# Targeted Proteomics goes clinical as primary endpoint: a case study.

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## Introduction

While proteomics tools are increasingly used in the early steps of the drug development journey, its use remains limited to exploratory endpoints in clinical phase. Successful MS methods used in the exploratory and preclinical phases are adapted to antibody-based assays. For the present study, no antibody-based assay to measure the level of the target engagement biomarker could be validated for the use in preclinical animal models. A MS-based protein quantification method was therefore developed in Research Use Only (RUO) and ultimately transferred to a Good Clinical Practice (GCP) environment for validation to support preclinical development and clinical trial Phase 1 as a primary endpoint. First, a high purity Stable Isotope Labelled (SIL) protein was produced in HEK293 cells cultured in SILAC medium and purified (anti-poly-histidine followed by IMAC and SEC). Second, a targeted MS assay was developed for the absolute quantification of the biomarker of interest. This GCP targeted proteomics method was validated according to FDA validation of bioanalytical methods for Industry and ICH-M10 guidelines. Specifically, the method validation included the assessment of precision and accuracy of the assay, surrogate matrix equivalence, lower limit of quantification (50 ng/ml), concentration range, short- and long-term stability of the samples. The validated, targeted MS method is routinely used to quantify a target engagement biomarker in a currently ongoing Phase 1 clinical trial.

## 1. Biological context

Bayer and Evotec partnered to develop a drug candidate in kidney disease.

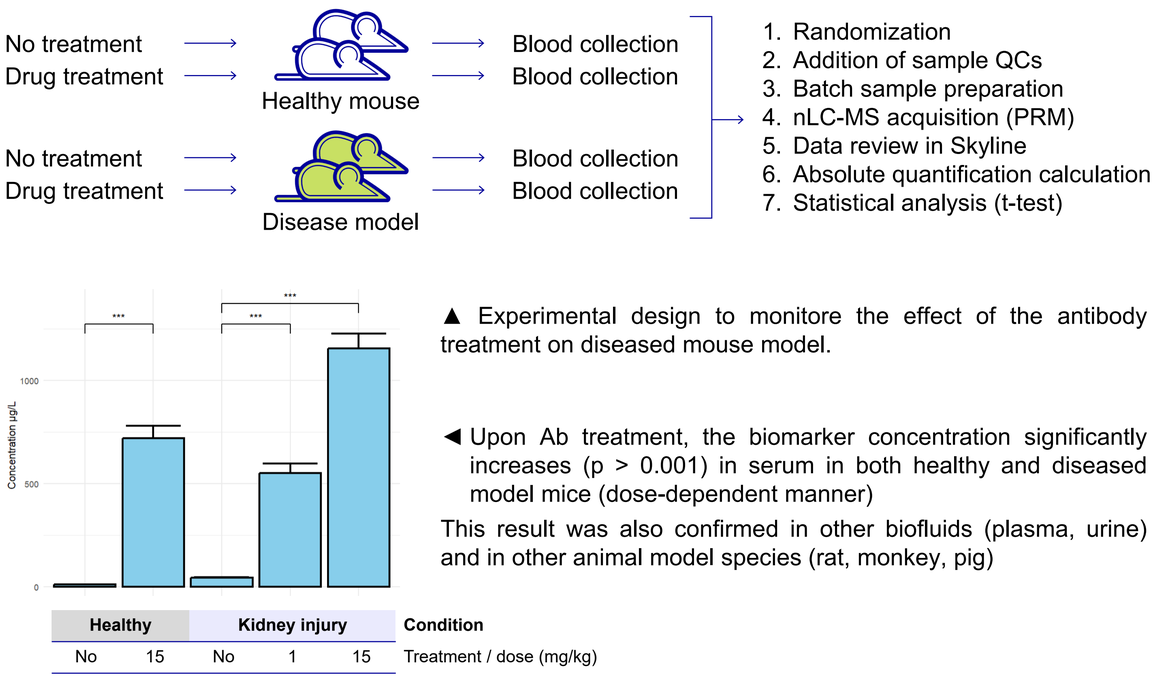
During the preclinical phase of drug development, Evotec developed a targeted method (RUO) for quantification of a biomarker that enabled the assessment of target engagement in animal models. In order to support clinical evaluation of the therapeutic candidate, Evotec adapted and transferred the targeted method to a GCP FDA-compliant environment with a fully validated MS-based targeted proteomics method.

## 2. RUO method for preclinical studies: PRM assay

### a. Method

The RUO method was successfully developed for mouse serum samples. Samples were prepared using the iST kit (Preomics GmbH) according to the supplier protocol. A pool of samples was prepared and processed in each plate as sample QC. Stable Isotope Label (SIL) peptides were added before peptide clean-up. 750 ng of peptides were injected on an in-house 40 cm x 75-µm C18 column with a U3000 (Thermo Fisher Scientific) and separated over a linear gradient of 25 min at a flow rate of 300 nL/min. Peptide transitions were measured with a scheduled PRM method on a Q-Exactive Plus (Thermo Fisher Scientific). Data were reviewed using Skyline (University of Washington) and further processed using MSstats package in R to keep sample. Peptides with an absolute quantity below the LOQ (2 µg/L) were considered as unquantifiable, and therefore were discarded from quantitative analysis.

