ATLAS ANTIBODIES QPrEST

QPrEST standards and endogenous proteins show similar digestion efficiency

Efficiency of protein digestion

One major source of variation in mass spectrometry (MS)-based proteomics experiments is incomplete protein digestion. This step is especially important in quantitative proteomics, where different types of internal standards are used as calibrants for determination of absolute protein concentrations[1]. A complete digestion resulting in a mixture of fully cleaved peptides is rarely possible, if not impossible, and fully cleaved peptides represent just a portion of the total number of peptides observed in a digested protein sample. The result is instead a mixture of peptides, with molar concentrations that in many cases do not represent the concentration of the endogenous protein from which they were generated. This variation can be decreased by using longer standards, added before trypsin digestion, that also need to be properly cleaved in order to release the target peptide(s). Apart from the digestion conditions, protelolytic cleavage is highly affected by the amino acid sequence surrounding the digestion site. Acidic residues close to the digestion site have been shown to suppress digestion and multiple K/R residues in close proximity can also alter the digestion efficiency. Hence, it is important to have a standard not only generating identical peptides as the endogenous protein, but the standard should also preferably contain a surrounding sequence identical to the target protein.

QPrEST multipeptide standards

QPrEST standards are heavy isotope-labeled multipeptide MS standards recombinantly produced in a bacterial host[2-4]. Each standard contains a stretch of 50-150 amino acids identical to a human protein sequence, including at least two unique tryptic peptides. In order to generate the tryptic peptides used for quantification, the QPrEST standards are added to the sample of interest prior to digestion. As a result, quantitative errors due to variation in digestion efficiency between standard and endogenous protein during the cleavage reaction are minimized. Figure 1 shows three examples of QPrEST sequences where selected tryptic peptides are highlighted. The criteria that were used for peptide selection are shown in table 1. All QPrEST standards contain a complete stretch of a human protein sequence, including also the amino acids between the tryptic peptide sequences.

Comparison of digestion kinetics between QPrEST standards and endogenous proteins

Based on RNA sequencing data, protein targets were selected that showed expression in HeLa cells. The selected QPrEST standards were of different lengths and contained different numbers of theoretical tryptic peptides. Samples were prepared in triplicate using a filter-aided sample preparation (FASP) procedure. The QPrEST standards were added to HeLa cell lysates directly after cell lysis and added in such amounts that the generated heavy to light (H/L) ratios were close to one. The digestion was stopped

...EVVSREVSGIK**AAYEAELGDAR**K**TLDSVAK**ERAR**LQLELSK**VREEFKELKARNTKK**EGDLIAAQAR**LK**DLEALLNS**
KEAALSTALSEKR**TLEGELHDLR**GQVAK**LEAALGEAK**K**QLQDEMLR**RVDAENRLQTMK**EELDFQKNIYSEELR**E... LMNA
QPrEST22525

...R**VLAPHLTR**AYAKDVKFGADAR**ALMLQGVDLLADAVAVTMGPK**GR**TVIIEQSWGSPK**VTK**DGVTVAK**SIDLKDKYK
NIGAK**LVQDVANNTNEEAGDGTTTATVLAR**SIAKEGFEKISK**GANPVEIR**RGVMLAVDAVIAEL... HSPD1
QPrEST23025

...AVDTYIPVPARDLEKPFLLPVEAVYSVPGR**GTVVTGTLER**GILKK**GDECELLGHSK**NIR**TVVTGIEMFHK**SLER**AEAG**
DNLGALVRGLKREDLRRGLVMVKPGSI... TUFM
QPrEST24739

Figure 1.
Amino acid sequences for three of the QPrEST standards included in the analysis. The QPrEST sequence is identical to a corresponding human protein sequence and contains multiple tryptic peptides. Selected tryptic peptides are highlighted. All standards have an N-terminal purification and quantification tag not shown in the figure.

Table 1.
Criteria for selection of tryptic peptides that can be used for quantification within QPrEST sequences.

