Development of MS- based immunoassays for transporter and cytochrome P450 quantification in human, rat and mouse

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During this time, I took over the supervision of a master student who worked on aspects of my thesis. Felix Schmidt performed the development of the TXP assays for murine CYPs and the application to murine samples. The data was re-analyzed in the context of this thesis.

Parts of this work have been done in cooperation:

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Abbreviations

AB	antibody
ABC transporter	ATP-binding cassette containing transporter
AGC	automatic gain control
AhR	aryl hydrocarbon receptor
AOP	adverse outcome pathway
Arnt	Ah receptor nuclear translocator
BCA	bicinchoninic acid
CAR	constitutive androstane receptor
ccRCC	clear cell renal cell carcinoma
CYP	cytochrome P450
Cypro	cyproconazole
dd	data dependent
DM	differentiation medium
d. water	deionized water
EN peptide	endogenous peptide
Epoxi	epoxiconazole
EST	expressed sequence tag
GPS	global proteome survey
HAP	high abundant protein
IP	immunoprecipitation
IS peptide	stable isotope labelled standard peptide
LC	liquid chromatography
LLOQ	lower limit of quantification
MALDI	matrix-assisted laser desorption/ionization
MRM	multiple reaction monitoring
MS	mass spectrometry
MSIA	mass spectrometric immunoassays
msx-tSIM	multiplexed targeted selected ion monitoring

OCTN	zwitterion/cation transporter
PC	pericentral
PCB	polychlorinated biphenyls
PE	propagated error
PP	periportal
Prz	prochloraz
PSAQ	Protein Standard Absolute Quantification
PXR	pregnane X receptor
RSD	relative standard deviation
RXR	retinoid X receptor
qPCR	quantitative polymerase chain reaction
SC	solvent control
SILAC	stable isotope labeling by/with amino acids in cell culture
SISCAPA	stable isotope standards and capture by anti-peptide antibodies
SLC	solute carrier
SRM	selected reaction monitoring
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TCPOBOP	1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene
tSIM	targeted selected ion monitoring
TXP	triple X proteomics
ULOQ	upper limit of quantification
WM	work medium

The abbreviations of all used chemicals and biochemicals as well as the CYPs and transporters covered in this thesis are listed in "Table 4: Chemicals and Biochemicals" and "Table 56: Proteins covered in this thesis."

1 Introduction

1.1 Proteomics

The proteome is defined as the entity of proteins expressed by a genome in a specific context and at a certain time [1]. In contrast to the genome which is distinct for an organism or cell, the proteome is dynamic and influenced by the environment [2, 3]. It varies between tissues, time points and disease states. Examining the proteome with high throughput approaches is referred to as proteomics [4]. Proteomics comprises methods for protein purification from complex matrices, their identification and quantification as well as elucidation of structural information and comparison to protein and DNA sequence databases [5]. Two-dimensional gel electrophoresis and chromatographic approaches such as ion-exchange chromatography and reversed phase chromatography are the major methods for protein purification [5, 6]. For protein identification, western blotting, Edman sequencing, and mass spectrometry (MS) can be employed [6, 7]. The listed protein content of a sample or a list of differentially expressed proteins are the typical result of a proteomics analysis [4]. Depending on the experimental setup and sample preparation, the proteomics workflow can also be used to investigate protein localization, turn-over, protein-protein-interaction and post-translational modification [3, 4]. No matter the question, approaches with MS - based read out prevail nowadays [2, 3, 8-13].

1.2 Mass spectrometry-based proteomics

For all MS-based methods, it is crucial to reduce the complexity of the biological samples because proteins are identified solely by the mass-to-charge ratios of their peptides and fragments thereof. Therefore, sensitivity and accuracy of MS-based analyses are linked tightly on efficient sample separation. Separation methods, which lead to fractionated samples are often coupled to matrix-assisted laser desorption/ionization (MALDI), while methods resulting in a continuous separation

such as liquid chromatography (LC), are typically combined with a continuous ionization source like electrospray ionization (ESI) [6]. Using the latter setup, insufficient separation can lead to ion suppression by coeluting analytes. Larger analytes as well as hydrophobic analytes suppress the ionization efficiency of smaller and more polar substances. Also, components of the sample matrix such as non-volatile or ion-pairing substances affect the ionization. They affect the droplet formation and evaporation efficiency and thereby the number of charged molecules reaching the gas phase. In order to minimize this effect, long gradients are used as well as thorough sample cleanup [14, 15]. There are several methods to reduce the complexity of biological samples utilizing different properties of the proteins. As a side effect detergents and non-protein components of the biological matrix are removed. Depending on the used chromatographic material, analytes can be separated according to their size (size exclusion), charge (ion exchange), hydrophobicity (reversed phase) or affinity to immobilized molecules [6]. Sample complexity can be further reduced by combining orthogonally methods for protein separation. Two-dimensional electrophoresis for example combines separation according to the isoelectric point and to the size. Another possibility to improve the sensitivity, is to deplete the sample of high abundant proteins by immunoprecipitation [16, 17]. This however, bears the risk of depleting also substances and proteins which bind either to these high abundant proteins or non-specific to the carrier material [16, 18]. However, every purification and fractionation step is time consuming and accompanied by loss of analytes. Thus, leading to a demand of larger sample amounts. Therefore, an efficient assay has to compromise between high purity and fast preparation of small sample amounts.

Depending on the sample preparation, two different types of MS analyses are distinguished: Top-down, which means analysis of whole proteins, or bottom-up in which case the proteins are digested enzymatically and MS-analysis takes place on the peptide level. Generally, it is more demanding to separate intact proteins than peptides for LC-MS approaches. [6]

The described approaches are well suitable for knowledge independent studies, for example to identify potential biomarkers or treatment-induced differences in the proteome. However, the identified proteins have to be validated in following studies [19]. Therefor knowledge driven MS-methods have been developed. These are selected reaction monitoring (SRM) and targeted selected ion monitoring (tSIM) as well as the multiplexed variants multiple reaction monitoring (MRM) and multiplexed tSIM (msx-tSIM) depending on the type of mass spectrometer. In both cases, parent ions are filtered for a certain mass-to-charge ratio, surveyed and fragmented for identification [15, 20]. Thereby increasing the number of scans and sensitivity of the measurement by one to two orders of magnitude in comparison to full-MS methods [20]. It also results in a linear correlation between peptide amount and signal with a dynamic range up to five orders of magnitude thereby allowing relative and absolute quantification [20].

1.3 Quantitative proteomics

The main objective of proteomics studies is not only to provide a list of proteins expressed under specific conditions but to quantify them, too [6]. The simplest method for relative quantification with a LC-MS setup is to compare extracted ion chromatograms of full-MS methods. SIM measurements improve the sensitivity by restricting the acquisition range to the mass-to-charge ratio of the target peptides. The quantification via SRM assays, on the other hand, is based on quantifying one or more fragments of the monitored peptide [20]. In each case, the method is based on quantifying a protein by analyzing one or more peptides thereof. Hence, the peptides have to be proteotypic, which means that its sequence is unique for one protein of the given species and can therefore be used as stoichiometric surrogate [20, 21]. Relative quantification can be improved and absolute quantification can be achieved by spiking isotopically labeled standards into the samples [15]. Coeluting standards correct for matrix effects such as ion suppression [20]. There are three types of standards used for absolute quantification: peptides (AQUA) and recombinant proteins (PSAQ) labeled

with stable isotopes. The third possibility is to use artificial concatemers, which can contain several labeled standard peptides (QconCAT) [15, 20, 22]. The type of standard defines at which step of the sample preparation the standard is added. While AQUA peptides are added after the enzymatic proteolysis, QconCATs and proteins are added before. This is done to diminish effects on the quantification by differing proteolysis efficiencies. However, it has been shown that peptides are often released more easily from QconCATs than from intact proteins [15, 23]. Labeled proteins can be spiked-in before the sample preparation in case of liquid samples or after lysis in case of tissues and cells. Thereby, using standard proteins allows to correct for analyte losses during fractionation and digestion. The earlier the spike-in of the reference substance, the more accurate the quantification will be [15]. However, production of high quality QconCAT and PSAQ standards is much more difficult than synthesizing AQUA peptides [15, 20]. The lower limits for quantification via LC-SRM assays is typically between 100 and 1000 ng protein per mL plasma. Sample through-put and sensitivity could be improved by enriching the proteins of interest during sample preparation or depleting high abundant proteins.

1.4 MS-based read - out of immunoassays

The enrichment of specific analytes during sample preparation is often done by immunoprecipitation. Typically, the antibodies are either immobilized on column material or the surface of beads is functionalized with the antibodies [24]. Functionalized columns can be used online, but complete analyte elution has to be ensured to avoid analyte carry-over. Functionalized beads, on the other hand, are not prone to carry-over between the samples, because the immunoprecipitation is performed offline and beads are not reused. Bead-based assays are especially convenient for low abundant proteins, because the reaction volume can be scaled up without scaling up the elution volume. Thereby the used sample volume becomes independent of the capacity of the analytical column [22].

The enrichment can be performed either at the protein or the peptide level. Mass spectrometric immunoassays (MSIA) use antibodies which target intact proteins. They can be coupled to top-down and bottom-up MS methods [24, 25]. As with the quantification strategies, it is easier to produce short peptides for the immunization than intact proteins and for bottom-up approaches it is sufficient to enrich the proteotypic peptide chosen for MS quantification. This approach has been realized as stable isotope standards and capture by anti - peptide antibodies assays (SISCAPA) [26]. Analytes can be enriched up to 1000 - fold by SISCAPA thereby achieving quantification in the ng / mL range [15, 27]. Nevertheless, high quality sandwich immunoassays are still more sensitive (fg / mL) [24, 28, 29]. The advantage of MS-based immnuoassays is that they are not hampered by unspecific bindings to the antibodies or the carrier material. Every analyte can be verified by the retention time, co-eluting standards and MS/MS before it is quantified. For this reason, it is also easier to multiplex MS-based immunoassays than sandwich immunoassays. With each additional analyte the background of the sandwich immunoassay is increased by cross-reactivity, while the analytes can still be quantified selectively by the MS-based read – out [21, 24].

The major drawback of both methods is that at least one antibody has to be produced per protein of interest [24]. Therefore, group-specific antibodies have been developed. One approach is to generate antibodies addressing modified amino acids such as phosphorylated tyrosine independent of the surrounding amino acids. These antibodies can be used to investigate for example signaling cascades which rely on protein phosphorylation and dephosphorylation [30, 31]. Antibodies addressing modified amino acids can be used to enrich proteins as well as peptides [24, 30]. Global proteome survey (GPS) and triple X proteomics (TXP), on the other hand, are designed to enrich groups of peptides sharing the same C-terminal epitope. The subsequent LC-MS measurement provides identification as well as quantification of the enriched peptides. [19, 24, 32-36].

1.5 Triple X proteomics (TXP)

Triple X proteomics is a bottom-up approach based on the immunoaffinity enrichment with antibodies addressing short C-terminal epitopes of four amino acids [27]. The fifth and sixth position are not included during the antibody generation, but it has been shown that they can influence the binding. Certain amino acids seem to be favored in this positions, even though hardly any amino acid is excluded [22]. Since sample preparation always includes proteolysis with trypsin, a C-terminal arginine or lysine is an essential part of the epitope [19]. TXP epitopes are too short for peptide specific binding, instead they address dozens to hundreds of peptides of a digested proteome [37]. This property can be facilitated to reduce the number of antibodies needed in comparison to peptide- or protein-specific antibodies. Based on the UniProtKB reference proteome, a proteome-wide study in human would require more than 70'000 conventional antibodies. This could be reduced tenfold by using TXP antibodies [27]. In the context of group specific antibodies, the concepts of specificity and absence of cross-reactivity cannot be used to characterize the quality of TXP antibodies [35]. Most TXP antibodies do not only enrich the desired epitope but also variants thereof [19]. While this is problematic for immunoassays with colorimetric read-out, it just enlarges the number of proteins which can be examined with MS-based read-outs.

To date, the TXP approach is used for knowledge driven proteomics studies. The epitopes are chosen in a manner to cover the proteins of interest with the smallest set of antibodies possible [37]. This is especially applicable for sets of homologous proteins such as Cytochrome P450 enzymes and G-protein coupled receptors [22, 34, 36]. By choosing proteotypic peptides whose C-terminus is located in a conserved region, the peptides derived from the target proteins can be enriched with a small number of antibodies and still be identified and quantified by the LC-MS read-out [19, 22, 36]. It has been shown that the sensitivity of LC-SRM assays can be increased drastically by preceding TXP enrichment [36]. Additionally, tSIM and tSIM with data dependent MS/MS (dd MS/MS) have been tested as read-out. While dd MS/MS increases the

specificity, it also decreases the sensitivity. It has been shown that after TXP enrichment and in combination with a coeluting reference peptide, tSIM is already sufficient to identify and quantify the surrogate peptides [22].

With minor adjustments, the TXP workflow (Figure 1) is used in our laboratory for fluid sample types such as urine, plasma and serum as well as tissue and cell culture preparations [22, 38]. In the case of tissue and cell pellets, the samples have to be lysed first, taking care that the conditions solubilize all proteins of interest for example transmembrane proteins. The subsequent steps are the same for all sample types: they are proteolyzed with trypsin. Subsequently, stable isotope labeled peptides (IS peptides) are added as reference for the quantification. During bead-based immunoprecipitation, TXP antibodies enrich all endogenous peptides (EN) with the respective epitope as well as the spiked-in reference peptides. Finally, eluted peptides are detected and quantified by LC-MS read-out. Since all physicochemical properties but the mass of the according EN and IS peptide are identical, EN and IS peptide pairs are precipitated stochiometrically and coelute from the analytical column. Therefore, quantification via the IS peptide takes into account losses during immunoprecipitation, chromatographic separation and ionization [22].

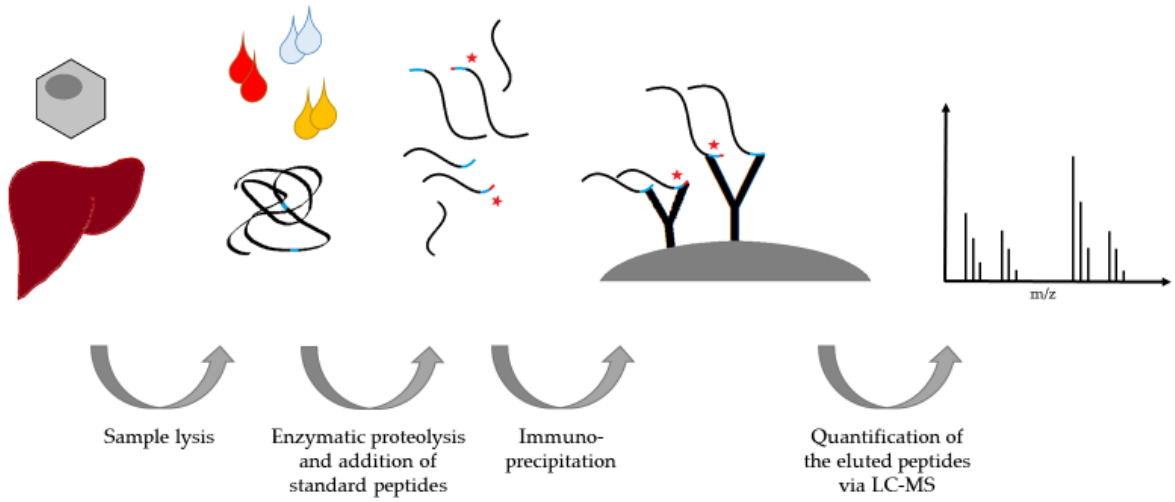


Figure 1: Workflow of Triple X proteomics (TXP). The TXP methodology is applicable for several sample types such as tissue, cell culture preparation, blood preparations and urine. Tissue and cell pellets must be lysed first. The following steps are for all sample types the same: Proteins are enzymatically proteolyzed and isotopically labeled standard peptides are added. TXP antibodies coupled to

magnetic beads enrich all peptides comprising the according C-terminal epitope. Subsequently, peptides are eluted and quantified via LC-MS.

The advantage of using the TXP workflow is that the complexity of the biological sample is reduced drastically with one purification step and peptides of interest are enriched simultaneously. Thereby the peptide signals are increased and suppression effects are minimized while allowing short LC gradients at the same time. Overall, the assays become more sensitive and high throughput feasible [22, 36]. Additionally, the immunoprecipitation removes detergents with high efficiency [34], which makes the method more tolerant towards the conditions of sample lysis and proteolysis. Therefore, the TXP methodology is suitable for transmembrane proteins, which are very hydrophobic [24, 34, 36]. They are solubilized with detergents to make them available for proteolysis. The resulting peptides are better soluble than the intact proteins and suitable for reverse phase chromatography with less or ideally no detergent, which is removed during the precipitation. Furthermore, the bead-based approach is very applicable for low abundant proteins because the amount of antibody and sample can be scaled up independently of the capacity of the analytical column as long as the elution volume is kept constant [22].

1.6 Cytochrome P450 enzymes

Cytochrome P450 enzymes (CYP) are heme containing mixed - function oxidases which are evolutionary conserved [39-41]. Even though they can be found amongst others in bacteria, fungi, plants, insects, fish and mammals [40], the sequence identity of the superfamily is below 20 % [39]. However, topology and folding of the enzymes are conserved. It is composed of a four helices bundle and a signature sequence containing a conserved cysteine, which is the proximal ligand of the heme-iron [39]. Additionally, they contain a N-terminal membrane anchor and a discontinuous membrane binding site, which orients the substrate binding pocket and the heme in the catalytic domain towards the membrane surface [39, 42]. In general, CYPs bind an oxygen molecule via the iron of the prosthetic group. One oxygen atom is reduced to water, the other is introduced into a lipophilic substrate making it more polar. This can

result in hydroxylation, dealkylation or oxidation of the substrate [43]. The required electrons for the reduction are provided by NADPH and a reductase protein such as cytochrome P450 reductase [44, 45].

