

TECHNICAL BRIEF

Tackling aspecific side reactions during histone propionylation: The promise of reversing overpropionylation

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Histone proteins are essential elements for DNA packaging. Moreover, the PTMs that are extremely abundant on these proteins, contribute in modeling chromatin structure and recruiting enzymes involved in gene regulation, DNA repair and chromosome condensation. This fundamental aspect, together with the epigenetic inheritance of histone PTMs, underlines the importance of having biochemical techniques for their characterization. Over the past two decades, significant improvements in mass accuracy and resolution of mass spectrometers have made LC-coupled MS the strategy of choice for accurate identification and quantification of protein PTMs. Nevertheless, in previous work we disclosed the limitations and biases of the most widely adopted sample preparation protocols for histone propionylation, required prior to bottom-up MS analysis. In this work, however, we put forward a new specific and efficient propionylation strategy by means of propionic anhydride. In this method, aspecific overpropionylation at serine (S), threonine (T) and tyrosine (Y) is reversed by adding hydroxylamine (HA). We recommend using this method for future analysis of histones through bottom-up MS.

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Histone analysis through bottom-up MS requires an additional step during sample preparation as compared to the traditional proteomics strategy: chemical derivatization by acylation reaction at primary amine groups. This is mainly because histones are highly enriched in basic amino acid residues, which comes as an advantage for the proper binding to DNA but results in generation of very short peptides for LC-MS analysis after trypsin digestion. Therefore, histones are mostly derivatized prior to and after trypsin-mediated cleavage by means of propionylation of accessible amine groups

to prevent trypsin-cleavage after lysine while maintaining trypsin efficiency over Arg-C efficiency [1, 2]. However, the technical framework of this propionylation step should not induce additional variation that obscures biological changes.

Previously, we performed an extensive investigation of side reactions for four commonly used propionylation protocols (method A till D), using data directed acquisition and peptide quantification performed via XICs after precursor alignment [3]. This analysis disclosed several pitfalls: side reactions on carboxyl groups (amidation, methylation), incomplete derivatization (underpropionylation) and aspecific propionylation at S, T and Y residues (overpropionylation). These pitfalls hinder identification and impair direct comparison of precursor intensities of biologically modified peptides. However, relative abundance proved to be a very robust measurement which quantified PTMs very similarly independent of the

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Abbreviations: HA, hydroxylamine; TEAB, triethylammonium bicarbonate; XIC, extracted ion chromatogram

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Table 1. Customizing a method in order to tackle known pitfalls

Based on - <i>pitfall</i>	Method name	Adjustments made	Pitfall			
			Amid	Meth	Und	Over
Method A	A.r	Reverse order of adding NH ₄ OH and propionylation reagent	↓	/	↑	/
- <i>Amid</i>	A.t	Add NH ₄ OH and propionylation reagent together	↓	/	↑	/
	A.n	Do not add any NH ₄ OH	↓	↑	/	↑
Method B and C	B.s	Do not mix methanol and propionic anhydride in advance	↑	/	↓	↑
- <i>Under</i>		Add NH ₄ OH at the end				
NEW	E	No buffer	/	/	/	↑
	F	Ethanolamine buffer	↑	/	/	↑
	G	Triethanolamine buffer	/	/	/	↑
	H	TEAB buffer	/	/	/	↑
Method H	H 42x	42x molar excess of propionic anhydride	/	/	/	↓
- <i>Over</i>	H 20x	20x molar excess of propionic anhydride	/	/	↑	↓
	H 5x	5x molar excess of propionic anhydride	/	/	↑	↓
Method H 42x	H 42x boil	Boil sample for 1h after propionylation	/	/	/	↓
- <i>Over</i>	H 42x HA	Reaction with HA after propionylation	/	/	/	↓

In order to address the known pitfalls, methods were customized. The effect of these adjustments on the average contribution of each pitfall was monitored. This contribution could be none (/), increase (↑), decrease (↓) or the adjustment could lead to complete reduction (↓) of the peptide form.

propionylation protocol applied. This implies that research groups using different protocols are likely to provide similar results when using this measurement to quantify changes between different biological samples [4]. Importantly however, correction factors for ionization efficiency are required when aiming at quantifying the true relative abundance in a single sample and the robustness here applies only to the comparison of different relative abundance values. Nevertheless, in order not to generate large amounts of uninformative new precursors that are being selected for MS/MS during data directed acquisition and at the same time enable “inter-run relative quantification” on biologically relevant precursors only (comparing changes in XICs directly), we set out to address these pitfalls in propionylation and develop a protocol that almost exclusively leads to correctly propionylated peptides. The methods investigated in both this and earlier work are described in more detail in Supporting Information (Tables 1, 2 and materials and methods). The impact of each adjustment made on propionylation as well as known side reactions is summarized in Table 1.

