Protocol

CAMCheck Kit

Easy to use Peptide Kit to monitor Efficiency of Carbamido-methylation during Proteomic Sample Preparation

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1 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 Figure 1. First, disulfide bonds within a protein 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 mass spectrometric analysis.

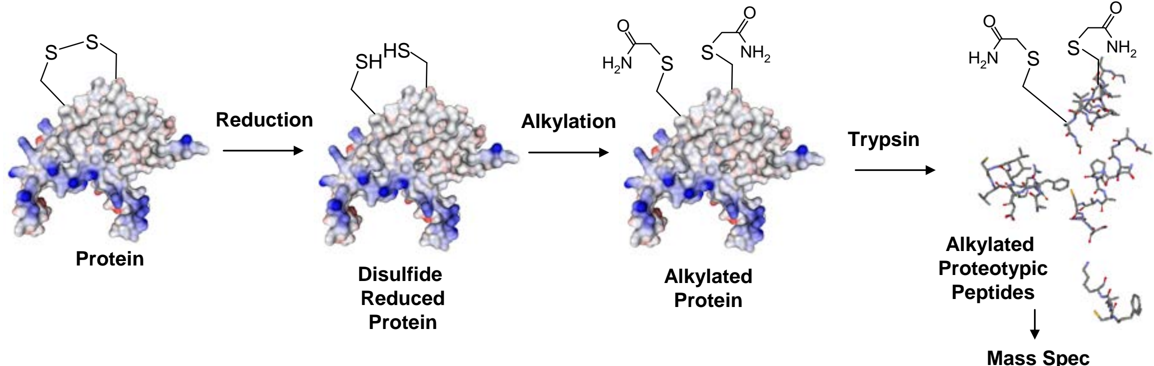

Figure 1: Standard workflow for mass-spectrometry-based proteomics.

In order to furnish reproducible results, MS based proteomics heavily relies on the high reproducibility of the standard workflow. Incomplete reduction and/or alkylation will impair the qualitative and quantitative results of such experiments. The same is true for undesired “overalkylation” (the alkylation of non-thiol moieties with IAA), which is a frequently neglected potential issue of proteomics workflows (e.g. a) Boja E. S. et al. “Overalkylation of a Protein Digest with Iodoacetamide”, *Anal. Chem.* **2001**, *73 (15)*, 3576-3582. b) Wang J. et al. “Influence of overalkylation in enzymatic digestion on the qualitative and quantitative analysis of proteins”, *Chin. J. Chrom.* **2013**, *31*, 927-933. c) Guo, M. et al. “Identification of the over alkylation sites of a protein by IAM in MALDI-TOF/TOF tandem mass spectrometry”, *RSC Adv.* **2015**, *5*, 103662-103668).

To address the issue of reduction/alkylation reproducibility, the present collection of peptides (CAMCheck Kit) was developed. It enables the *in-situ* determination of disulfide reduction and cysteine alkylation efficiency and reproducibility, including

potential overalkylation. The kit is compatible with routine sample digestion workflows and analysis can be performed using standard LC-MS protocols.

The principle of the CAMCheck Kit is shown in Scheme 1. The kit, which contains a mixture of equimolar amounts of peptides **1-1**, **2** and **3**, is spiked into the protein sample which is to be subjected to the proteomics workflow. Then the standard workflow will be performed. For readout the ratio of peptide **1-2** to **2** as well as the ratio of peptide **1-3** to **3** will be determined by mass spectrometry. The following deductions can be drawn:

- The ratio of the amount of peptide **1-2** to peptide **2** directly gives 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 designed to be 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.
- By determining the ratio of peptide **1-3** to peptide **3** the percentage of overalkylation can be determined. Please note that the absolute number obtained for percentage of overalkylation is true only for low percentages (e.g. 10-20%). When strong alkylation conditions lead to higher percentages of overalkylation (e.g. >20%), then the absolute number determined underestimates the real percentage. This is due to the fact that strong alkylation conditions can also lead to overalkylation of peptide **2** (to form peptide **3**).

SC-----AQTF-YYC-----K	Peptide 1-1
SC(CAM)AQTF*YYC(CAM)K	Peptide 2
(CAM)-SC(CAM)AQTF*YYC(CAM)K	Peptide 3

1) Reduction
2) Alkylation

SC(CAM)AQTF-YYC(CAM)K	Peptide 1-2	% Red+Alk
SC(CAM)AQTF*YYC(CAM)K	Peptide 2	
(CAM)-SC(CAM)AQTF-YYC(CAM)K	Peptide 1-3	% Red +Overalk
(CAM)-SC(CAM)AQTF*YYC(CAM)K	Peptide 3	

Scheme 1: Principle of the Kit. CAM = Carbamidomethyl.

<u>Experiment: Determination of Cysteine Alkylation Efficiency</u>

The kit was subjected to reduction and alkylation with iodoacetamide (IAA) under varying conditions.