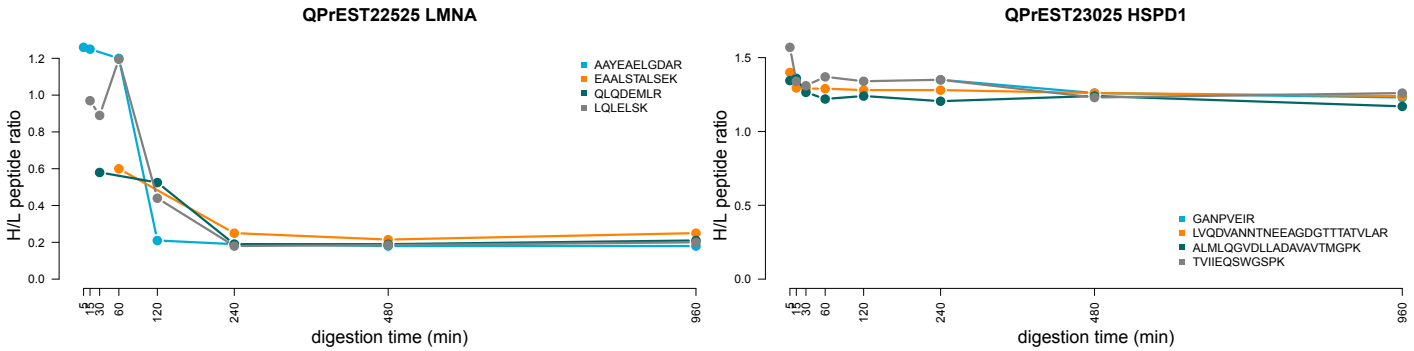
Tryptic peptide criteria
Peptide is between 7 and 35 amino acids long
Peptide ends with a K or R residue
Peptide contains a single K or R residue
Trypsin was assumed not to cleave at KP and RP sites
Peptide matches a single UniProt Swiss-Prot Human Proteome protein

at eight different time points, with digestion times ranging from 5 minutes to 16 hours. All samples were desalted and analyzed on a Bruker Impact II QTOF mass spectrometer using a data-dependent acquisition setup. The following data analysis was performed using the Proteinscape software. Median values for each peptide calculated from the three replicate experiments were used in the analysis. Figure 2 shows H/L ratios for pairs of QPrEST peptides and endogenous peptides from two different protein targets, prelamin A/C (LMNA) and mitochondrial 60 kDa heat shock protein (HSPD1). From this figure it can be seen that similar ratios are obtained for different peptides within the protein sequence, although a slight difference in digestion kinetics between QPrEST peptides and their endogenous counterparts can be noted in the beginning of the digestion time series. In general, peptides derived from the QPrEST standards are created earlier than the unlabeled endogenous peptides, resulting in high H/L ratios after short digestion times. This result can possibly be explained by the fact that the QPrEST standards are smaller and less structured than the corresponding endogenous proteins, which may result in a more efficient cleavage in the beginning of the digestion time series. However, after a certain digestion time an equilibrium has been reached where the ratios are stabilized and can be used to determine an accurate concentration of the target protein. A reasonable explanation for this result is that the QPrEST standards contain a larger sequence identical to the target protein, and not just the corresponding peptide sequences. Coefficients of variation between the different peptides for digestion times 4, 8 and 16 hours were 4.6%, 1.0% and 2.7% for HSPD1. For LMNA the corresponding values were 14.6%, 7.2% and 12.4%. The higher variation between the quantified peptides for this protein is mainly due to one of the peptides (EAALSTALSEK) that showed overall slightly higher ratios compared to the three other peptides. When excluding this peptide the variation dropped to 2.5%, 2.2% and 6.2% for the three digestion times.

Quantitative information from peptides with missed cleavage sites and partly oxidized methionines

Since digestion efficiency will vary depending on many factors such as digestion conditions, enzyme and amino acid sequence surrounding the cleavage site, not only fully cleaved peptides will be generated. Figure 3 shows two examples of peptides from the protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) where both fully cleaved peptides and peptides containing missed cleavage sites could be quantified. Also for these peptides higher H/L ratios are observed early in the digestion time series, but are later stabilized. The resulting quantitative data are very similar for the fully cleaved peptides compared to peptides containing missed cleavage sites. For one peptide (AENGKLVINGNPITIFQER) a slightly lower ratio is observed, which can possibly be explained by its location close to the N-terminal purification and quantification tag.

The enzyme trypsin is known to show low cleavage efficiency for cleavage sites directly followed by a proline residue, wherefore peptides containing an RP or KP motif can sometimes be excluded from the analysis. An example of such a peptide is shown in figure 4. Quantitative data for two peptides from the mitochondrial elongation factor Tu (TUFM) are shown, one of the peptides containing a KP site.



