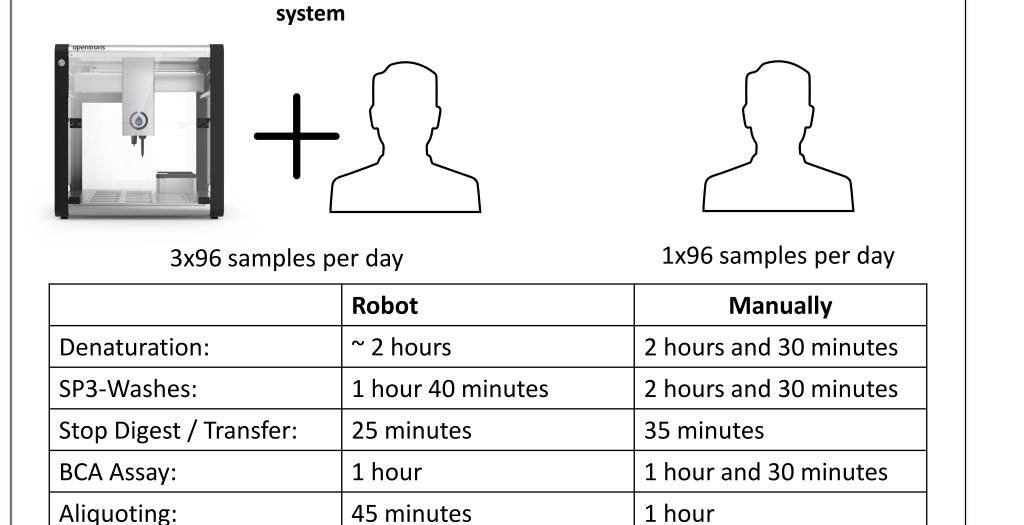
Cancer is one of the leading causes of death worldwide and the mortality of cancer is highly correlated with the disease stage at which cancer is diagnosed. This underscores the need for early cancer detection.

To discover novel biomarkers and improve patient care, blood specimens from patients at Massachusetts General Hospital were collected and blood plasma was extracted using gradient density centrifugation. The study had a cohort size of over 1,200 individuals and included patients with a high lung cancer risk (e.g., chain smokers, firefighters). Some of the participants had already developed lung cancer. The plasma samples were processed using the Opentrons liquid handler.



Differential protein expression of 478 proteins from 27 lung cancer and 19 control blood plasma samples analyzed on the Ascend platform. The highlighted dots indicated proteins with a Benjamini Hochberg p-value < 0.25. A moderated t-test was used to calculate the differential protein analysis p-values.

#### Benefits



#### References

1 hour

Liu, X. et al. A Semiautomated Paramagnetic Bead-Based Platform for Isobaric Tag Sample Preparation. J Am Soc Mass

8 hours

- Spectrom, 32(6):1519-1529 (2021). Leutert, M. et al. R2-P2 rapid-robotic phosphoproteomics enables multidimensional cell signaling studies. Mol Syst Biol.
- Hughes, C.S. et al. Single-pot, solid-phase-enhanced sample preparation for proteomics experiments. Nat Protoc 14, 68–