Protocol

SpikeTides™_TQL_PLUS

Peptides for relative and absolute quantification in SRM and MRM assays with additional options for peptide quantification by end-user

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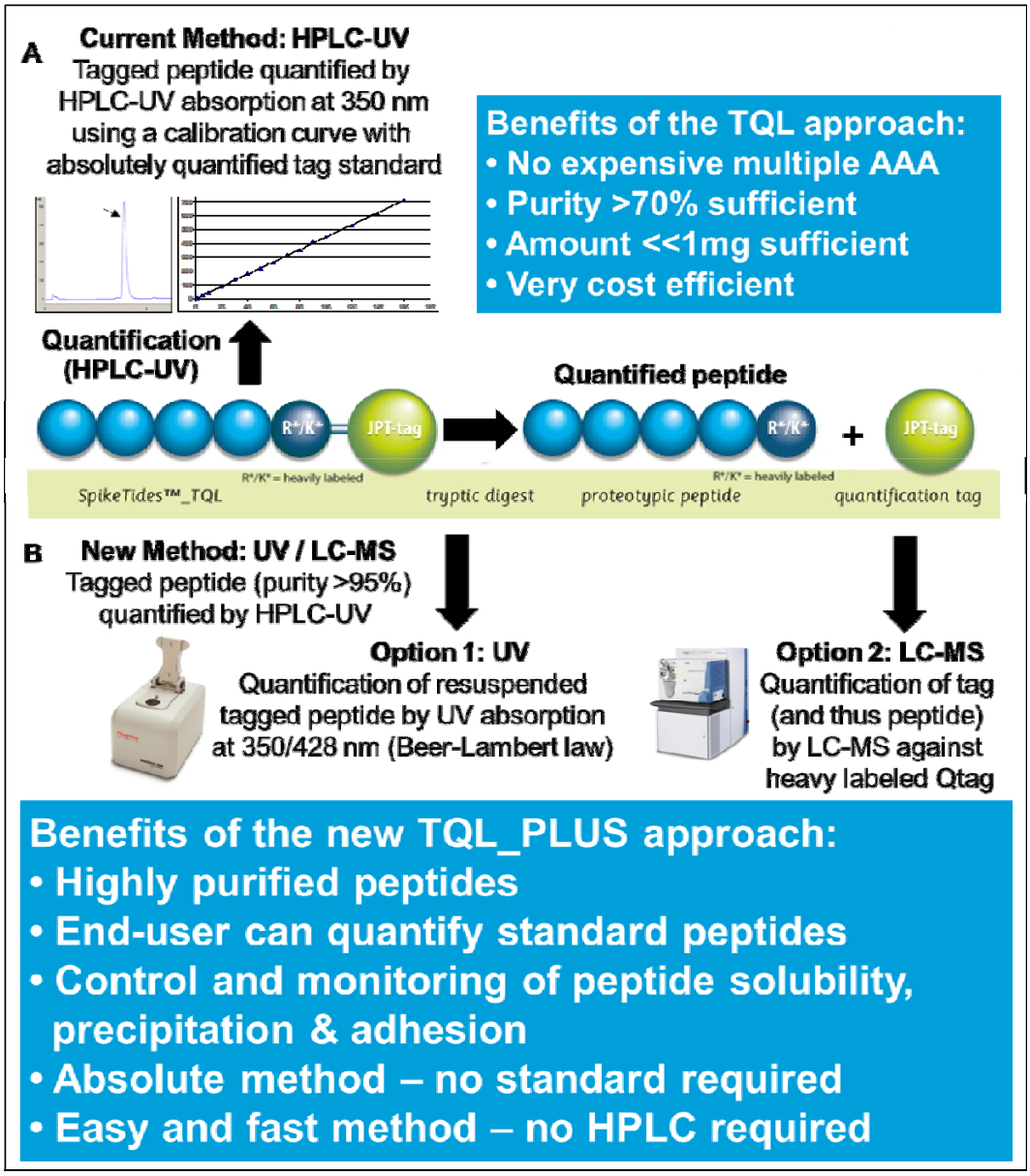
1 Introduction

Absolute protein quantification by targeted proteomics is dependent on the availability of stable isotope labeled (SIL) peptide standards. Traditionally, these standards have to be purified to high level enabling subsequent amino acid analysis or alternative peptide quantification methods 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.

