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Technical note

# G protein-coupled receptor quantification using peptide group-specific enrichment combined with internal peptide standard reporter calibration☆

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ABSTRACT

The G protein-coupled receptor (GPCR) super-family comprises the largest and most diverse group of membrane receptors in eukaryotes. GPCRs are involved in a plethora of physiological functions in all kinds of tissues. Detailed knowledge about GPCR presence and expression levels in tissues can be very helpful for drug development as the majority of drugs are designed to modulate membrane receptors. Furthermore, it is known that many adverse drug effects result from GPCR interactions. However, very few satisfactory methods are currently available for the detection and quantification of GPCRs. The detection is complicated by their three-dimensional structure, their hydrophobic properties, and their localization in the plasma membrane with 7-trans-membrane domains and small cytosolic and extracellular domains. Due to these properties it is very difficult to generate specific antibodies directed against GPCRs for sandwich immunoassays and Western blot. We therefore designed an immunoaffinity- and mass spectrometry-based approach to analyze GPCR-specific signature peptides in tryptic digests in rat tissue lysates. The expression levels of four different GPCRs were determined using chemically labeled synthetic standard peptides. Here, we demonstrate for the first time, that peptide immunoaffinity MS-based methods can render a reliable and quantitative analysis of multi-membrane spanning receptor molecules. This article is part of a Special Issue entitled: From Genome to Proteome: Open Innovations.





*Abbreviations:* 5-HT, 5-hydroxytryptamine (serotonin); ESI, electrospray ionization; GPCR, G-protein coupled receptor; IPA, isopropyl alcohol; iTRAQ®, isobaric tag for relative and absolute quantification; LC–MS, liquid chromatography–mass spectrometry; LLOD, lower limit of detection; LLOQ, lower limit of quantification; mAChR, muscarinic acetylcholine receptor; MALDI, matrix assisted laser desorption ionization; MFI, mean fluorescence intensity; PE, phycoerythrin; RSD, relative standard deviation; RT, retention time; S/N, signal-to-noise; TXP, Triple X proteomics

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## 1. Introduction

Peptide immunoprecipitation coupled with a mass spectrometric read-out of the captured peptides has proven to be a reliable method for the quantification of proteins in plasma [1–3]. In this approach, plasma proteins are enzymatically digested after which distinct signature peptides specific for the target proteins are enriched using single peptide-specific antibodies and subsequently quantified by mass spectrometry as a surrogate for the corresponding protein. The advantage of this mode of detection is that the complex tertiary protein structure is reduced to soluble linear peptides, whose primary sequence can be unambiguously confirmed using tandem mass spectrometry. For this reason, immunoaffinity MS approaches are an excellent choice for efficient quantification of difficult target proteins present in biological samples. In a recent study this methodology was applied to the measurement of the tumor biomarker EGFR 2 (Her2), a receptor tyrosine kinase, and progesterone receptor (PR), an intracellular receptor, in normal and tumor tissue [4]. Although highly sensitive sandwich immunoassays have already been described for both target proteins in literature [5] this study nevertheless highlighted the potential of immunoaffinity-based MS strategies for protein expression analyses. In particular, immunoaffinity-based MS has been shown capable of providing unambiguous results in the analysis of very large proteins, insoluble membrane proteins and protein complexes, where other antibody-based quantification methods such as sandwich immunoassays are unreliable [6].