Figure 2 shows that the peptide set is well suited to determine differences in reduction and alkylation conditions. While under standard conditions (5 mM TCEP, 10 nM IAA, entry 1) the reduction and alkylation efficacy was measured to be approximately 95 %, too small amounts of IAA led to incomplete conversion (entries 2-6). This also happened when TCEP was omitted (bar 8). Stronger alkylation conditions (bars 9-12) drove the alkylation efficacy to 100 %.

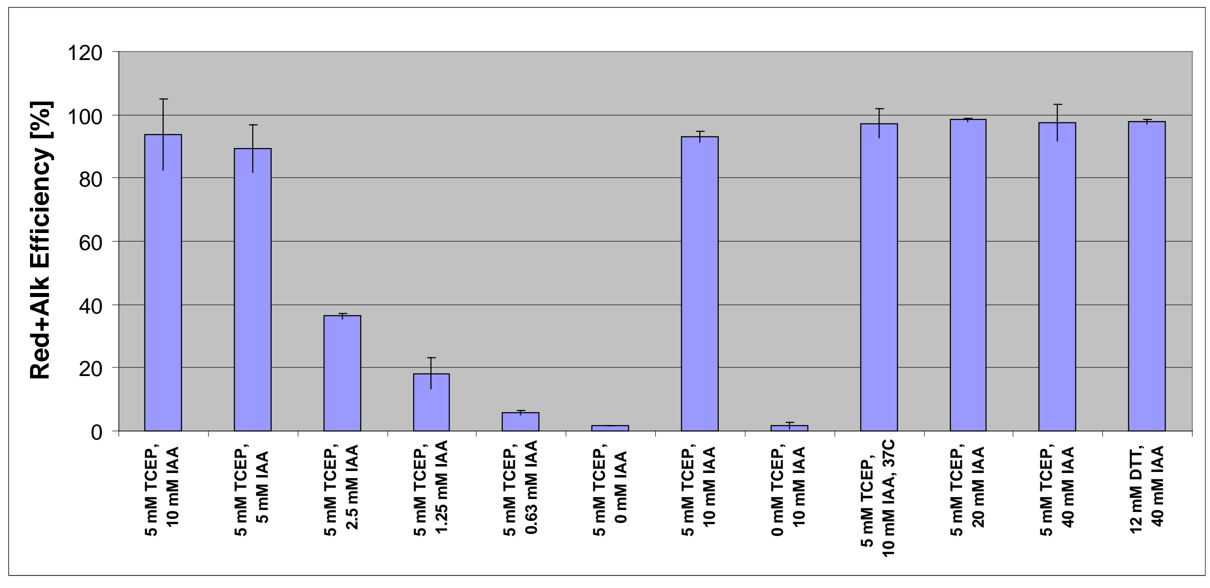

Figure 2: Reduction and alkylation efficacy (percent conversion) of the kit peptide under varying conditions. The average of two independent experiments is shown.

Experiment: Determination of Overalkylation

Figure 3 shows the degree of (undesired) overalkylation under the same conditions as above. Although in most cases overalkylation occured only to a small extent (entries 1-6), forcing conditions (entries 9-12) can drive overalkylation to a remarkable level above 20 %.

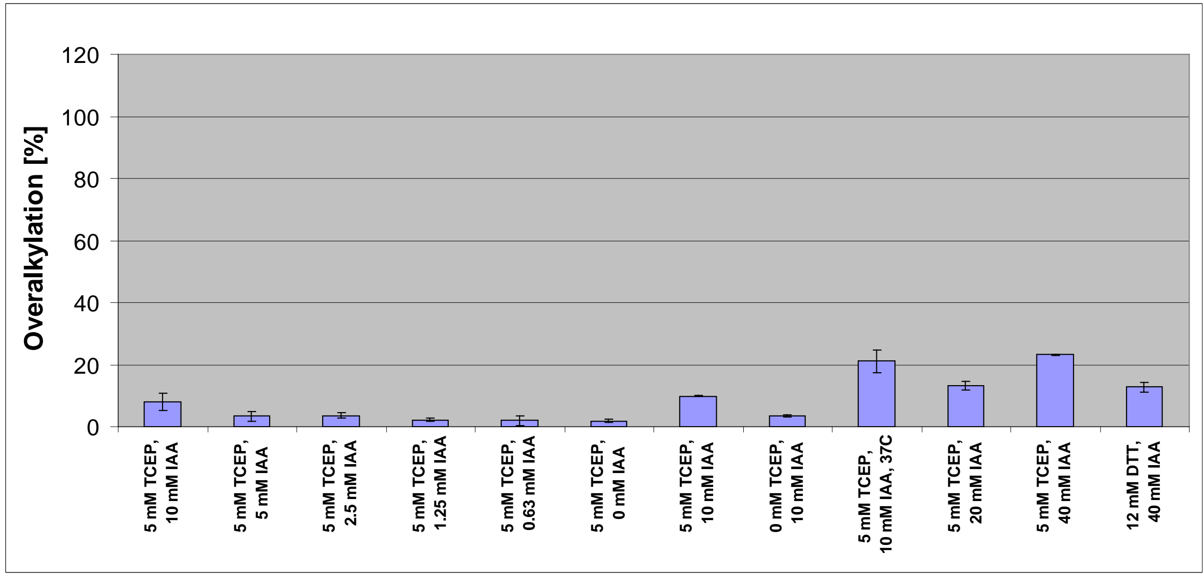

Figure 3: Percent overalkylation of the kit peptide under varying conditions. The average of two independent experiments is shown.