To overcome this situation, JPT recently developed a new method for absolute peptide quantification (SpikeTide™_TQL concept).[1-2] The method (outlined in Scheme 1A) relies on peptide quantification by HPLC-UV using a quantification tag (Qtag) with a specific UV absorption. Upon addition of trypsin, the peptide - Qtag bond is cleaved, thus furnishing the desired absolutely quantified proteotypic peptide. SpikeTides_TQL peptides are purified to a level of >70 %. A higher purity is not required, because during the quantification by HPLC-UV possible contaminants are separated and only the target peptide is absolutely quantified.

SpikeTides_TQL peptides are labeled with stable isotopes (usually C-terminal Arg U-$^{13}C_6$;U-$^{15}N_4$ or Lys U-$^{13}C_6$;U-$^{15}N_2$) and typically provided as aliquots of 5 x 1 nmol of quantified target peptide. To minimize known peptide stability issues in solution, the quantified peptide is provided as a dry powder.



Scheme 1: A) Concept of SpikeTide™_TQL quantification. B) Concept of the new SpikeTide™_TQL_PLUS quantification.

SpikeTide™_TQL_PLUS (Scheme 1B) is a newly developed extension of the SpikeTide™_TQL approach. SpikeTide™_TQL_PLUS peptides are specifically designed to enable direct quantification of reconstituted UV-tagged peptides by the end-user. The resulting accurate quantification of peptide solutions allows to control and monitor known problems related to peptide solubility, precipitation and adhesion to vials.

Scheme 1B shows the concept of the new approach. SpikeTide_TQL_PLUS peptides are purified to a high level (purity >95%) and aliquoted to provide precise absolute amounts of peptide as dry powder. After reconstitution, the peptides can be re-quantified by

• **Option 1: Quantification by UV measurement** at specific wavelength using a NanoDrop Spectrophotometer. The peptide quantity is calculated based on the Beer-Lambert law. This is done using the specific UV absorption of the Qtag at 350 nm (acidic solutions) or 428 nm (basic solutions) – wavelengths at which the proteotypic peptide does not absorb light.

and/or

• **Option 2: Quantification by LC-MS measurement** after tryptic cleavage of the Qtag. An aliquot of the reconstituted SpikeTide™_TQL_PLUS peptide is added to a precisely quantified amount of heavy-labeled Qtag (in the following termed "Qtag*"). After tryptic digestion, the ratio of Qtag to Qtag* is determined, thus furnishing the concentration of the Qtag - and thus the proteotypic peptide.

It is important to keep in mind that both quantification approaches (Option 1 and Option 2) require highly pure peptides, because (unlike SpikeTide™_TQL peptides) contaminants containing the Qtag would compromise the quantification of the target peptide. This is the reason why SpikeTide™_TQL_PLUS peptides are always provided in purities of >95 %.

To show the general applicability and accuracy of the method, several experiments were performed, the results of which are depicted in the following. First, different SpikeTide™_TQL_PLUS peptides were prepared and absolutely quantified by the standard methodology used for SpikeTide™_TQL peptides (HPLC-UV *vs.* an absolutely quantified reference standard). Aliquots of 8-10 nmol of dry powder were prepared. Second, several different quantification methods were performed.

Option 1: Quantification by UV

SpikeTide™_TQL_PLUS peptides were resuspended in different solvent systems that are frequently used for peptide reconstitution (Figure 1, solvent A, B or C). Absolute quantification was performed by measuring the UV absorption at specific wavelengths in a standard UV Spectrophotometer (NanoDrop™ 2000). Figure 1 shows the obtained quantification results from two independent experiments compared to the respective quantities obtained by amino acid analysis (AAA).

