Protocol

SpikeTides^{TM} Set Metabolic Enzymes – heavy – quantified

Collection of stable isotope labeled proteotypic peptides for absolute quantification of 24 enzymes of the human central energy metabolism.

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1 Introduction

Metabolic transformation is a major event during cancer development. Specifically the de-regulation of the central energy metabolism, a phenomenon already observed a century ago, contributes to the cancer phenotype. Alterations in the central energy metabolism are thought to induce metabolic vulnerabilities and thus are of therapeutic interest.

One highly efficient method for the multiplexed detection and quantification of proteins like metabolic enzymes is targeted proteomics by mass spectrometry. The method makes use of one or more proteotypic peptide(s) from protein(s) of interest that are generated by tryptic digestion of the protein(s) and monitored by a selected reaction monitoring (SRM) or by a multiple reaction monitoring (MRM) assay.
Relative and absolute quantification by targeted proteomics requires stable isotope-labeled proteotypic peptides as internal standards. For absolute quantification these standards need to be absolutely quantified. The traditional way to prepare the quantified standards is the purification of the peptide to a very high level enabling subsequent amino acid analysis or alternative peptide quantification methods (LavaPep, Ninhydrin, Lowry) for peptide content determination. The drawbacks of these methods are low throughput and high costs for a) consumables for peptide synthesis (especially for peptides with incorporated heavy amino acids), and b) for efforts to purify and quantify peptides for absolute protein quantification.

JPT overcomes the traditional laborious and expensive purification and peptide content determination by attachment of a proprietary small chemical tag to the proteotypic peptide (proteotypic peptide + chemical tag, Figure 1).
Subsequent to the addition of a defined amount of tagged SpikeTide to the sample of interest, the digesting protease will release the desired proteolytic fragment from the SpikeTides in a defined 1:1 ratio, thus enabling the exact absolute quantification of the peptide. Alternatively to adding the SpikeTide to the sample of interest that is to be digested, the SpikeTide_TQL can also be proteolytically cleaved before addition to the sample.

The chemical tag is designed to be rapidly cleaved by trypsin that is commonly used for protein digestion. The concept of SpikeTides_TQL has been summarized (1) and was applied in a number of successful studies (2).

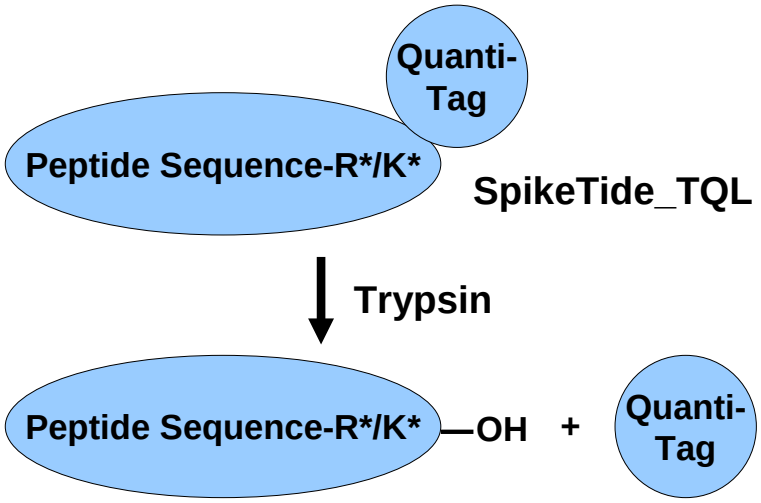

Figure 1: Release of a proteotypic peptide from the SpikeTide_TQL by proteolysis. Because the proteotypic peptide contains a Lys or Arg residue at the C-terminus, trypsin is used for processing the tagged SpikeTide_TQL.

Due to the high promise of metabolic enzymes for the stratification of cancer, the concept of SpikeTides_TQL was used for the development of a kit of absolutely quantified peptides for multiplexed absolute quantification of these enzymes.
The proteotypic peptides of 24 major metabolic enzymes were selected based on experimental data and bioinformatics predictions. The peptides were synthesized and absolutely quantified with the help of the SpikeTides_TQL concept. For all peptides highly reproducible MRM-based assays were set up and the LOD and the LOQ were determined.

