Fast and Accurate Determination of Cysteine Reduction and Alkylation Efficacy in Proteomics Workflows

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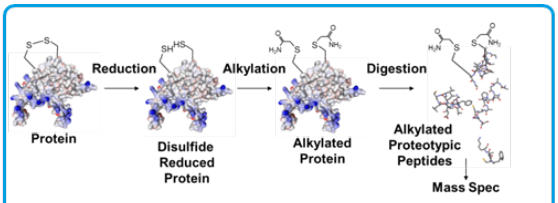
Cysteine reduction and alkylation is an integral part of all bottom-up proteomics workflows. To address the issue of reduction/alkylation reproducibility, a kit of peptides was developed. It enables the *in-situ* determination of disulfide reduction and cysteine alkylation conditions and reproducibility, including potential over-alkylation. The kit is compatible with routine sample digestion workflows and analysis can be performed using standard LC-MS protocols.

Introduction

Mass-spectrometry-based proteomics is one of the most powerful methods for the multiplexed detection of proteins in complex samples. The typical workflow of a mass-spectrometry-based proteomics experiment is shown in Scheme 1. First, disulfide bonds within proteins are reduced (e.g. with DTT or TCEP), followed by alkylation of the resulting thiol groups with iodoacetamide (IAA) or chloroacetamide (CAA). Finally, the alkylated proteins are digested (usually with trypsin) to yield peptides that are amendable to LC-MS/MS analysis.



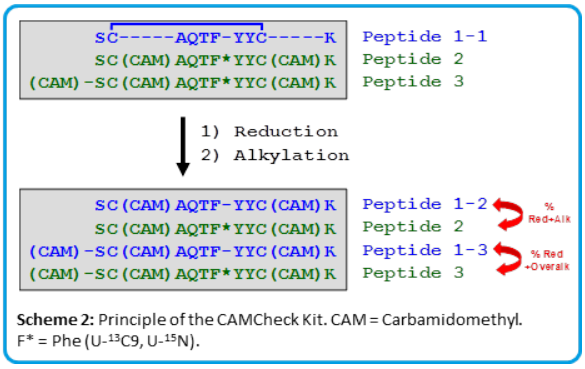
Scheme 1: Standard workflow for mass-spectrometry-based proteomics.

Incomplete reduction and/or alkylation will impair the qualitative and quantitative results of such experiments. The same is true for undesired "over-alkylation" (the alkylation of non-thiol moieties), which is known from several studies[1] but still a frequently neglected potential issue of proteomics workflows.

To address the issue of reduction/alkylation reproducibility, a collection of peptides was developed, the **CAMCheck Kit** that enables the *in-situ* determination of disulfide reduction and cysteine alkylation efficiency and reproducibility. The principle of the developed peptide set is shown in Scheme 2. A pre-manufactured mixture of equimolar amounts of peptides **1-1**, **2** and **3** is spiked into a protein sample and subjected to the standard proteomics workflow. During the workflow, the disulfide bond in peptide **1-1** is reduced and the resulting thiol groups are alkylated. This leads to the formation of the carbamidomethylated peptide **1-2** and – if too harsh alkylation conditions are applied – to the over-alkylated peptide **1-3**.

For the determination of disulfide reduction and alkylation efficacy, the amount of peptide **1-2** relative to its heavy-labelled counterpart peptide **2** is determined by mass spectrometry. The extracted ion chromatograms indicate the percentage of peptide **1-1** that was successfully reduced and alkylated. For example, when [Amount **1-2**] / [Amount **2**] = 0.95, then the efficacy of the reduction and alkylation procedure was 95 %. Because peptide **1-1** is non-naturally occurring, and thus undesired interferences with components of biological samples are in the main ruled out, the degree of conversion of kit peptide **1-1** can be used as a measure of protein conversion efficiency.

Analogous to the assessment of alkylation efficacy, the percentage of over-alkylation can be determined by measuring the ratio of peptide **1-3** to its over-alkylated counterpart peptide **3**.



Scheme 2: Principle of the CAMCheck Kit. CAM = Carbamidomethyl. F* = Phe (U-^{13}C9, U-^{15}N).

Materials & Methods

The CAMCheck Kit was subjected to reduction and alkylation with IAA or CAA under varying conditions. One kit unit (1000 pmol) was resuspended in 0.1 M ammonium bicarbonate (1000 µL). An aliquot of the solution (10 µL, 10 pmol) was transferred to an Eppendorf tube and diluted with 90 µL of 0.1 M ammonium bicarbonate. The reduction was performed using the following conditions: 10 mM DTT, 50 ˚C, 40 min., shaker. Subsequently, the peptides were alkylated under different conditions with IAA or CAA (10-50 mM, 30 min, RT, dark or 50 mM, 30 min, 37 °C). After concentration *in vacuo*, the sample was resuspended in 1% formic acid (FA) and analyzed by LC-MS. The LTQ Orbitrap Velos mass spectrometer was operated in data dependent mode using FTMS readout. The samples were separated on in-house packed C18 columns using a nano flow UHPLC with buffer A (Water, 5% DMSO) and a 60 min gradient from 4 to 32% buffer B (Acetonitrile, 5% DMSO). Extracted ion chromatograms were generated using Xcalibur 4.0.