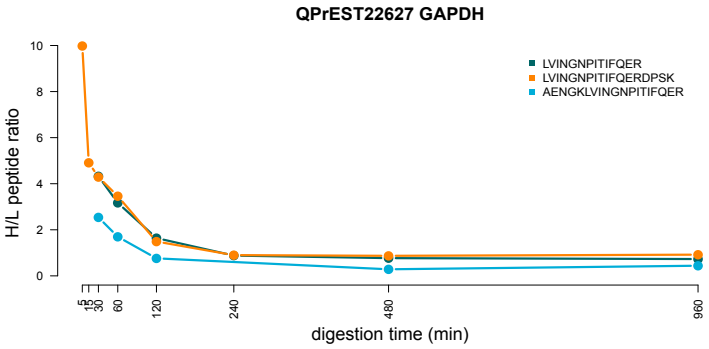
LMNA
QPrEST22525

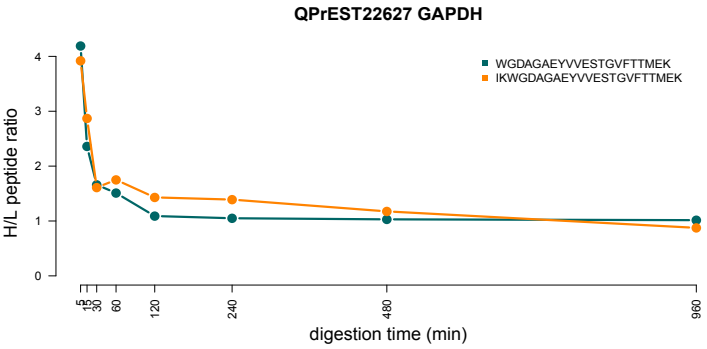
...EVVSREVSGIK**AAYEAELGDAR**KTLDSVAKERAR**LQLELSK**VRE
EFKELKARNTKKEGDLIAAQARLKDLEALLNSK**EAALSTALSEK**R
TLEGELHDLRGQVAKLEAALGEAKK**QLQDEMLR**RVDAENRLQTM
KEELDFQKNIYSEELRE

HSPD1
QPrEST23025

...RVLAPHLTRAYAKDVKFGADAR**ALMLQGVDLLADAVAVTMGPK**
GR**TVIIEQSWGSPK**VTKDGVTVAKSIDLKDKYKNIGAK**LVQDVANN**
TNEEAGDGTTTATVLARSIAKEGFEKISK**GANPVEIR**RGVMLAVDA
VIAEL

Figure 2.
Heavy to light (H/L) ratios for peptides originating from prelamin A/C (LMNA) and mitochondrial 60 kDa heat shock protein (HSPD1) after different digestion times. QPrEST sequences are shown at the bottom and quantified peptides are highlighted.





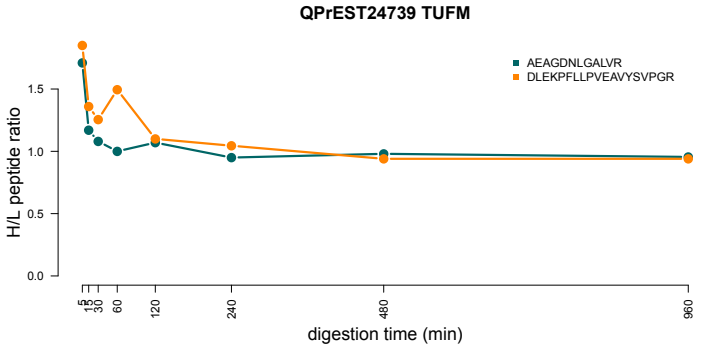
GAPDH
QPrEST22627

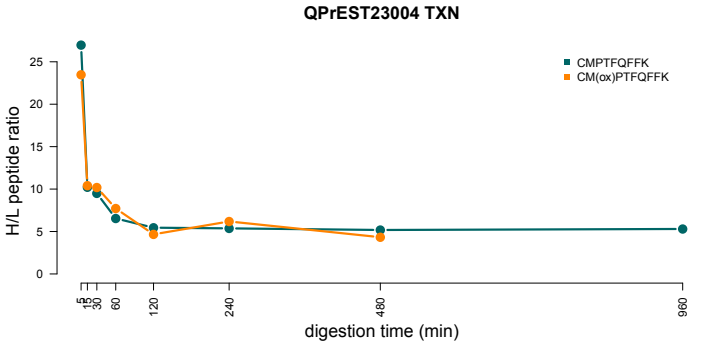
...TVK**AENGK**LVINGNPITIFQER**DPSK**IKWGDAGAEYVVESTGVFT TMEKAGAHLQGGAKRVIIS

GAPDH
QPrEST22627

...TVKAENGKLVINGNPITIFQERDPSK**IK**WGDAGAEYVVESTGVFT **TMEK**AGAHLQGGAKRVIIS

Figure 3.
Heavy to light (H/L) ratios after different digestion times for fully cleaved peptides and corresponding peptides with a missed cleavage site originating from glyceraldehyde-3-phosphate dehydrogenase (GAPDH). QPrEST sequences are shown at the bottom and quantified peptides are highlighted.