In summary, this is a collection of a large number of absolutely quantified stable isotope labeled peptides. The efficient assembly of the kit was made possible by the high efficiency of the peptide quantification approach. The kit enables the multiplexed absolute quantification of 24 central metabolic enzymes from complex protein mixtures in a concentration range spanning several orders of magnitudes in biological fluids and tissues to support diagnosis and stratification of cancer and other applications.

2 List of Components

Component	Quantity	Format
Micronics rack	51 SpikeTides_TQL labeled with stable isotopes (C-terminal Arg U-$^{13}C_6$;U-$^{15}N_4$ or Lys U-$^{13}C_6$;U-$^{15}N_2$). Tryptic digestion releases respective proteotypic peptides. **Aliquots: 1 x 1nmol** quantified target peptide	96 well format (Micronics Roborack 1.4mL tubes)
Product Documentation	1	Microsoft Excel File

3 Storage

- All SpikeTides products 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 Additional Materials required

- Protease of adequate enzymatic activity which is able to cleave the peptide–tag peptide bond (like trypsin for peptides with Lys or Arg residues C-terminally of the proteotypic peptide): JPT recommends to use “Promega Sequencing Grade Modified Trypsin”.
- 0.1M Ammonium bicarbonate
- Dithiotreitol
- Iodoacetamide
- Formic acid

5 Experimental part

5.1 Micronics rack layout

The product documentation provided by email or available for download contains all information needed for easy allocation of peptide sequence to the Micronics rack tube number. The numbering starts with well A1 in the upper left corner, counting the first 12 peptides up to well A12. Peptide 13 is deposited in well B1 and so on. All other columns and rows are filled likewise.

The Micronics rack tubes are delivered with a yellow lid, keeping environmental air and humidity out of the tubes.

5.2 Experimental protocol

The tagged Spiketide peptides cannot be used as Spike-in controls for your assay solution without prior digestion to release the tryptic peptide.

1. Solubilize the peptides in a solution consisting of 80% of 0.1M ammonium bicarbonate and 20% acetonitrile.

As the peptides were synthesized with alkylated cysteine residues, steps 2 and 3 can be skipped

2. Add DTT to a final concentration of 12 mM in order to reduce all cysteine residues in your protein-containing sample. Incubate sample for 30 minutes at 32 °C.
3. Alkylate all Cys residues by adding iodoacetamide resulting in a final concentration of 40 mM. Incubate sample for 30 minutes at 25 °C in the dark. Dilute your solution by a factor of 3-4 with 0.1 M ammonium bicarbonate.
4. Add the tagged SpikeTides to your sample followed by the addition of protease for generation of proteotypic peptides. JPT recommends to use a weight-oriented dilution of 1/100-1/15 enzyme/substrate followed by an incubation of the sample for 16 h at 30°C (shaken) and rotation wheel for 4h at 30 °C.
5. Add formic acid to a final pH value of 3 to stop the enzymatic reaction.
6. Dry down the sample and resolubilize in 0.1 % formic acid (make sure that the pH value is acidic!).

	If protease concentration is too high, the enzyme might start cleaving amino acid bonds not typical for its proteolytic activity. Make sure to keep the enzyme concentration in the recommended range to get optimal results.

6 References

(1) Schnatbaum, K. et al. non-peer-reviewed application note in *Nature Methods* **2011**, 8.

(2) (a) Martínez-Morillo, E. et al. *J. Proteome. Res.* **2012**, *11*, 3880−3887. (b) Simicevic, J. et al. *Nat. Methods* **2013**, *10*, 570-576. (c) Kim J. S. et al. *J. Proteome. Res.* **2013**, *12*, 2582−2596. (d) Martínez-Aguilar, J. et al. *J. Proteome. Res.* **2013**, *12*, 3679−3688. (e) Saito, M. A. et al. *Science* **2014**, *345*, 1173-1177.