Evaluating Sample Preparation methods in a Core facility environment for samples with low amounts of protein

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INTRODUCTION

This study tested several recent automated digestion devices to determine:

- sensitivity for small starting total protein amounts as low as 1ug: we used suspension-trap (S-trap), protein aggregation capture (PAC) and surfactant-assisted one pot
- enhancement of extravesicular (EV) proteins in human plasma via two methods: perchloric acid depletion and EV enrichment via strong-anion exchange (SAX) magnetic beads.

Digestion of all samples was done either manually or using recent automated devices like Opentrons-2 and King Fisher APEX.

METHODS –see Fig 1 for details

Digestion technique evaluation:
Tryptic digestions were done to assess the lowest amount of total protein we can start with and still obtain good number of IDs. Mouse liver homogenates were subjected to reduction / alkylation / tryptic proteolysis, except the MagReSyn™ hydroxyl beads PAC digestion.

Plasma extracellular vesicle (EVs):
Tryptic digestions on human plasma was done to assess the best method for enhancement of extravesicular proteins. Plasma samples were subjected to reduction / alkylation / tryptic proteolysis, except the Perchloric Acid method.

All tryptic peptides were then lyophilized, and resuspended in 0.1% TFA, and subjected to LC/MS analysis.

LCMS

Exploris480 (ThermoFisher) / Ultimate3000RSLC (ThermoFisher)
-data-independent analysis

timsTOF HT (Bruker Daltronics) / Evosep nanoLC (Evosep)
-data-independent analysis-Parallel Accumulation Serial Fragmentation (DIA-PASEF)

Data Analysis

DIA files were analyzed with Spectronaut v.18.6 software (Biognosis), using reviewed FASTA database for Homo Sapiens, UP00005640, and Mus Musculus, UP0000000589.

Results of Digestion technique evaluation, Fig2, Fig3

1) surfactant-assisted one-pot digestion, using a non-ionic detergent n-dodecyl-b-D-maltoside (DDM).
2) PAC using Hydroxyl magnetic beads (ReSyn Biosciences) and processed on a KingFisher Apex (Thermo Fisher) sample prep device
3) PAC using Hydroxyl magnetic beads (ReSyn Biosciences), processed manually
4) suspension-trap spin columns (ProtiFi, Farmingdale, NY)

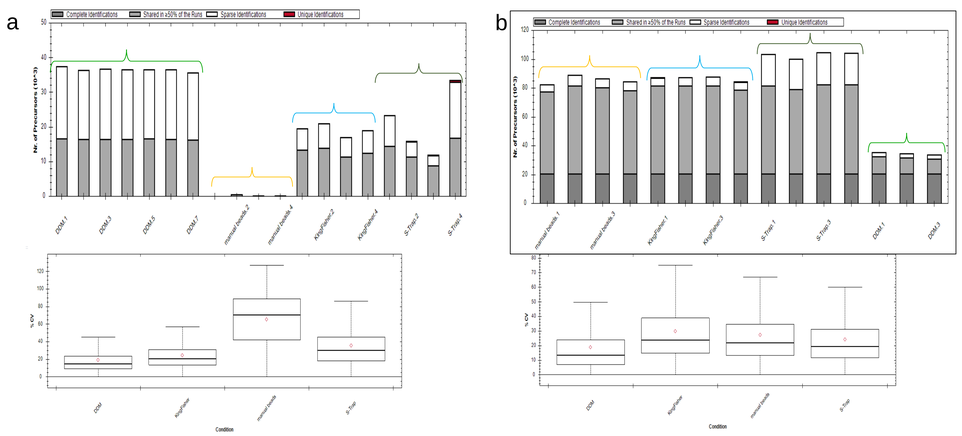


Fig2. Evaluation of the digestion four techniques' sensitivity and reproducibility: a) number of precursors and CVs for starting material of a) 10ug and b) 50ug

Fig1. The four digestion techniques evaluated:

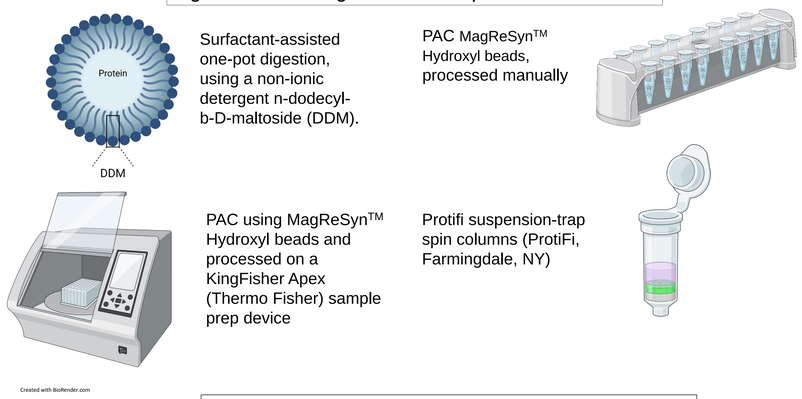


Table1: Number of precursors found in the four digestion techniques evaluated, comparing three different starting material amounts: 1ug, 10ug, and 50ug.