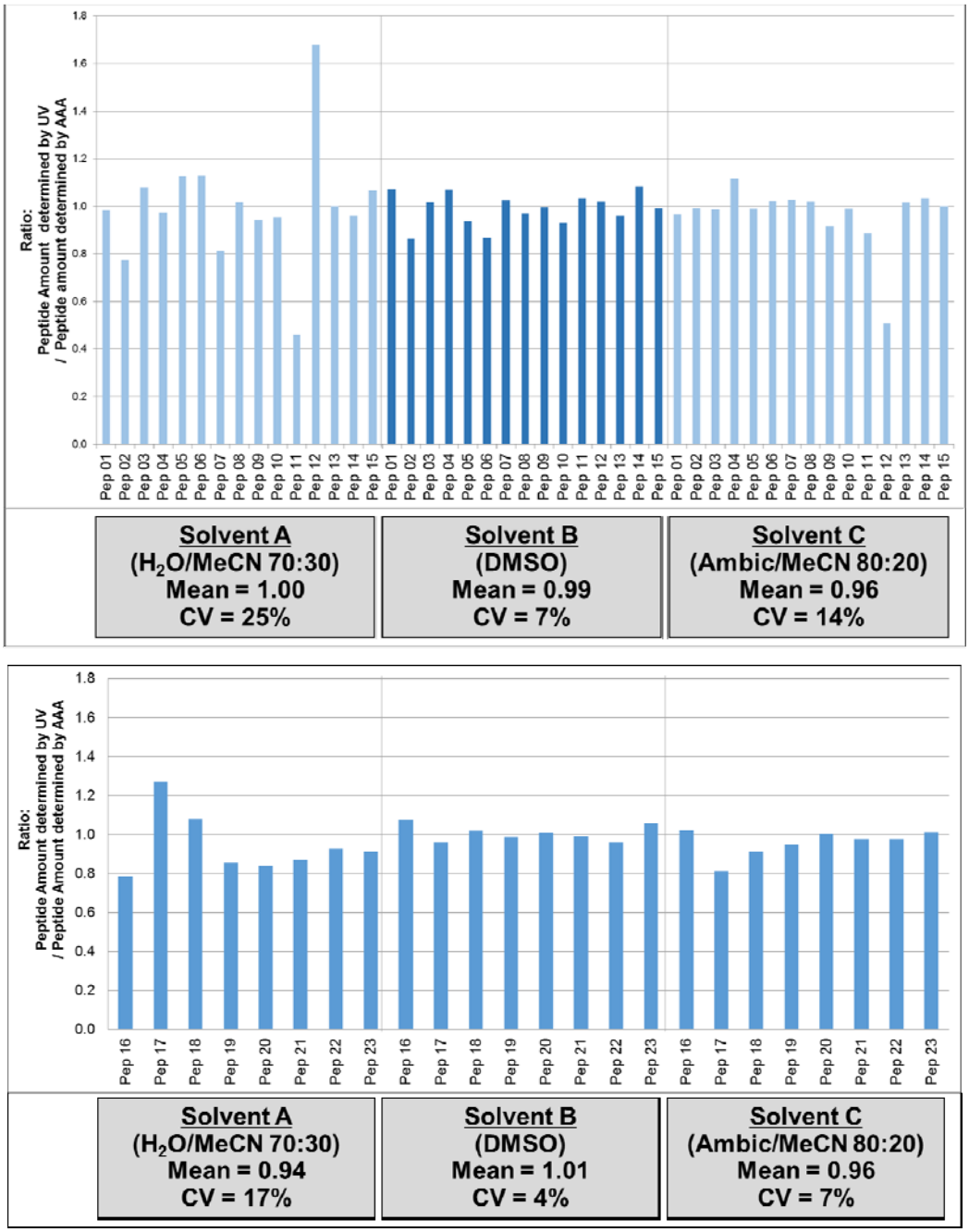


Figure 1: Comparison of quantification results by UV and AAA (2 datasets).