Whereas the development of sandwich immunoassays for the quantification of Her2 – a receptor family with a single trans-membrane domain – has been achieved [7], the approach fails when applied to the quantification of ion channels or multiple trans-membrane proteins including GPCRs. GPCRs contain long stretches of hydrophobic amino acids in their primary structure, enabling them to form seven trans-membrane domains. Their hydrophobicity, combined with their location, in the double lipid layer of the cell membrane, severely restricts the number and accessibility of potential epitopes, thus hampering the generation of antibodies and hence limiting the availability of GPCR specific immunoassays. Typically, only short regions of the receptors are accessible in their native surrounding in the membrane bi-layers and these short protein loops are usually not large enough for the two-site binding required in a sandwich immunoassay. In addition, the high number of GPCR isoforms complicates the generation of isoform-specific antibodies and consequently the development of specific assays for GPCR differentiation. Therefore, most assays for the detection of GPCRs in cells are functional or mRNA-based [8,9]. To the authors' knowledge, there are currently no assays available for the absolute quantification of GPCRs at the protein level.

Nevertheless, GPCRs remain one of the most important classes of protein drug targets due to their role in regulating a vast diversity of biological processes. The number of pharmaceuticals designed to modulate GPCRs reveals the need for more detailed knowledge regarding this protein group. It is estimated that up to 80% of all drugs modulate membrane proteins [10] and as much as 50% of the currently marketed drugs are directed towards GPCRs [11]. Approximately 800 different GPCR-genes are known in humans [12] and are classified in 6 families. Serotonin receptors (5-hydroxytryptamine receptors, 5-HT R) are a prominent example of GPCRs. These are primarily expressed in platelets, gut and the central nervous system. Here, they are involved in the regulation of processes such as behavior, sleep, appetite or anxiety [13–16]. In platelets and the gut, serotonin mediates cardiovascular functions or motility respectively [17–20]. Another important family is the muscarinic acetylcholine receptor family (mAChR). The isoform M2, for instance, is predominantly located in cardiac tissue and is involved in regulating the heart rate [21,22]. As such, mAChR is not only important as a drug target, but is also highly relevant in safety pharmacology [23,24]. At lower levels, it is also expressed in the lung, uterus, and many parts of the brain (hippocampus, cortex, thalamus, basal forebrain, brainstem and others).

Members of these GPCR families were chosen for immunoaffinity MS-based quantitative investigations in most relevant tissues because of their great regulatory relevance and their pharmaceutical potential as drug targets. We adopted the Triple X proteomics (TXP) methodology [25] which employs antibodies directed against short terminal epitopes sharing a common terminal amino acid motif present in peptides derived from in silico GPCR digests. These antibodies are capable of enriching groups of peptides with the same terminal sequence motif directly from tryptic digests. We generated antibodies against the C-terminal end of tryptic fragments present in the sequences of four GPCRs. For the determination of these proteins, we integrated a quantification strategy developed by Dayon and colleagues [26]. Tryptically digested tissue and synthetic standard peptides of known concentrations were labeled with different iTRAQ reagents and combined. Initially, TXP antibodies were used to capture GPCR peptides in a first immunoprecipitation step which were subsequently eluted and analyzed via reversed phase nano-liquid chromatography tandem mass spectrometry (nLC–MS/MS). Chemical labeling of analyte peptides and peptide standards with different isobaric tags, combined with TXP antibody-based peptide enrichment permitted multiplexed GPCR quantification via an internal multipoint calibration curve.

## 2. Materials and methods

Acetonitrile, acetic acid, ammonium bicarbonate, dithiothreitol, iodoacetamide and isopropyl alcohol were purchased from Sigma Aldrich (Steinheim, Germany). Dynalbeads Protein G were purchased from Life Technologies (Carlsbad, CA, USA), ethanol from Roth (Karlsruhe, Germany) and formic acid from Promochem (Wesel, Germany). iTRAQ® reagents were purchased from AB Sciex (Foster City, USA), n-octyl β-D-glucopyranoside from AppliChem (Darmstadt, Germany), PBS from PAA (Pasching, Austria) and trifluoroacetic acid from Biosolve (Valkenswaard, Netherlands). Modified Trypsin Gold (mass spectrometry grade) was acquired from Promega (Madison, USA) and synthetic peptides were synthesized by Intavis (Cologne, Germany). One signature peptide was chosen for quantification for each of the receptors investigated.



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