CYPs are classified according to their sequence identity. The CYP superfamily is divided into families, which are denoted by a number, and subfamilies, which are indicated by letters. The individual proteins of a subfamily are numbered consecutively. Proteins of the same family share over 40 % of their primary structure and subfamilies over 55 % in the case of mammalians [43, 46]. For some isoforms, there are homologs in other species, they were named accordingly, otherwise the isoforms were numbered in the order of their discovery irrespective of the species [46]. In case of gene clusters there are not always exact orthologs for every gene [46, 47]. In the human genome 57 CYPs have been identified, 87 in rats and 102 in mice [41, 47]. Examples for orthologous CYPs from are listed in Table 1 [47, 48]:

Table 1: Selected orthologous CYP genes in human, rat and mouse [47, 48].

human	rat	mouse
CYP1A1	*Cyp1a1*	*Cyp1a1*
CYP1A2	*Cyp1a2*	*Cyp1a2*
CYP2B6	*Cyp2b1*	*Cyp2b10*
	Cyp2b2	*Cyp2b13*
	Cyp2b3	*Cyp2b9*
	Cyp2b12	*Cyp2b19*
	Cyp2b15	
	Cyp2b31	
	Cyp2b21	*Cyp2b23*
CYP2C8	*Cyp2c55*	*Cyp2c55*
CYP2C9		
CYP2C18		
CYP2C19		
CYP2E1	*Cyp2e1*	*Cyp2e1*
CYP3A4	*Cyp3a9*	*Cyp3a13*
CYP3A5		
CYP3A7		

In mammals, CYPs are located at the cytosolic side of the endoplasmic reticulum and the inner membrane of the mitochondria and serve two major functions [42]. While all CYP families are part of the biosynthesis and metabolism of endogenous substances such as hormones, bile acids and vitamins, the CYP families 1, 2 and 3 are major parts of the oxidative metabolism of xenobiotics [42, 43].

The subfamily Cyp1a consists of two proteins in human, mouse and rat which are highly conserved. Cyp1a1 and Cyp1a2 recognize planar substances like polycyclic aromatic hydrocarbons and arylamines as substrates. Similarly, Cyp1b1 and Cyp2e1 are strongly conserved between species and are the only members of their respective subfamily. The Cyp2a subfamily includes three human, three rattine and four murine isoforms. Even though, CYPs are classified across species, minor differences may lead to drastic changes in substrate specificity and catalytic activity. The rodent Cyp2a isoforms, for example, catalyze the hydroxylation of steroids, while human Cyp2a6 oxidizes substances like aflatoxin B1 and nicotine and shows a great substrate overlap with Cyp2e1. Cyp2c is the most diverse subfamily harboring four human, seven rattine and nine murine enzymes. As for Cyp2a, the substrate specificities differ greatly between human and rodent Cyp2c isoforms. Additionally, the expression of some isoforms is gender dependent in adult rats: Cyp2c12 and Cyp2c13 are female – and male – specific isoforms respectively. [43]

The subfamily Cyp3a recognizes a very broad range of substrates and is therefore very important in xenobiotic metabolism. Humans express four and rats and mice each express six Cyp3a isoforms [43]. It was estimated that together, CYPs are involved in the metabolism of 70 -80 % of all clinically used drugs [41].

1.7 Transporters

Transporters control the traffic of substances such as sugars, amino acids, inorganic ions and xenobiotics across membranes [49]. There are estimations that 2-5 % of the human genes are transporters or transport related proteins [49, 50]. They can be divided into five groups: Channels, carriers, group translocators and primary as well as secondary active transporters. Channels and carriers facilitate protein-mediated diffusion in the direction of a concentration gradient. Primary active transporters use a direct energy source such as ATP hydrolysis or light to transport substrates and generate a concentration gradient. Secondary active transporters can also give rise to a concentration gradient, but they use a secondary source of energy such as proton or sodium gradients which are maintained using primary energy sources. Group translocators modify their substrates in the course of the transport [51]. In the following ATP-binding cassette (ABC) transporters and solute carriers (SLC) which are primary and secondary active transporters respectively, will be discussed.

The nomenclature of the CYP superfamily has been transferred to other gene families including the ABC and SLC transporter families [49, 50, 52]. A letter identifies the subfamily and a consecutive number the individual transporter in case of the ABC transporters [52]. For SLC transporters, the families are indicated by Arabic numerals followed by an A as spacer and a number identifying the individual transporter. In contrast to CYPs, SLC proteins with more than 20 % amino acid identity are already assigned into the same family [49]. The subfamily SLC21 has been renamed to SLCO to be able to classify its members exactly like CYPs [53]. For the proteins, the nomenclature is not well standardized. For most proteins, several synonyms are in use. Therefore, all CYPs and transporters discussed in this thesis are identified by the UniProt ID in the supplemental information (Table 56).

In general, ABC transporters have four conserved domains and can be found in all living organisms [50, 54]: Two transmembrane domains, which consist of six helices and two intracellular nucleotide binding domains containing the ATP – binding cassette. The multidrug resistance associated proteins MRP1, MRP2 and MRP3 differ

in that respect that they have an additional N-terminal transmembrane domain consisting of five helices [50, 54]. The breast-cancer-resistance-protein (BCRP), on the other hand, is a half-transporter. It consists of one transmembrane domain and one nucleotide binding domain. Therefore, it has to form a dimer to become functional [50, 54, 55]. ABC transporters are efflux pumps, which use the consecutive hydrolysis of two ATP molecules to transport their substrates independent of concentration gradients [50, 51, 55]. So far 49 different ABC transporters which belong to seven subfamilies have been described in humans: Multidrug resistance protein 1 (MDR1) and bile salt export pump (BSEP) are the most prominent of the ABCB family. They are located in the apical membrane of the cells and transport various hydrophobic and cationic substances such as phenobarbital (PB) and bile acids respectively [50, 56]. It has been reported that the amount of MDR1 can differ 50-fold in humans [56, 57]. In contrast to humans, rodents have two closely related isoforms of MDR1 [50, 56]. They have overlapping expression patterns, but while the loss of *Mdr1b* can be compensated by *Mdr1a* in knockout mice, *Mdr1a* is essential for the maintenance of the blood – brain – barrier [58-61]. The ABCC family consists of twelve members in humans, nine of which are transporters [50]. The gene products are designated multidrug resistance associated proteins (MRP). MRP1 is located in the basolateral membrane and transports organic anions such as glutathione conjugates as well as positive and neutral amphiphilic substances. In addition, it can also co-transport ions with unconjugated glutathione [50, 55, 56]. MRP2, on the other hand, has a similar substrate specificity as MRP1 but is located in the apical membrane. Like BSEP, it is involved in the secretion of bile acids. In contrast to BSEP, MRP2 only transports sulfated bile acids [50]. The basolaterally expressed MRP3 transports similar substances to MRP1, MRP2 and BSEP, but binds them with a lower affinity. In contrast, MRP4 is localized differentially. In the liver, it is expressed basolaterally and apically in the kidney. Thereby, facilitating the clearance of a substance from the liver via the blood into the urine. [50].

In contrast to the ABC transporters, the SLC transporter superfamily consists of at least 43 families and the structure has yet to be resolved [49, 62]. For the SLC22A family, twelve alpha-helical transmembrane segments are predicted with intracellularly located N- and C-termini as well as intracellular consensus sequences for phosphorylation [63, 64]. The family consists of organic anion transporters (OAT) and organic cation transporters (OCT) as well as zwitterion/cation transporters (OCTN). They can function as uniporters, symporters or antiporters [51, 64]. In case of the symporters and antiporters, they utilize an existing gradient to drive uphill transport of one substrate [65]. OAT1, OAT3 and OAT4 are examples of antiporters. They mediate the uptake of extracellular substances such as prostaglandins in exchange for 2-oxoglutarate [64, 66]. OAT1 and OAT3 are expressed basolaterally while OAT4 is expressed apically. Together, they are involved for example in the renal excretion and reabsorption process of prostaglandins [64]. The members of the SLCO family, on the other hand, transport amphiphilic organic substances such as bile acids and steroid conjugates in exchange for intracellular substances such as bicarbonate [66]. While in the intestinal tract, SLCO transporters are expressed in the luminal membrane, they mediate uptake from the blood in hepatocytes, proximal tubule cells and the endothelial cells of the brain capillaries [62]. In contrast to other transporter families, they are not conserved well between species and for some human SLCO transporters, there are no orthologs described in rodents, yet [66].

In summary, it can be said, that there is a great overlap in the substrate specificity of individual transporters within transporter families as well as between ABC and SLC transporters. This allows the net movement of substances through cells as well as from one organ to another [50, 62]. But it may also allow very tight regulation and specialization by expressing transporters with similar but different substrate specificities and affinities only in specific tissues or subcellular localizations [63].

1.8 Regulation of CYP and transporter expression

The tightly regulated expression of CYPs and transporters has already been addressed in the context of subcellular localization and organ differences. But they are also differentially expressed within organs. For murine kidney for example it has been shown that MRP1 is expressed in cells of Henle's loop and the cortical collecting duct but not proximal tubular cells [50, 67]. Even though it is not as obvious as in the kidney, the liver tissue is also not uniform. The liver is composed of structural and functional units called lobules. The afferent blood vessels, the portal vein and the hepatic artery, are localized at the corners of the lobule and the efferent central vein in the middle. Along the blood flow, the hepatocytes can be divided into periportal, midzonal and pericentral cells [68-70]. The hepatocytes differ also with respect to their expression pattern and metabolic activity. Bile synthesis and glutamine synthesis is restricted to pericentral hepatocytes, while cholesterol synthesis is restricted to periportal cells. Gluconeogenesis and fatty acid degradation, on the other hand, take place in all hepatocytes but is gradually reduced in the direction of pericentral cells. Glycolysis is regulated contrarily and picks up towards the pericentral hepatocytes [68]. The expression of enzymes belonging to the xenobiotic metabolism, on the other hand, is dynamic. Under normal conditions they are expressed in a few layers of pericentral hepatocytes, but the expression can expand toward the periportal cells in the presence of inducing agents such as phenobarbital. [69]

The xenobiotic metabolism is highly adaptable to environmental influences such as food components. Two receptor types are mainly involved in the regulation of the xenobiotic metabolism which are also transcription factors: The aryl hydrocarbon receptor (AhR) and orphan nuclear receptors [71]. The AhR binds next to halogenated aryl hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) also other hydrocarbon ring systems such as polychlorinated biphenyls (PCB) and prochloraz (Prz) as long as they can assume a planar conformation [71-73]. The AhR is a cytosolic receptor which translocates into the nucleus upon ligand binding. There it dimerizes with Ah receptor nuclear translocator (Arnt), which is restricted to the nucleus. The

translocation of AhR to the nucleus does not dependent on Arnt, but the dimerization is required for DNA binding [71]. The expression of Cyp1a and Cyp1b1 as well as BCRP, BSEP, NTCP and MDR1 is influenced by AhR (Table 2) [43, 50, 74].

Table 2: Regulation of CYP and transporter expression by nuclear receptors. CAR, PXR and AhR can induce as well as repress the expression of CYPs and transporters. Target genes are indicated in italics, the according protein in brackets.

gene (protein)	CAR	PXR	AhR
Abcg2 (BCRP)	induction [74]	induction [74]	induction [74]
Abcb11 (BSEP)	repression [74]	repression [74]	repression [74]
Cyp1a (Cyp1a)			induction [50]
Cyp1b1 (Cyp1b1)			induction [43]
Cyp2a5/6 (Cyp2a5/6)	induction [43]	induction [43]	
Cyp2b (Cyp2b)	induction [43, 50]	induction [43]	
Cyp3a (Cyp3a)	induction [43]	induction [43]	
Abcb1 (MDR1)	induction [74]	induction [56, 74]	induction [74]
Abcc2 (MRP2)	induction [74]	induction [74] repression [50]	
Abcc3 (MRP3)	induction [50]	induction [74]	
Slc10a1 (NTCP)	repression [74]	repression [74]	repression [74]
Slc22a7 (OAT2)	repression [65, 74]	repression [74]	repression [74]
Slc22a1 (OCT1)	repression [74]	repression [74]	repression [74]
SLCO1B3 (SLCO1B3)	repression [74]		repression [74]
SLCO2B1 (SLCO2B1)	repression [74]	induction [74]	repression [74]

Orphan nuclear receptors bind steroid based ligands and consist of a highly conserved DNA-binding domain and a ligand binding domain. Pregnane X receptor (PXR), constitutive androstane receptor (CAR) and retinoid X receptors (RXR) are examples for orphan nuclear receptors. While RXR can also bind as homodimer to its response element, CAR and PXR form heterodimers with RXR. CAR, PXR and RXRα are

predominantly expressed in the liver [71], which is the main site for xenobiotic metabolism [43]. Like AhR, CAR and PXR translocate into the nucleus subsequently to ligand binding [75]. While PB and rifampicin are model agonists of CAR and PXR respectively [75], there are also substances which can activate both receptors such as Clotrimazole and the azole fungicides cyproconazole (Cypro) and epoxiconazole (Epoxi) [73, 76]. PXR and CAR address overlapping targets such as *Abcg2, Abcb1, Cyp2b* and *Cyp3a* (Table 2) [43, 74].

The expression pattern cannot only be influenced by environmental impacts but it can also be changed under pathological conditions. OCT1 has been reported to be down regulated in the liver during obstructive cholestasis [63]. The expression pattern of tumors can also be very different from the surrounding tissue. Cyp1b1, for example, is expressed much higher in breast cancer than the surrounding tissue [43]. In the context of multidrug resistance, ABC – transporters are frequently found to be overexpressed [55, 56, 77].

1.9 Biological and medical relevance of CYPs and transporters

CYPs and transporters are involved in processes, such as xenobiotic metabolism, barrier maintenance, bioavailability, drug-drug interaction and multidrug resistance [55, 71, 78-82]. The xenobiotic metabolism can be divided into four steps. First, the substances have to enter the cell to enable intracellular metabolism. This is dramatically accelerated by uptake transporters such as SLC22 transporters [64]. During phase I a functional group is introduced into the substrates by oxygenases. Among others, Flavin – containing monooxygenases, monoamine oxidases and CYPs are counted to the phase I enzymes. Phase II enzymes include transferases such as sulfotransferases, glutathione S-transferases, UDP-glucuronosyltransferases and N-acetyl transferases, but also epoxide hydrolases and reductases. The conjugated products of the phase II metabolism are in general better soluble. Thereby phase II metabolism improves the excretion via urine and bile. Phase III describes the secretion of the metabolized xenobiotics into the blood or the bile. This is mediated by export

transporters such as MDR1 and MRP proteins. Secretion into the blood ultimately leads to excretion via renal clearance. [71]

Chemotherapeutics are common drugs for the treatment of cancer. However, while some patients can be cured, others respond temporarily or incompletely. This phenomenon is called cancer drug resistance[55]. It can be due to impaired drug delivery or to genetic or epigenetic alterations of the tumor cells [55, 83]. Cellular drug resistance is often gained during treatment and shows cross-resistance to other therapeutics which is called multidrug resistance. The classical multidrug resistance is mediated by overexpression of ATP transporters such as MDR1 and MRP which reduces the drug concentration in the tumor cell drastically [55, 83, 84]. The same effect is achieved by down-regulating influx transporters and inducing detoxifying enzymes such CYPs. Additionally, the tumors cells become more resistant by increasing the DNA repair and evading apoptosis. [55]

CYPs and transporters have great impact on the pharmacokinetic and the toxicity of a substance. At the same time, drugs can change the expression levels of CYPs and transporters as well as act as inhibitors. Thereby they also change their own bioavailability, metabolism and elimination as well as the pharmacokinetics of co-administered drugs. Therefor they are examined thoroughly during drug development [85-87]. In the preclinical phase, *in vitro* as well as animal *in vivo* models are used to predict kinetics and toxicity in man [43, 85, 88]. However, even though CYPs and transporters are highly conserved, the exchange of one amino acid can alter the substrate specificity and the catalytic activity [43]. Therefore, it is important to choose the right animal model with respect to the drug metabolism as well as the study objective. Drug development involves studies in mouse, rat, rabbit, dog, pig, monkey and man [85]. In addition, humanized mouse models have become important in recent years [89].

2 Objective

The objective was to develop TXP assays for the quantification of CYPs and transporters. Based on previously developed assays for human CYPs [22], TXP assays for human, rat and mouse ABC and SLC transporters should be developed. Additionally, the approach was expanded to also address CYPs in rat and mouse.

The TXP methodology is very suitable for the quantification of CYPs and transporters. Both protein classes consist of very homologous superfamilies. They can be addressed efficiently by TXP antibodies by choosing conserved epitopes. At the same time, the methodology is not limited by unspecific antibody binding because of the MS – based read – out. The immunoprecipitation with TXP antibodies is a sample preparation method which allows to address very low abundant analytes like transporters as well as highly inducible proteins like CYPs. Additionally, by addressing surrogate peptides instead of proteins, solubility issues of intact transmembrane proteins such as transporters are circumvented. Furthermore, conserved TXP epitopes cannot only be used to address several proteins in the same species with one antibody, but to analyze target proteins in several species using the same antibody.

The project included the selection of suitable TXP epitopes, the development of multiplexed TXP assays as well as the analysis of several studies in man, rat and mouse (Figure 2). The developed assays were applied to compare the protein expression profiles of sample sets, e.g. normal versus tumor tissue, as well as to investigate the induction potential of fungicides in different *in-vitro* and *in-vivo* models.

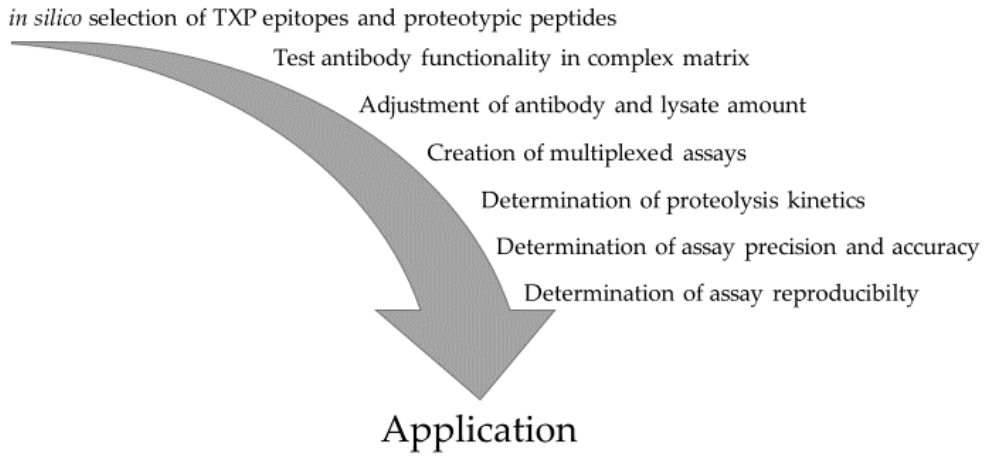


Figure 2: Workflow of the TXP assay development.