Starting protein amount	DDM In-Solution	King-Fisher hydroxyl beads	Manual hydroxyl beads	S-Trap
1ug	37,377	19,543	0	23,246
1ug	36,380	20,979	519	15,812
1ug	36,733	17,043	122	11,795
1ug	36,434	18,894	32	33,414
1ug	36,592			
1ug	36,579			
1ug	35,656			
10ug	37,314	85,177	717	105,489
10ug	32,388	86,079	11	107,440
10ug	38,004	79,972	40,271	107,161
10ug	38,629	82,353	44,883	107,922
10ug	38,788			
50ug	35,419	87,042	82,483	103,311
50ug	34,674	87,207	88,773	99,961
50ug	33,679	87,548	86,571	104,457
50ug		84,213	84,520	104,154

Note: *approximately 600ng of peptide was loaded for each*

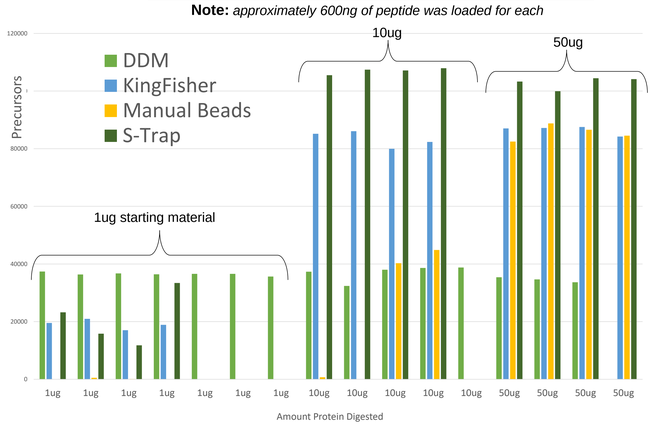
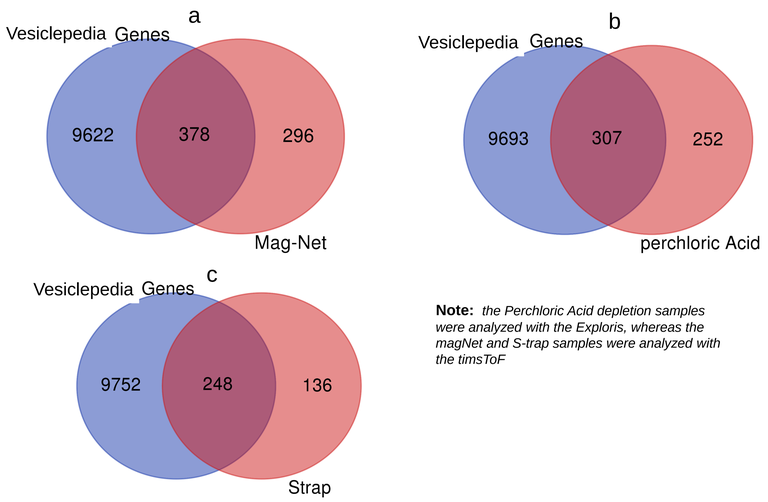


Fig.3 Number of identified precursors from comparing three different starting material amounts: 1ug, 10ug and 50ug for the four digestion techniques.

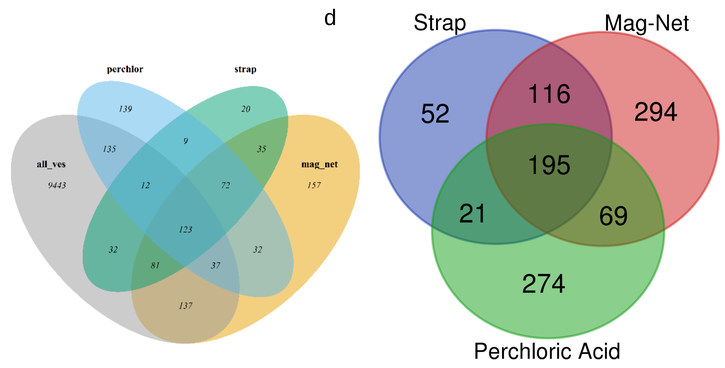
Results of Plasma extracellular vesicle (EVs) enrichment evaluation, Fig.4

1. suspension-trap spin columns.
2. magReSyn™ SAX magnetic beads (ReSyn Biosciences) processed on a KingFisher Apex (Thermo Fisher) sample prep device. The one-step enrichment strategy uses hyper-porous strong-anion exchange magnetic microparticles. Enrichment is based on electrostatic interactions between MagReSyn SAX microparticle and negatively charged phosphoplipids located on EV membrane surfaces.
3. perchloric acid aided EV enrichment, based on depletion of the most abundant proteins

Fig4. Plasma EV a) Venn diagrams of the identified genes coming from proteins we detected in the four plasma sample preps, all compared to the Homo Sapiens list of Evs from Vesiclepedia (http://www.microvesicles.org/) vs. a) MagReSyn™ SAX bead enrichment b) perchloric acid-aided enrichment c) neat plasma with no EV enrichment d) the three EV analyses summed up



Note: *the Perchloric Acid depletion samples were analyzed with the Exploris, whereas the magNet and S-trap samples were analyzed with the timsToF*



Discussion / questions

The Daedelian labyrinth of sample processing evaluations yielded following questions:

- Why do the number of precursors remain constant across digestion amounts for DDM in-solution? With larger staring material amounts, is there a proportion of DDM to protein which should be used?
- Why are the #of IDs for SAX and perchloric acid methods so low compared to other published reports?