Summary

- The kit enables the *in-situ* determination of disulfide reduction and cysteine alkylation efficiency and reproducibility, including potential overalkylation.
- The kit is compatible with routine sample digestion workflows and analysis can be performed using standard LC-MS protocols.
- Kit peptides do not occur in native proteins (to enable application of the kit in native protein digests).

2 List of Components

Polypropylene vial with a mixture of the following compounds:

#	Sequence	Formula	Monoiso. Mass [g/mol]	Monoiso. Mass [M+2H]2+ [g/mol]	Amount [pmol]
A	cyclo-(SCAQTFYYCK)	$C_{54}H_{74}N_{12}O_{16}S_2$	1210.48	606.25	10/1000*
B	SC(CAM)AQTF*YYC(CAM)K	$C_{49}{}^{13}C_9H_{82}N_{13}{}^{15}NO_{18}S_2$	1336.56	669.29	10/1000*
C	(CAM)-SC(CAM)AQTF*YYC(CAM)K	$C_{51}{}^{13}C_9H_{85}N_{14}{}^{15}NO_{19}S_2$	1393.59	697.80	10/1000*

* Depending on kit. CAM = Carbamidomethyl, F* = Phe (U-^{13}C9,^{15}N)

3 Storage

- The CAMCheck Kit should be stored at -20°C.

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING YOUR EXPERIMENTS!

PLEASE CONTACT JPT PEPTIDE TECHNOLOGIES´ TECHNICAL SERVICES FOR ASSISTANCE IF NECESSARY.

4 Experimental Protocol

1. Solubilize the kit in a solution of 0.1 M ammonium bicarbonate and 0-20% acetonitrile (at least 50 µL). Please note that the kit contains 1000 pmol or 10 pmol of each peptide, so dilution of this solution is likely to be necessary.
2. Add an aliquot of the resulting solution (e.g. 1-10 µL depending on the sensitivity of the mass spectrometer) to a sample (the sample for which the reduction and alkylation efficiency is to be monitored).

Perform a standard proteomic workflow, i.a. steps 3-7

3. Add TCEP to a final concentration of 5 mM or DTT to a final concentration of 10 mM in order to reduce all cysteine residues in your protein-containing sample. Incubate sample for 40 minutes at 50°C on a Thermoshaker.
4. Alkylate all Cys-residues by adding iodoacetamide resulting in a final concentration of 10 mM or CAA to a final concentration of 50 mM. Incubate sample for 30 minutes at 25°C in the dark.
5. Add activated trypsin (e.g. to produce an enzyme/substrate ratio of 1:50) and incubate (e.g. at RT for 16 hours).
6. To stop the enzymatic reaction, acidify the sample, e.g. by addition of formic acid or 2 M HCl to a final concentration of 50 mM or TFA to a final concentration of 2 % (the final pH of the solution should be <2).
7. Analyze sample by LC-MS.

5 Readout

Determination of Alkylation Efficiency

Fill out the blank fields in the following table.

Peptide **1-2** Resulting from Alkylation of Kit Peptide **1-1**			Peptide **2**			Calculate Alkylation Efficacy [%]
Sequence	Monoisot. Mass [M+2H]2+ [g/mol]	Area EIC ***(Column A)*** *fill out*	Sequence	Monoisot. Mass [M+2H]2+ [g/mol]	Area EIC ***(Column B)*** *fill out*	***Ratio Column A/B*** *calculate*
SC(IAM)AQTF-YYC(IAM)K	664.28		**SC(IAM)AQTF*-YYC(IAM)K**	669.29		

Determination of Overalkylation

Fill out the blank fields in the following table.

Peptide **1-3** Resulting from Overalkylation of Peptide **1-1**			Peptide **3**			Calculate Alkylation Efficacy [%]
Sequence	Monoisot. Mass [M+2H]2+ [g/mol]	Area EIC ***(Column A)*** *fill out*	Sequence	Monoisot. Mass [M+2H]2+ [g/mol]	Area EIC ***(Column B)*** *fill out*	***Ratio Column A/B*** *calculate*
(IAM)-SC(IAM)AQTF-YYC(IAM)K	692.79		**(IAM)-SC(IAM)AQTF*-YYC(IAM)K**	697.80		

6 Appendix

The following abbreviations were used:

CAA = Chloroacetamide

DTT = Dithiothreitol

IAA = Iodoacetamide

RT = Room temperature

TCEP = Tris(2-carboxyethyl)phosphine