Objective

3 Materials and Methods

3.1 Materials

3.1.1 Expendable items

Table 3: Expendable items

Item	Type denotation	Manufacturer
96 tip comb for PCR magnets	KingFisher®™ 96 tip comb	Thermo Fisher Scientific, Waltham, MA, USA
analytical column	Acclaim PepMap RSLC 75 µm x 15 cm, nanoViper	Thermo Fisher Scientific, Waltham, MA, USA
autosampler glass bottle	Vial short thread, 1.5 mL, amber glass + label	VWR, Darmstadt, DE
autosampler glass insert	Micro-Insert, 0.1 mL, clear glass 15mm, top	VWR, Darmstadt, DE
Disposable bag		Sarstedt. Nümbrecht, DE
glass bottle 500 mL, 1000mL	DURAN® Laboratory bottle with DIN thread, GL 45	Duran Group GmbH, Wertheim/Main, DE
Glass pasteur pipette		WU Mainz, Mainz, DE
Heat Sealing Foil Sheets	Peelable Heat Sealing Foil Sheets	Thermo Fisher Scientific, Waltham, MA, USA
hypodermic needles on Luer connectors	Sterican® Gr. 1, G 20 x 1 1/2" / ø 0,90 x 40 mm	B. Braun Melsungen AG, Melsungen, DE
Ligand-Coupled magnetic beads	Dynabeads® Protein G	Thermo Fisher Scientific, Waltham, MA, USA
Microplate sealing tape	Axygen® AxySeal	Corning Incorporated, Corning, NY, USA
microtiter plate, 96 well, F bottom	Microplate, 96 well, PS, F-Bottom, clear	Greiner Bio-One, Frickenhausen, DE
microtiter plate, 96 well, V bottom	0.2 mL Skirted 96-well Robotic Plate	Thermo Fisher Scientific, Waltham, MA, USA
nano electrospray emitter	Stainless Steel Nano-bore emitters	Thermo Fisher Scientific, Waltham, MA, USA
Needle collecting box	Medibox	B. Braun Melsungen AG, Melsungen, DE
nitrile gloves	Nitrile, unsterile, powder free, structured surface	VWR, Darmstadt, DE
pH indicator paper	Universal indicator paper pH 1-14	Carl Roth, Karlsruhe, DE
pipette tips 10 µL	epT.I.P.S. Standard 0.1-10 µL	Eppendorf, Hamburg, DE
pipette tips 1000 µL	epT.I.P.S. Standard 50-1000 µL	Eppendorf, Hamburg, DE
pipette tips 1200 µL	Tips, 1250 µL, QUICKRACK Tip Transfer System	Biozym Scientific, Hessisch Oldendorf, DE
pipette tips 200 µL	epT.I.P.S. Standard 2-200 µL	Eppendorf, Hamburg, DE
pipette tips 250 µL	LTS tips 250 µL	Mettler-Toledo, Columbus, OH, USA
pipette tips 300 µL	LTS tips 300 µL	Mettler-Toledo, Columbus, OH, USA
pipette tips 5000 µL	epT.I.P.S. Standard 100-5000 µL	Eppendorf, Hamburg, DE

Item	Type denotation	Manufacturer
pipette tips for positive displacement pipette	25 µL Drummond Microdispenser Replacement tubes	Drummond Scientific Company, Broomall, PA, USA
plastic medical syringe, 2 mL	Injekt®-F	B. Braun Melsungen AG, Melsungen, DE
Plastic bottles	Wash Bottles Narrow-Neck, Technical Grade-PFA	Brand GMBH + CO KG, Wertheim, DE
Precolumn	µ-Precolumn 300µm i.d. x 5 mm C18 PepMap 100, 5 µm, 100 Å	Thermo Fisher Scientific, Waltham, MA, USA
reaction tube 0.2 mL	PCR Tube Strips 0.2 mL	Eppendorf, Hamburg, DE
reaction tube 0.65 mL	Mµlti®-safety microcentrifuge tubes	Carl Roth, Karlsruhe, DE
reaction tube 1.5 mL	Eppendorf Tubes® 3810X	Eppendorf, Hamburg, DE
reaction tube 15 mL	Cellstar tubes, 15 mL, PP, graduated, conical bottom	Greiner, Frickenhausen, DE
reaction tube 50 mL	Cellstar tubes, 50 mL, PP, graduated, conical bottom with support	Greiner, Frickenhausen, DE
screw cap for autosampler glass bottle	PP screw cap 9 mm, tr. Natural Rubber red-orange/TEF, 1mm	VWR, Darmstadt, DE
screw cap for reaction tubes (1.5 mL and 2 mL)	screw cap	neoLab, Heidelberg, DE
screw cap glass bottle	GL 45 Screw Caps	Duran Group GmbH, Wertheim/Main, DE
screw cap reaction tube 1.5 mL	neoLab-Reaction vessels with screw thread, 1.5 ml, conical	neoLab, Heidelberg, DE
screw cap reaction tube 2 mL	neoLab-Reaction vessels with screw thread, 2.0 ml, self-standing	neoLab, Heidelberg, DE

3.1.2 Chemicals and Biochemicals

Table 4: Chemicals and Biochemicals

Reagent	Abbreviation	Manufacturer
2-Propanol, LC-MS Grade	IPA	BioSolve BV, Valkenswaard, NL
3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate	Chaps	Carl Roth, Karlsruhe, DE
Acetic Acid 100 %		Carl Roth, Karlsruhe, DE
Acetonitrile, LC-MS Grade	ACN	LGC Promochem, Wesel, DE
Albumin fraction V (protease-free)	BSA	Carl Roth, Karlsruhe, DE
Ammonia solution 25 %, Rotipuran		Carl Roth, Karlsruhe, DE
Ammonium bicarbonate	ABC	Sigma-Aldrich, St. Louis, MO, USA
Benzonase Nuclease, >99 % purity		Merck, Darmstadt, DE
Blocking Reagent for ELISA		Roche Diagnostics, Mannheim, DE
Complete (protease inhibitor cocktail) tablets	Complete	Roche Diagnostics, Mannheim, DE
Customized polyclonal antibody sera		Pineda Antikörper Service, Berlin, DE
Customized synthetic standard peptides		Intavis AG, Tübingen, DE

Reagent	Abbreviation	Manufacturer
Deoxycholic Acid sodium salt	DOC	Carl Roth, Karlsruhe, DE
Dimethyl sulfoxide	DMSO	Sigma-Aldrich, St. Louis, MO, USA
Dionex™ Cytochrome C Digest		Thermo Fisher Scientific, Waltham, MA, USA
Di-sodium hydrogen phosphate 2-Hydrate		Carl Roth, Karlsruhe, DE
Ethylenediamine tetraacetic acid	EDTA	Carl Roth, Karlsruhe, DE
Formic Acid, 99 %	FA	Thermo Fisher Scientific, Waltham, MA, USA
Hydrochloric acid fuming 37 %	HCl	Carl Roth, Karlsruhe, DE
Iodoacetamide	IAA	Sigma-Aldrich, St. Louis, MO, USA
LTQ Velos ESI Positive Calibration Solution		Thermo Scientific, Waltham, MA, USA
Magnesium chloride		Sigma-Aldrich, St. Louis, MO, USA
Methanol, ROTISOLV		Carl Roth, Karlsruhe, DE
n-octyl-β-glucopyranoside	NOG	AppliChem, Darmstadt, DE
NP40 Surfact Amps Detergent Solution	NP-40	Thermo Scientific, Waltham, MA, USA
phenylmethanesulfonyl fluoride	PMSF	Roche Diagnostics, Mannheim, DE
Phosphate Buffered Saline, 10x	PBS	Thermo Fisher Scientific, Waltham, MA, USA
Pierce Trypsin Protease, MS-Grade		Thermo Fisher Scientific, Waltham, MA, USA
Pierce™ BCA Protein Assay Kit		Thermo Scientific, Waltham, MA, USA
Sequencing Grade Modified Trypsin (with 50 mM Acetic acid as resuspension buffer)		Promega Corporation, Fitchburg, WI, USA
Sodium bicarbonate		Merck, Darmstadt, DE
Sodium chloride	NaCl	Carl Roth, Karlsruhe, DE
Sodium dihydrogen phosphate dihydrate		Carl Roth, Karlsruhe, DE
Sodium dodecyl sulfate	SDS	Sigma-Aldrich, St. Louis, MO, USA
Sodium hydroxide	NaOH	Carl Roth, Karlsruhe, DE
Technical buffer solution pH 4.01		Mettler-Toledo, Columbus, OH, USA
Technical buffer solution pH 7.00		Mettler-Toledo, Columbus, OH, USA
Technical buffer solution pH 9.21		Mettler-Toledo, Columbus, OH, USA
Triethanolamine	TEA	Carl Roth, Karlsruhe, DE
Trifluoroacetic acid ULC/MS Optigrade	TFA	LGC Promochem, Wesel, DE
Tris(2-carboxyethyl)phosphine	TCEP	Carl Roth, Karlsruhe, DE
Triton X-100 Surfact Amps Detergent Solution		Thermo Scientific, Waltham, MA, USA
Trypsin from bovine pancreas		Sigma-Aldrich, St. Louis, MO, USA
Water (LC-MS grade)		Carl Roth, Karlsruhe, DE

3.1.3 Laboratory equipment

Table 5: Laboratory equipment

Apparatus	Type denotation	Manufacturer
analytical balance	explorer	OHAUS Waagen, Bad Hersfeld, Deutschland
analytical balance	CPA225D-0CE	Sartorius Stedim Biotech, Göttingen, DE
Automated magnetic-particle processor	KingFisher™ 96	Thermo Scientific, Waltham, MA, USA
Automated magnetic-particle processor	KingFisher™ Flex Purification System	Thermo Scientific, Waltham, MA, USA
Ball mill	Mikro-Disembrator U	Sartorius, Göttingen, DE
centrifuge for PCR plates	Universal 30 F	Hettich, Tuttlingen, DE
centrifuge for PCR tubes	MiniStar silverline	VWR, Darmstadt, DE
centrifuge for reaction tubes	Rotilabo®-mini-centrifuge "Uni-fuge"	Carl Roth, Karlsruhe, DE
centrifuge for reaction tubes	5415 D	Eppendorf, Hamburg, DE
centrifuge for reaction tubes and PCR plates, cooled	5810R	Eppendorf, Hamburg, DE
centrifuge for reaction tubes, cooled	5417 R	Eppendorf, Hamburg, DE
Electronic Pipette 5-200µL	eppendorf research pro	Eppendorf, Hamburg, DE
Electronic Pipette 5-300µL	E4 XLS	Mettler-Toledo, Columbus, OH, USA
Filter-based multi-mode microplate reader	FLUOstar Optima Microplate Reader	BMG Labtech, Ortenberg, DE
Flake ice maker	Scotman AF40	Frimont S.p.A., Pogliano Milanese, IT
heat sealer	Abgene Combi Thermo Plate Heat Sealer	Thermo Scientific, Waltham, MA, USA
magnet for microtiter plate	Dynal -96 Side Skirted	Thermo Scientific, Waltham, MA, USA
Magnet for of Dynabeads in 1.5mL reaction tubes	DynaMag Spin	Thermo Scientific, Waltham, MA, USA
Magnetic comb for King Fisher system	KingFisher®™ 96 PCR head	Thermo Scientific, Waltham, MA, USA
Magnetic stirrer	RCT basic	IKA®-Werke, Staufen, DE
Mass Spectrometer	Q Exactive™ - Orbitrap Mass Spectrometer	Thermo Scientific, Waltham, MA, USA
Mass Spectrometer	Q Exactive Plus™ - Orbitrap Mass Spectrometer	Thermo Scientific, Waltham, MA, USA
micropipette 0.1 - 2.5 µL	eppendorf research	Eppendorf, Hamburg, DE
micropipette 1 - 10 µL	eppendorf research	Eppendorf, Hamburg, DE
micropipette 10 - 100 µL	eppendorf research	Eppendorf, Hamburg, DE
micropipette 100 - 1000 µL	eppendorf research	Eppendorf, Hamburg, DE
micropipette 2 - 20 µL	eppendorf research	Eppendorf, Hamburg, DE
micropipette 20 - 200 µL	eppendorf research	Eppendorf, Hamburg, DE
micropipette 50 - 5000 µL	eppendorf research	Eppendorf, Hamburg, DE
Multichannel Electronic Pipette 1-10µL	eppendorf research pro	Eppendorf, Hamburg, DE

Apparatus	Type denotation	Manufacturer
Multichannel Electronic Pipette 50-1200µL	eppendorf research pro	Eppendorf, Hamburg, DE
Multichannel Electronic Pipette 5-200µL	eppendorf research pro	Eppendorf, Hamburg, DE
Multichannel Pipette 5-50µL	Pipet-Lite XLS	Mettler-Toledo, Columbus, OH, USA
pH-meter	pH-Meter 766	Knick, Berlin, DE
porcelain mortar	mortar with spout, glazed, Size 0 a	Morgan Advanced Materials Haldenwanger GmbH, Waldkraiburg, DE
porcelain pestles	pestle, grinding surface unglazed, size 0 a	Morgan Advanced Materials Haldenwanger GmbH, Waldkraiburg, DE
positive displacement pipette 2-25 µL	Positive Displacement Digital Microdispensers	Drummond Scientific Company, Broomall, PA, USA
Rotating mixer	RM5	Assistent, Sondheim, DE
shaking incubator with temperature control	Thermo Mixer Comfort	Eppendorf, Hamburg, DE
Sonication bath	Sonorex RK 31	Bandelin, Berlin, DE
Sonication bath	Transsonic T780/H	Elma, Singen, DE
SWC Safety Weighing Cabinet	SWC Safety Weighing Cabinet	Sartorius Stedim Biotech, Göttingen, DE
Ultra High Performance Liquid Chromatography System	UltiMate 3000 RSLCnano System	Thermo Scientific, Waltham, MA, USA
vibrating and rotating sample mixer	Hulamixer Sample mixer	Life Technologies, Carlsbad, CA, USA
vortex mixer	Vortex-Genie 2	Scientific Industries, Bohemia, NY, USA
Water purification system	Milli Q Plus	Merck Millipore, Billerica, MA, USA
Water purification system	arium® 611VF	Sartorius Stedim Biotech, Göttingen, DE

3.1.4 Software

Table 6: Software

Software	Distributor
Adobe Illustrator CS5	Adobe, San Jose, CA, USA
Adobe Photoshop CS5	Adobe, San Jose, CA, USA
Chromeleon 6.8	Thermo Scientific, Waltham, MA, USA
Chromeleon Client 6.8	Thermo Scientific, Waltham, MA, USA
Endnote X7	Thomson, Philadelphia, PA, USA
etiLABEL	ETISOFT, Delmenhorst, DE
MS Office 2010, 2013 and 2016	Microsoft, Redmond, WA, USA
Origin 2015G	OriginLab Corporation, Northampton, MA, USA
Pinpoint 1.4	Thermo Scientific, Waltham, MA, USA
Proteome Discoverer 1.3	Thermo Scientific, Waltham, MA, USA

Skyline 3.5.0.9319	MacCoss Lab, Department of genome sciences, University of Washington, Seattle, WA, USA
Tune 2.5	Thermo Scientific, Waltham, MA, USA
TXP-Tools	Internal script by Hannes Planatscher
ultraVNC viewer 1.2.0.4	www.uvnc.com
Windows XP, Windows 7	Microsoft, Redmond, WA, USA
Xcalibur 3.0	Thermo Scientific, Waltham, MA, USA

3.1.5 Databases

Table 7: Databases

Database	Date	Distributor
Mascot	Version 2.3.02	Matrix Science Ltd., London, GB
PaxDb (Protein Abundance Database)	Version 4	University of Zurich, Zurich, CH
SEQUEST	Version 28.0.0.0	University of Washington, Seattle, WA, USA
unigene	September 2015	National Center for Biotechnology Information, U.S. National Library of Medicine, Bethesda, MD, USA
UniProtKB (UniProt Knowledgebase)	specified in the figure legends	UniProt Consortium

3.1.6 Buffers and solutions

3.1.6.1 Lysis

Table 8: SDS stock solution

reagent	final concentration
SDS	10 % (w/v)
d. water	

Table 9: Lysis buffer 1 -/-

reagent	final concentration
Triton (10 % (v/v))	0.5 % (v/v)
SDS (10 % (w/v))	0.01 % (v/v)
NaCl	0.15 M
Sodium dihydrogen phosphate dihydrate	0.01 M
EDTA	0.002 M
d. water	
pH	7.2

Table 10: Protease- Inhibitor stock solution

reagent	final concentration
Complete	10 x
Lysis buffer X -/-	

Table 11: Lysis buffer 1 +/-

reagent	final concentration
Protease- Inhibitor stock solution	1 x
Lysis buffer 1 -/-	

Table 12: Lysis buffer 1 +/+

reagent	final concentration
Benzonase	1 U
Lysis buffer 1 +/-	

Table 13: Lysis buffer 2 -/-

reagent	final concentration
NP-40 (10 % (w/v))	1 % (v/v)
SDS (10 % (w/v))	0.01 % (v/v)
NaCl	0.15 M
Sodium dihydrogen phosphate dihydrate	0.01 M
EDTA	0.002 M
d. water	
pH	7.2

Table 14: Lysis buffer 2 +/-

reagent	final concentration
Complete	1 x
Lysis buffer 2 -/-	

Table 15: Lysis buffer 3

reagent	final concentration
Deoxycholic acid sodium salt	1 % (w/v)
SDS (10 % (w/v))	0.01 % (v/v)
NaCl	0.15 M
Sodium dihydrogen phosphate dihydrate	0.01 M
EDTA	0.002 M
Complete	1 x
d. water	
pH	7.2

3.1.6.2 Enzymatic proteolysis

Table 16: TEA buffer

reagent	final concentration
TEA	200 mM
d. water	
pH	8.5

Adjust pH with 25 % ammonia solution.

Table 17: TCEP stock solution

reagent	final concentration
TCEP	1 M
d. Water	

Aliquots can be stored at -20°C.

Table 18: NOG stock solution

reagent	final concentration
NOG	10 % (w/v)
d. water	

Table 19: IAA solution

reagent	final concentration
IAA	0.5 M
d. water	

Prepare IAA solution always right before use.

Table 20: Trypsin solution

reagent	final concentration
Trypsin	1 mg /mL
Resuspension buffer	

Aliquots can be stored at -20°C.

Table 21: PMSF stock solution

reagent	final concentration
PMSF	200 mM
Ethanol	

Aliquots can be stored at -20°C.

