Reproducibility Analysis of Clinically-relevant Targets Identifies Durable Phosphopeptide Ions

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Background

When considering the creation of a Laboratory Developed Test (LDT) or an in vitro diagnostic (IVD), a sufficiently-high accuracy, reproducibility and robustness are required. The use of Liquid Chromatography Mass Spectrometry (LC-MS) within the clinical context differs from its use in the research environment. LC-MS paired with the latest machine learning (ML) techniques is poised to play an important role in the routine testing within precision oncology. Due to the labile nature of protein phosphorylation and the multistep process of phosphoproteomics analysis, the establishment of reproducible clinical phosphoproteomic methods is a challenge.

Here, we build a targeted method to quantify phosphopeptides that have previously been shown to be predictive of treatment response in leukemia patients, when used as part of ML-based predictive models *(1)*. Using this targeted method we assess the analytical and technical reproducibility of these phosphopeptides in both cell lines and primary patient material.

Methods and Experimental design

- LC-MS analysis: using a Thermo Scientific Exploris 240, a PRM method was created targeting 118 phosphopeptide ions. For each experiment, 24 inter-technical replicates and 24 bio-replicates were acquired. Data was analysed using Skyline or an inhouse generated software, Au2Select ***(Figure 1)***. Reproducibility of phosphopeptide quantification was assessed from three fragments per ion. Calculated coefficient of variation (CV) for quantification values across replicates.

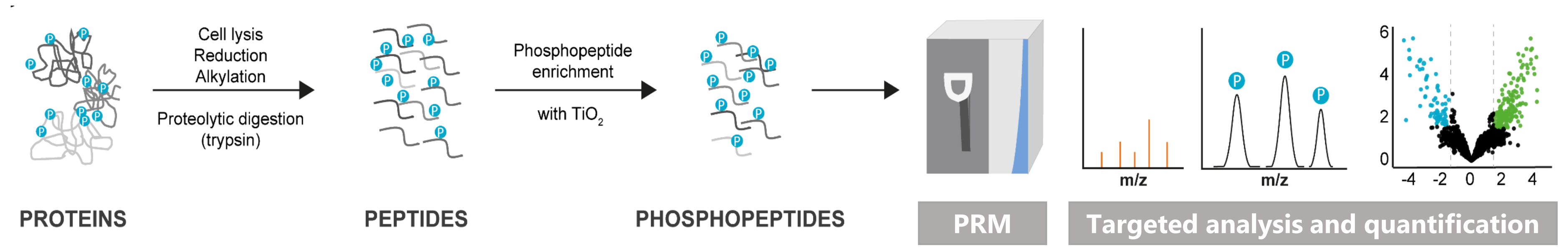


Figure 1. Phosphoproteomics workflow

- Lysates from three cell lines were collected in three different weeks (1, 2, and 3). Each cell line lysate from each week were combined in a 1:1:1 ratio to create a "super sample". Each super sample set were prepared to generate enough material to assess analytical, technical and biological reproducibility. Additionally, one set was prepared from pooled primary patient material ***(Figure 2)***.

