MODULAR, SCALABLE AND AUTOMATABLE ON-BEAD PIPELINE FOR BOTTOM-UP PROTEOME AND PHOSPHOPROTEOME PROFILING WITH BUILT-IN PEPTIDE AND PHOSPHOPEPTIDE FRACTIONATION

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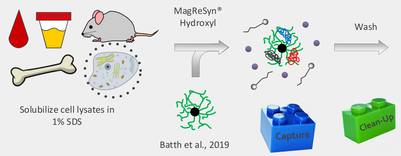
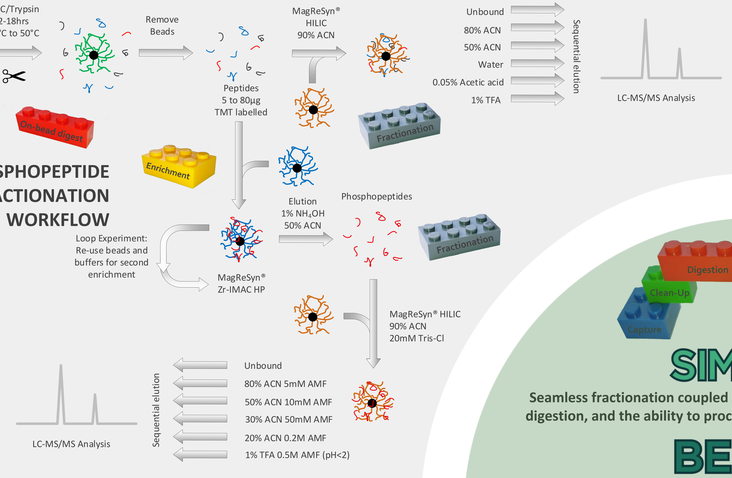
DIGEST PREPARATION WORKFLOW



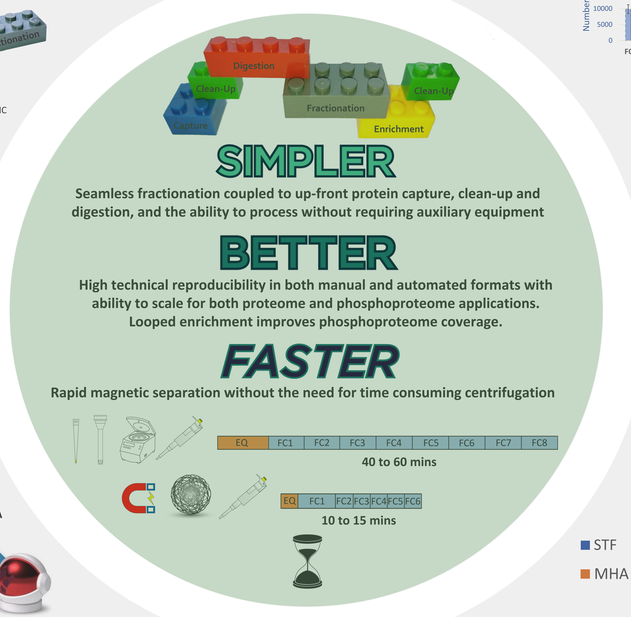
PHOSPHOPEPTIDE FRACTIONATION WORKFLOW

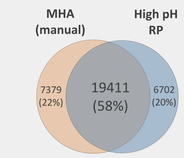


PEPTIDE FRACTIONATION WORKFLOW

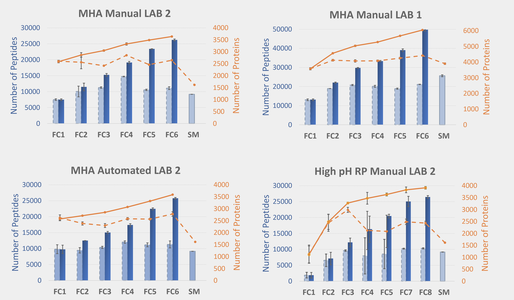
INTRODUCTION

- Peptide fractionation is a well established strategy in bottom-up proteomics to increase the depth of proteome coverage
- Recently a method for peptide fractionation was reported by Deng *et al.* (2021) using caboxylate magnetic microparticles
- Here we evaluate the use of HILIC magnetic particles with zwitterionic functionality for application to peptide and phosphopeptide fractionation
- The new magnetic HILIC affinity (MHA) method of peptide fractionation is benchmarked against the commonly used high pH reverse phase (HpRP), either on cartridges (peptide fractionation), or StageTips (phosphopeptide fractionation)
- We outline the benefits of the method, including speed of generating fractions for analysis, simple automation (including up front on-bead protein digestion), high technical reproducibility in manual and automated formats, and scalability for both proteome and phosphoproteome applications.
- In a separate experiment, we evaluate the use of a second looped enrichment to increase the yield of phosphopeptides from the biological sample, re-enriching using the same beads and buffers after the first phosphopeptide elution.

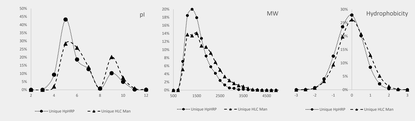




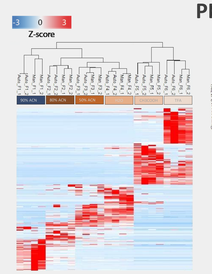
Peptide overlaps showed over 20% uniqueness, indicating the two methods are complementary.