3.1.6.3 Immunoprecipitation

Table 22: Blocking buffer

reagent	final concentration
Blocking Reagent for ELISA	1 x
d. water	

Table 23: CHAPS stock solution

reagent	final concentration
CHAPS	10 % (w/v)
d. water	

Table 24: ABC stock solution

reagent	final concentration
ABC	100 mM
d. water	
pH	7.4

Adjust pH to 7.4

Table 25: PBSC

reagent	final concentration
PBS (10 x)	1 x
CHAPS (10 %)	0.03 % (w/v)
d. water	

Table 26: ABCC

reagent	final concentration
ABC	50 mM
CHAPS (10 %)	0.03 % (w/v)
d. water	

Table 27: Elution buffer

reagent	final concentration
FA	1 %
d. water	

3.1.6.4 LC-MS measurement

Table 28: Loading buffer

reagent	final concentration
ACN	2 %
TFA	0.05 %
water (LC-MS grade)	

Table 29: Mobile phase A

reagent	final concentration
FA	0.1 %
water (LC-MS grade)	

Table 30: Mobile phase B

reagent	final concentration
ACN	80 %
FA	0.1 %
water (LC-MS grade)	

Table 31: Rear piston flush solution

reagent	final concentration
IPA	10 %
water (LC-MS grade)	

3.1.7 Biological samples

3.1.7.1 Cell pellets for human cell culture blend

Frozen cell pellets of HepG2, HEK293 and HCT116 were kindly provided by Dr. Tränkle.

3.1.7.2 Set of human liver tissue and preparations thereof

Prof. Schwab and Prof. Zanger kindly provided a set of human liver tissue and preparations thereof, membrane enriched fractions, microsomes and cytosol. The set contained ten samples from five female and five male patients aged between 47 and 75 years which were diagnosed with primary liver cancer or liver metastasis.

3.1.7.3 Healthy human liver tissue

Christine Wegler and Prof. Artursson kindly provided a set liver biopsies from twelve males and three females aged between 42 and 79 years. The diagnoses were, clear cell carcinoma, hepatocellular carcinoma, colorectal cancer or renal cell carcinoma. The biopsies do not contain tumor tissue, but healthy liver tissue. The medication is given in Table 32. Pooled lysates were used for development of human TXP assays (4.2.5, 4.2.7, 4.2.8, 4.2.9).

Table 32: Patient information

Patient	Medication
1	
2	metformin, gabapentin, atorvastatin, alfuzosin, paracetamol, warfarin, tiotropiumbromide, budesonide, formoterol
3	candesartan, metoprolol, citalopram, acetyl salicylic acid, bicalutamide, insulin
4	acetylsalicylic acid, atorvastatin, omeprazole, metoprolol, glyceryl trinitrate
5	
6	omeprazole
7	candesartan, felodipine, omeprazole
8	metoprolol
9	
10	
11	metformin
12	omeprazole, propranolol
13	
14	omeprazole, hydroxyzine, sumatriptan, zolpidem
15	acetylsalicylic acid, metoprolol, amlodipine, ezetimibe

3.1.7.4 Paired human kidney samples

This study contains healthy kidney tissue as well as kidney tumor samples from the same patients. Patients were aged between 51 and 82 years and diagnosed with clear cell renal cell carcinoma (ccRCC) or adrenocortical carcinoma. The relevant medication at the time of operation is given below. Samples were kindly provided by Prof. Stevanović.

Table 33: Patient information

patient	gender	age	subtype	medication
1	male	61	adrenocortical carcinoma	
2	male	61	ccRCC	
3	female	57	eosinophile ccRCC	
4	female	82	eosinophile ccRCC	Acerbon 10; Cynt 0,2; Aquaphor 20; Norvasc 5; Cibacen 5; ASS 100
5	male	61	ccRCC	
6	male	72	ccRCC	Norvasc, Lasix
7	female	59	ccRCC	Eferox, Furosemide, Exjade, Citalopram, Simvastatin, BisoHexal, Neupro-patch, Lyrica, Folcur, Marcumar, Tramadol, Omeprazole, Amineurin, Xipamide, Allobeta
8	male	58	ccRCC	
9	male	61	ccRCC	Tarivid, Concor, Fortecortin, Saroten, Blopress, Omeg, vitamine B, Novalgin, Omnic, Durogesic
10	female	51	ccRCC	Amaryl, Ferrosanol
11	male	80	ccRCC	Beloc zok

3.1.7.5 Liver tissue of phenobarbital-treated mice

Dr. Singh and Prof. Schwarz kindly provided frozen liver tissue of phenobarbital-treated mice for TXP method development (4.2.5, 4.2.7, 4.2.9). Male C3H/He mice were injected N-nitrosodiethylamine at the age of six weeks. After three treatment - free weeks, mice were fed with a diet containing 0.05 % phenobarbital for 27 weeks and sacrificed either 2 or 45 days thereafter. Animals were part of a study published 2013 [90].

3.1.7.6 Primary mouse hepatocytes

Pericentral and perivenous primary hepatocytes were isolated from male C3H/HE mice via digitonin perfusion [69]. Cells were lysed and proteolyzed with trypsin. Proteolyzed samples were provided by Simon Kling.

3.1.7.7 Fungicide-treated samples

Dr. Braeuning and Dr. Marx-Stölting kindly provided a set of samples to study the effects of fungicides on protein expression in the liver. It contained rattine and murine liver tissue samples as well as lysates of cultivated HepaRG cells.

The human HepaRG cells were differentiated and consequently treated with different fungicides and combinations thereof for 24 hours (Table 34). CITCO was used as positive control with either differentiation medium (DM) or work medium (WM). In the last case, cells were switched to work medium fifteen hours before the treatment. Harvested cell pellets were lysed with lysis buffer 2 -/-.

Table 34: Conditions for HepaRG treatment.

condition	test substance	concentration
differentiation medium (DM)		9 % FCS + 1.8 % DMSO
DM solvent control		9 % FCS + 1.9 % DMSO
work medium (WM)		2 % FCS +0.5 % DMSO
WM solvent control		2 % FCS + 0.6 % DMSO
DM + CITCO		1.9 % DMSO + 0.6 µM, 2.5 µM, 10 µM CITCO
WM + CITCO		0.6 % DMSO + 0.6 µM, 2.5 µM, 10 µM CITCO
cyproconazole	cyproconazole	0.6 µM, 2.5 µM, 10 µM, 40 µM
epoxiconazole	epoxiconazole	0.6 µM, 2.5 µM, 10 µM, 40 µM
prochloraz	prochloraz	0.6 µM, 2.5 µM, 10 µM, 40 µM
mixture I	cyproconazole+ epoxiconazole	0.3 µM, 1.25 µM, 5 µM, 20 µM each
mixture II	cyproconazole+ epoxiconazole +prochloraz	0.2 µM, 0.83 µM, 3.33 µM, 13.33 µM each

Male wistar rats were treated with different fungicides and combinations thereof for 28 days and phenobarbital was used as positive control (Table 35). All substances were administered via the feed. At the beginning of the study, rats were nine weeks old. Animals were part of published studies [73, 91]. Liver tissue of phenobarbital-treated animals was also used for TXP method development (4.2.2, 4.2.4, 4.2.5, 4.2.7, 4.2.9).

Table 35: Additives of rat diet.

condition	test substance	concentration
control		
phenobarbital	phenobarbital	500 ppm
cyproconazole	cyproconazole	100 ppm, 1000 ppm
epoxiconazole	epoxiconazole	90 ppm, 900 ppm
prochloraz	prochloraz	100 ppm, 1000 ppm
mixture I	cyproconazole + epoxiconazole	100 ppm + 90 ppm, 1000 ppm + 900 ppm
mixture II	cyproconazole + epoxiconazole + prochloraz	100 ppm + 90 ppm + 100 ppm, 1000 ppm + 900 ppm + 1000 ppm

The murine set included samples from C57BL/6 mice as well as transgenic C57BL/6 mice expressing only humanized forms of the receptors CAR and PXR (hCAR/hPXR). At the age of eight weeks, mice were treated with two fungicides and phenobarbital for 28 days. Fungicides were administered via the feed while phenobarbital was given via drinking water (Table 36).

Table 36: Additives of mouse diet.

condition	test substance	concentration
control		
phenobarbital	phenobarbital	0.05 % (w/v)
cyproconazole	cyproconazole	50 ppm, 500 ppm
prochloraz	prochloraz	50 ppm, 500 ppm

3.2 Methods

3.2.1 Selection of suitable peptides and TXP-epitopes

For the selection of suitable peptides, the amino acid sequences of all target proteins are fragmented *in-silico* based on tryptic cleavage. The resulting peptides are filtered to exclude all peptides with undesirable characteristics: All peptides which cannot be assigned uniquely to one protein are excluded, as well as all peptides either shorter than eight amino acids or longer than 25 amino acids. Peptides containing methionine will be excluded, if there is an alternative peptide which meets all criteria. Depending on the target protein, one to eleven peptides meet these criteria.

This peptide screening is performed for each species separately. The subsequent selection of at least one peptide per target protein is done for all species together. The key aspect of the selection is to minimize the number of TXP antibodies needed to cover all target proteins.

3.2.2 Sample preparation

3.2.2.1 Preparation of peptide standards

Between 0.5 and 1 mg lyophilized standard peptide is weighed with an analytical balance. A 5 mM solution is prepared by adding DMSO, mixing it vigorously and, if necessary, sonicating it. Subsequently it is diluted to 1 mM by adding LC-MS grade water. Peptide stock solutions are stored at -20°C.

3.2.2.2 Tissue Lysis

For tissue lysis, all equipment and samples have to be cooled with liquid nitrogen. Tissue samples are transferred into cooled cryovials and weighed. The sample should weigh between 8 and 80 mg. In case of larger tissue samples, they have to be fragmented in a mortar filled with liquid nitrogen.

Samples are pulverized using a ball mill at 2000 rpm for 1.5 min. The pulverized tissue can either be stored at - 80°C or directly used for lysis. The samples are incubated with the 20 to 50-fold volume lysis buffer (20-50 µL buffer : mg sample) for one hour at

room temperature under constant rotation. The lysate is transferred into a new vial and centrifuged for 5 min at 16'000 g to sediment cell debris. The supernatant is stored at - 80°C until analysis.

3.2.2.3 Cell lysis

At the time of harvest, cells are washed twice with ice cold PBS, scraped of the cell culture dish and sedimented by centrifugation. The cell pellets can be stored at – 80°C. At the time of lysis, pellets are thawed on ice. Subsequently, they are re-suspended in double volume of lysis buffer and incubated for one hour at room temperature while mixing it vigorously every 15 min.

3.2.2.4 Protein quantification

The amount of extracted protein is determined in every lysate using the Pierce™ BCA Protein Assay Kit according to the manufacturer's manual.

3.2.2.5 Enzymatic proteolysis

For enzymatic proteolysis 100 – 400 µg extracted protein is diluted with distilled water and TEA buffer. TCEP and NOG are added before the samples are heated for 5 min to 95°C. Subsequently the samples are cooled to room temperature and IAA is added. After 30 min incubation in the dark while shaking continuously, trypsin is added in such a manner that it is the twentieth part of the used protein amount. Standard proteolysis takes place over night for 16 h while shaking the samples at 37°C. The enzymatic proteolysis is terminated by a 5 min heating step at 95°C and subsequent protease inhibitor addition (PMSF). The total volume of the proteolysis is 425 µL. If not stated otherwise, TPCK treated and methylated trypsin is used.

Table 37: Reagents needed for enzymatic proteolysis

reagent	final concentration
lysate	0.2 – 1 µg/µL
d. water	
TEA buffer	44 mM
0.1 M TCEP	5 mM
NOG stock solution	0.4 % (w/v)
IAA solution	10 mM
Trypsin solution	1 : 20 (trypsin : protein)
PMSF stock solution	1 mM

3.2.2.6 Determination of assays accuracy and precision

For recovery plots, all IS peptides of a multiplex are diluted with blocking buffer to the concentration which is used to spike samples for quantification. This solution is used to prepare a serial dilution of the respective endogenous peptides from 1000 fmol down to 0.05 fmol. After immunoprecipitation (3.2.3) and LC-MS-analysis (3.2.4), the results are used to evaluate precision and accuracy of the assays. Additionally, equal volumes of all IS and EN peptides are mixed and diluted to 5 fmol/µL with loading buffer. This input is used as positive control for LC-MS hardware performance as well as to determine the ratio between matching IS and EN peptide. The concentration of EN peptide stock solutions was adjusted by the input ratio.

3.2.3 Immunoprecipitation

The immunoprecipitation is performed semi-automated at room temperature using a magnetic particle processor. To do so, the proteolyzed samples are distributed into a 96-well microtiter PCR plate (sample plate). Antibody stocks are diluted with PBSC and IS peptide stocks are diluted with blocking buffer. The corresponding TXP-antibody and standard peptide dilutions are added and the volume is filled up to 100 µL with PBSC. After 1 h incubation at room temperature with regular mixing intervals, magnetic beads are transferred into the sample plate. They are coated with

protein G to trap the TXP antibody – peptide complex. Therefor the beads are incubated for another hour while swirling them up every 10 min. Subsequently the beads are transferred into two washing plates containing PBSC and three containing ABCC. Finally, the protein G - TXP antibody – peptide complex is degraded in 20 µL elution buffer and the beads are removed from the elution plate. The eluate is transferred manually into a new microtiter plate to remove remnants of beads and heat sealed for LC-MS analysis.

Table 38: Protocol for semi-automated immunoprecipitation using the KingFisher.

action	plate content	duration	cycle	speed	number of cycles
pick up comb	sample plate				
mix sample	sample plate	1 h	mix 2 min pause 8 min	middle	6 x
collect beads	beads + PBSC		mix 10 s collect 3 x	middle	
release beads	sample plate		mix 10 s	middle	
mix sample	sample plate	1 h	mix 2 min pause 8 min collect 3 x	middle	6 x
wash 1	PBSC	4:30 min	mix 1:50 min pause 25 s collect 3 x	middle	2 x
wash 2	PBSC	4:30 min	mix 1:50 min pause 25 s collect 3 x	middle	2 x
wash 3	ABCC	4:30 min	mix 1:50 min pause 25 s collect 3 x	middle	2 x
wash 4	ABCC	4:30 min	mix 1:50 min pause 25 s collect 3 x	middle	2 x
wash 5	ABCC	4:30 min	mix 1:50 min pause 25 s collect 3 x	middle	2 x
elution	elution buffer	4:30 min	mix 1:50 min pause 25 s collect 3 x	middle	2 x
release and leaf comb	PBSC	5 s	mix 5 s	middle	

3.2.4 LC-MS analysis

3.2.4.1 LC

After immunoprecipitation, the eluate is separated by high performance liquid chromatography. A precolumn is used to desalt the samples and to remove antibodies from the eluate. Therefor the eluate is mixed with loading buffer which is used to retain the peptides on the precolumn. Subsequently, the remaining peptides are transferred to and separated by an analytical C18 – column using a linear gradient of a changing mobile phase A and B ratio. This general LC method is adjusted to full MS and tSIM measurement requirements (Table 39): For full MS measurements, 10 µL eluate is injected and separated with a 20 min gradient. For the quantification of the target peptides by tSIM measurement, a 2.75 min gradient and 5 µL are sufficient. When multiplex assays are compiled, the tSIM gradient is optimized further to separate the increasing number of peptides efficiently.

Table 39: Parameters of LC methods

parameter	full MS	tSIM
column oven temperature	40°C	55°C
injected volume	10 µL	5 µL
flow rate on precolumn	20 µL/min	20 - 120 µL/min
flow rate on analytical column	0.3 µL/min	1 µL/min
method duration	45 min	10 min
gradient duration	20 min	2.75 min
gradient composition	4 – 55 % mobile phase B	4 – 45 % mobile phase B

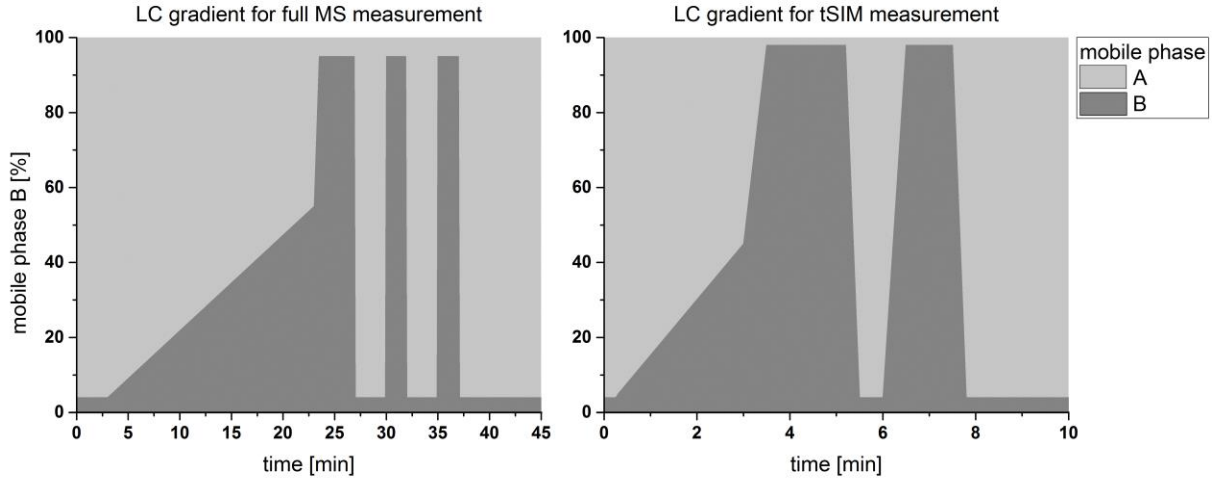


Figure 3: LC gradients for full MS and tSIM measurements

3.2.4.2Full MS

The full MS method is used for experiments in which the identification of peptides is the main focus. It is set to the positive mode with data dependent MS/MS of the top ten peaks with charge states two and three. The instrument settings are listed in Table 40.

Table 40: Properties of full MS / dd-MS/MS

full MS	
Resolution	70 000
AGC target	3e6
Maximum IT	100 ms
Scan range	300 to 2000 m/z
dd-MS/MS / dd-SIM	
Resolution	17 500
AGC target	5e5
Maximum IT	60 ms
Loop count	10
TopN	10
Isolation window	2.0 m/z
Fixed first mass	---
NCE / stepped NCE	25
dd settings	
Underfill ratio	0.0%
Intensity threshold	0.0
Apex trigger	---
Charge exclusion	unassigned, 1, 5-8, >8
Peptide match	---
Exclude isotopes	on
Dynamic exclusion	5.0 s

3.2.4.3 tSIM

tSIM methods are employed to quantify a set of known target peptides. Coeluting IS and EN peptides confirm the peptide identity as well as the correct charge state and data dependent MS/MS which is triggered by signals higher than 20'000. Mass - to - charge – ratios and charge states of the target peptides are provided in the inclusion list. Method settings are listed in Table 41.