First, we tried to avoid amidation of carboxyl groups by making adjustments to method A (which was investigated in more detail in foregoing work) [3]. This resulted in method A.r, A.t and A.n. In method A.r and A.t, we mixed the same amount of ammonium hydroxide prior to or simultaneous with propionic anhydride, which no longer led to the generation of amidated carboxyl groups. However, due to an increase in underpropionylated peptides we refrained from further developing this strategy. Leaving out ammonium hydroxide as such, as done in method A.n, did also prevent amidation of carboxyl groups, but on the other hand induced methylation of these groups and led to aspecific propionylation at S, T

and Y residues. The former is due to an O-acylation reaction between the very reactive “mixed anhydride” and methanol (Supporting Information Fig. 1). This reaction did not occur in the presence of ammonium hydroxide (method A, A.r and A.t) because amine groups are more reactive than hydroxyl groups. In conclusion, the adjustments made to method A failed to provide an optimal propionylation method.

Next, we tried to convert underpropionylation in methods B and C of our previous work [3]. Despite the fact that all underpropionylation was countered by omitting mixing of methanol with propionic anhydride prior to the reaction and at the same time adding ammonium hydroxide after propionylation reagent, method B.s was deemed undesirable because new pitfalls such as amidation and overpropionylation arose.

In a third attempt, we investigated the effect of interfering with the buffer system by either using no buffer at all or changing the buffer system to contain ethanolamine, triethanolamine and triethylammonium bicarbonate (TEAB), resulting in four new methods: E till H. Unfortunately, all these methods led to an increase in overpropionylation, probably due to the reactivity of an anhydride towards hydroxyl groups (Supporting Information Fig. 2). Nevertheless, if overpropionylation is the only unwanted reaction, it is worth trying to address this issue specifically.

Hence we tried to tackle this aspecific overpropionylation in two ways: (i) preventing it from happening by lowering the molar excess of propionic anhydride or (ii) reversing it by means of boiling or the addition of hydroxylamine (HA) after the final propionylation step. This strategy was performed on aforementioned method H, which suffered from no other issues than overpropionylation based on our analysis. The