For experiments in the presence of cell lysate, the following changes were made to the above conditions: An aliquot of the solution (20 µL, 20 pmol) was transferred to an Eppendorf tube containing 100 µg of cell lysate (U2-OS cell line) in 8 M Urea and 40 mM Tris/HCL pH 7.6. The reduction was performed in a shaker using 10 mM DTT at 50 ˚C for 40 min. Subsequently, the peptides were alkylated under different conditions. The sample was diluted with 3 volumes of 40 mM Tris/HCl and digested using trypsin 1:25 (enzyme/substrate ratio) at 37˚C overnight in a shaker. The samples were desalted using C18 Empore material in a StageTip format. The equivalent to 1 µg digest/200 fmol kit was analyzed by LC-MS.

Results

Fig. 1 depicts the reduction and alkylation efficacy under different conditions as determined by the CAMCheck Kit. Two reagents were compared for their alkylation behavior: Iodoacetamide (IAA) and Chloroacetamide (CAA).

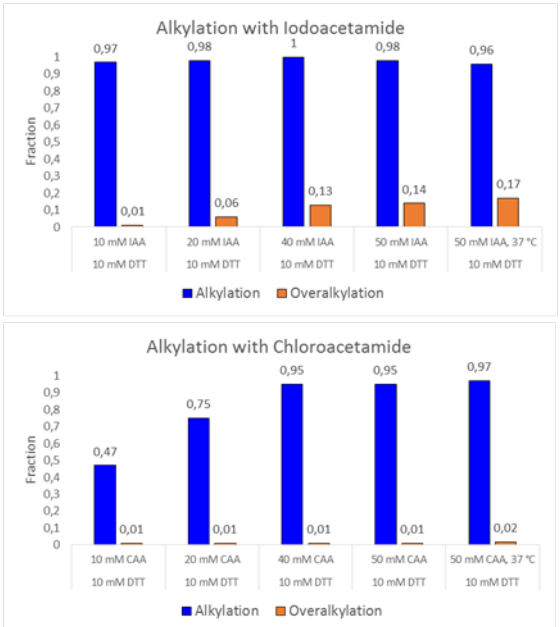


Figure 1: Reduction and alkylation efficacy (percent conversion of the kit peptide) under varying conditions. IAA or CAA used as alkylation reagent.

Figure 2 shows results for experiments where the kit was applied to a complete proteomics workflow including the reduction, alkylation and tryptic digestion of cell lysate.

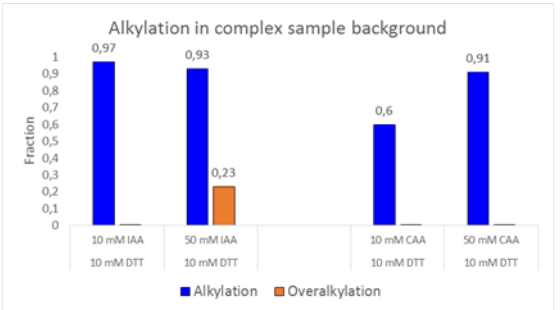


Figure 2: Reduction and alkylation efficacy (percent conversion of the kit peptide) under varying conditions in complex sample background (U2-OS cell lysate).

Discussion & Conclusions

The obtained data show that IAA is a powerful alkylation reagent (>95 % alkylation efficacy under all conditions, 10-50 mM, Fig. 1). CAA is less potent in alkylation of cysteine, because a minimum concentration of 40 mM was required to achieve complete conversion. However, the high reactivity of IAA results in over-alkylation of non-cysteine residues (1-17 %, Fig. 1, upper chart, orange bars). In contrast to IAA, CAA shows only minor over-alkylation, even at 50 mM and 37 °C (Fig. 1, lower chart, orange bars).

Based on the obtained data it can be concluded that CAA is – compared to IAA – a superior reagent for cysteine alkylation in proteomics workflows. This is in agreement with previous results[2] and still a significant finding, as some of the applied conditions (i.a. 10 mM DTT followed by 40 mM IAA) are widely used in proteomics experiments. While for shotgun proteomics over-alkylation can be included in the database search, targeted workflows will suffer from imprecise quantification or loss of identification if substantial over-alkylation occurs.

The kit was also applied to a complete proteomics workflow including the reduction, alkylation and tryptic digestion of cell lysate. The peptides were reliably detectable at 200 fmol in complex background and the respective quantitative results were in good agreement with the data above (Fig.2). To ease readout in complex background it is advisable to identify the retention times of the full length peptides in a previous experiment in order to ensure correct peak assignment. We conclude, that the kit is compatible with the widely applied bottom up proteomic workflow.

In summary, the kit enables the *in-situ* determination of disulfide reduction and cysteine alkylation efficiency and reproducibility, including potential over-alkylation and therefore allows the optimization of experimental conditions. The kit is compatible with routine sample digestion workflows and analysis can be performed using standard LC-MS protocols. The data analysis is very straightforward as only four peptide ion chromatograms need to be analyzed. As the peptides were chosen to be non-naturally occurring, the kit can be applied in native protein digests.

References

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