Table 41: Properties of tSIM / dd-MS/MS

SIM	
Resolution	35 000
AGC target	5e6
Maximum IT	60 ms
Loop count	1 / 2
MSX count	1 / 2
Isolation window	3.0 m/z
Scan range	300 to 1200 m/z
dd-MS/MS	
Resolution	17 500
AGC target	2e5
Maximum IT	60 ms
Loop count	1
TopN	1
Fixed first mass	---
NCE / stepped NCE	20
dd settings	
Underfill ratio	0.6%
Intensity threshold	2.0e4
Apex trigger	---
Charge exclusion	---
Peptide match	---
Exclude isotopes	
Dynamic exclusion	2.0 s

3.2.4.4 Data analysis

The Proteome Discoverer 1.3 is employed to analyze results of full MS measurements. Peptides are identified using Mascot and SEQUEST as reference data bases and medium peptide filter. The search parameters are specified in Table 42 and Table 43. Pinpoint, on the other hand, is used to analyze raw files of tSIM measurements. Import and analysis parameters are listed in Table 44. To ensure a robust analysis, peaks within 50 % intensity of base peak are used to calculate total peak areas and peptide amounts. The analysis is double-checked for random samples and low intensity signals by manually surveying the isotope pattern, charge state and peak form with Xcalibur.

Table 42: Search settings for Mascot - Version 2.3.02

parameter	specification
protein database	complete proteome Set (08/2013)
enzyme name	Trypsin
maximum missed cleavage sites	1
instrument	ESI-TRAP
taxonomy	all entries
precursor mass tolerance	5 ppm
fragment mass tolerance	0.05 Da
use average precursor mass	false
dynamic modifications	oxidation (M), oxidation (HW)
static modifications	carbamidomethyl (C)

Table 43: Search settings for SEQUEST - Version 28.0.0.0

parameter	specification
Protein Database	Complete Proteome Set (08/2013)
Enzyme name	Trypsin
Maximum Missed Cleavage Sites	1
Precursor Mass Tolerance	5 ppm
Fragment Mass Tolerance	0.05 Da
Use Average Precursor Mass	false
Use Average Fragment Masses	false
Use Neutral Loss a Ions	True
Use Neutral Loss b Ions	True
Use Neutral Loss y Ions	True
Weight of a Ions	0
Weight of b Ions	1
Weight of c Ions	0
Weight of x Ions	0
Weight of y Ions	1
Weight of z Ions	0
Dynamic Modifications	Oxidation / +15.995 Da (H, M, W)
Static Modifications	Carbamidomethyl / + 57.021 Da (C)

Table 44: Parameters of analysis with Pinpoint 1.3

parameter	setting
MS1 accuracy	5 – 15 ppm
Scan filter	SIM or Full (as available)
MSMS accuracy	1000 ppm
Isolation mode	MSMS isolation width 0.2 u
Peak width	0.05 min
minimum signal threshold	100
possible alignment error	2
What area option to use?	Peaks within 50% intensity of base peak
number of smoothing points	5

3.2.5 Statistical analysis

For analysis of possible effects ANOVA analyses with Fisher LSD or Bonferroni's correction were performed using Origin 2015G. The cross-species study was analyzed with a Student's-t-test (two tailed for heteroscedastic data) using Excel2016 to handle the size of the sample set. Bonferroni's correction for multiple testing was applied.

If results are evaluated as fold change, error propagation will be applied:

$$\Delta f = \sqrt{\left(\frac{\partial f}{\partial x} \cdot \Delta x\right)^2 + \left(\frac{\partial f}{\partial y} \cdot \Delta y\right)^2 + \cdots} \quad [92]$$

The general formula was adopted as follows:

$$\Delta f = \sqrt{\left(\frac{\sigma_x}{\bar{x}_x}\right)^2 + \left(\frac{\sigma_c}{\bar{x}_c}\right)^2} \cdot \left(\frac{\bar{x}_x}{\bar{x}_c}\right)$$

Δf = propagated error (PE)

σ_x = standard deviation of the treatment

$\bar{x}_x$ = mean value of the treatment

σ_c = standard deviation of the control

$\bar{x}_c$ = mean value of the control

4 Results

4.1 Selection of epitopes and peptides

For this project, 26 transporters in up to three different species (human, rat and mouse) and 45 CYPs for up to two different species (rat and mouse) were covered. In total this amounted to 109 different proteins (Table 56). To select suitable peptides and TXP epitopes, the sequences of all targets were fragmented *in silico* at tryptic cleavage sites. Resulting peptides were filtered to eliminate those with undesired characteristics, such as very long or methionine containing peptides (see 3.2.1 for specifications). This resulted in one to eleven peptide candidates per protein. Subsequently at least one peptide per protein was chosen in such a manner as to minimize the number of TXP antibodies needed to cover them all (Table 57). Therefor 72 different epitopes were necessary. Out of these, ten TXP epitopes covered more than four targets. The most versatile epitope could be used to analyze twelve different target proteins (FSGR). The majority of epitopes could be used to analyze two to four different targets of the set. Another 21 epitopes had to be selected to cover the remaining targets individually. The efficiency of the chosen TXP epitopes is visualized in Figure 4 and listed in Table 58. As a side effect of this approach, some proteins are covered by up to four different TXP epitopes of the set. Each of these epitopes is required for at least one additional target protein of the set, which is addressed by only one epitope of the set.

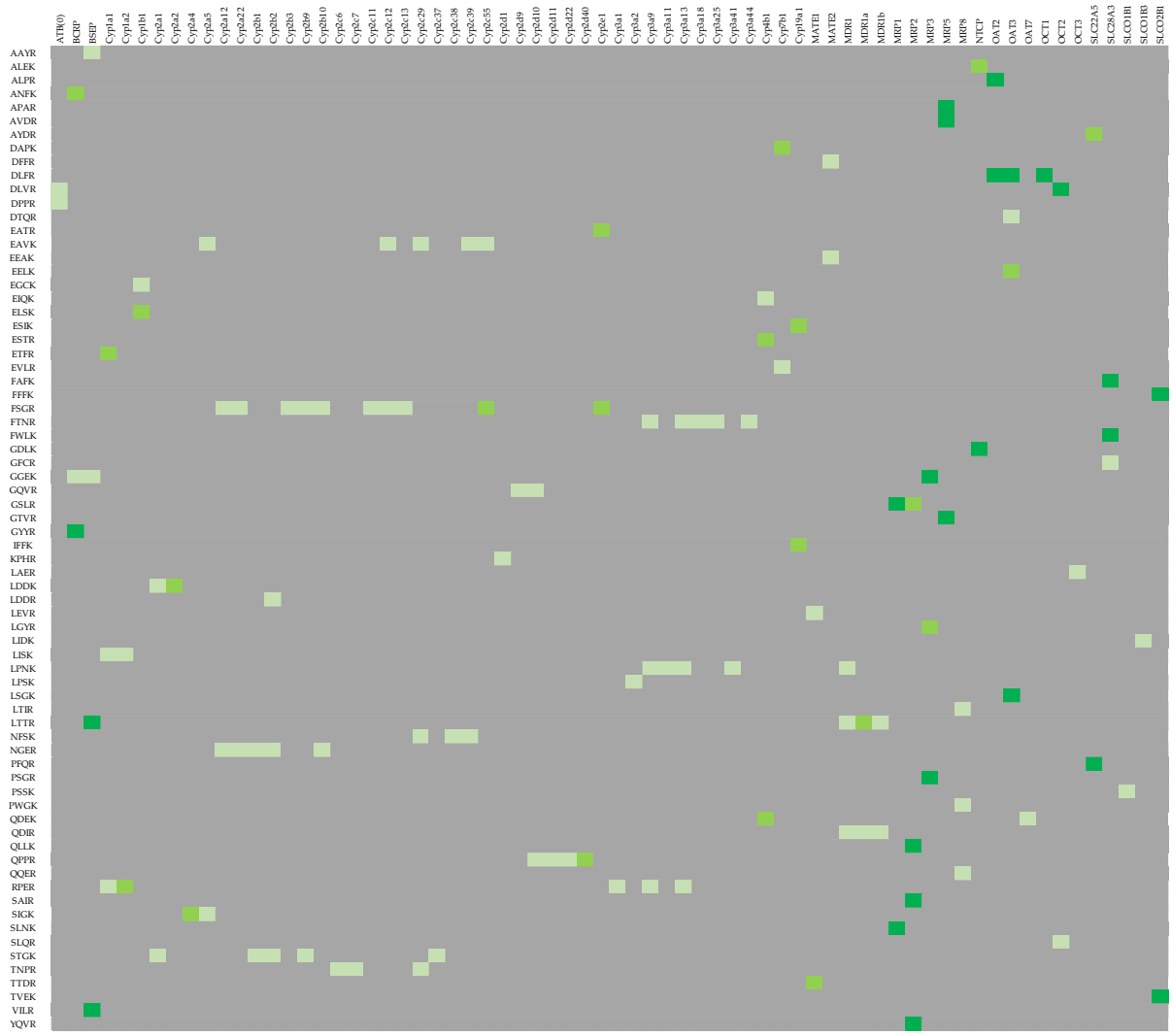


Figure 4: Heat map displaying the efficiency of TXP epitopes. The number of target proteins covered by each TXP epitope is indicated in green. The color saturation corresponds to the number of species which are covered (one species, two species, and three species).

There are two aspects which have been discussed to be included in the selection process: The number of proteins per proteome which carry this epitope and whether high abundant proteins are targeted by the epitope [22]. Both features were not considered during the selection process but it was analyzed retrospectively whether they could be linked to successful assay development. The development is considered successful for epitopes which are part of the final TXP assay set.

The number of proteins which can be addressed by a TXP epitope was determined using an in-house script called TXP tool. Only entries of the UniProtKB reference proteome were considered for this analysis. The percentage of proteins carrying the

TXP epitope with respect to the proteome was calculated with following formula for each species:

$$\frac{number\ of\ proteins\ (TXP, species)}{number\ of\ all\ reference\ proteome\ entries\ (species)} * 100 = protein\ ratio$$

The chosen epitopes address between 0.03 and 20 % of the tested proteomes. Subsequently, the epitopes were grouped according to successful and non-successful assay development during this thesis. The outcome is presented in Figure 5: The groups of one species cannot be distinguished by the protein ratio.

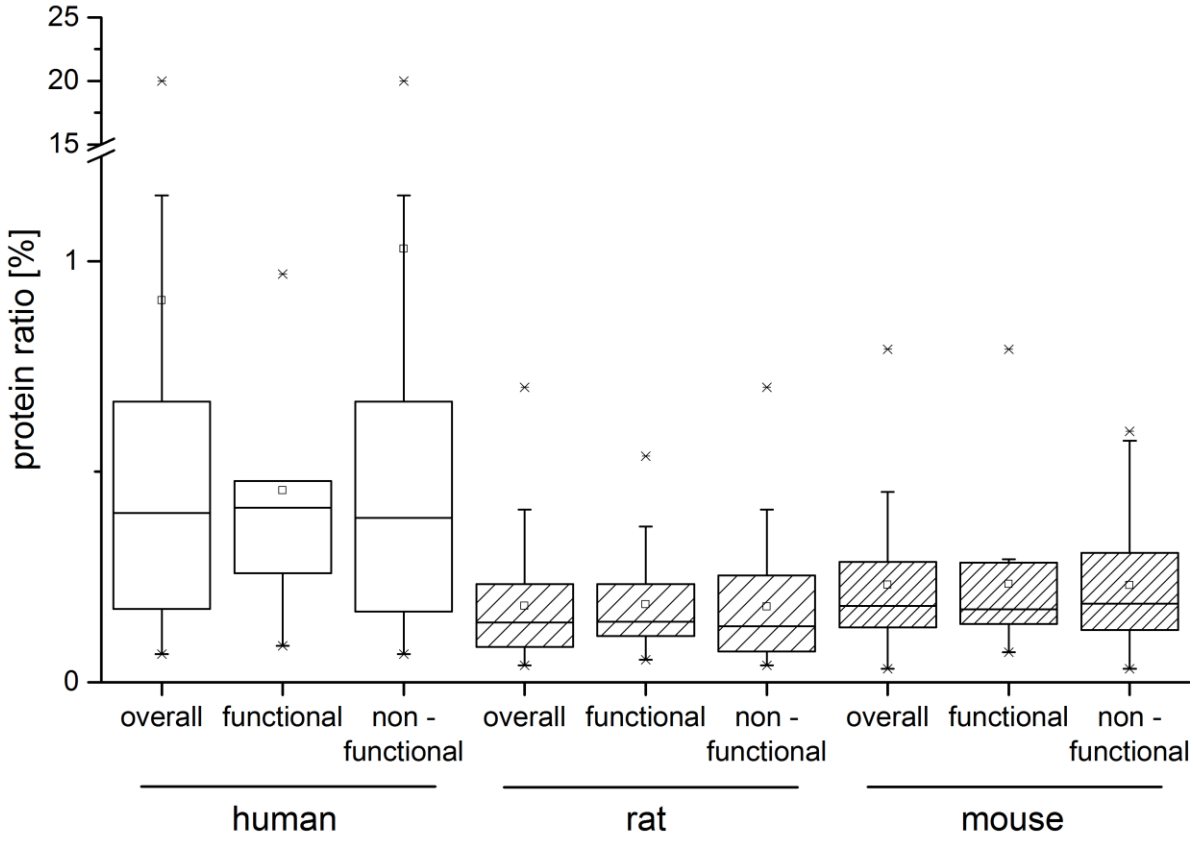


Figure 5: Comparison of TXP epitopes with respect to assay development. The percentage of proteins which contain a TXP epitope in relation to the size of the proteome was calculated. Then the epitopes were additionally classified according to whether a functional assay could be developed during this thesis or not. Each species was analyzed separately. (UniPortKB reference proteome 20.12.2015)

Table 45: High abundant proteins and their coverage by TXP epitopes in all species. The fifteen most abundant proteins are indicated in gray according to the PaxDb entries for human liver (integrated), mouse liver (integrated) and rat whole organism. Only proteins with Swiss-Prot entries were included. The canonical sequences as well as all isoforms were checked whether they include any of the chosen TXP epitopes.

high abundant protein	human	rat	mouse
10 kDa heat shock protein, mitochondrial		LDDK	
3-ketoacyl-CoA thiolase, mitochondrial			
60S ribosomal protein L19			
60S ribosomal protein L21			
60S ribosomal protein L3			
60S ribosomal protein L39			
78 kDa glucose-regulated protein			DLFR
Actin, cytoplasmic 1			
Alcohol dehydrogenase 1			
Alcohol dehydrogenase 1A			
Alcohol dehydrogenase 1B			
Alcohol dehydrogenase 4			
Argininosuccinate synthase			
ATP synthase subunit beta, mitochondrial			
Carbamoyl-phosphate synthase [ammonia], mitochondrial		EATR	EATR
Carbonic anhydrase 3			
Cytochrome b5		EIQK, EVLR	
Elongation factor 1-alpha 1			
Endoplasmin			
Fatty acid-binding protein, liver			
Ferritin light chain			
Fructose-bisphosphate aldolase B			
Glutathione S-transferase A1			
Glutathione S-transferase A3			
Heat shock cognate 71 kDa protein			DLFR
Hemoglobin subunit alpha			
Hemoglobin subunit beta			
Hemoglobin subunit beta-1			
Hemoglobin subunit delta			
Myelin basic protein			
Peroxiredoxin-1			
Phosphatidylethanolamine-binding protein 1	LSGK		
Protein disulfide-isomerase			EAVK, NGER
Serum albumin			
Superoxide dismutase [Cu-Zn]			
Thymosin beta-4		LPSK	
Ubiquitin-60S ribosomal protein L40			

High abundant proteins are the second aspect which might be used to improve the epitope selection process. The hypothesis is that all peptides bind competitively to the binding sites of the antibodies. Peptides of high abundant proteins could block the antibodies and suppress the binding of low abundant peptides. To investigate this, the fifteen most abundant proteins in liver were chosen for each species according to PaxDb [93]. For rat, whole organism data was used, because organ specific data was not available. Nine TXP epitopes are present in at least one of the proteins (Table 45). It was analyzed whether the presence of TXP epitopes in these proteins correlates with the success rate of assay development (Table 46): Overall it was possible to develop at least one functional assay for 35 % of the epitopes. By excluding epitopes which address a high abundant protein, the ratio would have been decreased by 5 %. 13 % of the assays in the final assay set would be missing.

Table 46: Ratio of TXP epitopes with which a successful assay was developed. The TXP epitopes were analyzed in total as well as only TXP epitopes which did or did not address a HAP. Following ratio is given:
epitopes for which assay development was successful / all tested epitopes

	human	**rat**	**mouse**
all epitopes	10 / 43	20 / 55	14 / 48
epitopes which address no HAP	10 / 42	16 / 50	12 / 44
epitopes which address HAPs	0 / 1	4 / 5	2 / 4

4.2 Assay development

The development of new TXP assays was initially based on the PhD thesis of Frederik Weiss [22]. His approach was further optimized and additional experiments were included in the development. The critical components of a TXP assay are the peptides and antibodies. Both were produced customized by Intavis AG and Pineda Antikörper Service respectively. The antibodies were delivered as rabbit sera and purified in-house. Each serum got an identifier which contains following information: clonality (monoclonal mAB / polyclonal pAB), antigen, host species and a consecutive number. During assay development, the purified antibodies were characterized and the

antibody amount used for immunoprecipitation was adjusted individually. On this basis multiplex assays were compiled of which the accuracy, precision and reproducibility was determined. Additionally, the sample preparation was optimized. Since polyclonal antibodies purified from serum were used, the assay development had to be performed for each serum individually. Therefore, characterization of the purified antibodies was the first step of the development.

4.2.1 Antibody characterization and functionality in complex matrix

The antibody was characterized in two aspects: What is the actual binding motif of the antibody? Is the antibody able to enrich the according standard peptide in complex matrix?

Both questions have been addressed by using human cell line blend as an artificial matrix. Human cell line blend was generated from human cell lysates: HepG2, HEK293 and HCT116 were cultivated under standard conditions and harvested at 80-100 % confluency. After lysis with lysis buffer 1 +/-, the protein concentration was determined and lysates were mixed in a 1 : 1 : 1 protein ratio. Enzymatic proteolysis and immunoprecipitation (20 µg protein and 5 µg AB / IP) were performed as needed. To examine the antibody epitopes, the immunoprecipitation was performed without standard peptides and the eluate was analyzed with the full MS method. A monoclonal antibody against Myc proto-oncogene protein (mAB_cMyc_ms1) was used as negative control, as well as matrix processed without Protein-G coupled beads and antibodies. Identified peptides were used to generate sequence logos to depict the C-termini which lead to retention during immunoprecipitation [22, 35]. Detailed information for all antibodies is given in Table 64.

