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Direct infusion-SIM as fast and robust method for absolute protein quantification in complex samples

Christina Looße[a], *Sara Galozzi*[a], *Linde Debor*[b], *Mattijs K. Julsing*[b], *Bruno Bühler*[b], *Andreas Schmid*[b], *Katalin Barkovits*[a], *Thorsten Müller*[a,1], *Katrin Marcus*[a,*,1]

[a] *Medizinisches Proteom-Center, Ruhr-University Bochum, D-44801 Bochum, Germany*

[b] *Laboratory of Chemical Biotechnology, Department of Biochemical and Chemical Engineering, TU Dortmund University, D-44227 Dortmund, Germany*

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ABSTRACT

Relative and absolute quantification of proteins in biological and clinical samples are common approaches in proteomics. Until now, targeted protein quantification is mainly performed using a combination of HPLC-based peptide separation and selected reaction monitoring on triple quadrupole mass spectrometers. Here, we show for the first time the potential of absolute quantification using a direct infusion strategy combined with single ion monitoring (SIM) on a Q Exactive mass spectrometer. By using complex membrane fractions of *Escherichia coli*, we absolutely quantified the recombinant expressed heterologous human cytochrome P450 monooxygenase 3A4 (CYP3A4) comparing direct infusion-SIM with conventional HPLC-SIM. Direct-infusion SIM revealed only 14.7% (±4.1 (s.e.m.)) deviation on average, compared to HPLC-SIM and a decreased processing and analysis time of 4.5 min (that could be further decreased to 30 s) for a single sample in contrast to 65 min by the LC–MS method. Summarized, our simplified workflow using direct infusion-SIM provides a fast and robust method for quantification of proteins in complex protein mixtures.

1. Introduction

Quantification of proteins is a common approach in proteomics. Using heavy isotope labeled peptides, absolute quantification of specific proteins by selected reaction monitoring (SRM), a targeted mass spectrometric approach measured on a triple quadrupole mass spectrometer, is a well-established method [1–6]. Although SRM measurements are sensitive and highly selective, a triple quadrupole does not show high resolution and high mass accuracy. Furthermore, absolute quantification with SRM requires method optimization for every new protein of interest or peptides representing the protein with regard to selection of transitions and collision energy optimization. Utilizing the Q Exactive mass spectrometer (Thermo Scientific), which combines a quadrupole mass filter and an orbitrap mass analyzer, targeted approaches can be carried out with high resolution and accurate mass (HR/AM)

* *Corresponding author*. Tel.: +49 0234 3228444; fax: +49 0234 3214554.
E-mail address: katrin.marcus@rub.de (K. Marcus).
[1] These authors contributed equally to this work.

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detection [7]. Furthermore, quantification can be performed by single ion monitoring (SIM) at MS level or parallel reaction monitoring (PRM) at MS/MS level [8,9]. In the SIM mode, the quadrupole isolates the precursor mass of interest in a narrow window of e.g. 2 *m/z*. Then, ions are accumulated in the C-Trap and analyzed in the orbitrap. In a standard proteomics workflow prior to mass spectrometry (MS) measurement an HPLC is used for pre-fractionation of the peptide mixture, which usually takes about 1–2 h depending on sample complexity. So far, the direct infusion MS setup is applied for characterization and quantification of metabolites, for example in lipid profiling to investigate the progression of colorectal cancer [10] or to detect metabolite changes in liver extracts from mice after exposure to polychlorinated biphenyls [11]. To our knowledge a direct infusion-SIM approach has not been used to absolutely quantify proteins from complex biological samples until now. This method might be of special interest e.g. for protein quantification in biotechnological systems, such as whole-cell biocatalysts as well as other biological systems like cells or tissues. In order to establish an efficient biocatalyst and process setup, a fast and reliable absolute quantification of the target protein from the biological matrix is important to define or monitor optimal reaction conditions to achieve high amounts of active protein. Here, we applied the direct infusion-SIM approach to quantify the heterologously expressed human cytochrome P450 monooxygenase 3A4 (CYP3A4), which was produced in *Escherichia coli*. Human cytochrome P450 enzymes are a family of membrane-bound heme-containing monooxygenases responsible for the metabolism of many endogenous compounds as well as xenobiotics, mainly in the liver [12,13]. The enzymes are of special interest as biocatalysts in order to synthesize gram-scale amounts of drug metabolites [14]. The overall aim of this study was to develop a fast, robust and reproducible method for protein quantification that can be used to (absolutely) quantify proteins from complex protein mixtures, e.g. *E. coli* membrane fractions. We performed direct infusion-SIM with the Q Exactive mass spectrometer and compared results to data obtained with a conventional HPLC-SIM approach. Direct infusion-SIM appeared to be a fast and accurate tool for the determination of absolute abundance of proteins in complex biological samples.