### b. Proteomics absolute quantification in mouse plasma

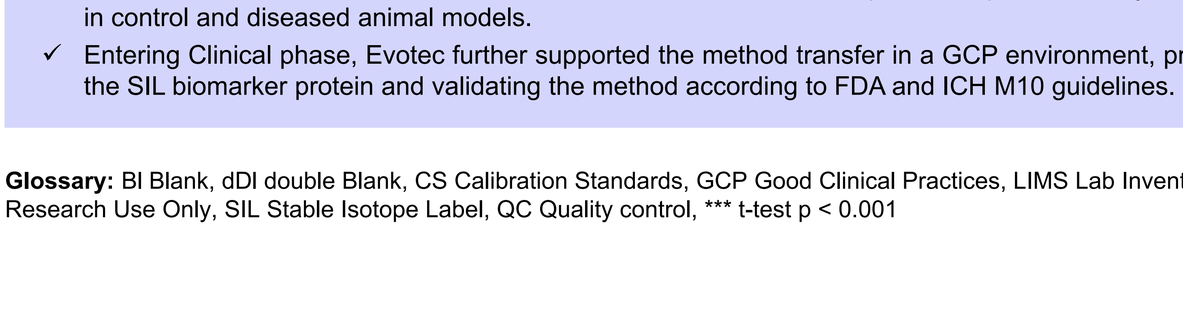



▲ Experimental design to monitore the effect of the antibody treatment on diseased mouse model.

◄ Upon Ab treatment, the biomarker concentration significantly increases ($p > 0.001$) in serum in both healthy and diseased model mice (dose-dependent manner)

This result was also confirmed in other biofluids (plasma, urine) and in other animal model species (rat, monkey, pig)

## 3. Method transfer from RUO to GCP environment




**Needs:**
- ✓ Synthesis of SIL protein
- ✓ Validated systems
- ✓ Validated LIMS
- ✓ GCP facility

**Guidelines**:
- FDA Bioanalytical Method Validation Guidance for Industry
- ICH M10 Bioanalytical Method Validation

**Challenge 1.** Typically, the MS-based proteomics targeted assay uses SIL peptides as surrogates for the whole protein for the absolute quantification. According to the FDA guidance "*The reference standard should be identical to the analyte*". Therefore, in MS-based proteomics, the reference standard should be a SIL protein. However, such SIL protein is often not available on the shelf. Here, we produced the SIL protein from transfected HEK293 cells.

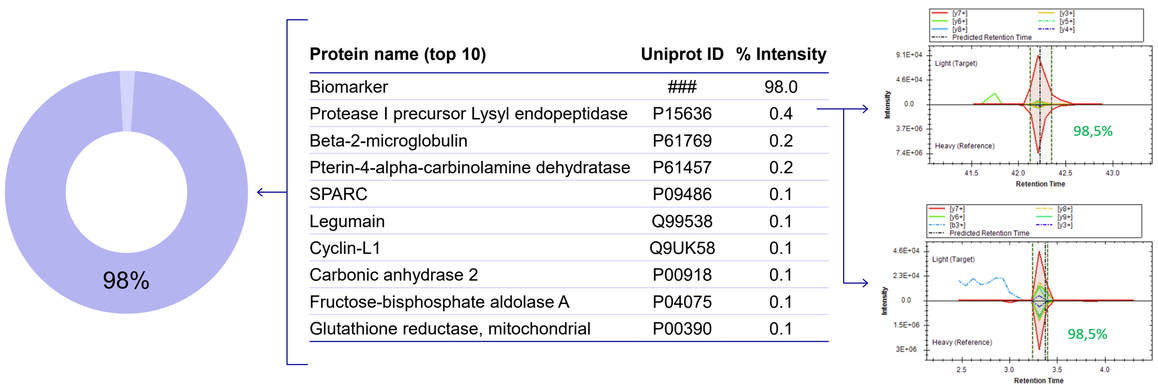
**Challenge 2.** The whole proteomics process must be validated: protocol, instruments, LIMS. Here, we adapted the RUO proteomics workflow into an already validated GCP environment for LCMS bioanalysis based on ICH M10 guidelines. We leveraged the internally validated Analyst software and LIMS. We implemented the sample preparation in a Hamilton liquid handler and validated the procedure.