The sequence logos of all antibodies which are part of the final TXP-assay set (4.2.6) are presented in Figure 6. Additionally, the number of peptide sequences and tags the logos based on are listed. The tags are defined as the four C-terminal amino acids of a peptide. Furthermore, the ratio of enriched peptides is given. This ratio is obtained by referring the number of enriched peptides with the target TXP epitope to the number

of peptides theoretically present in the proteome sharing this epitope (UniProtKB reference proteome June 2014). 80 % of the generated antibodies significantly enriched peptides and a binding motif could be determined. Up to 38 % of the theoretically possible peptides containing the target TXP epitope were precipitated. The antibodies chosen for the final TXP assay set enriched up to 33 % of all peptides in the human proteome with the target TXP epitope. For two antibodies, pAB_TXP_LDDK_rbt1 and pAB_TXP_QPPR_rbt1, it was not possible to determine a binding motif. Nevertheless, it was possible to establish TXP – assays using these antibodies. The most diverse binding motif of a single antibody comprised up to 31 C-terminal amino acid sequence variations. The antibodies chosen for the final TXP assay set precipitated up to 22 different tags. For 15 antibodies, the experiment was not performed, because a preliminary experiment showed, that they do not precipitate the desired peptides in buffer. Therefore, the functionality in complex matrix was not tested. This experiment was also not performed for the peptide specific antibodies.

To ensure the transferability of the results between different species and tissues, sequence logos for three selected antibodies were also generated using mouse and rat liver tissue as well as rat heart and kidney tissue. The similarity of motifs obtained from different human cell line samples had already been shown [35]. The sequence logos of all sample types and species were similar (Figure 7). In general, the murine and rattine sequence logos were based on less peptide sequences and fewer C-terminal tags were included. In the case of pAB_TXP_GYYR_rbt2, the first and fourth position were preserved. In the second and third position, hydrophobic amino acids were preferred. Between 7 -14 % of the known GYYR peptides were enriched. In the binding motif of pAB_TXP_QDIR_rbt1, the second and fourth positions were preserved, while the first position tolerated seven different amino acids. On the third position the hydrophobic amino acids isoleucine, leucine and valine were preferred. 15 – 20 % of the known QDIR-peptides were enriched, with the exception of the heart sample. Here only 8 % of the known QDIR – peptides were precipitated. For pAB_TXP_SLNK_rbt4 only the first position is variable and precipitated 5 -13 % of the possible peptides.

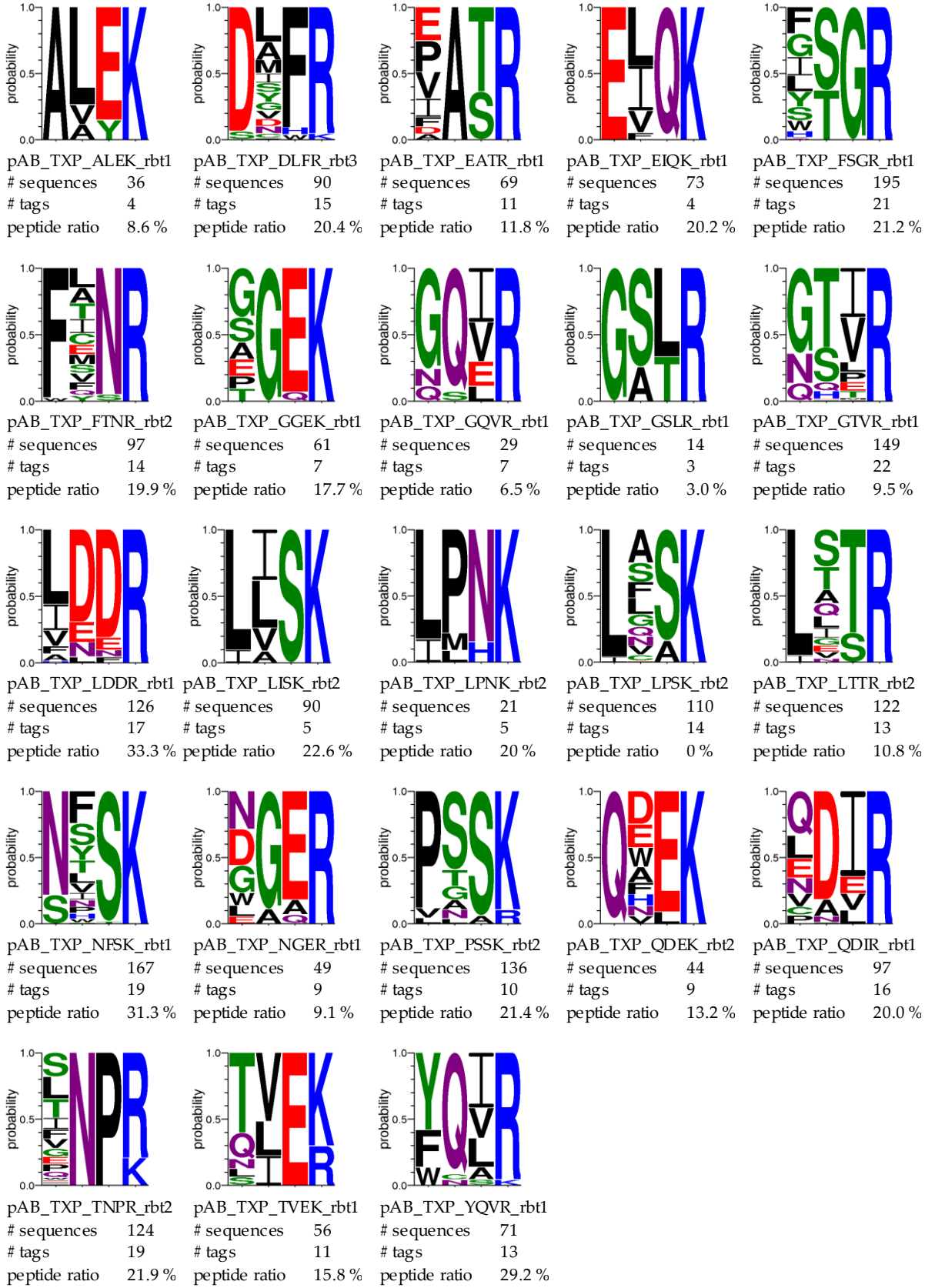


Figure 6: Binding motifs characterizing the actual epitopes of TXP antibodies. The antibody ID specifies amongst others the target epitope. The actual binding motif is presented as sequence logo. The number of peptide sequences and C-terminal tags the logo is based on are listed. Additionally, the ratio of enriched peptides with the expected TXP epitope is given in percent (UniProtKB ref. prot. June2014).

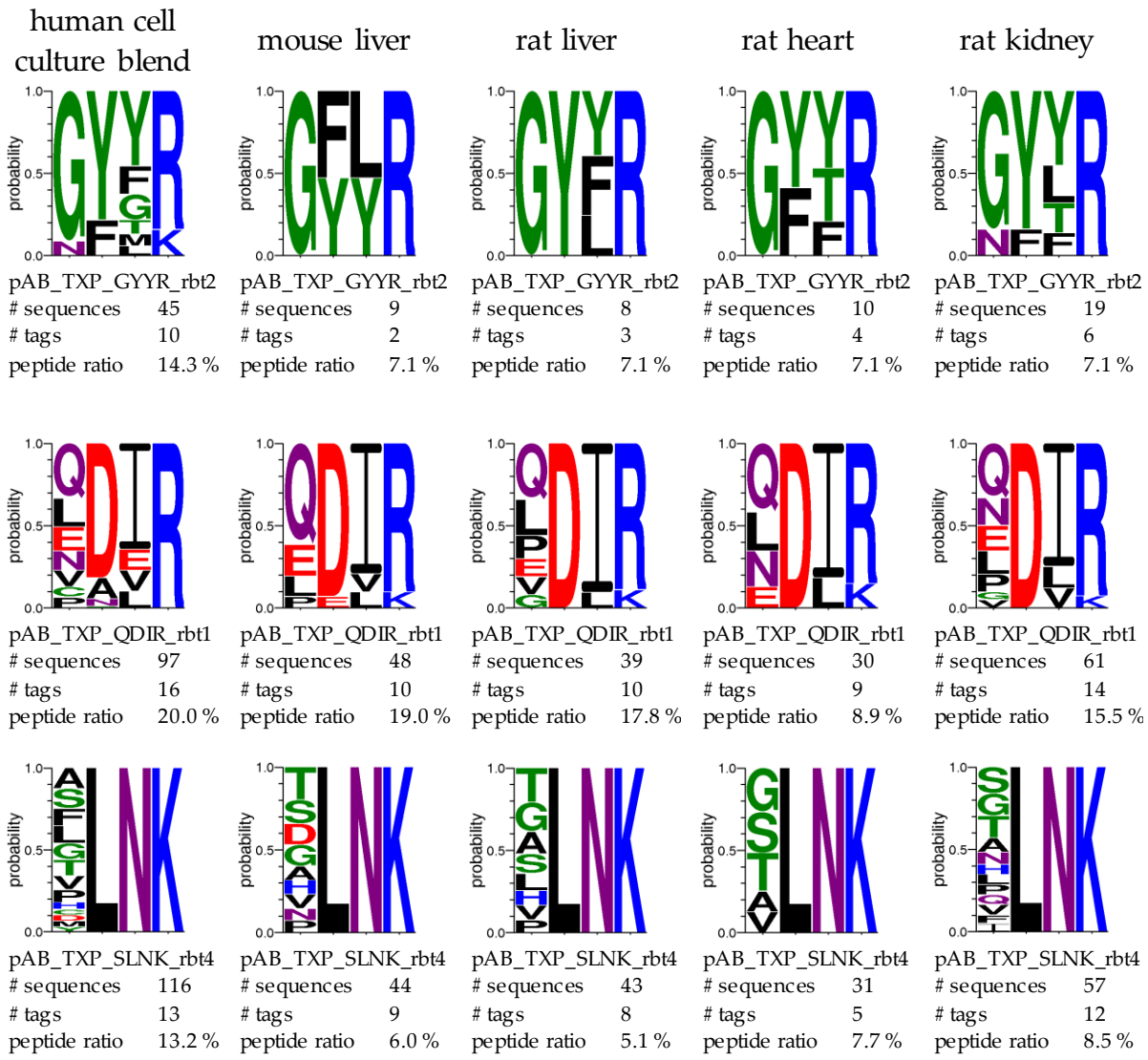


Figure 7: Comparison of binding motifs resulting from different species and tissues. The sequence logos of three antibodies generated with human cell culture blend are compared to sequence logos generated with mouse and rat tissue samples. Additionally, the number of peptide sequences and C-terminal tags logos are based on are listed as well as the ratio of enriched peptides with the expected TXP epitope (rat and mouse: UniProtKB reference proteome December 2014).

The sequence logos characterize the binding motif of the antibodies, but nevertheless it was observed, that some peptides with the targeted C-terminal epitope were not precipitated. Hence, the complex matrix human cell culture blend was spiked with 1 pmol of each standard peptide, to test whether it is enriched during immunoprecipitation with 5 µg antibody. The eluate was analyzed with the tSIM method. If the total file area exceeded 10^4, the peptide was considered enriched.

Antibodies which enriched none of the spiked-in target peptides and peptides which were not enriched by any antibody were excluded from further assay development. 85 % of the standard peptides passed the total file area threshold (Table 65) and 87 % of the TXP – epitopes are covered with at least one functional antibody (Table 64). Only a subset of the spiked-in standard peptides was enriched for the following six TXP epitopes: DLFR, EAVK, EELK, QLLK, STGK, and TFDR. All others either precipitated all offered peptides or none.

4.2.2 Optimization of lysis conditions

Lysis buffer 1 +/- had been established before [22] and was used for human cell culture blend production. It contained Triton as main detergent and a protease inhibitor cocktail to prevent protein degradation during lysis. It was tested whether the lysis process could be further optimized for transmembrane proteins by either additional sonication of the samples or use of additives and different detergents. Therefor a liver tissue sample of a phenobarbital treated rat was fragmented into pieces of about 10 mg. For each condition, three pieces were lysed with the 50-fold volume of lysis buffer. Subsequently the amount of extracted protein was determined by BCA assay and a subset of nine peptides was quantified by immunoprecipitation and tSIM measurement (Figure 8):

Samples lysed with lysis buffer 1 +/- were additional sonicated for 1.5 min in a sonication bath. This did not influence the amount of quantified target proteins. Likewise, it was also not altered significantly by addition of Benzonase Nuclease (lysis buffer 1 +/+). The omission of the protease inhibitor cocktail (lysis buffer 1 -/-), on the other hand, increased the quantified amount of the majority of targets. Especially the detectable amount of Cyp2b1/2 and NTCP via the GDLK peptide were enhanced. The third tested aspect was the detergent: Beside Triton the detergents NP-40 (lysis buffer 2 +/-) and DOC (lysis buffer 3) were tested. NP-40 and DOC slightly improved the quantified amount of eight and seven target proteins respectively. In comparison

to DOC, the quantified amount was increased or equal with NP-40. The conclusion of the optimization was that further experiments were performed with NP-40 containing lysis buffer 2 -/- without additives and sonication.

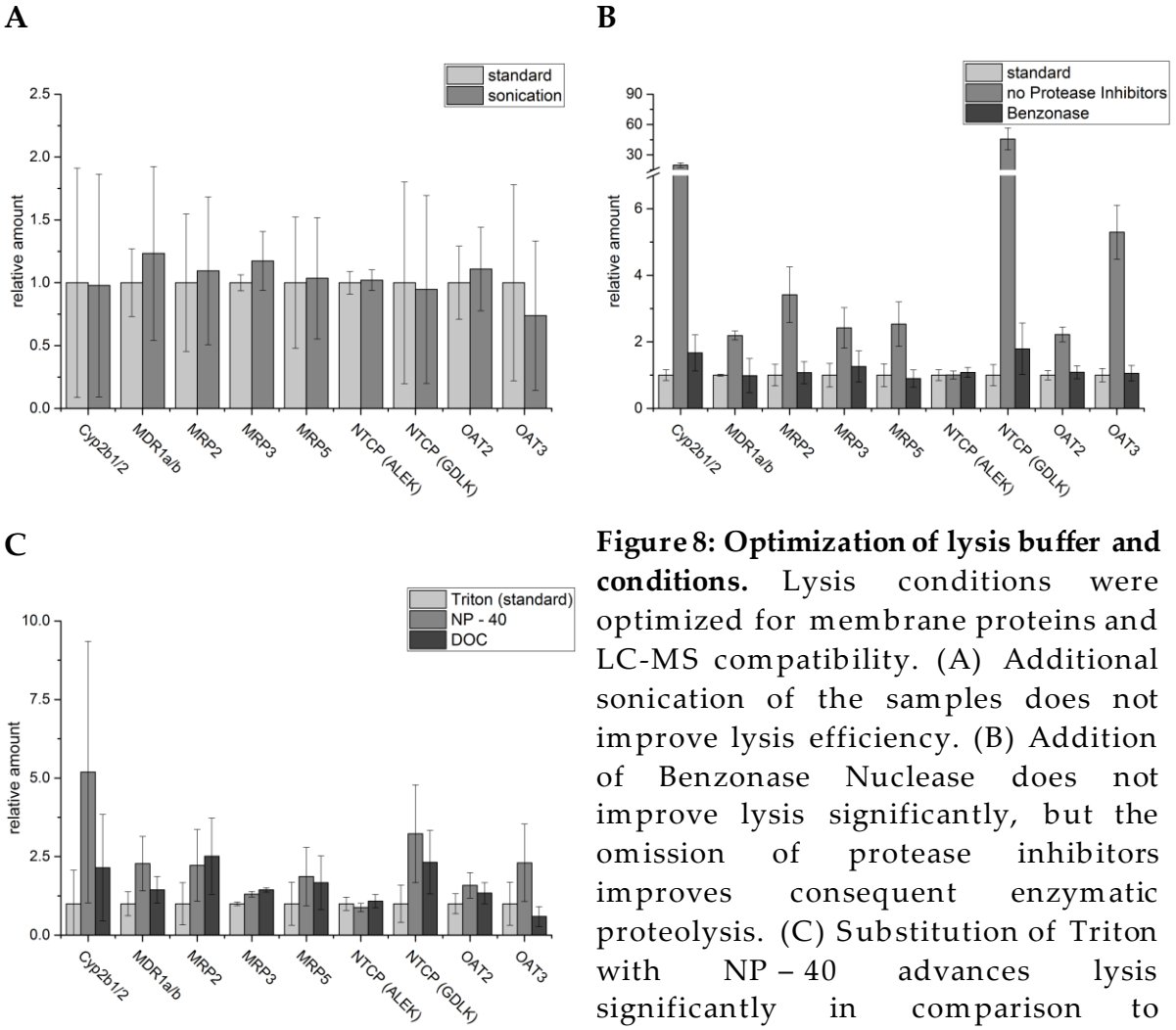


Figure 8: Optimization of lysis buffer and conditions. Lysis conditions were optimized for membrane proteins and LC-MS compatibility. (A) Additional sonication of the samples does not improve lysis efficiency. (B) Addition of Benzonase Nuclease does not improve lysis significantly, but the omission of protease inhibitors improves consequent enzymatic proteolysis. (C) Substitution of Triton with NP – 40 advances lysis significantly in comparison to exchange with DOC. Fold change to standard condition and PE are shown, n=3. ANOVA Fisher LSD analysis was performed ($p \leq 0.05$).

The optimized lysis buffer 2 -/- was used to investigate the impact of the lysis buffer volume and the temperature at which lysis was performed. Pulverized tissue samples were mixed with 10-, 50- or 100-fold volume lysis buffer (x µL lysis buffer : mg tissue). The amount of lysis buffer did not affect quantification of the target peptides significantly (Figure 9A). Last but not least, it was tested whether the quantified peptide amount will be altered if lysis is performed at room temperature or at 4°C. The temperature did not influence the quantification of the target peptides (Figure 9B).

For convenience, in all following experiments lysis was performed using 20- to 50-fold volume of lysis buffer and at room temperature.

