Comprehensive Profiling of Data Acquisition Strategies to Optimize Clinically-feasible Approaches for Monitoring Predictive Phosphorylation Markers

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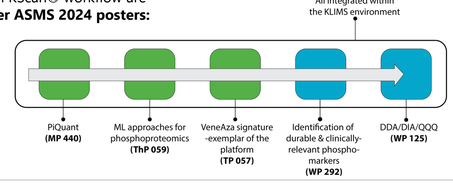
Background

- Quantitative phosphoproteomics maps the cell signalling landscape and has the potential to transform precision medicine[1-4]
- Kinomica have successfully identified and validated panels of phosphopeptide markers to accurately predict treatment response in different cancers.
- A significant hurdle in realising the potential of phosphopeptide markers to inform patient treatment is the translation from untargeted discovery LC-MS approaches to the clinic, where more targeted methods focusing on quantitative accuracy, robustness and higher throughput are key.
- Proving the accuracy, robustness and agreement of phosphopeptide quantification across these approaches is essential in translating phosphopeptide biomarkers to clinically-relevant diagnostic tests.

Kscan® workflow for biomarker discovery

- Clinical patient samples are processed using a streamlined phosphoproteomics protocol in which proteins are extracted, digested, enriched for phosphopeptides then analysed by LC-MS
- Differential expression analysis, machine learning and kinase-substrate enrichment analysis (KSEA) are performed to gain biological insight and identify candidate biomarkers.
- Predictive models are built using candidate biomarkers and validated with independent blinded sample cohorts.

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



Experimental Design

- Starting material;
  - Cell lines [DIA method development]
  - Untreated and pervanadate treated cell lines mixed in different ratios [DDA vs DIA]
  - Acute Myeloid Leukemia (AML) patient peripheral blood mononuclear cells [DDA/DIA concordance]
- Proteins were extracted, digested, desalted and enriched for phosphopeptides using titanium dioxide.
- Data was acquired using different MS systems run with different flow rates/acquisition modes (Fig. 1);
  - Thermo Orbitrap Exploris 240 with Vanquish Neo UPLC [Nanoflow DDA]
  - SCIEX ZenoTOF 7600 with Waters M-Class UPLC [Microflow DDA & DIA]
  - Thermo TSQ Altis Plus QqQ with Vanquish Neo UPLC [Microflow MRM]
- DDA data was analysed using an in house pipeline.
- DIA data was analysed using DIA-NN[5].

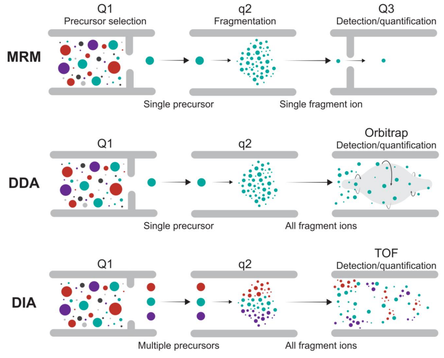


Figure 1. Schematic of different MS acquisition modes tested

Results

Developing a DIA method for phosphopeptide quantification

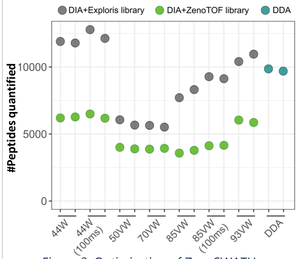


Figure 2. Optimisation of ZenoSWATH windows and spectral library generation

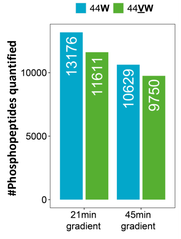


Figure 3. Optimisation of gradient length and windows

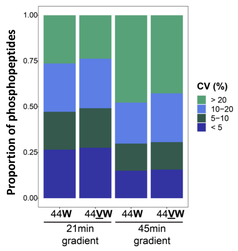
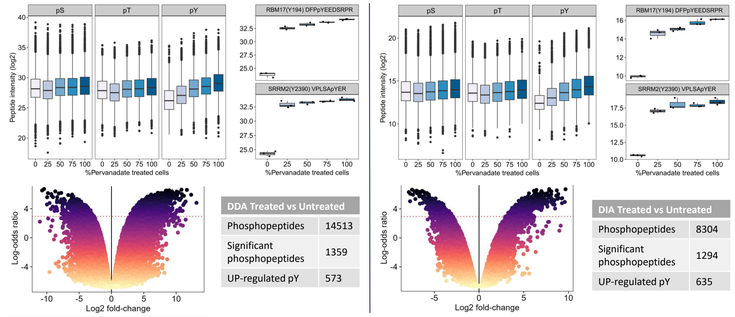