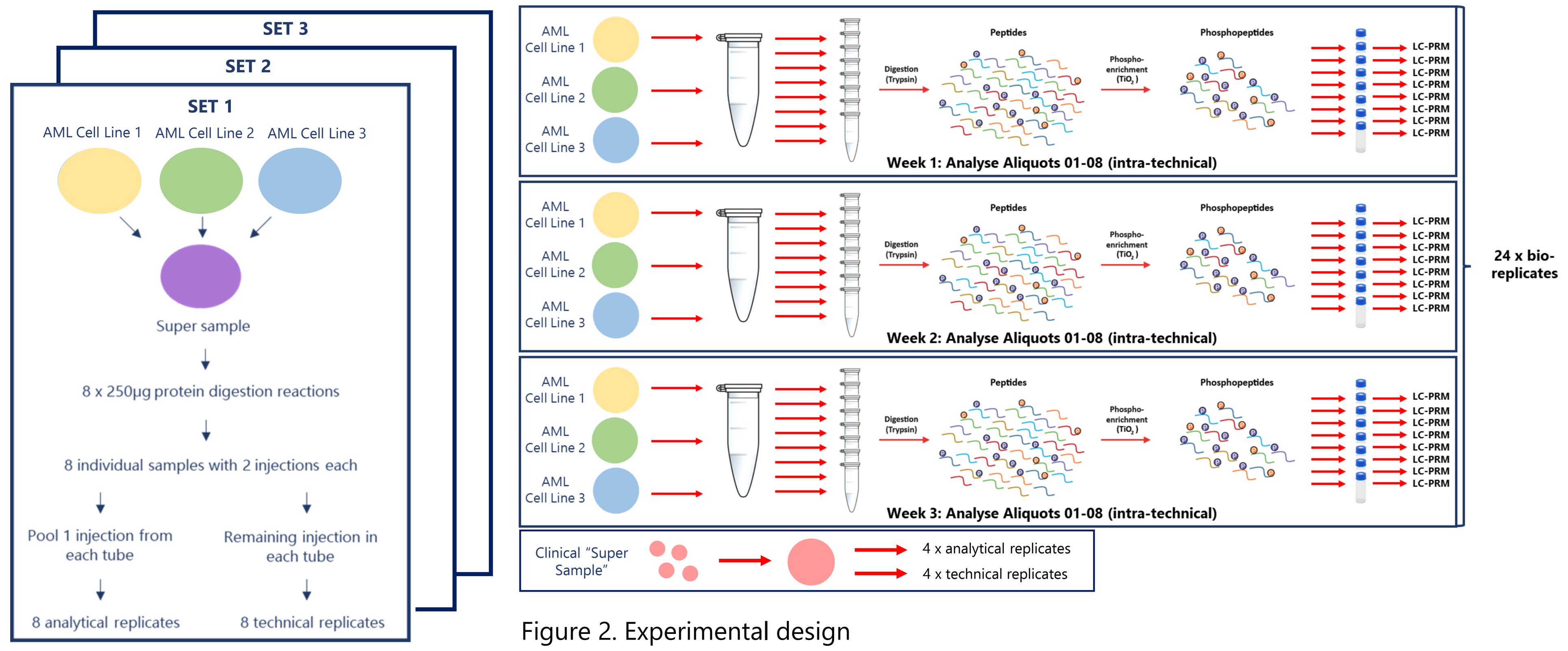


Figure 2. Experimental design

Targeted methods can **reproducibly quantify phosphopeptides** suitable for **clinical translation**

