Sample Preparation for Protein Quantification by LC MS/MS

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EBF, June 2011

About Alphalyse

Alphalyse is a specialized contract research organization (CRO) providing advanced protein analysis to the life science community.

The company is situated in Odense, Denmark in one of the strongest mass spectrometry clusters in the World, and in Palo Alto, CA, USA.

We have today more than 1000 customers in North America and Europe including 8 of top-10 pharma companies.

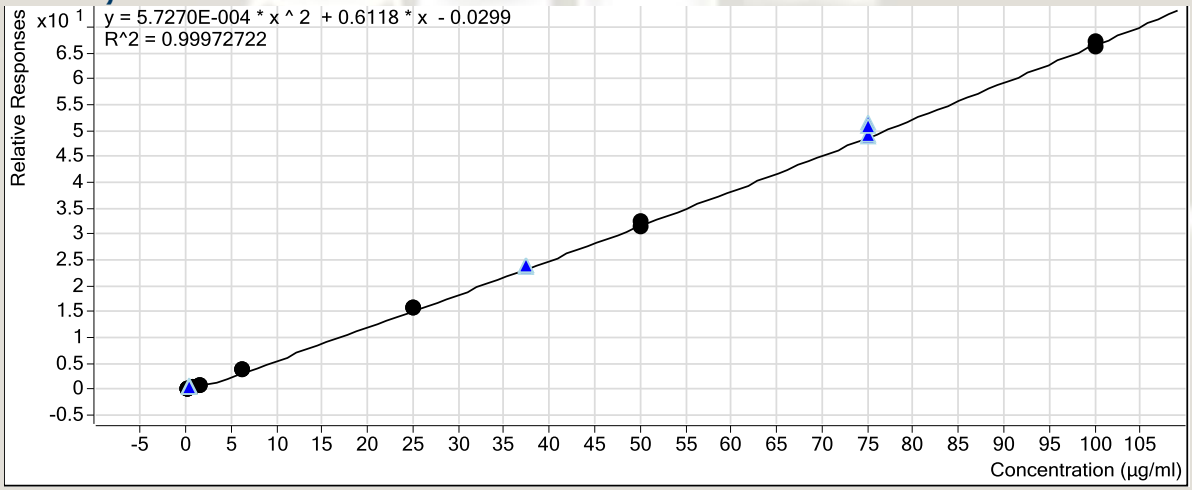
Member of the Global CRO Council (GCC) for Bioanalysis.

LC-MS Quantification of proteins in clinical development by Alpha-Quant™ assays

Assay

- LC MS with MRM quantification using isotope labeled internal standard peptides
- Measurement range 0.01-100 ug/ml in human serum
- 8 point calibration curve
- 3 QC standards

TIC, MRM



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Mass Spectrometry (MS)
Protein Quantification
Relative & Absolute Quantification

Relative quantification

- Isobaric labeling (iTRAQ)
- Stable isotope labelling by amino acids in cell culture (SILAC)
- Isotope-coded affinity tags (iCAT)
- Protein digest in O18 water
- Label-free (HI3, spectra counting)

Absolute quantification

- LC-MS with stabile isotope peptides (AQUA)
- LC-MS with isotope labeled protein standard