## 4. Synthesis of SIL protein biomarker

### a. Method

Plasmids expressing the SIL protein biomarker were transfected in HEK293 cells using lipofectamine 3000 in SILAC culture medium for 4 days (10 % Dialized FBS SILAC, 10 mM HEPES, 1% NEAA, 100 U/mL Penicillin/streptomycin, 0.1 mM Proline, 0.4 mM L-Arginine, 0.8 mM L-Lysine in SILAC DMEM). Proteins were pulled down on Ni-NTA phynexus resin. Protein purity was assessed with anti-his tag, protein yield was assessed by nanodrop, and SIL incorporation was assessed by MS (DIA global profiling)

### b. MS QC



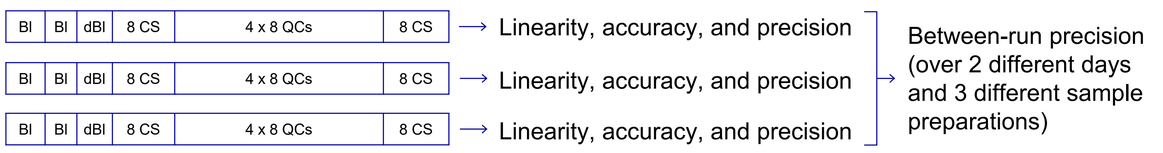

▲ After purification, the SIL protein biomarker yield was 98.0 % based on protein intensity. The subsequent targeted peptides had a 98.5 % heavy-to-light ratio demonstrating excellent SIL amino acid incorporation.

## 5. GCP-ready method validation

### a. Method

The GCP method was successfully developed for human plasma samples. SIL biomarker was added to each sample, calibrant, and QC level. Two µL of samples were extracted using the iST kit (Preomics GmbH) according to supplier protocol using a validated protocol on a Hamilton liquid handler. Five µL of extracts of peptides (5 µg) were injected on a nanoEase M/Z Peptide CSH C18 column (Waters) (130Å, 1.7 µm, 300 µm X 50 mm) with a Acquity µLC (Waters over a linear gradient of 10.6 min at a flow rate of 14 µL/min. Peptide transitions were measured with a scheduled MRM method on a Q-Trap 6500+ (Sciex) mass spectrometer. Data were processed using Analyst (Sciex), QC review was performed using a dedicated LIMS.

### b. Method validation



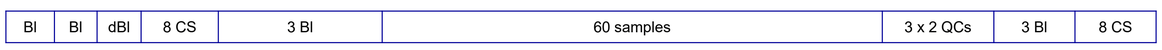

▲ A method validation analytical run for in-run and between-run linearity, accuracy, and precision consisted of 2 blanks (Bl, the surrogate matrix and SIL protein), 1 double blank (dBl, surrogate matrix), 8 calibration standards (CS), 8 replicates of 4 levels of QC (QCs: high, medium, low, LLQ), and 8 CS. For each concentration level of calibrant standards and QC, the accuracy and precision thresholds were ± 15 %, except for the LLQ at ± 20 %.

Maximum batch size, Biomarker stability in plasma, Biomarker stock solution, freeze-thaw cycle stability, injection repeatability were also assessed and validated.

### c. Figure of merit

| Title | Method for the Determination of Biomarker in Human Plasma |
|---|---|
| Range | 50 – 10 000 ng/mL |
| Accuracy | 87.8 – 108.0 % |
| In-run Precision | 15.4 % at LLQ |
| Between-run Precision | 5.4 % |
| Carry-over | < 26.4 % at LLQ |
| Matrix effect | No |
| Biomarker stability in plasma | 24 h at RT, 7 months at -80 °C, Freeze/thaw 3 cycles |
| Maximum batch size | 96 injections, 60 samples |

### d. Application of the method



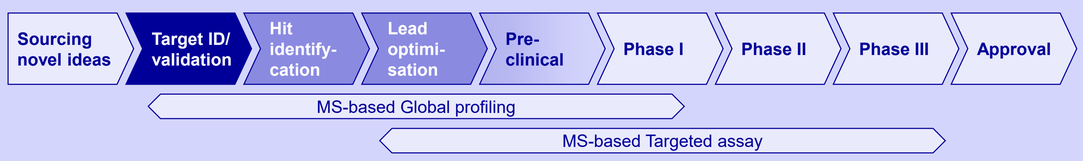

▲ A typical analytical run consisted of 2 Bl, 1 dBl, 8 CS, 60 samples and 2 replicates of 3 QC levels, and 8 CS.

The method is currently applied on over 1200 human plasma samples for the Phase I clinical trial.

## 6. Conclusion

To support the development of an antibody drug candidate, we leveraged MS-proteomics throughout the whole journey:

- ✓ A Biomarker was identified using proteomics global profiling
- ✓ This biomarker was then leveraged to support the pre-clinical phase in RUO environment. Specifically, the biomarker concentrations were increased in serum and plasma upon antibody treatment in control and diseased animal models.
- ✓ Entering Clinical phase, Evotec further supported the method transfer in a GCP environment, producing the SIL biomarker protein and validating the method according to FDA and ICH M10 guidelines.




▲ While the clinical phase is typically supported by ligand-binding assay, Evotec supported MS-based proteomics for the project needs throughout the whole drug development value chain.

**Glossary:** Bl Blank, dDl double Blank, CS Calibration Standards, GCP Good Clinical Practices, LIMS Lab Inventory Management System, LLQ Lower Limit of Quantification, QC Quality Control, RT Room temperature, RUO Research Use Only, SIL Stable Isotope Label, QC Quality control, *** t-test $p < 0.001$