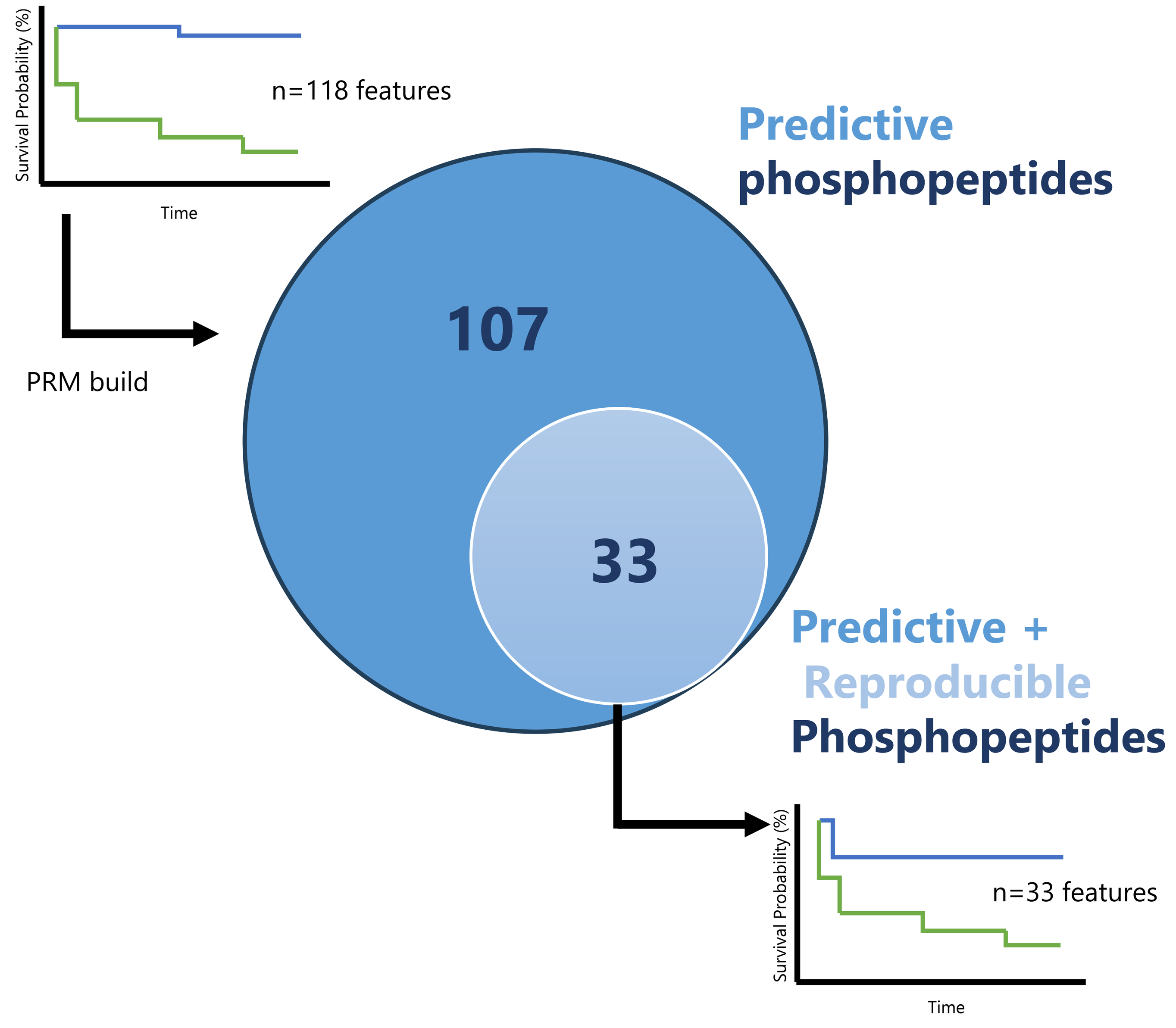
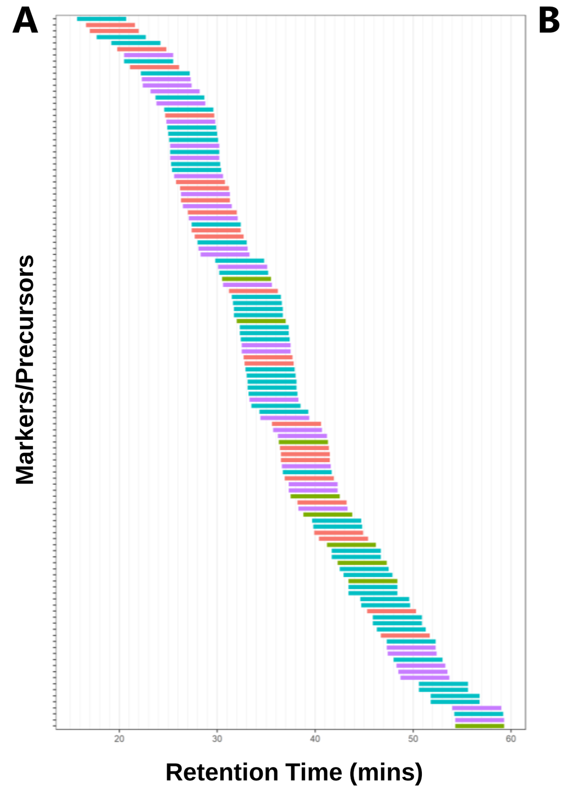


Figure 3. Identification of reproducible phosphopeptides that can predict treatment response. Targeted assay developed to monitor 118 phosphopeptide-based features; 107 optimised for reproducibility analysis; and 33 determined to be reproducible and suitable for further model development.

Results

PRM method build

A



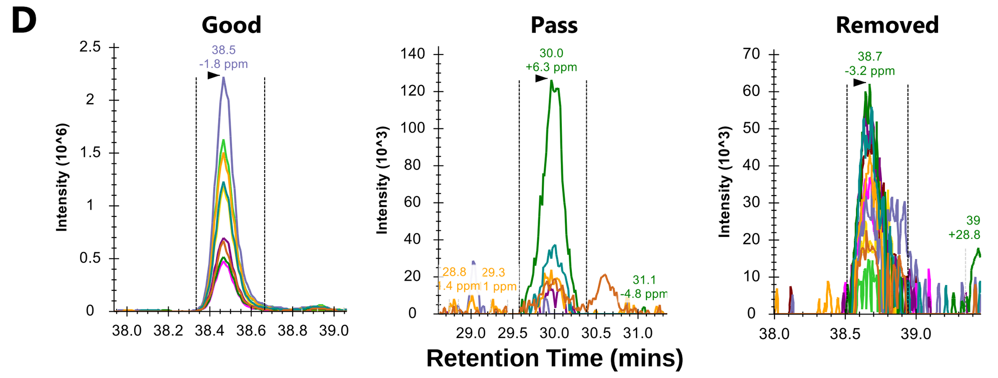
B

Performance	No. of markers
Quantified in all samples	96
Not quantified in >1 sample	11
Not quantified in any samples	11

C

No. of fragment ions quantified across all samples	No. of markers	Proportion of all markers (%)
0	11	9
3	60	51
4	34	29
5	8	7
6	4	3
7	1	1
Total	118	100

D



- Method development initially targeted a build comprising 118 precursor ions **(Figure 4)**.
- Selected peptides were scheduled in a single PRM method utilizing:
  - a minimum of 3 fragment per peptide;
  - fragment m/z > 200;
  - minimum of 6 scans per XIC; and
  - top 3 fragment areas averaged.
- Following optimisation it was possible to develop a method that included 107/118 of the phosphopeptide precursors.