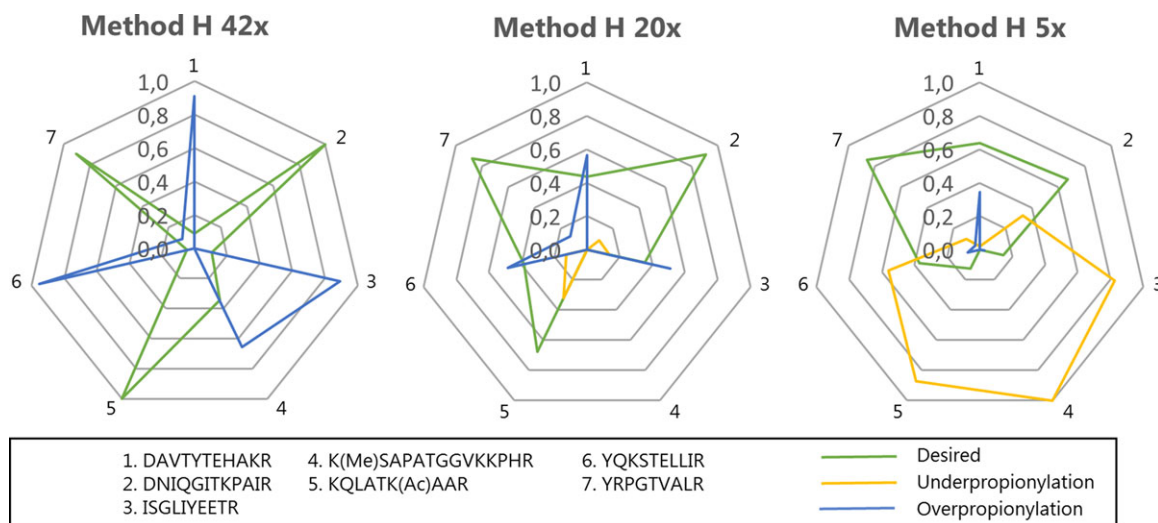


Figure 1. Lowering the molar excess (42x, 20x and 5x) of propionic anhydride per primary amine prevents most overpropionylation but increases underpropionylation. Each method is represented by a radar chart showing the average contribution of over- (blue), under- (yellow) and desired propionylation for seven peptides monitored. Each peptide is located on one angle of the radar chart and each peptide form is represented by another color. The abundance of a specific peptide form is shown on the radius, whereby a conversion rate of 0 is located in the center, increasing outwards.

first approach (method H42x, H20x and H5x) was partially successful, since overpropionylation decreased along with the reduction in propionic anhydride added to the samples. However, Fig. 1 shows underpropionylation increased simultaneously and finding the optimal molar excess to balance between both pitfalls appeared to be difficult. Furthermore, researchers often do not know the exact amount of sample – let alone primary amines – they have on their hands, which would be required for converting an ideal molar excess into a specific volume of propionic anhydride. Given the latter conclusion, we set out to reverse overpropionylation instead of avoiding it. Based on the literature we found two strategies: (i) boiling of propionylated peptides during 1 h (method H42x boil) [5] and (ii) adding hydroxylamine (method H 42x HA) [6–8]. Note that lower amounts of HA were recently used by other groups for quenching the acylation reaction with primary amines [9, 10]. Boiling as well as the use of HA, were implemented using method H42x, since overpropionylation is the only known side reaction for this approach. By boiling the sample we were able to break the ester bond on most peptides but unfortunately the amount of overpropionylated peptides remained too high for some peptides, such as for DAVTYTEHAKR where 41% of the peptides were left overpropionylated. The HA-mediated acyl removal on the other hand was more efficient, resulting in an average conversion rate of 95% (Fig. 2A). The addition of this very reactive nucleophile reversed overpropionylation, without removing propionylation on primary amines (Fig. 2B). The absence of non-wanted in vitro generated side products when using this protocol was confirmed by means of an error tolerant search, as described in Meert et al. [3]. Next to the very abundant propionylation at K- and N-terminus, no remarkable in vitro

modifications were identified. From another point of view, the unchanged intensity of the DNIQGITKPAIR peptide depicted in Supporting Information Fig. 3B strongly suggests that not even a modification other than the 1000+ searched in the error tolerant search is modifying the precursors during this reversal of overpropionylation. Hence, all methods generating only overpropionylated products as unwanted side products can be turned into reliable propionylation methods by addition of HA post propionylation. Moreover, the reaction with HA will facilitate identification of ADP-ribosylated D or E residues as it generates a hydroxamic acid derivative with an addition of 15.0109 Da. This increment can be more easily identified by MS than ADP-ribosylation as such [11]. Albeit, when using a method to reverse overpropionylation, one should always take into account that acetyl groups on S, T and Y residues will no longer be identified. This modification has been detected in several biological settings, as extensively reviewed in [12]. If researchers specifically aim at characterizing acetylation at S, T or Y residues, one should therefore use a propionylation method without boiling or reaction with HA. For correct identification it will then be of importance to include propionylation at S, T or Y in the search parameters. Correct quantification on the other hand can then only be obtained by using relative abundances.

In conclusion, we were able to develop a protocol (method H 42x HA) that attains efficient propionylation on primary amines, has no remaining aspecific propionylation of S, T and Y residues and displays no side reactions such as amidation or methylation of carboxyl groups. Despite the loss of information on in vivo acetylated S and T residues, this propionylation protocol has several advantages over other methods. First, sensitivity will increase as the MS signal

