Synthetic Peptide Reference Standards for PTM Proteomics

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Introduction

Despite recent improvements, PTM proteomics is still challenging.[1] Typical problems are a) low endogenous abundance, b) low ionization intensity, c) changed fragmentation, d) limited stability during proteomics workflows, and/or e) complex fragmentation spectra interpretation, especially regarding correct PTM site localization.

Defined synthetic PTM reference peptides support PTM proteomics and help to overcome challenges. Accordingly, the recently launched ProteomeTools project[2] will incorporate reference spectra for hundred thousands of PTM modified peptides. The goal of the current study is the development of defined sets of PTM reference standards that should be valuable tools for optimizing and standardizing proteomics workflows.

Methods and Results

Two sets of PTM reference peptides were synthesized in a stable-isotope labeled (SIL) form and analyzed by LC-MS/MS (Figure 1, Tables 1 and 2). Peptides for set A were non-quantified, while peptides for set B were purified to high purity and absolutely quantified.

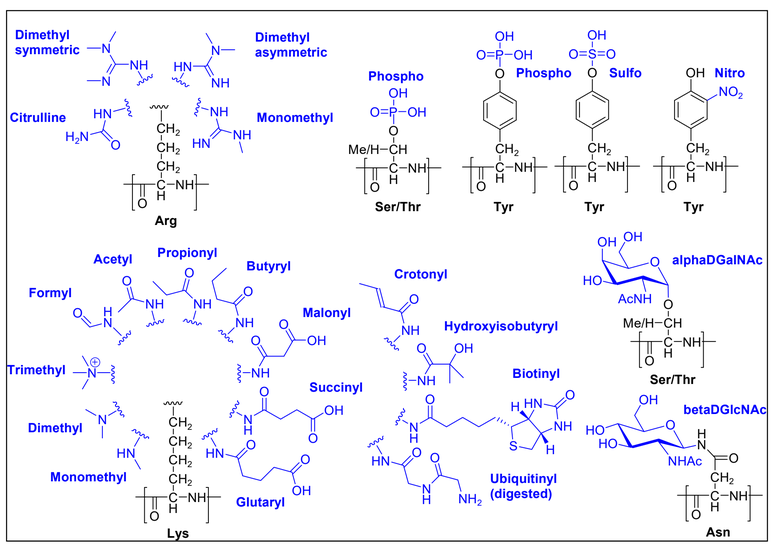

Figure 1: Overview of PTMs included in the study.

The peptide sequences were chosen from available MS datasets or – when this was not possible – taken from Uniprot. The PTMs were incorporated by using respective pre-synthesized modified amino acid building blocks for SPOT[3] (set A) or classical resin-based synthesis (set B). LC-MS/MS analysis was done on a Fusion Lumos instrument, data analysis with MaxQuant.

Tables 1 and 2: Synthesized peptide sets.

Final Peptide Set A: Non-quantified peptides	
Name	**# Peptides**
Lys Unmodified	100
Lys(Methyl)	100
Lys(Dimethyl)	100
Lys(Trimethyl)	100
Lys(Formyl)	100
Lys(Acetyl)	100
Lys(Propionyl)	100
Lys(Butyryl)	100
Lys(Malonyl)	100
Lys(Succinyl)	100
Lys(Glutaryl)	100
Lys(Crotonyl)	100
Lys(Hydroxyisobutyryl)	100
Lys(Biotinyl)	100
Lys(GG) (Ubiquitinyl)	100
Arg Unmodified	100
Arg(Methyl)	100
Arg(Dimethyl asymm)	100
Arg(Dimethyl symm)	100
Cit	100
Nitrotyr Unmodified	100
Nitrotyr	100
Hyp Unmodified	100
Hyp	100
Glycosyl Unmodified	100
Glycosyl Ser/Thr(alphaDGalNAc)	100
Glycosyl Ser/Thr(betaDGlcNAc)	100
	∑ 2700

Final Peptide Set B: Absolutely quantified peptides	
Name	**# Peptides**
Lys(Methyl)	6
Lys(Dimethyl)	6
Lys(Trimethyl)	6
Lys(Formyl)	6
Lys(Acetyl)	6
Lys(Propionyl)	6
Lys(Butyryl)	6
Lys(Malonyl)	6
Lys(Succinyl)	6
Lys(Glutaryl)	6
Lys(Crotonyl)	6
Lys(Hydroxyisobutyryl)	6
Lys(Biotinyl)	6
Lys(GG) (Ubiquitinyl)	6
Arg(Methyl)	6
Arg(Dimethyl asymm)	6
Arg(Dimethyl symm)	6
Phospho Unmodified	4
Phospho-Ser	5
Phospho-Thr	5
Phospho-Tyr	4
Tyr Sulfatation Unmodified	5
Tyr Sulfatation	6
Glycosyl Unmodified	3
Glycosyl Ser(alphaDGalNAc)	4
Glycosyl Thr(alphaDGalNAc)	3
Glycosyl Asn(betaDGlcNAc)	1
	∑ 142

For the development of set A, 170 or more peptides were synthesized for each PTM in non-isotope labelled form. It turned out (Figure 2) that the recovery for all peptides in LC-MS/MS experiments was very high. This encompassed not only frequently analyzed PTMs like Lys(Ac) (in fact 49,586 previously reported Lys(Ac) modified proteotypic peptides were synthesized), but also less frequently analyzed PTMs like crotonylated or hydroxyisobutyrylated Lys.