Figure 4. PRM setup. A) The quality of all identified markers were assessed along with their retention time. B) The number of markers (precursors) quantified in the samples analysed. C) The number of fragment ions quantified across all samples for each marker (precursor). D) Representative examples of ion traces acquired and acceptable chromatography for target inclusion.

Reproducibility testing

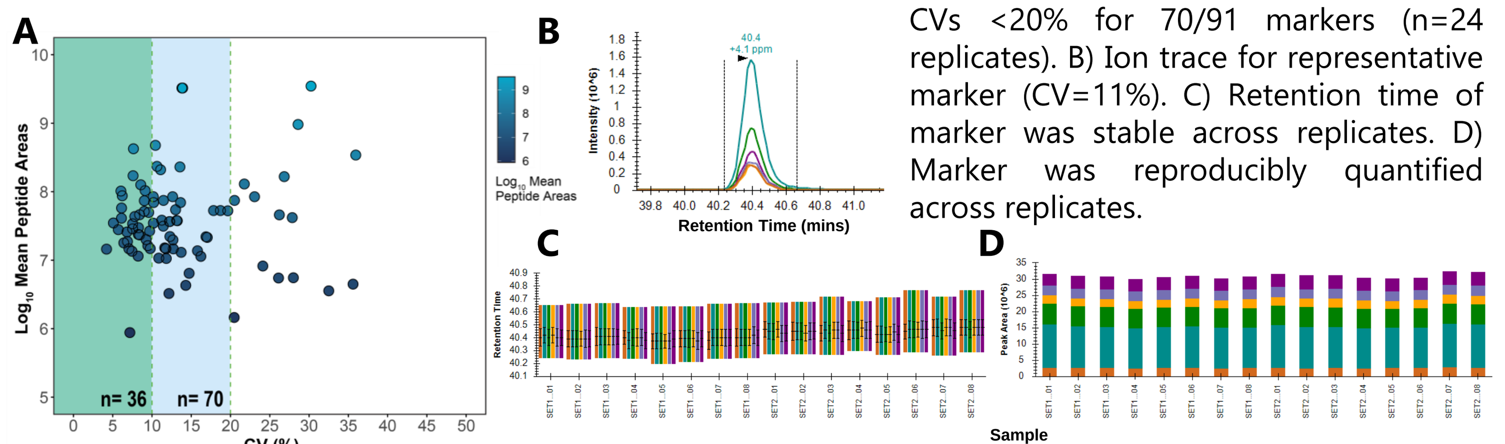


Figure 5. Technical reproducibility. A) CVs <20% for 70/91 markers (n=24 replicates). B) Ion trace for representative marker (CV=11%). C) Retention time of marker was stable across replicates. D) Marker was reproducibly quantified across all replicates.