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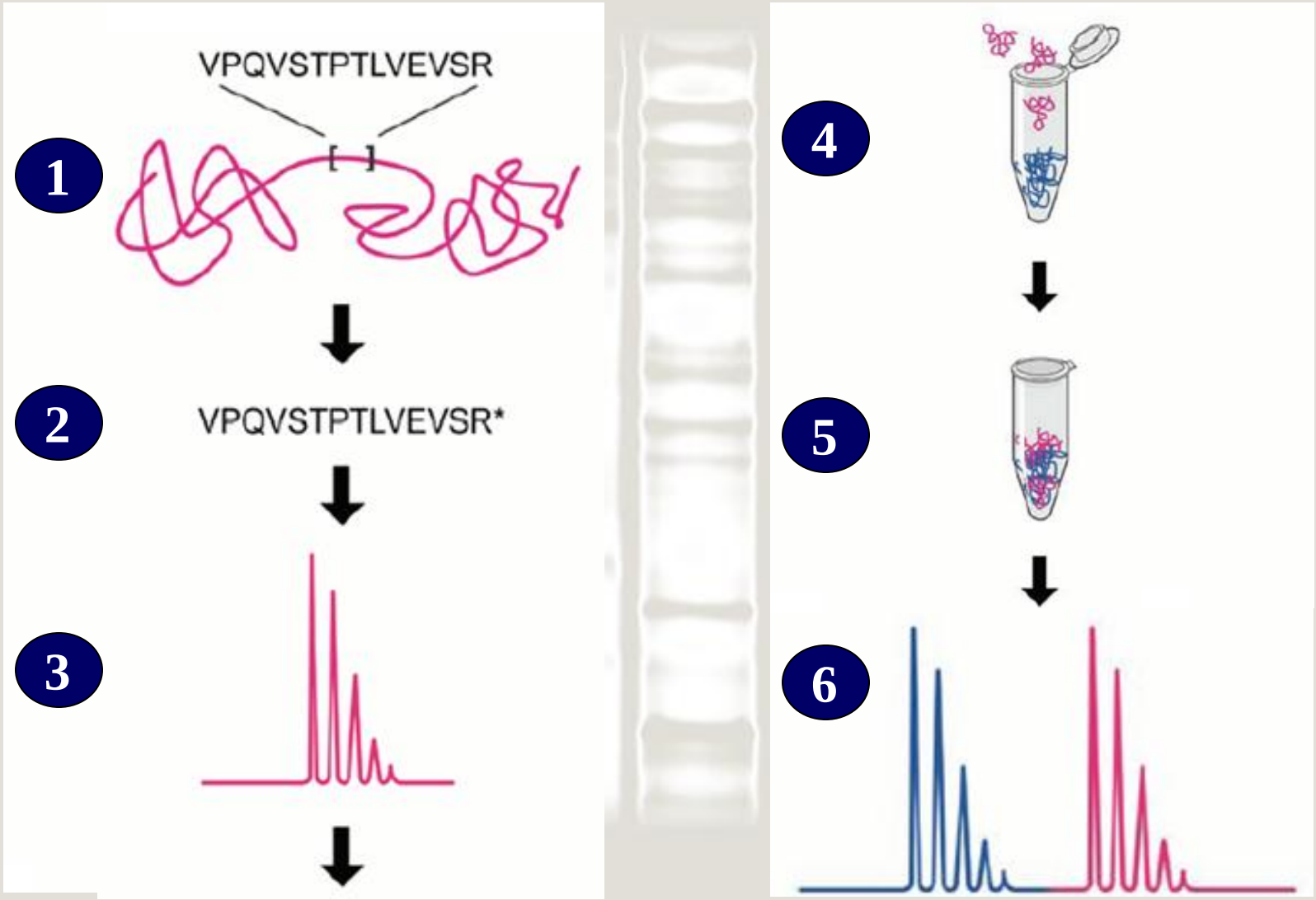
AQUA - Quantification

Principle



Ref. Stemmann, Gygi et al. Cell 2001, 107, 715-726

Sample Preparation Troubleshooting in LC MS protein quantification

- Variable and incomplete digestion. Different yields for different peptides.
- The peptide is unstabile in solvent. It binds to surfaces, to the sample matrix, or it aggregates. Problem observed both for internal standard peptide, and the natural peptide when released by digestion.
- Matrix interference with digestion, peptide purification and MS analysis.
- The peptide is partly modified by deamidation, oxidation, pyroglutamic acid,….

These issues are related to individual protein structures and peptide sequences. They depend on solvents, buffers and the sample matrix.

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Sample Prep Solutions

- Protein digestion is optimized using a range of digestion procedures
- Peptide purification from Matrix using a range of purification steps, e.g. centrifugation, SPE, filters, etc.
- Select peptides without common modifications: Cys, Met, Trp etc.
- Use of AQUA peptides as internal standards
- Use of pure reference protein as external calibration standard

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Alpha-Quant™ Workflow

Digestion – test and optimize cleavage conditions

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Peptide cleanup – purify peptides and remove salt, detergents

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LC-MS/MS peptide identification

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Select optimal MRM transitions

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Evaluate, synthesize isotope labeled peptides

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LC-MS/MS peptide quantification

↓

Integrate workflow: digestion, peptide cleanup, LC-run, MRM transitions

↓

Method qualification:

QC standards & Calibration standards are prepared using reference protein spiked into relevant matrix.