TUFM
QPrEST24739

...AVDTYIPVPAR**DLEKPFLLPVEAVYSVPGR**GTVVTGTLERGILKK GDECELLGHSKNIRTVVTGIEMFHKSLER**AEAGDNLGALVR**GLKR EDLRRGLVMVKPGSI

TXN
QPrEST23004

...SLSEKYSNVIFLEVDVDDCQDVASECEVK**CMPTFQFFK**KGQKVG EFSGA

Figure 4.
Heavy to light (H/L) ratios after different digestion times for two peptides originating from the mitochondrial elongation factor Tu (TUFM), one of which contains a KP site. The QPrEST sequence is shown at the bottom and quantified peptides are highlighted.

Figure 5.
Heavy to light (H/L) ratios after different digestion times for a peptide from thioredoxin (TXN) that was quantified both in an unmodified and an oxidized state. The QPrEST sequence is shown at the bottom and the quantified peptides is highlighted.

As for the previous examples, the H/L ratios are stabilized after a certain digestion time and similar quantitative information is obtained for both peptides.

Peptides containing methionines are also often excluded from quantitative analysis due to partial oxidation of this residue during sample preparation. This can result in unreliable quantitative data if there is a variation in the relative amount of oxidation between the endogenous protein and internal standard. Figure 5 shows an example of a peptide from thioredoxin (TXN) that was quantified both in an unmodified and an oxidized state with very similar H/L ratios. Included QPrEST standards are shown in table 2.

Summary

- QPrESTs are heavy isotope-labeled multipeptide standards that contain an amino acid sequence identical to a stretch from a human protein target
- QPrEST standards are added to the sample of interest prior to proteolytic digestion, which results in a similar digestion efficiency between the QPrEST standard and the endogenous protein when letting the digestion reaction reach equilibrium
- Peptides containing missed cleavage sites, KP or RP sites and methionines can also be used for quantification

REFERENCES

1. Villanueva, J., Carrascal, M. and Abian, J. (2014) **Isotope dilution mass spectrometry for absolute quantification in proteomics: Concepts and strategies**. *J Proteomics* 96, 184-199
2. Edfors, F., Bostrom, T., Forsstrom, B., Zeiler, M., Johansson, H., Lundberg, E., Hober, S., et al. (2014) **Immunoproteomics using polyclonal antibodies and stable isotope-labeled affinity-purified recombinant proteins**. *Mol Cell Proteomics* 13, 1611-1624
3. Zeiler, M., Straube, W. L., Lundberg, E., Uhlen, M. and Mann, M. (2012) **A Protein Epitope Signature Tag (PrEST) library allows SILAC-based absolute quantification and multiplexed determination of protein copy numbers in cell lines**. *Mol Cell Proteomics* 11, O111 009613
4. Zeiler, M., Moser, M. and Mann, M. (2014) **Copy number analysis of the murine platelet proteome spanning the complete abundance range**. *Mol Cell Proteomics* 13, 3435-3445

Table 2.
QPrEST standards included in the analysis.

Product name	Product number	Gene name	Protein name
QPrEST LMNA	QPrEST22525	LMNA	Prelamin-A/C
QPrEST CH60	QPrEST23025	HSPD1	60 kDa heat shock protein, mitochondrial
QPrEST G3P	QPrEST22627	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
QPrEST EFTU	QPrEST24739	TUFM	Elongation factor Tu, mitochondrial
QPrEST THIO	QPrEST23004	TXN	Thioredoxin

atlasantibodies.com

Our website provides you with easy access to all characterization data, and online ordering via our web shop.

You can also send your order to order@atlasantibodies.com or send an e-mail to support@atlasantibodies.com to discuss any matters regarding protein quantification.

You'll find we're Totally Human.

2016-03-07

ATLAS ANTIBODIES

Atlas Antibodies AB
AlbaNova University Center
SE-106 91 Stockholm, Sweden
atlasantibodies.com

Phone +46(0)8 54 59 58 50
Fax +46(0)8 54 59 58 51
support@atlasantibodies.com
contact@atlasantibodies.com