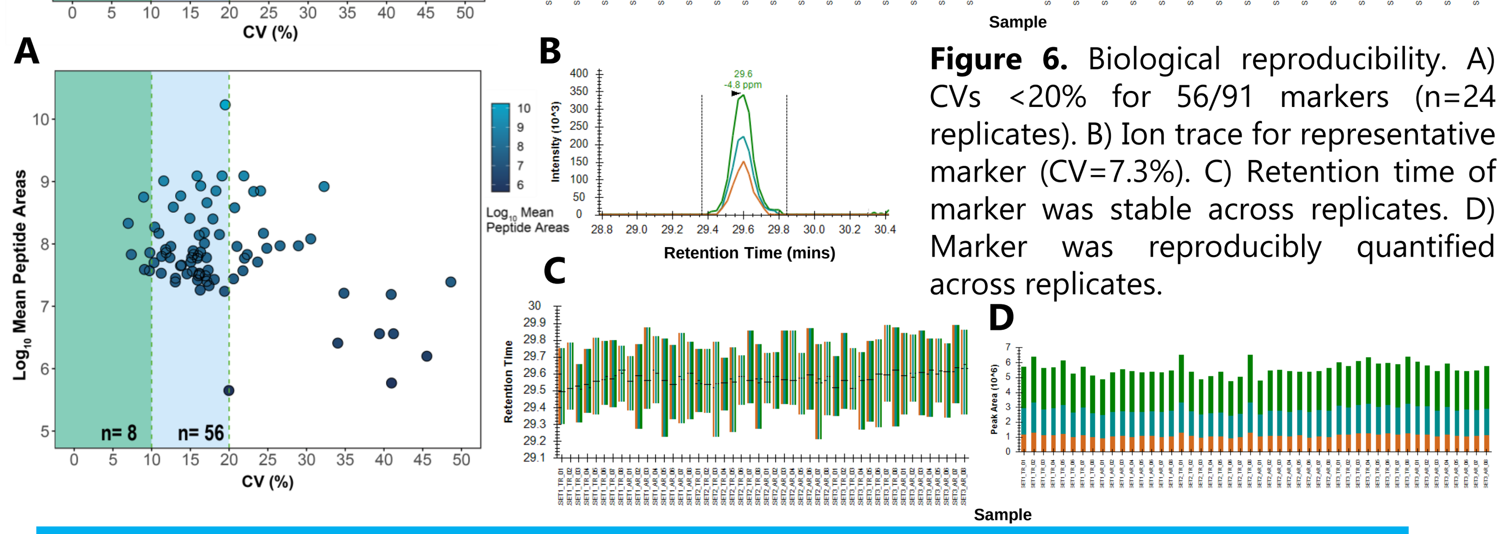


Figure 6. Biological reproducibility. A) CVs <20% for 56/91 markers (n=24 replicates). B) Ion trace for representative marker (CV=7.3%). C) Retention time of marker was stable across replicates. D) Marker was reproducibly quantified across all replicates.

Testing with primary material

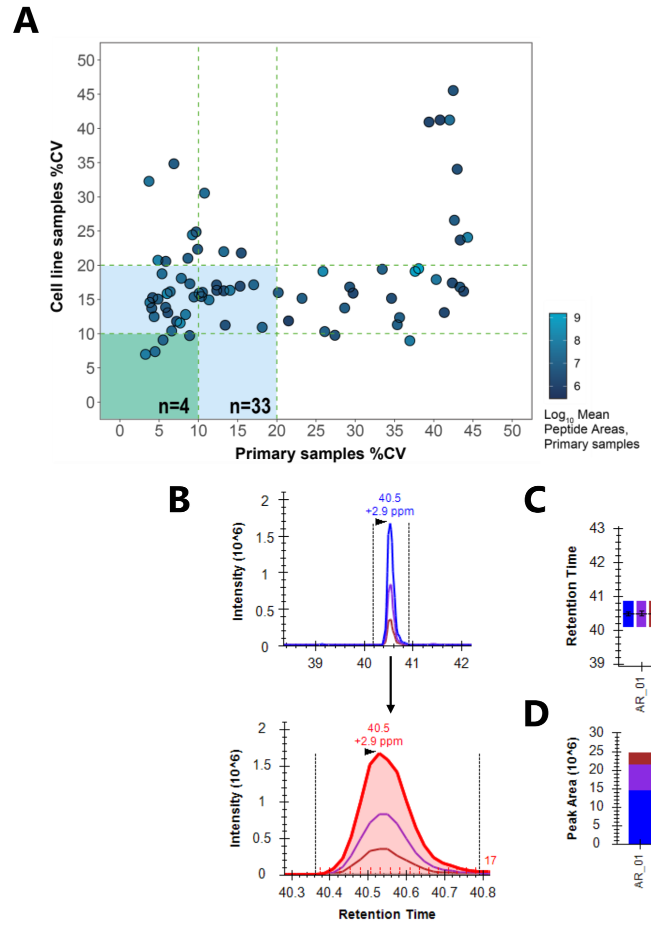


Figure 7. PRM performance with primary samples. A) CVs <20% for 50/91 markers (n=8) & 33/50 markers in both primary and cell line experiments. B) Ion trace for representative marker (CV=4.8%) & lower panel illustrates 17 points across the peak. C) Retention time of marker was stable across replicates. D) Marker was reproducibly quantified across all replicates.

Conclusions and future directions

- PRM method developed for 107/118 phosphopeptide biomarkers identified by DDA *(1)*.
- Technical and biological reproducibility of 91/107 phosphopeptide precursors was assessed and 33 were determined to be reproducible across all sample types.
- Phosphopeptides that were both predictive and durable across testing can now be used to tune and continue development of clinically-robust treatment response model.
- New 33-feature predictive model and targeted method will be employed to validate these findings in a retrospective analysis of suitable patient samples.

References:

1. Dokal, A. et al. *J. Clin. Oncol.* 39 (Suppl.), 7019-7019 (2021).

Other aspects of the full KScan® workflow are covered in **several other ASMS 2024 posters:**