Demonstrate linearity within measurement range, precission and accuracy

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Quantification of monoclonal antibodies

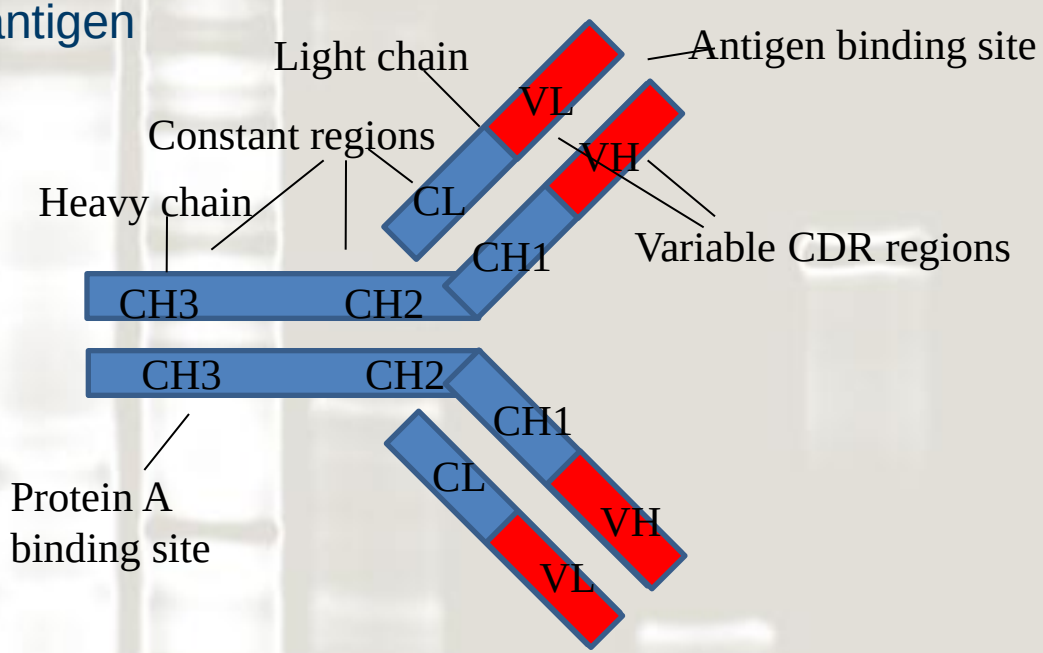
Sample preparation strategies

- Brute force strategy – digestion of whole sample
- Controlled proteolysis
- Target affinity purification
- Protein A purification
- Reduction/Alkylation, denaturation

What is being measured?

Therapeutic antibodies may exist in different forms in the blood stream and LBA measure:

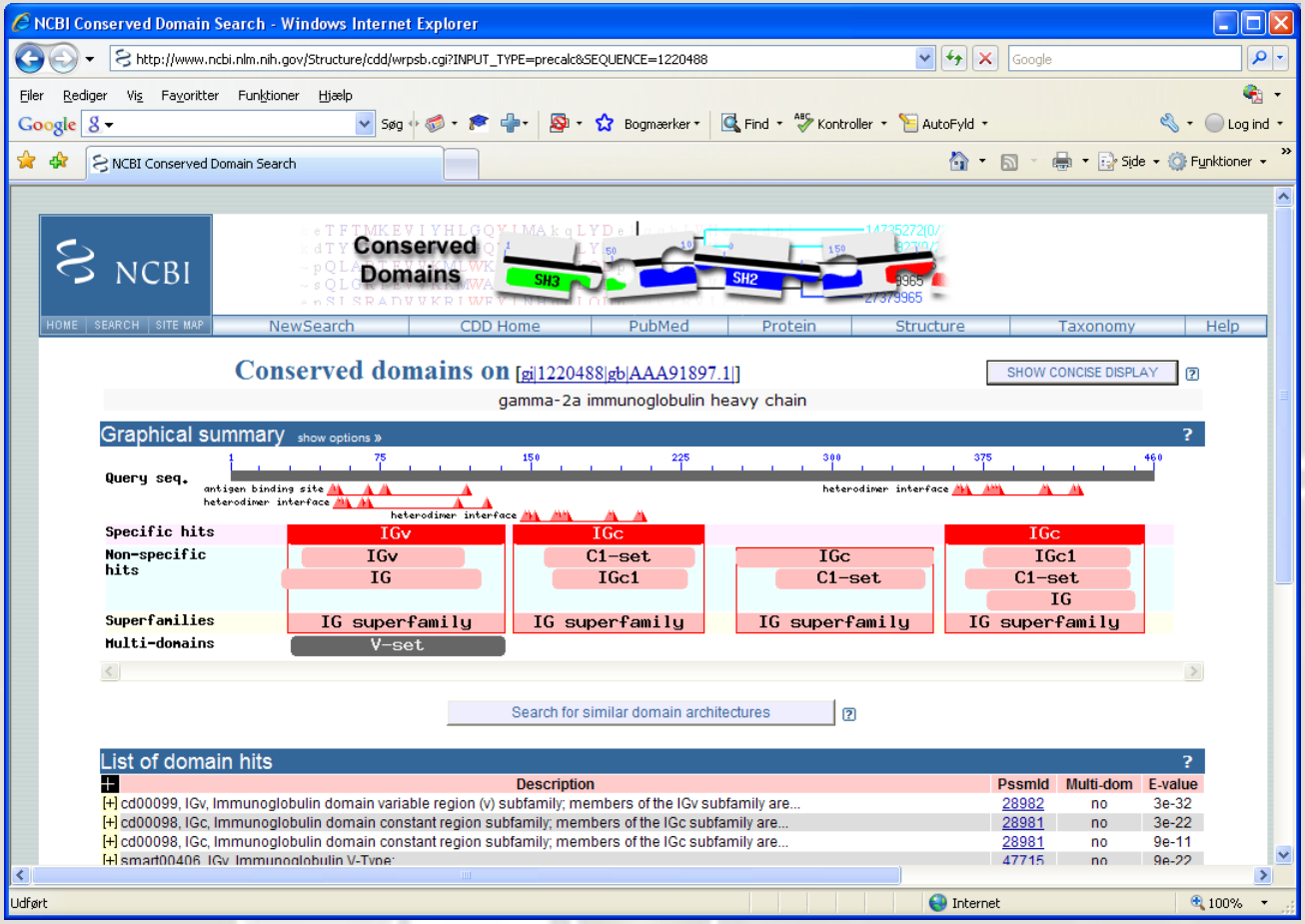
- Free
- Partially bound to antigen
- Fully bound



Measured by LC MS/MS:

- Total protein
- Intact ab when combined with Protein A purification

Choice of signature peptides in antibody



- Both heavy and light chain
- Variable regions for quantification in same species, ie human ab in human serum
- Conserved regions in other species, ie human ab in preclinical animal serum

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Case example
Quantification of protein in clinical development