The data show that quantification by specific UV absorption yielded high concordance with quantities obtained by AAA. Depending on the solvent system used, mean values differed by only 0-6%, while the coefficient of variation (CV) ranged from 17-25% (H_2O/MeCN 70:30) to only 4-7% (DMSO). These low values substantiate UV absorption as an efficient means for quantification of UV-tagged SIL peptide standards.

Option 2: Quantification by LC-MS

SpikeTide™_TQL_PLUS peptides were absolutely quantified by the following procedure: a) Resuspension in solvent system A, B or C; b) Addition to a known amount of Qtag*; c) Tryptic digestion; d) Quantification of the obtained Qtag *vs.* the Qtag* reference standard by LC-MS. The results (Figure 2) show a high consistency between the quantification results by the new method and quantities obtained by HPLC-UV and by AAA, respectively.

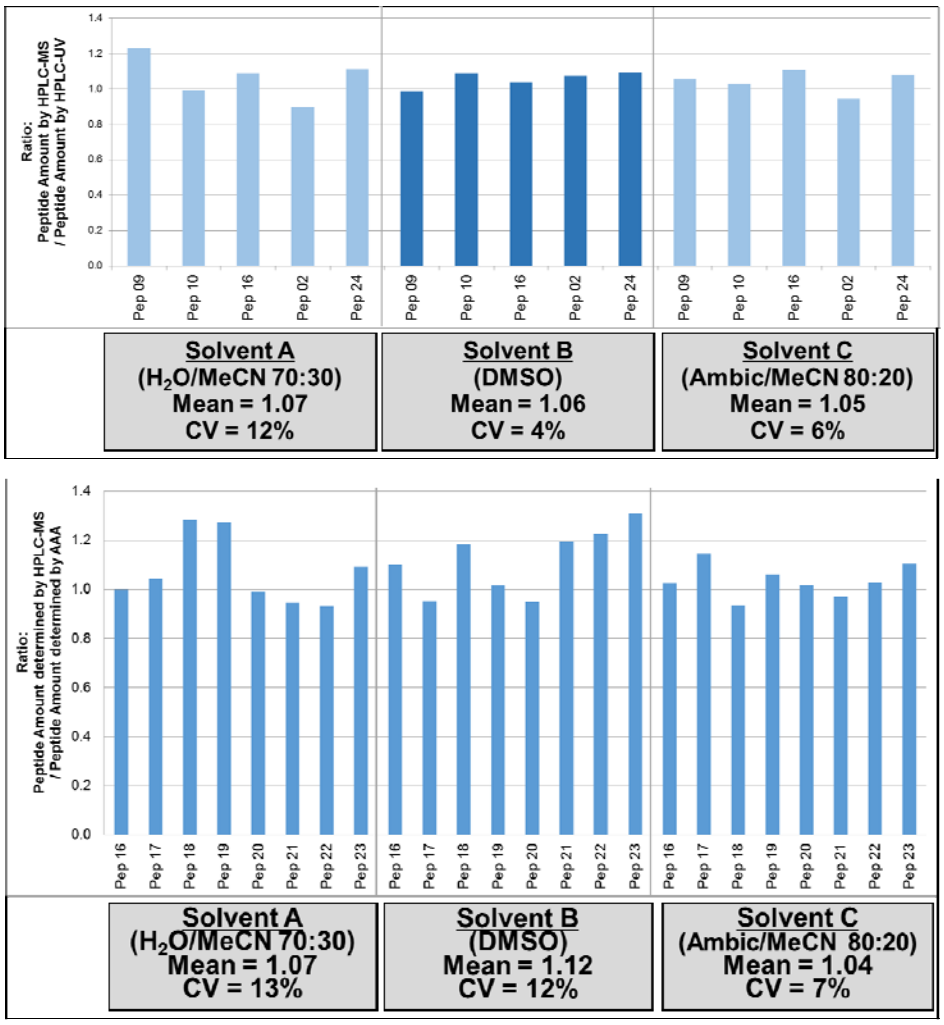


Figure 2: Results of MS based quantification of SpikeTide™_TQL_PLUS peptides.