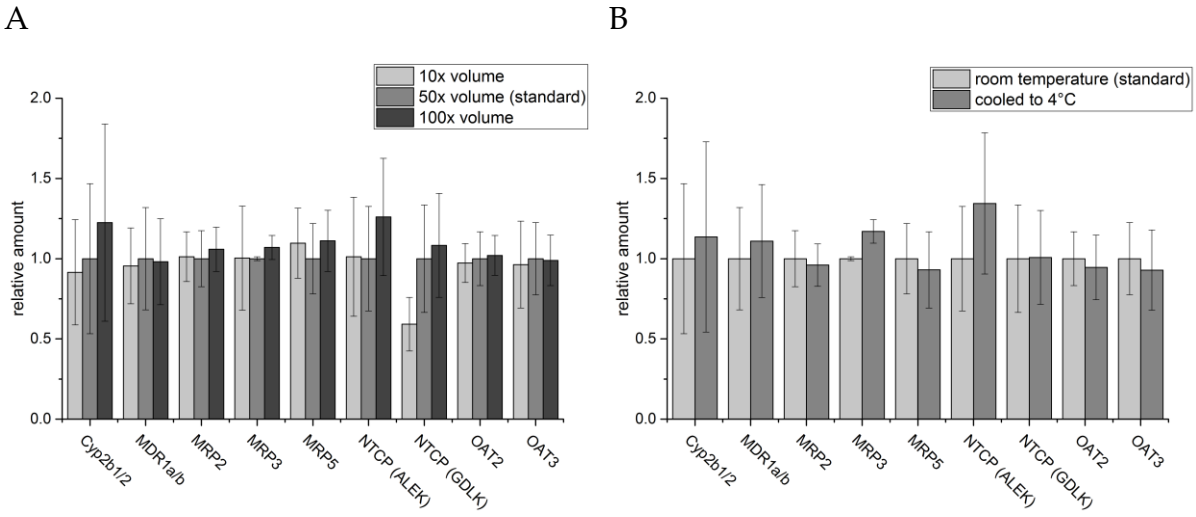


Figure 9: Optimization of lysis conditions. The effect of lysis in different volumes and at different temperatures was tested. Neither the tested ratios of lysis buffer and tissue weight (A) nor the temperature at which lysis was performed (B) affected lysis efficiency. Fold change to standard condition and PE are given. n=3, ANOVA Fisher LSD analysis was performed ($p \leq 0.05$).

4.2.3 Analysis of subcellular liver fractions

Despite the optimized lysis conditions, some target proteins could not be detected even though they were expected to be expressed in the sample, e.g. overexpressing cell lines (data not shown). Since many other MS-based quantification methods for transporters and CYPs rely on enrichment of the analytes by subcellular fractionation [26, 94-99], it was tested whether subcellular fractionation improves the sensitivity of the TXP approach. Therefor a set of human liver tissue and preparations thereof, membrane enriched fractions, microsomes and cytosol, were examined. The tissue samples were processed according to the optimized lysis protocol. The preparations were incubated for one hour with the same volume of double concentrated lysis buffer 2 -/- before determining the protein concentration and followed by the immunoprecipitation and the MS-analysis. Immunoprecipitation was performed with 50 µg extracted protein.

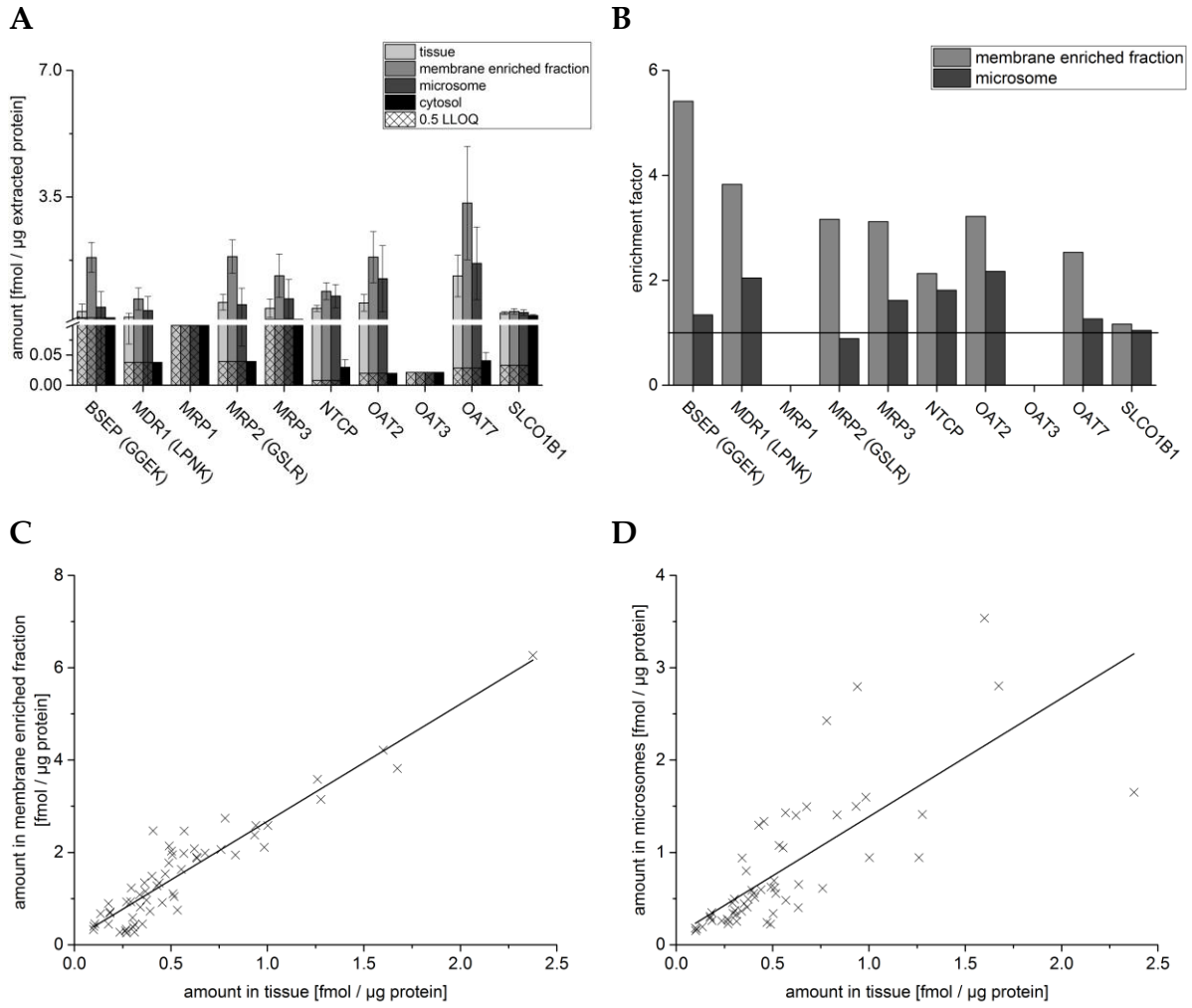


Figure 10: Analysis of subcellular liver fractions. Human liver tissue and three preparations thereof, membrane enriched fraction, microsome and cytosol, were analyzed. The quantified amount was in the same range in the different sample types. Values below LLOQ were set to 0.5 LLOQ for further analysis. Mean and SD are given (A). The analytes were enriched two to fivefold in the membrane enriched fraction and up to twofold in the microsomes (B). The quantified amounts from tissue and membrane enriched fractions correlate well (Pearson R = 0.93) while the amount from tissue and microsomes do correlate slightly (Pearson R = 0.74). Values below LLOQ were excluded from the correlation (C, D). n=8

Eight out of ten targets could be quantified in all sample types (Figure 10A). For BSEP, some tissue samples revealed levels below the LLOQ (see 4.2.8), while all membrane enriched fractions could be quantified. Seven transporters were enriched two or fivefold in the membrane enriched fraction (Figure 10B). One was only slightly enriched. All targets were less enriched in the microsomal fraction. Four targets were not enriched in the microsomal preparation. In the cytosolic fraction, all target proteins

but SLCO1B1 were strongly depleted. SLCO1B1 was quantified in all sample types within in the same range.

It was analyzed whether the analyte amounts quantified in the tissue correlate with the preparations thereof (Table 47). Since most of the transporters could not be quantified in the cytosol preparations, they did not correlate with the amounts quantified from tissue. The quantified amounts of five proteins correlated very well between tissue and the membrane enriched fraction ($R > 0.9$). Between tissue and the microsomes, six proteins correlated well ($R > 0.8$). The data points of all analytes are depicted as scatter plots (Figure 10, C-D). The quantified amounts from tissue and membrane enriched fractions correlated well (Pearson $R = 0.93$) while the amount from tissue and microsomes did correlate slightly (Pearson $R = 0.74$).

Table 47: Correlation of analyte quantification in different sample preparations. The quantified amounts of nine analytes from three different sample preparations were compared to the amounts quantified from tissue. Pearson R is given. n=8

analyte	membrane enriched fraction	microsome	cytosol
BSEP (GGEK)	-0.75	0.96	
MDR1 (LPNK)	0.93	0.98	
MRP1			
MRP2 (GSLR)	0.66	0.87	
MRP3	0.98	0.92	
NTCP	0.39	0.37	0.76
OAT2	0.94	0.88	
OAT3	0.94	0.87	
OAT7	0.99	0.54	0.75
SLCO1B1	0.75	0.77	0.52

4.2.4 Analyte stability on protein and peptide level

For a small sample set, it is possible to perform sample preparation without further storage. Sample lysis and BCA assay are executed on the first day. Enzymatic proteolysis is done overnight, followed by immunoprecipitation on the next morning. MS analysis can be started on the afternoon of the second day. But most of the times it is more convenient or even necessary to be able to pause the sample preparation and

store the samples. Therefore, analyte stability after several freeze-thaw cycles was tested on protein and peptide level. Liver tissue of a phenobarbital treated rat was lysed for this purpose and split into aliquots after determination of the protein content. To test the stability of the proteins, enzymatic proteolysis was started either directly or after up to three freeze-thaw cycles. The freezing periods lasted at least four hours at - 20°C, the thawing periods one hour at room temperature. Subsequently the proteolyzed samples were stored at - 20°C. Analyte stability was determined by quantifying ten transporter peptides after immunoprecipitation by tSIM measurement. To examine the peptide stability on the other hand, the proteolyzed sample was subjected to freeze-thaw cycles. Lysates were frozen once before enzymatic proteolysis was started. Immunoprecipitation was either performed directly or after one freeze-thaw cycle. The experiment was performed twice with three technical replicates each.

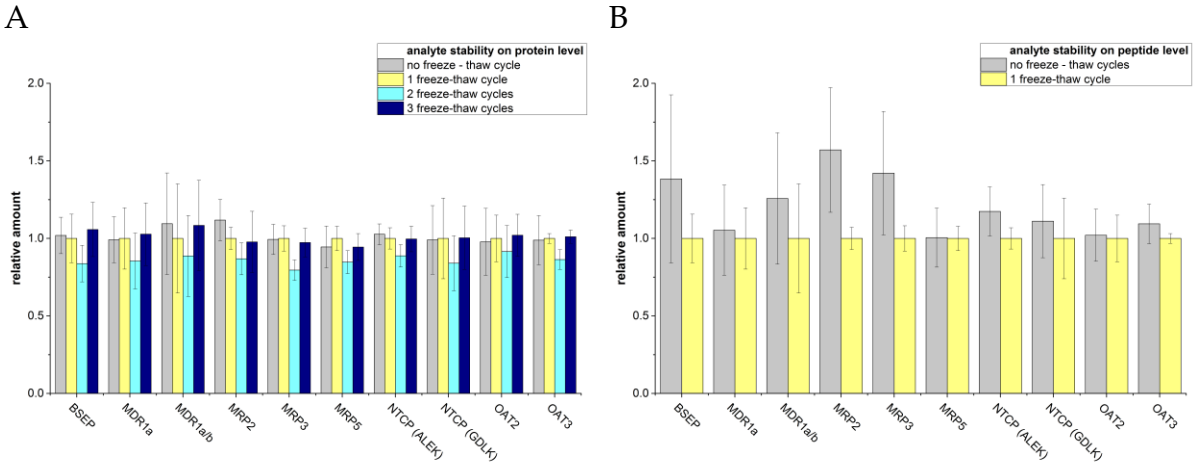


Figure 11: Analyte stability on protein and peptide level. The analyte stability on protein level was examined by subjecting rat liver lysate to up to three freeze-thaw cycles (A). The stability on peptide level was determined by testing the proteolyzed sample (B). Fold change to 1 freeze-thaw cycle and PE are given (n=6). ANOVA Bonferroni analysis was performed ($p \leq 0.05$).

The examined analytes were stable on protein level. The quantified amount of all analytes was slightly decreased after two freeze-thaw cycles but it did not meet the significance criteria (ANOVA Bonferroni ($p \leq 0.05$)). Furthermore, no decrease was detected after three freeze-thaw cycles (Figure 11A). The stability of the examined analytes in the proteolyzed samples was decreased significantly after one freeze-thaw

cycle (Figure 11B). The quantified amount of all tested peptides was slightly increased, after immediate immunoprecipitation. For BSEP, MRP2 and MRP3 this effect was more pronounced.

4.2.5 Adjustment of antibody and sample amount

This experiment served the purpose to determine the minimum amount of antibody and proteolyzed sample which was sufficient for stable quantification, as well as to choose the best antibody serum, if more than one was available and functional. Three different amounts of antibody (1, 2 and 5 µg) were tested as well as three amounts of digested sample (10, 20 and 40 µg). The optimal conditions may vary for the same antibody – peptide combination between the species. Therefore, all assays were tested with pooled human liver samples as well as liver tissue of phenobarbital-treated rats and mice.

The protein amount of tissue lysates was determined before tryptic proteolysis. For each purified antibody, nine different immunoprecipitations were performed testing each antibody amount with every protein amount. 50 fmol of all respective IS peptides were spiked-in. In some cases, when earlier experiments indicated so, 100 fmol (LTIIPQDPILFSGSLR, ITIIPQDPVLFSGSLR) or 150 fmol (SPSFADLFR, VQQEIDEVIGQVR and VQQEIDAVIGQVR) were spiked in. Eluates were measured with tSIM method.

The amount of quantified target protein per µg proteolyzed protein was determined. Data was further analyzed by calculating mean and relative standard deviation of all measurements with 1, 2 or 5 µg antibody as well as 10, 20 or 40 µg protein. For better discrimination, this was repeated with exclusion of measurements with 10 µg protein and 1 µg antibody respectively. A relative standard deviation below 20 % was set as criterion for reproducible quantification. All conditions which could not be quantified because of EN or IS peptide signal quality, were defined as 0 fmol / µg protein.

The results for human assays are given below (Table 48), results for rat and mouse assays are given in the supplemental information (Table 66 and Table 67). Because

human sample material was very limited, antibody sera were excluded from this experiment in case of other experiments indicating so (data not shown). Thirteen purified antibodies were tested with human material. Six led to very reproducible results and can be used with 1 µg antibody and 10 µg extracted protein. Three assays can be run with 20 µg protein and either 2 or 5 µg antibody. Two assays required the maximal protein amount of 40 µg and at least 5 µg antibody. Only two target proteins could not be quantified with any of the tested conditions.

Table 48: Adjustment of antibody and proteolyzed protein amount for human samples. Three different antibody and protein amounts were tested for reproducible analyte quantification. For each purified antibody 1, 2 and 5 µg were tested with 10, 20, and 40 µg proteolyzed protein. Results are given as % RSD. If not stated otherwise, the conclusion column gives the minimal amount of antibody and protein necessary. Antibodies which did not enrich EN or IS peptide sufficiently for quantification were not used further (n.u.f.). Antibodies which lead to suitable IS signals and should be tested again when a sample containing this target is available are additionally indicated with (#).

antibody	peptide	10-40 µg protein AB: 1 –	2 -	5µg	20-40 µg protein AB: 1 –	2 -	5µg	1-5 µg AB protein: 10 –	20 - 40µg		2-5 µg AB protein: 10 –	20 - 40µg		conclusion protein / AB
…DLFR_rbt3	LSPSFADLFR	6.0	3.2	4.2	5.9	1.2	4.6	4.2	0.7	6.8	5.8	0.5	2.9	20 µg / 2µg
	RPSYLDLFR	38	31	23	53	5.0	11	36	10	36	12	4.7	0.9	
	YTASDLFR	—	—	—	—	—	—	—	—	—	—	—	—	
…FFFK_rbt2	FIGLQFFFK	20	51	28	0.9	19	24	36	33	16	14	17	12	40 µg / 6.5 µg
…GDLK_rbt2	GIYDGDLK	1.6	9.8	2.8	1.8	1.5	3.1	11	6.0	2.5	16	2.1	0.5	10 µg / 1 µg
…GSLR_rbt1	LTIIPQDPILFSGSLR	24	28	14	27	0.7	9.2	38	20	25	6.1	27	17	40 µg /5 µg
	ITIIPQDPVLFSGSLR	—	—	—	—	—	—	—	—	—	—	—	—	
…LEVR_rbt1	GGPEATLEVR	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f. (#)
…LIDK_rbt2	TLGGILAPIYFGALIDK	—	43	4.4	—	1.4	2.4	106	87	87	58	0.2	4.0	20 µg / 2 µg
…LTTR_rbt2	NSPGALTTR	9.6	4.0	6.2	5.2	5.5	2.5	16	1.9	6.0	10	2.1	5.9	10 µg / 1 µg
	NTTGALTTR	3.3	11	1.2	0.9	5.8	0.4	9.0	8.6	5.5	11	2.9	2.5	
…PSSK_rbt1	YVEQQYGQPSSK	21	6.6	3.3	28	2.2	2.9	13	17	5.9	9.8	1.6	3.5	use other AB
…PSSK_rbt2	YVEQQYGQPSSK	11	4.5	6.5	9.9	4.3	7.5	12	3.6	2.0	7.0	0.4	2.8	10 µg / 1 µg
…QDEK_rbt2	NKPLFDTIQDEK	6.0	3.5	4.7	3.8	4.2	4.4	6.4	1.8	4.8	6.4	0.1	0.2	10 µg / 1 µg
…QDIR_rbt1	LYDPTEGMVSVDGQDIR	2.5	2.7	2.3	3.4	3.2	2.4	2.6	0.8	0.5	3.5	0.2	0.6	10 µg / 1 µg
…TVEK_rbt1	SSISTVEK	42	17	1.4	26	19	1.8	53	38	17	4.4	30	9.1	20 µg / 5 µg
…YQVR_rbt1	IQFNNYQVR	5.9	2.4	3.7	2.5	3.2	4.2	2.2	3.7	3.6	0.5	5.0	2.4	10 µg / 1 µg

52 polyclonal antibodies were tested for rattine assays: 23 antibodies met the criteria for further method development. Thirteen purified antibody sera were excluded from further experiments because another antibody produced better results. For ten TXP-

epitopes, none of the antibody sera met the criteria. The antibody pAB_TXP_LPSK_rbt2 was used further for the assay development, because spiked-in IS amount was too low for stable signals, but endogenous peptide could be detected in 40 µg proteolyzed protein with 5 µg antibody.

For murine assay development, 66 purified antibody sera were tested: Nineteen antibodies met the criteria and were chosen for further assay development and fourteen antibodies were not used further, because another one produced better results. Sixteen TXP-epitopes could not be covered by any of the purified antibodies.