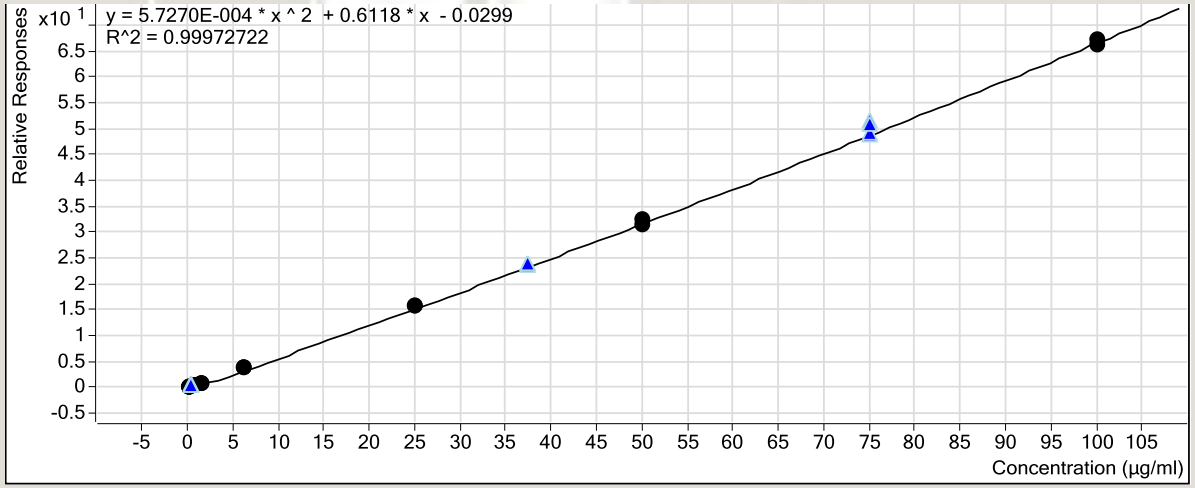
Assay Validation

- EMA Guideline on Validation of Bioanalytical Methods
- 8 point calibration curve, 0.01-100 ug/ml. Curve fit R2>0.9995
- 2 x 3 QC stds: Precision 2-5%, Accuracy 99-101%

GLP Sample analysis

- 1800 human serum samles (double determination) run in 6 weeks
- 5 ul serum per analysis
- 6 outlier samples reanalyzed. 2 samples RSD>15%, 2 analysis failures of one sample, 2 samples had 1 sample < LLOQ

TIC, MRM



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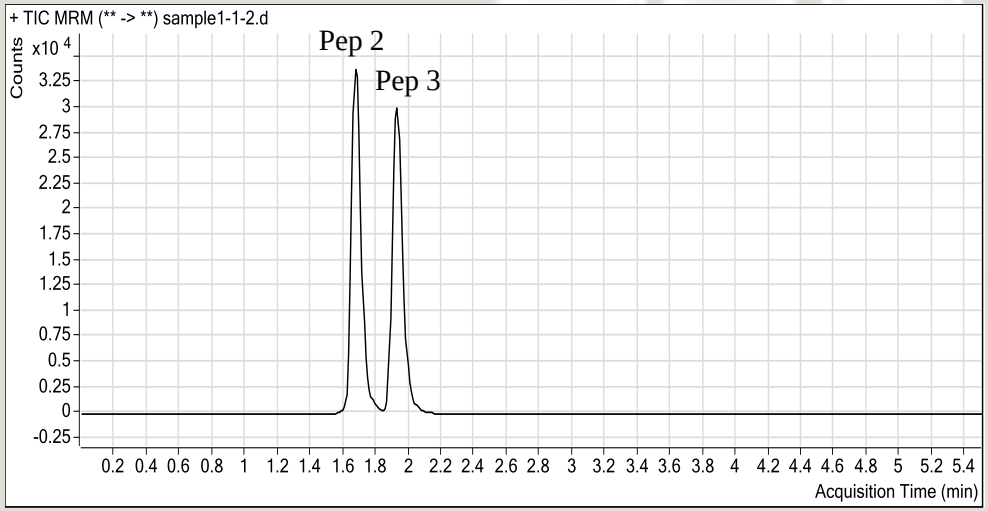
Case example
Quantification of recombinant protein vaccine in manufacturing process development

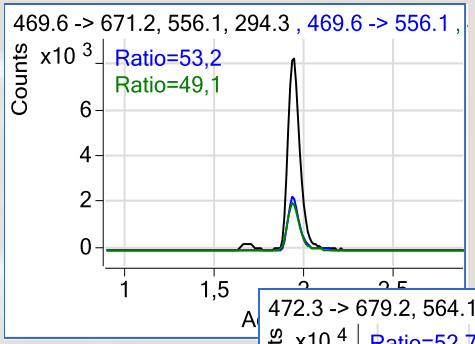
AQUA peptides

- 3 peptides were selected and synthesized as AQUA peptides with heavy isotope labeled amino acids.
- AQUA Peptide 1 was discarded due to observation of partial deamidation of the natural peptide