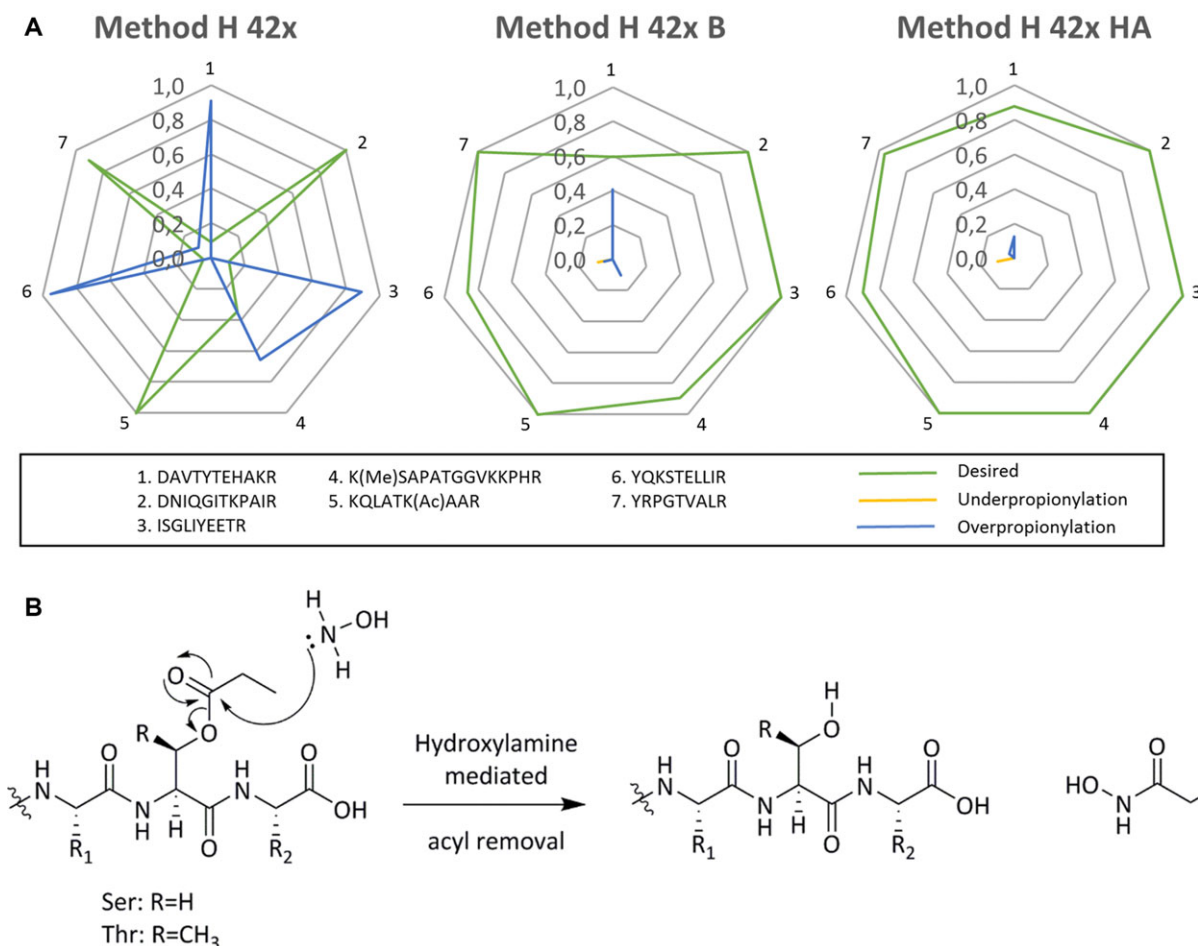


Figure 2. Reversing overpropionylation. (A) Overpropionylation in method H 42x can be (partially) reversed by boiling the sample for 1 h or adding HA. Each method is represented by a radar chart showing the average contribution of over- (blue), under- (yellow) and desired propionylation for seven peptides monitored. Each peptide is located on one angle of the radar chart and each peptide form is represented by another color. The abundance of a specific peptide form is shown on the radius, whereby a conversion rate of 0 is located in the center, increasing outwards. (B) Reaction mechanism of reversing overpropionylation by means of HA. HA is a strong nucleophile which will attack the carbonyl group and thereby induce acyl removal.

of the same peptide is no longer spread over different MS precursors, generated by unwanted side reactions or inefficient propionylation. Additionally, no precious acquisition time will be lost by generating MS/MS spectra of differentially in vitro modified peptides instead of spectra belonging to peptides with the interesting in vivo modifications. But not only data acquisition will benefit from using both efficient and specific propionylation protocol: also the confidence of peptide identifications will increase, considering that less variable modifications such as propionylation on S, T and Y have to be taken into account when performing a database search. Consequently, more in vivo modifications can be permitted before causing a combinatorial explosion. This in turn will lead to more biologically relevant conclusions. Finally, sample preparation will require less time as only one round of propionylation is required prior to as well as post digestion.

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