In summary, a new and easy approach for end-user driven quantification of SIL peptide solutions in targeted proteomics was developed. The extended method provides a number of benefits:

- Highly purified peptides
- End-user driven quantification of peptide solutions
- Control and monitoring of peptide solubility, precipitation & adhesion
- Absolute method - no standard required
- Easy and fast method - no HPLC required

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

(2) Recent applications: e.g. (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. (f) Martínez-Aguilar, J. et al. *BMC Cancer* ***2015***, *15*, 1-14. (g) Korbakis, D. et al. *Mol. Cell Proteomics* **2015**, *14*, 1517-1526. (h) Saito, M. A. et al. *Proteomics* **2015**, *15*, 3521-3531.

2 List of Components

Component	Quantity	Format
CD-ROM	1	Microsoft Excel file with peptide list and quantification calculator tool

Product	Component	Quantity	Format
SpikeTide™_TQL_PLUS	Micronics rack	Depends on number of peptides and required rack layout **Aliquots: 5 x 10 nmol** per peptide, other aliquotation formats upon request	96 well format (Micronics Roborack 1.4mL tubes)

Heavy labeled Qtag for quantification by LC-MS:

Reference Standard (Qtag*) for quanti-fication by LC-MS$	Vial	**1 x 1 nmol** heavy labeled Qtag (Qtag*)	Polypropylene Vial (Sarstedt, 2mL PP, 72.664.711)

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 to cleave the peptide-tag peptide bond. JPT recommends to use Promega Sequencing Grade Modified Trypsin
- Solvents systems: i.a. 0.1M Ammonium bicarbonate, Acetonitrile, DMSO, water
- Dithiotreitol
- Iodoacetamide
- Formic acid

5 Experimental Protocols

5.1 Quantification by UV (NanoDrop)

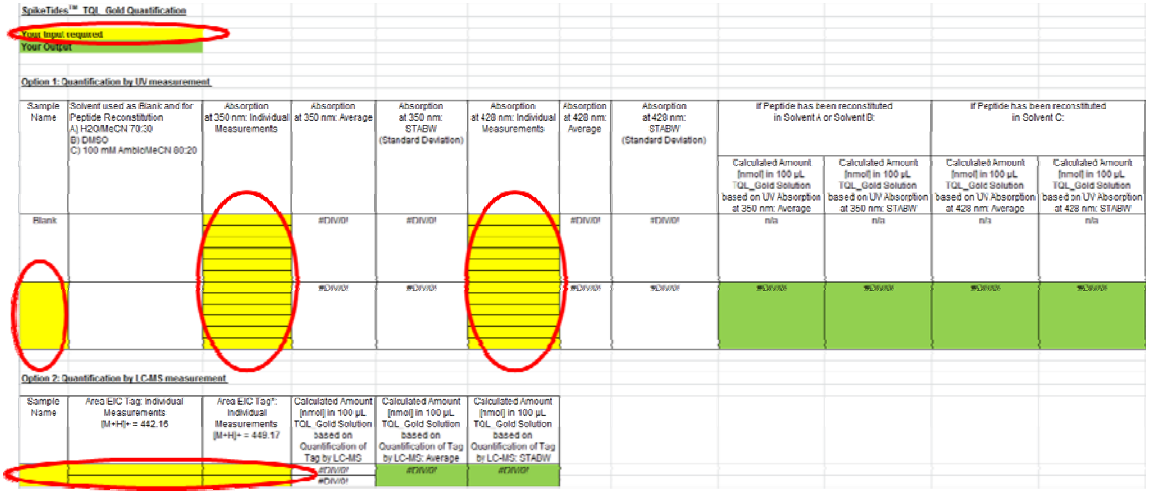
⚠	Please note that the following quantification procedure can only be done with SpikeTide™_TQL_PLUS peptides ! Do not perform this protocol with SpikeTide™_TQL peptides, as this can lead to serious mis-quantifications !