TIC, MRM

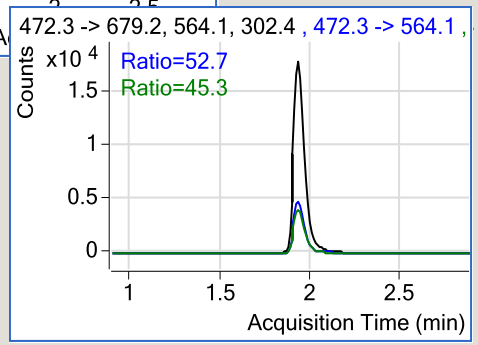


3 MRM transitions per peptide



Natural peptide

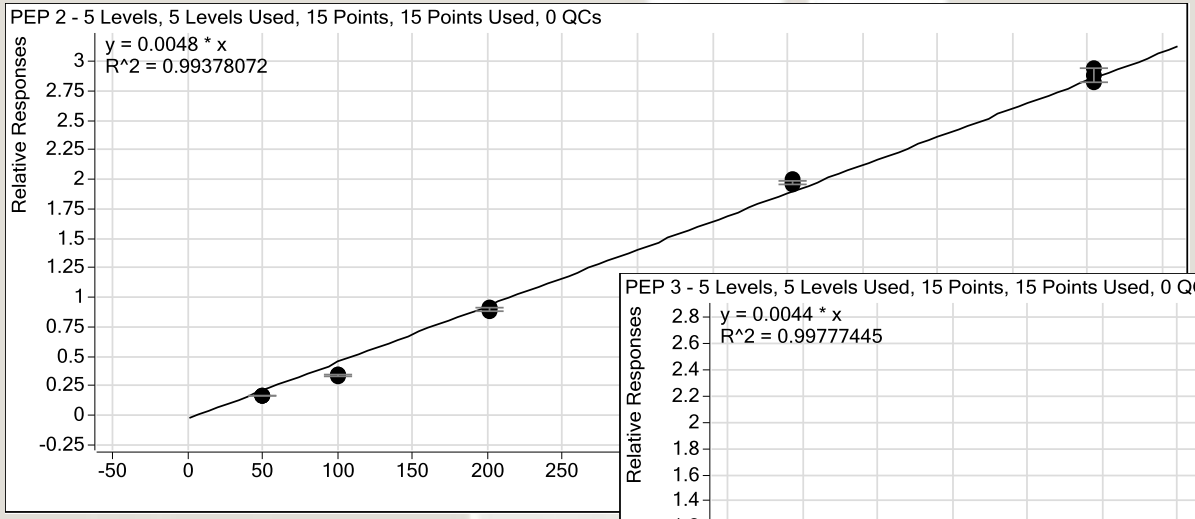
AQUA peptide



External calibration curves on ref. protein

- External calibration curves for Peptide 2 and Peptide 3
- Triplicate analysis, fixed AQUA concentration
- Demonstrates assay linearity and precision within measurement range

Peptide 2. R^2= 0.994



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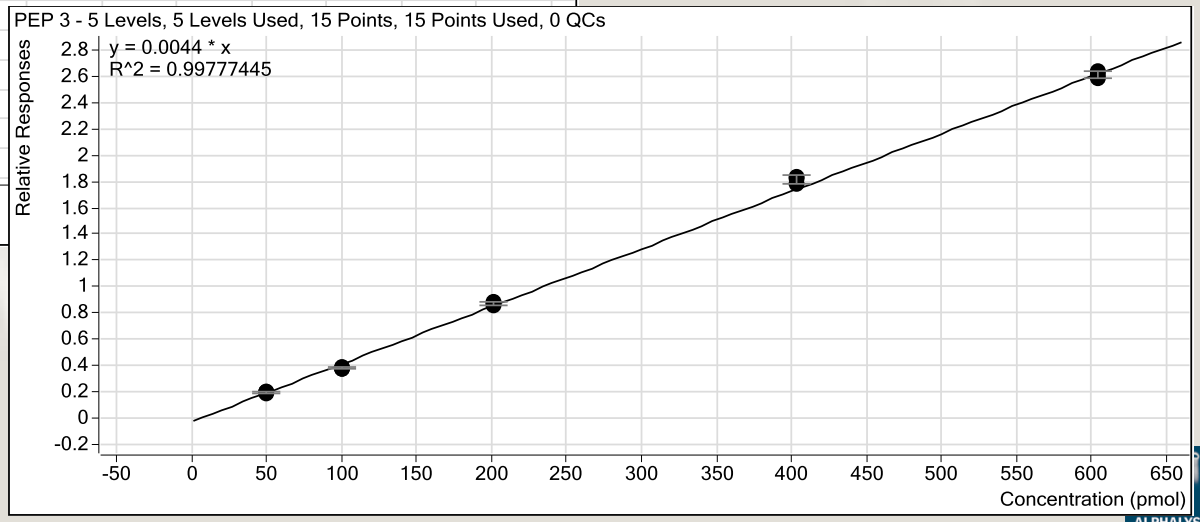


Applications

- LC MS/MS protein quantification is applied to recombinant and natural proteins & peptides in complex sample matrices.