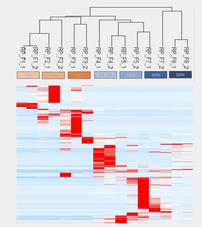
Above: MHA peptide fractionation resulted in a **gain of 2-3 fold in protein and peptide ID's** in comparison to the original starting material (SM). This was consistent for manual and automated formats, and across laboratory sites. Similar gains were observed with high pH RP fractionation, but with a higher number of fractions, and lower reproducibility. *Below:* possible complementarity is confirmed by analyzing the peptide properties, unique MHA peptides were generally more basic, higher MW and more hydrophobic as compared to HpHRP

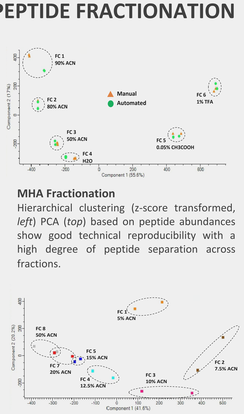


PEPTIDE FRACTIONATION



MHA Fractionation
Hierarchical clustering (z-score transformed, *left*) PCA (*top*) based on peptide abundances show good technical reproducibility with a high degree of peptide separation across fractions.





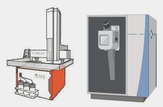
HpRP Fractionation
High degree of peptide separation was also observed across peptide fractions collected from the high pH fractionation kit, but technical replicates clustered less tightly likely due to the lower technical reproducibility.

METHODS

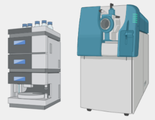
MANUAL

AUTOMATED

LAB 1



LAB 2



Manual peptide fractionation
- Evosep 1, 30 SPD, 40 min gradient
- Exploris 480, DIA, 45k, 2s

Phosphopeptide fractionation
- Zr-IMAC HP enriched, TMT labelled
- KingFisher™ Flex (auto)
- *Fractionated*: Evosep1, 40 SPD, 30 min gradient, Exploris 480, DIA, 45k, 2s

Peptide fractionation
- Manual and KingFisher™ Duo (auto)
- Dionex nanoRSLC, 30min gradient, Sciex 6600, SWATH 60VW

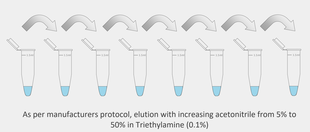
Re-enrichment by looping beads
- KingFisher™ Duo
- Evosep 1, 40 SPD, 40 min gradient
- Exploris 480, DIA, 30k, 2s

DirectDIA

Total Proteome
- The MHA method for peptide fractionation was benchmarked against a HpRP peptide fractionation kit (Pierce)
- The manual MHA workflow took 10 to 15 min to complete, while the HpRP kit required 40 to 60 min due to the centrifugation steps
- The MHA method required a lower elution volume per fraction (⅓ of the volume), and potentially lower for coupling directly to LCMS analysis

Phosphoproteome
- Similarly, the manual MHA workflow took 10 to 15 minutes to complete, while the 3M™ Empore™ C18 StageTip fractionation (STF) required 40 to 60 minutes (centrifugation, and excluding StageTip assembly of ~60 minutes)
- Phosphopeptides were eluted in 200µl for the automated MHA workflow, and StageTips were eluted with 50µl. A reduced elution volume of 50µl can be achieved by using low volume plates with the MHA method

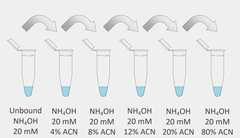
HIGH PH RP CARTRIDGE: PEPTIDE FRACTIONATION

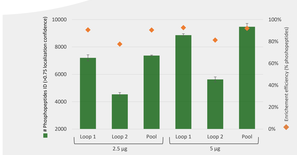


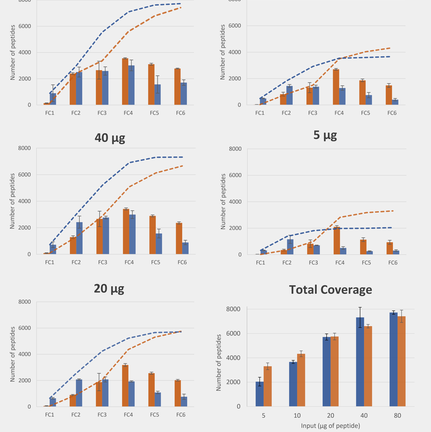
As per manufacturers protocol, elution with increasing acetonitrile from 5% to 50% in Triethylamine (0.1%)

STAGE TIP: PHOSPHOPEPTIDE FRACTIONATION

Conditioned







PHOSPHOPEPTIDE FRACTIONATION

The binding and elution conditions of the MHA method were adapted for phosphopeptide fractionation, after phosphopeptide enrichment using MagReSyn® Zr-IMAC HP. The workflow was compared against an established STF method for various peptide input amounts (5–80 µg input prior to phosphopeptide enrichment). Both methods employ the same number of total fractions for analysis. The number of phosphopeptides identified were comparable between the two methods. Notably at lower input amount (<10 µg), the MHA method started to outperform the STF method. Moreover, MHA is significantly faster to complete, and can be automated on a KingFisher™ magnetic bead handling station.

LOOPED PHOSPHOPEPTIDE ENRICHMENT

We evaluated a looped enrichment strategy in an attempt to improve the yield from low input material, by repeating of the phosphopeptide enrichment protocol using the same beads and buffers from the first enrichment. This data (far left) showed that additional phosphopeptides can be enriched from the sample after the first enrichment. With 5 µg of input peptide, we achieve an approximately 9500 phosphopeptide identifications, an increase of 6.9% when pooling the 2 elutions from the looped experiment (2 enrichments), versus our standard protocol employing a single enrichment step. For 2.5 µg of peptide input an average of 7500 peptides was achieved.

CONFLICT OF INTEREST DISCLOSURE
Stoyan Stoychev, Justin Jordaan, and Ireshyn Govender are employed by ReSyn Biosciences, proprietors of MagReSyn® technology.

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- Some equipment images in methods courtesy of bioreneder.com