Figure 4. Summary of phosphopeptide CV's

- **Libraries built from nanoflow Exploris DDA work well for ZenoTOF DIA (Fig. 2).**
- **Shorter gradients with fewer windows and longer accumulation times quantified more phosphopeptides (Fig. 3) with lower CVs (Fig. 4).**

Phosphopeptide quantification in the same samples analysed by…

Nanoflow DDA **vs** **Microflow DIA**



DDA Treated vs Untreated	
Phosphopeptides	14513
Significant phosphopeptides	1359
UP-regulated pY	573

DIA Treated vs Untreated	
Phosphopeptides	8304
Significant phosphopeptides	1294
UP-regulated pY	635

Figure 5. Analysis of the same phosphopeptide samples by nanoflow DDA (left) and microflow DIA (right), comparing intensities of different phosphopeptides (top) and differential expression analysis between 100% treated and untreated (bottom).

- **DIA quantified fewer phosphopeptides overall but biological trends are preserved (Fig. 5).**

QqQ MRM for Phosphopeptide quantification

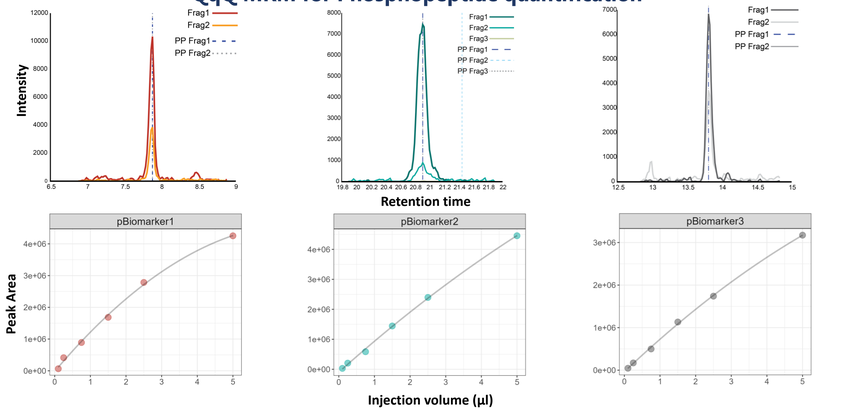


Figure 6. Targeted microflow MRM data for 3 phosphopeptide biomarkers with XIC for monitored fragment ions (top) and peak areas at different injection volumes (bottom).

- **Phosphopeptide biomarkers are robust across MS instruments and detectable with <10% of a standard injection (Fig. 6).**

Concordance of peptide quantification across different approaches

	AML Patients treated with M+IC		AML Patients treated with ICa	
	All Peptides	Biomarkers	All Peptides	Biomarkers
Sample 1	0.58	0.84	0.6	0.86
2	0.67	0.86	0.6	0.7
3	0.66	0.67	0.58	0.73
4	0.65	0.65	0.58	0.6
5	0.7	0.71	0.55	0.93
6	0.69	0.74	0.56	0.72
7	0.69	0.77	0.57	0.74
8	0.21	0.68	0.62	0.74
9	0.22	0.69	0.59	0.68
Average	0.56	0.73	0.58	0.74

Figure 7. Correlation coefficients between DDA and DIA runs from 9 AML patients across 2 treatment regimes, calculated across either all peptides or just candidate biomarkers.

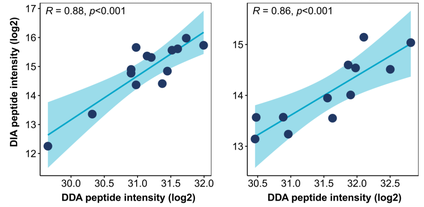


Figure 8. Correlation of phosphopeptide quantification between DDA and DIA data acquired from the same AML patients for phosphopeptide biomarkers from two different treatment regimes.

- **The overall correlation of all peptides is reasonable but correlations are stronger for candidate biomarkers (Fig. 7)**
- **Some biomarkers are extremely robust across different acquisition modes (Fig. 8)**

Conclusions

- **We have optimised a microflow zenoSWATH method for phosphopeptide quantification from an equivalent amount of input material to that achievable from patients …Microflow zenoSWATH with a 20 min gradient quantifies more phosphopeptides than DDA with 90 min gradients.**
- **Whilst not true for all phosphopeptides, we have identified phosphopeptide biomarkers which can be reproducibly quantified across different MS instruments, acquisition modes and flow rates.**
- **Filtering candidate biomarkers to those robust across different acquisition modes will facilitate translation to clinically viable assays used to inform patient treatment.**

References
1. Dokal A., *ASCO Annual Meeting*, 2021
2. Casado P., *Leukemia*, 2021
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5. Demichev, V., *Nat Methods*, 2020