- Of specific importance are Monoclonal antibodies, Protein vaccines, and Therapeutic proteins.

- Pre-clinical and clinical development – PK /TK analysis

- Protein production/manufature – process development and documentation

Summary

Alphalyse has sucessfully developed LC MS/MS protein quantification assays for a range of protein biologics.

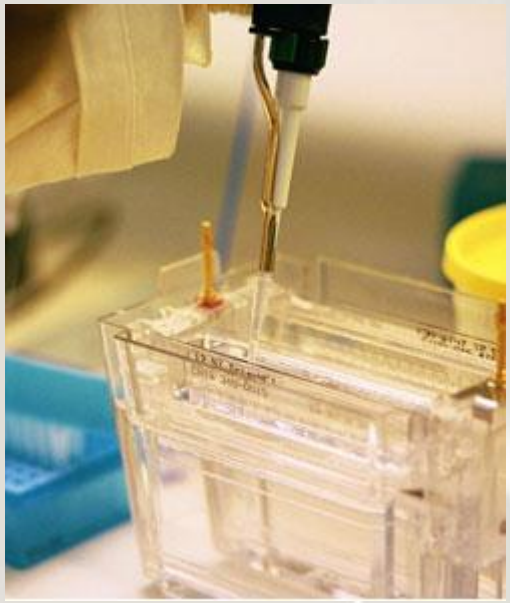
Sample preparation should be adjusted to the specific protein and the selected peptides. The assay should be qualified using pure reference protein in the relevant matrix.

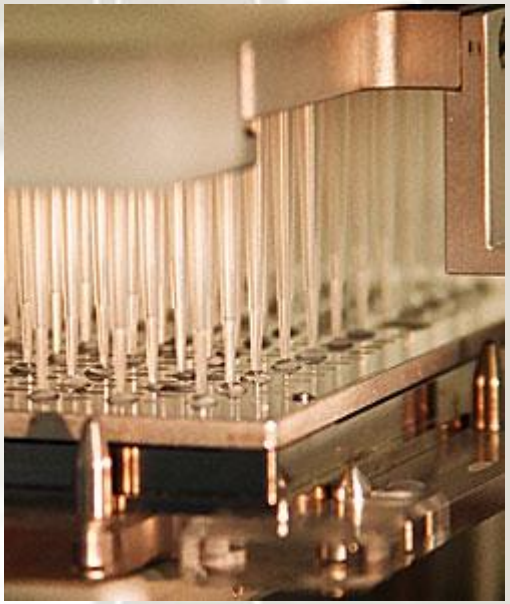
Advantages

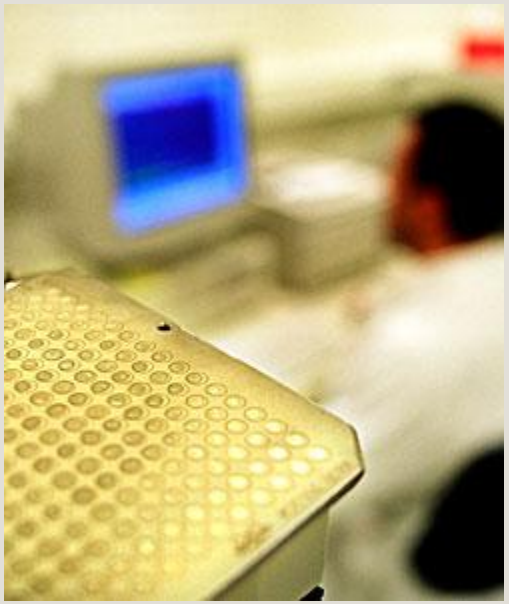
- Direct measurement of analyte
- High dynamic range, precision and sensitivity
- Applicable on complex biological matrices , e.g. serum

- Multiplexing
- Protein modifications
- HTP assay with rapid resolution HPLC, 5-10 min cycle time
- Fast assay development (2-3 months)

Questions?







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