2. Experimental procedures

2.1. Reagents

$^{13}C_6/^{15}N_2$-Lys labeled peptides were purchased from Thermo. Solvents used for mass spectrometry were all HPLC/MS grade. Acetonitrile (ACN) was purchased from Biosolve, trifluoroacetic acid (TFA) and formic acid (FA) from Sigma Aldrich.

2.2. *E. coli* cultivation and CYP3A4 synthesis

E. coli DH5α [15] containing the genes encoding human CYP3A4 and cytochrome P450 reductase on the plasmid pCW3A4 [16] was used. *E. coli* DH5α (pCWori(+)) was used as control strain without CYP3A4. A single colony of *E. coli* DH5α from an LB agar plate [17] was used to inoculate 3 ml LB medium, which were grown overnight at 37 °C and 200 rpm to a biomass concentration of about 1 $g_{CDW}\,l^{-1}$. The precultures were diluted to a biomass concentration of 33 $mg_{CDW}\,l^{-1}$ in 40 or 100 ml TB medium (0.89 M KPO_4 buffer (potassium phosphate buffer), pH 7.4, 24 $g\,l^{-1}$ yeast extract, 12 $g\,l^{-1}$ tryptone, 2 $g\,l^{-1}$ peptone, 4 $ml\,l^{-1}$ glycerol, 1 mM thiamin, 25 $\mu l\,l^{-1}$ trace elements and 100 $mg\,l^{-1}$ ampicillin) [18] in non-baffled, capped 250 ml Erlenmeyer flasks. The cultures were incubated at 30 °C and 200 rpm (rotary incubator) until a biomass concentration of 83 $mg_{CDW}\,l^{-1}$ was obtained. Then, gene expression was induced by the addition of 1 mM IPTG, 0.5 mM δ-aminolevulinic acid (δ-ALA), and 25 μM $FeCl_3$ and incubation was continued for another 24 h. Cells were harvested by centrifugation (20 min at 4700 × *g* and 4 °C) and resuspended in 100 mM KPO_4 (pH 7.4) containing 20% glycerol, 1 mM EDTA, 0.1 mM dithiothreitol (DTT), and 0.2 mM Pefabloc (Sigma–Aldrich) at 17 $g_{CDW}\,l^{-1}$ and stored at −20 °C until usage.

2.3. Membrane isolation

Cell lysis was achieved by two passages through a French Press, followed by centrifugation (20 min, 4 °C, 4700 × *g*) and ultracentrifugation of the supernatant (2 h, 4 °C, 40,800 × *g*). Membranes were resuspended in 100 mM KPO_4 buffer (pH 7.4) containing 20% glycerol, 1 mM EDTA and 0.1 mM dithiothreitol (DTT) and stored at −20 °C.

2.4. Sample preparation

Membrane fractions containing human CYP3A4 were subjected to tryptic digestion. For each tryptic digest 10 μg protein were used and 110 $fmol\,\mu l^{-1}$ of each heavy peptide were spiked. Samples were reduced for 20 min at 56 °C with 5 mM DTT and alkylated with 15 mM iodoacetamide in the dark for 15 min. Digestion was carried out over night at 37 °C with trypsin (trypsin:protein ratio 1:5.5) in the presence of 0.01% (wt/vol) ProteaseMAX (Promega), 15 mM bicine and 30 mM NH_4HCO_3. After stopping the digestion by addition of 0.5% TFA, samples were purified using OMIX columns (Agilent Technologies) and eluted in water containing 55% ACN and 0.1% TFA. Eluates were concentrated in a vacuum concentrator and resolved in 100 μl water containing 40% ACN/0.1% FA.

2.5. Direct infusion-SIM with HESI source

Samples were loaded in a 250 μl Hamilton syringe, injected by a syringe pump with a flow rate of 5 $\mu l\,min^{-1}$ into the HESI (heated electrospray ionization) source and measured for 4.5 min with a SIM method on a Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific). For ionization a spray voltage of 3.6 kV and capillary temperature of 320 °C was used and sheath gas flow rate was set to 6 units. The acquisition method consisted of two scan events, Full MS and targeted SIM (t-SIM). The Full MS was monitored from *m/z* 350–1400, with an orbitrap resolution of 35,000 (at *m/z* 200), a maximum injection time of 120 ms and an automatic gain control (AGC) value of 3e6. The t-SIM was performed with a resolution of 70,000 (at *m/z* 200), a maximum injection time of 250 ms and an automatic gain control (AGC) value of 1e5.