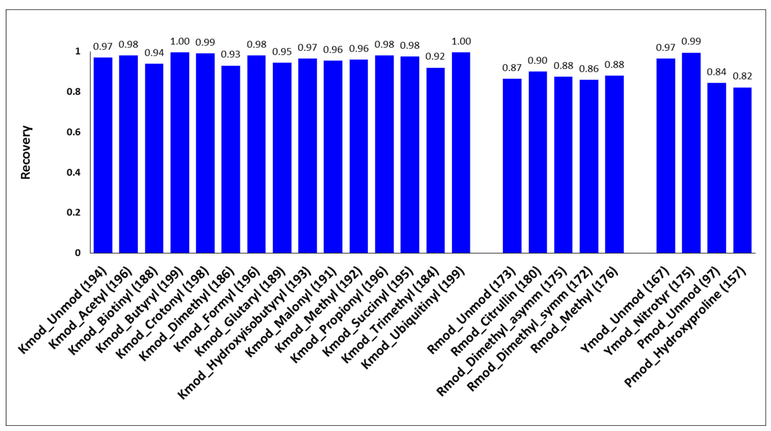

Figure 2: Recovery (proportion of successfully detected peptides) for PTM modified peptides to be included in set A. The number of synthesized peptides is shown in brackets below the bars.

Based on the obtained data the optimal peptides for set A were selected (100 peptides per PTM). All analyzed peptides showed very high andromeda scores (average >100 in all cases, Figure 3a). Although still high, the scores for some PTMs were somewhat lower. These can be explained by lower synthetic accessibility (i.e. Lys Malonyl) or a shift of the preferred ionization state from 2+ to 3+ (e.g. Lys Acetyl to Lys Methyl, Figure 3b).

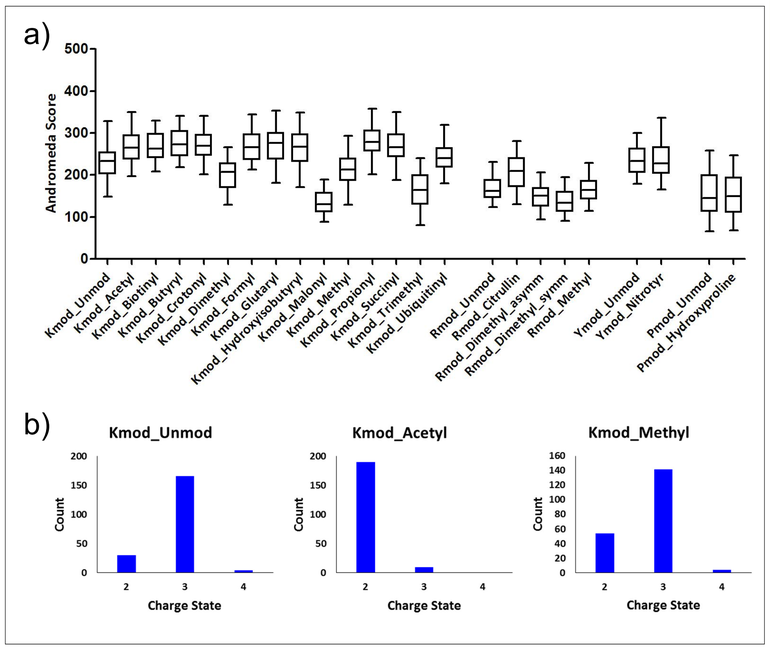

Figure 3: a) Determined MaxQuant Andromeda scores for all peptides from final set A. b) Determined charge distribution for three selected Lys modifications.

We are currently working on the use of the peptide sets for optimizing MS conditions and on the analysis of retention time shifts.

To support the PTM proteomics community it is planned to make both peptide sets commercially available as SIL labelled peptides. Customized sets can be synthesized quickly at reasonable costs with the high-throughput SPOT peptide synthesis platform. These peptides sets can be used as reference standards or for answering specific questions.

Conclusion

Synthetic peptide reference standards were prepared as tools for optimizing and standardizing proteomics workflows and analysis conditions.
The catalogue of modifications can easily be extended as new modifications are described.

References

(1) (a) Olsen, J. V. et al. *Mol. Cell. Proteomics* **2013**, *12*, 3444-2452. (b) Doll, S. et al. *ACS Chem Biol.* **2015**, *10(1)*, 63-71.
(2) Zolg, D. P. et al. *Nat. Methods* **2017**, *14(3)*, 259-262.
(3) Wenschuh, H., et al.. *Biopolymers* **2000**, *55*, 188-206.

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