1. Solubilize the SpikeTide™_TQL_PLUS peptide that has been provided as a 10 nmol aliquot in **100 µL** of your preferred solvent:
   a) H_2O/MeCN 70:30 (70% water, 30% acetonitrile)
   or
   b) DMSO
   or
   c) Ambic/MeCN 80:20 (80% of 0.1M ammonium bicarbonate, 20% acetonitrile).
2. **Vortex** for **at least 1 minute** or use ultrasound for dissolution.
3. Start the **NanoDrop** software and chose the “UV-VIS” option. Chose the sub-menue “Measure UV-Vis” and use settings “Baseline correction -> No” and “Auto-Pathlength -> No”. Add the wavelengths **350 and 428 nm** to the table of measurement wavelengths.
4. Perform repeated **UV measurements** according to the NanoDrop user manual using 2.0 µL of solvent for each measurement.
   a) Add **blank solvent** (the same solvent system that the peptide was reconstituted in) to the tip of the properly cleaned NanoDrop. **Press “Blank”**. When finished, clean the tip of the NanoDrop with a small piece of wiping paper.
   b) To determine the absorption at baseline, add **blank solvent**, **measure** the UV absorption by pressing the icon “Measure”, and clean the tip of the NanoDrop. Repeat this cycle of solvent addition, measurement and cleaning **at least three times**. **Do not omit this step b) as this adds additional precision to your measurement!**

c) If necessary, repeat steps a) and b) until the obtained mean UV absorption of the blank solution (average of at least 3 measurements) is smaller than 0.005.

d) Add the **peptide solution** to the tip of the NanoDrop. **Measure** the UV absorption by pressing the icon “Measure” and clean the tip of the NanoDrop. Repeat this cycle of solvent addition, measurement and cleaning **at least three times**. If the obtained signal appears not sufficiently stable then do additional measurements.

5. Export the measured UV absorption values according to the NanoDrop user guide by selecting “Reports” on the left side of the screen and pressing the icon “Export”.
6. For data analysis, copy the relevant data into the table in the provided Excel sheet to calculate the concentration of the SpikeTide™_TQL_PLUS peptide (yellow boxes in the following picture).



SpikeTides™ TQL Gold Quantification

Your Input required

Your Output

Option 1: Quantification by UV measurement

Sample Name	Solvent used as Blank and for Peptide Reconstitution A) H2O/MeCN 70:30 B) DMSO C) 100 mM AmbicMeCN 80:20	Absorption at 350 nm: Individual Measurements	Absorption at 350 nm: Average	Absorption at 350 nm: STABW (Standard Deviation)	Absorption at 428 nm: Individual Measurements	Absorption at 428 nm: Average	Absorption at 428 nm: STABW (Standard Deviation)	If Peptide has been reconstituted in Solvent A or Solvent B:		If Peptide has been reconstituted in Solvent C:	
								Calculated Amount [nmol] in 100 µL TQL_Gold Solution based on UV Absorption at 350 nm: Average	Calculated Amount [nmol] in 100 µL TQL_Gold Solution based on UV Absorption at 350 nm: STABW	Calculated Amount [nmol] in 100 µL TQL_Gold Solution based on UV Absorption at 428 nm: Average	Calculated Amount [nmol] in 100 µL TQL_Gold Solution based on UV Absorption at 428 nm: STABW
Blank			#DIV/0!	#DIV/0!		#DIV/0!	#DIV/0!	n/a	n/a	n/a	n/a
			#DIV/0!	#DIV/0!		#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!

Option 2: Quantification by LC-MS measurement

Sample Name	Area EIC Tag: Individual Measurements [M+H]+ = 442.16	Area EIC Tag*: Individual Measurements [M+H]+ = 449.17	Calculated Amount [nmol] in 100 µL TQL_Gold Solution based on Quantification of Tag by LC-MS	Calculated Amount [nmol] in 100 µL TQL_Gold Solution based on Quantification of Tag by LC-MS: Average	Calculated Amount [nmol] in 100 µL TQL_Gold Solution based on Quantification of Tag by LC-MS: STABW
			#DIV/0!	#DIV/0!	#DIV/0!

Proceed with the standard proteomics workflow shown below. In principle, SpikeTides™_TQL_PLUS peptides are also compatible with other/similar digestion protocols, however, care should be taken as to use harsh enough digestion conditions. If the peptides were ordered with alkylated cysteine residues, steps 7 and 8 can be skipped.

7. Add DTT to a final concentration of 12 mM or TCEP to a final concentration of 5 mM in order to reduce all cysteine residues. Incubate sample for 30-60 minutes at 32-37°C.

8. Alkylate all cysteine residues by adding iodoacetamide or chloroacetamide resulting in a final concentration of 40 mM. Incubate sample for 30 minutes at 25°C in the dark. Dilute your solution by factor 3-4 with 0.1M ammonium bicarbonate.
9. Add the SpikeTide™_TQL_PLUS peptide to your sample. Add activated trypsin to obtain an enzyme/substrate ratio of 1:50 to 1:15 and incubate at 20-37 °C for 5-16 hours for the generation of proteotypic peptides. The SpikeTide™_TQL_PLUS peptides will be digested in parallel to your protein sample.
10. Add formic acid to a final pH value of 3 to stop the enzymatic reaction.
11. Dry down the sample and resolubilize in 0.1% formic acid (make sure that the pH value is acidic!)
12. Perform LC-MS analysis.

5.2 Quantification by Mass Spectrometry

⚠	Please note that the following quantification procedure can only be done with SpikeTide™_TQL_PLUS peptides ! Do not perform this protocol with SpikeTide™_TQL peptides, as this can lead to mis-quantifications !

SpikeTides™_TQL_PLUS peptides can in principle be quantified before or after addition to the sample. In the following, the first option is described.

1. Solubilize the SpikeTide™_TQL_PLUS peptide that has been provided as a 10 nmol aliquot in **100 µL** of your preferred solvent:
   a) H_2O/MeCN 70:30 (70% water, 30% acetonitrile)
   or
   b) DMSO
   or
   c) Ambic/MeCN 80:20 (80% of 0.1M ammonium bicarbonate, 20% acetonitrile).
2. **Vortex** for **at least 1 minute** or use ultrasound for dissolution.
3. Add 10.0 µL (1000 pmol) of the solution from step 2 to a vial containing the absolutely quantified **Qtag*** (1000 pmol, such a vial is provided by JPT if ordered via JPT's webshop). Attention! It is important to keep an equimolar ratio of both peptides! Therefore **do not use other amounts than the ones specified here!**
4. For **tryptic digestion** dilute the solution from step 3 with fresh ammonium bicarbonate (100 µL, 100 mM, prepared by dissolving 79.1 mg in 10 mL water). Add **activated trypsin** to obtain an enzyme/substrate ratio of 1:15 to 1:10. Shake at 37 °C for 16 hours. The SpikeTide™_TQL_PLUS peptides will be digested in parallel to your protein sample.
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!)
7. Perform LC-MS analysis (at least duplicate measurement of each sample) to obtain the EIC area values for the light tag ("Tag", [M+H]+ = 442.16) and the heavy labeled tag ("Tag*", [M+H]+ = 449.17).

8. For data analysis, fill in the table in the provided Excel sheet to calculate the concentration of the SpikeTide™_TQL_PLUS peptide.

Proceed with the standard proteomics workflow shown below. In principle, SpikeTides™_TQL_PLUS peptides are also compatible with other/similar digestion protocols, however, care should be taken as to use harsh enough digestion conditions.

9. Add the SpikeTide™_TQL_PLUS peptide to your sample. Add activated trypsin to obtain an enzyme/substrate ratio of 1:50 to 1:15 and incubate at 20-37 °C for 5-16 hours for the generation of proteotypic peptides. The SpikeTide™_TQL_PLUS peptides will be digested in parallel to your protein sample.
10. Add formic acid to a final pH value of 3 to stop the enzymatic reaction.
11. Dry down the sample and resolubilize in 0.1% formic acid (make sure that the pH value is acidic!)
12. Perform LC-MS analysis.