4.2.6 Compilation of multiplex assays

The results of the antibody and protein amount adjustment were used to compile multiplexed assays. Following criteria were applied to arrange the assays:

1. The mass-to charge ratios of all peptides must differ by more than 1 due to data analysis requirements.
2. The antibody amount must not exceed 7 µg.
3. Each peptide is measured during a 0.6 min time frame. To generate enough data points per peak, less than five time frames should overlap at a time.
4. Minimal sample amount needed for quantification should match.
5. In case of more than one possible combination, the variant with the highest chromatographic resolution is chosen.

There was an exception of criteria one, for peptides with the same TXP – epitope. In case they were separated well in the gradient, they could still be measured in the same multiplex. This was the case for the FTNR peptides.

The slope of the LC step gradient was adjusted to improve peptide separation. The limiting factor however, was the amount of beads which can be transferred by the magnetic particle processor. This confines the total amount of antibody per assay to 7 µg. During the following experiments, some of the assays were further improved by increasing the antibody amount or switching a pair of antibodies between multiplexed

assays. The amount of spiked-in IS peptide was also adapted to the expected level of endogenous peptide. Here the final assay sets are described.

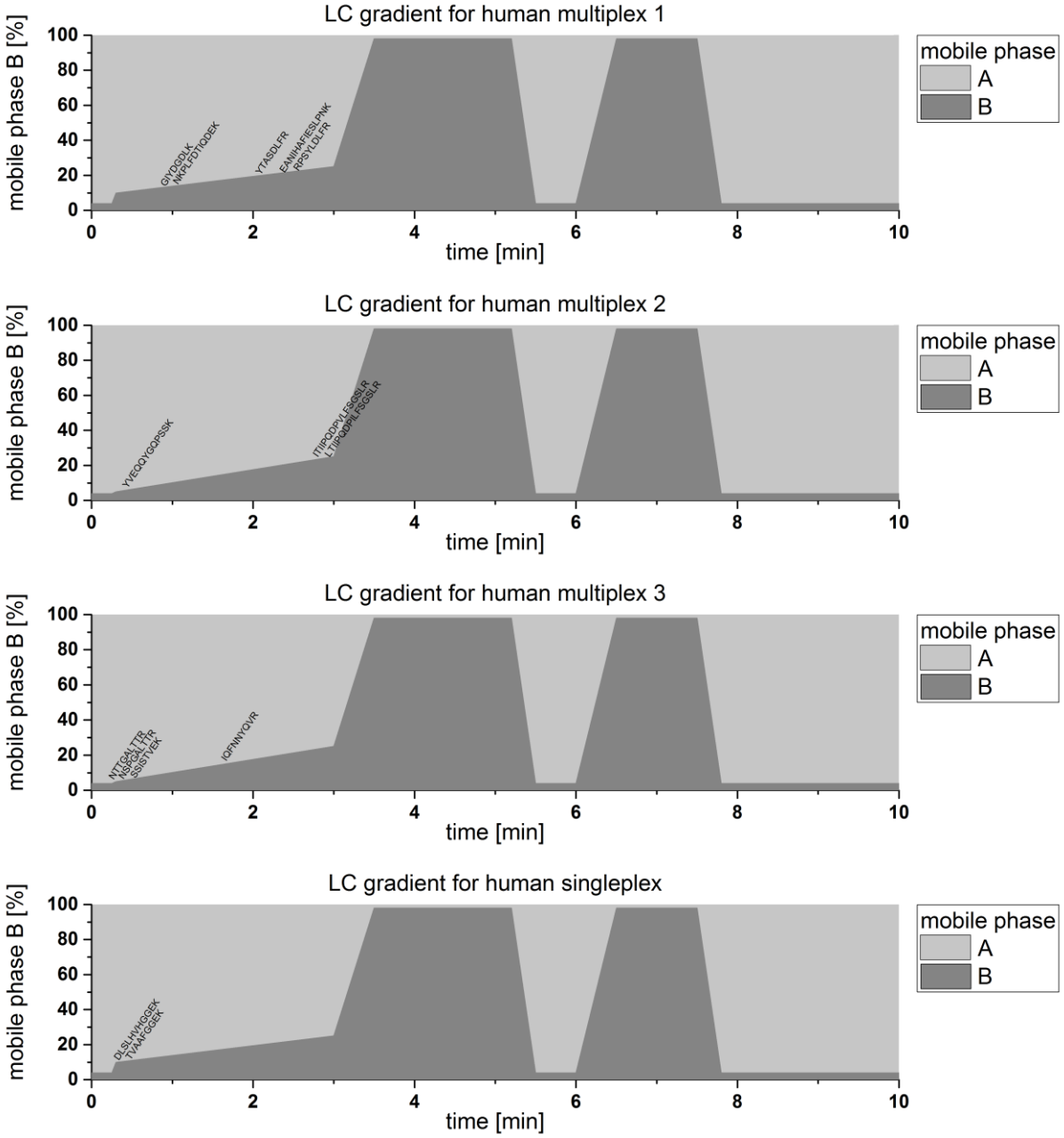


Figure 12: Adjusted LC gradients for multiplexed human assays. The LC gradients for tSIM measurements were flattened to ensure the separation of the increased number of peptides per assay. Peptide sequences indicate the retention times corrected for the dead time.

For human samples, three multiplex and one singleplex assays were compiled. They allow quantification of eleven transporters, three of which can be analyzed with two independent assays. The improved LC step gradients are displayed in Figure 12. The percentage of mobile phase B at the time point of peptide elution was estimated by

means of the retention time and peptides are depicted at the respective time point. Further details are given in Table 49.

Table 49: Multiplexed transporter assays for human samples. Three multiplex (MPh) and one singleplex (SPh) assays were created. The percentage of mobile phase B at the time point of peptide elution was estimated by means of the retention time. The antibodies are sorted in alphabetical order, while the peptides are sorted by increasing retention time / eluent B percentage.

	gradient [% B]	antibody ID	AB amount	peptide	target	spike-in [fmol]	% B at elution
MPh1	10-25	...DLFR_rbt3	2 µg	GIYDGDLK	NTCP	50	15
		...LPNK_rbt1	1 µg	NKPLFDTIQDEK	OAT7	30	16
		...QDEK_rbt2	1 µg	YTASDLFR	OAT3	100	20
		…GDLK_rbt2	1 µg	EANIHAFIESLPNK	MDR1	50	22
				RPSYLDLFR	OAT2	50	22
MPh2	5-25	...GSLR_rbt1	5 µg	YVEQQYGQPSSK	SLCO2B1	50	9
		...PSSK_rbt2	2 µg	ITIIPQDPVLFSGSLR	MRP1	50	25
				LTIIPQDPILFSGSLR	MRP2	50	25
MPh3	5-25	...LTTR_rbt2	1 µg	NTTGALTTR	MDR1	50	7
		...TVEK_rbt1	5 µg	NSPGALTTR	BSEP	100	7
		...YQVR_rbt1	1 µg	SSISTVEK	SLCO2B1	50	8
				IQFNNYQVR	MRP2	50	16
SPh1	10-25	...GGEK_rbt1	5 µg	DLSLHVHGGEK	MRP3	50	11
				TVAAFGGEK	BSEP	50	12

For the analysis of rat samples, 21 antibodies were arranged into seven multiplex and one singleplex assays (Table 50). The antibody pAB_TXP_QDIR_rbt1 was part of two multiplexed assays depending on the expected amount of endogenous peptide. By this means eleven transporters and seventeen CYPs could be quantified, three of which were covered by two independent assays.

Table 50: Multiplexed TXP assays for rattine samples. Seven multiplex (MPr) and one singleplex (SPr) assays were compiled. The percentage of mobile phase B at the time point of peptide elution was estimated by means of the retention time. The antibodies are sorted in alphabetical order, while the peptides are sorted by increasing retention time / eluent B percentage.

	gradient [%B]	antibody ID	AB amount	peptide	target	spike-in [fmol]	% B at elution
MPr1	10-25	...GGEK_rbt1	2 µg	NLTLHVQGGEK	MRP3	50	12
		...NGER_rbt1	1 µg	GIYDGDLK	NTCP	50	14
		...QDIR_rbt1	2 µg	EYGVIFANGER	Cyp2B1/2	150	19
		…GDLK_rbt2	1 µg	LYDPIEGEVSIDGQDIR	MDR1a/b	100	24
MPr2	5-25	...GTVR_rbt1	5 µg	NTTGSLTTR	MDR1b	50	14
		...LTTR_rbt2	2 µg	NTTGALTTR	MDR1a	50	15
				NNPGVLTTR	BSEP	50	16
				LTIIPQEPVLFSGTVR	MRP5	100	23
MPr3	5-25	...ALEK_rbt1	2 µg	AAATEDATPAALEK	NTCP	150	11
		...DLFR_rbt3	5 µg	ALQRPSYLDLFR	OAT2	50	18
				YGLSDLFR	OAT3	30	19
MPr4	5-30	...LDDK_rbt1	1 µg	EANHLISK	Cyp1a2	50	6
		...LDDR_rbt1	1 µg	EAEYLISK	Cyp1a1	40	14
		...LISK_rbt2	2 µg	EIDQVIGSHRPPSLDDR	Cyp2b2	50	15
		...QDIR_rbt1	2 µg	DFNPQHFLDDK	Cyp2a2	50	22
				LYDPIEGEVSIDGQDIR	MDR1a/b	50	28
				DFDPQNFLDDK	Cyp2a1	50	29
MPr5	5-30	...EATR_rbt1	5 µg	EALVDHGEEFSGR	Cyp2c13	50	12
		...FSGR_rbt1	2 µg	EALVDHAEAFSGR	Cyp2b3	50	14
				FINLVPSNLPHEATR	Cyp2e1	50	18
				EALDDLGEEFSGR	Cyp2c55	50	24
				EALIDYGEEFSGR	Cyp2c12	50	24
				EALVDLGEEFSGR	Cyp2c11	100	27
hMPr6	5-20	...FTNR_rbt2	1 µg	ECYSTFTNR	Cyp3a9	50	11
		...LPNK_rbt2	1 µg	TWDPDQPPR	Cyp2d3	50	12
		...QPPR_rbt1	1 µg	HGEIQFNNYQVR	MRP2	50	14
		...YQVR_rbt1	1 µg	ECYSVFTNR	Cyp3a18	50	15
				LQDEIDAALPNK	Cyp3a9	50	18
MPr7	10-40	...EIQK_rbt1	1 µg	LQEEIDGALPSK	Cyp3a2	200	17
		...LPSK_rbt2	5 µg	AMDSFPGPPTHWLFGHALEIQK	Cyp4b1	50	28
SPr1	5-25	...GSLR_rbt1	5 µg	ITIIPQDPVLFSGSLR	MRP1	50	25
				LTIIPQDPILFSGSLR	MRP2	50	25

Five multiplexed and two single assays were arranged, for murine samples. Thereby five transporters and sixteen CYPs could be quantified with fourteen antibodies. Three proteins could be analyzed with two independent assays.

Table 51: Multiplexed TXP assays for murine samples. Five multiplex (MPm) and two singleplex (SPm) assays were created. The percentage of mobile phase B at the time point of peptide elution was estimated by means of the retention time. The antibodies are sorted in alphabetical order, while the peptides are sorted by increasing retention time / eluent B percentage.

	gradient [%B]	antibody ID	AB amount	peptide	target	spike-in [fmol]	% B at elution
MPm1	5-30	...ALEK_rbt1	1 µg	NVTVHVQGGEK	MRP3	50	8
		...GGEK_rbt1	2 µg	NTTGALTTR	MDR1a	50	8
		...LTTR_rbt2	1 µg	NNPGVLTTR	BSEP	50	11
				AAATEDATPAALEK	NTCP	150	13
MPm2	5-25	...TVEK_rbt1	5 µg	SSISTVEK	SLCO2B1	50	8
		…GDLK_rbt2	1 µg	GIYDGDLK	NTCP	50	14
MPm3	5-20	...GQVR_rbt1	2 µg	TWDPDQPPR	Cyp2d40	100	13
		...QPPR_rbt1	5 µg	NTWDPDQPPR	Cyp2d10	50	13
				TTWDPTQPPR	Cyp2d22	100	15
				VQQEIDEVIGQVR	Cyp2d9	300	20
				VQQEIDAVIGQVR	Cyp2d10	300	20
MPm4	10-40	...EATR_rbt1	5 µg	NVSQSLTNFSK	Cyp2c39	50	16
		...NFSK_rbt1	2 µg	NFNQSLTNFSK	Cyp2c38	50	19
				NISQSFTNFSK	Cyp2c29	50	19
				FINLVPSNLPHEATR	Cyp2e1	100	23
MPm5	5-25	...FSGR_rbt1	2 µg	ESLDVTNPR	Cyp2c29	100	12
		...LDDK_rbt1	2 µg	EALVDHAEAFSGR	Cyp2b9	50	15
		...TNPR_rbt2	2 µg	EALVGQAEAFSGR	Cyp2b10	100	18
				DFNPQHFLDDK	Cyp2a12	50	23
				EALDDLGEEFSGR	Cyp2c55	50	24
SPm1	5-45	...NGER_rbt1	5 µg	GYGVAFSNGER	Cyp2a12	50	16
				GYGVTFSNGER	Cyp2a22	50	16
SPm2	5-45	...FTNR_rbt2	5 µg	ECYSVFTNR	Cyp3a25	50	17
				DCLSVFTNR	Cyp3a44	50	22

4.2.7 Kinetic of enzymatic proteolysis

The TXP-method used here quantifies proteins on the basis of proteotypic surrogate peptides. Trypsin is highly effective, but the kinetic of proteolysis can vary strongly between proteins [100]. Therefore, it was essential to optimize the reaction time of the enzymatic proteolysis.

The objective of this experiment was to determine the optimal duration, which allows to quantify all target proteins out of one sample preparation. In order to do so, enzymatic proteolysis of liver lysates was terminated at different time points after 2 to 96 hours. Immunoprecipitation was performed with all antibodies chosen for further method development after the adjustment of antibody and lysate amount as well as all antibody sera which enriched a suitable amount IS peptide but no quantifiable amount endogenous peptide. In addition to the tryptic peptides chosen for quantification, the according peptides with one N-terminal tryptic missed cleavage site were monitored, but not quantified. The highest quantified amount of each peptide was set as one and all other results were given as ratios thereof. The majority of proteins, showed an increase up to six or sixteen hours, followed by a plateau or slow decrease.

The digestion kinetics of the human proteins are given in Figure 13. Seventeen out of 23 tested peptides could be quantified at least at one time point. BSEP was analyzed with two peptides, which both reach a plateau between 6 and 66 hours. MDR1 was determined via three different peptides. The LPNK and QDIR peptides peak after 6 and 16 hours respectively followed by a rapid decrease of the quantified amount. The third peptide, on the other hand, fluctuates between 70 and 90 % of the maximum amount during the complete time course. Both peptides used for MRP2 quantification peak at six hours but the decrease is much stronger for the GSLR peptide. No missed cleavage peptides were detected at any time point (Table 68). To quantify all human targets out of one sample preparation, the best proteolysis duration is between 16 to 18 hours.

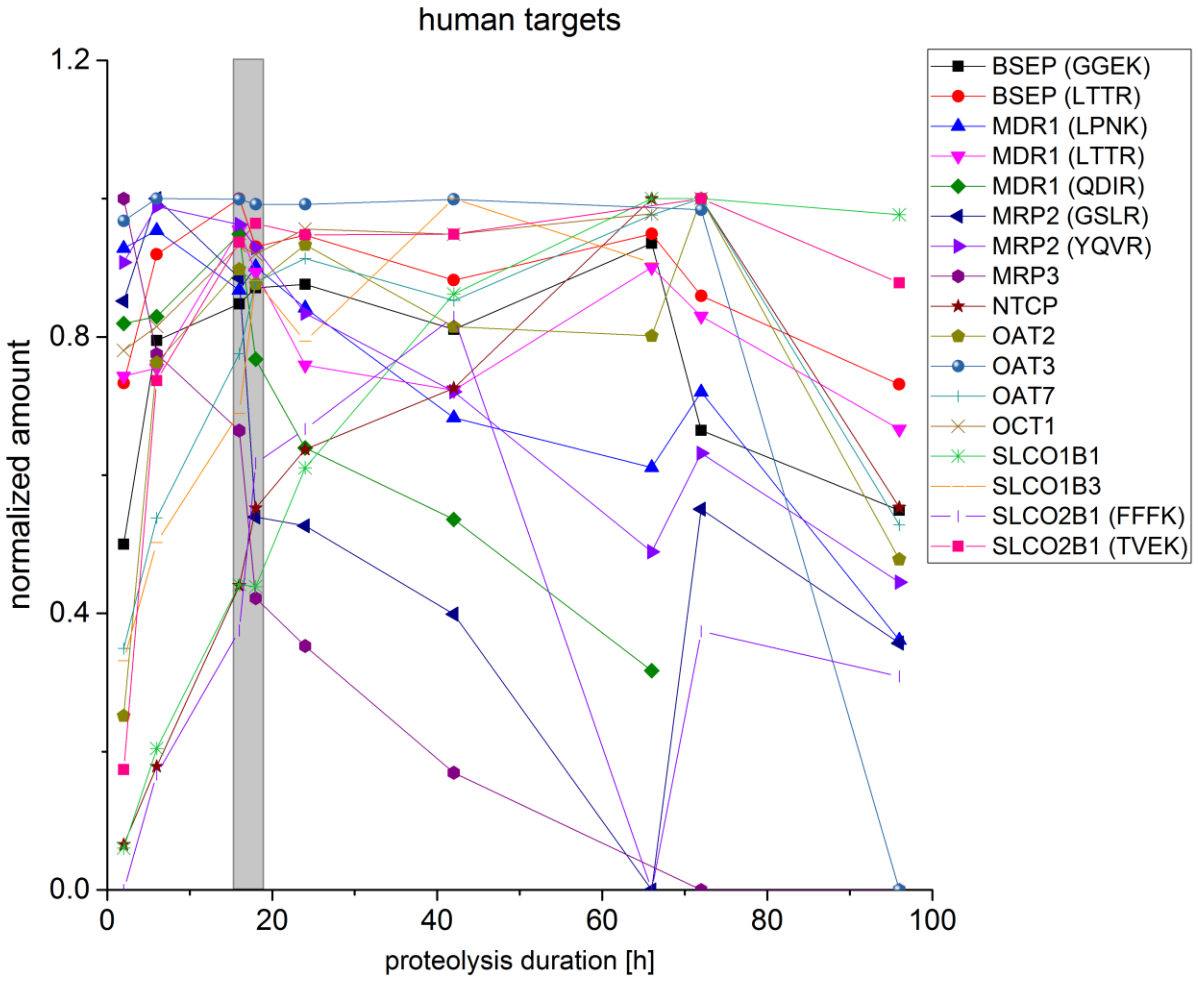


Figure 13: Kinetics of tryptic proteolysis for human target proteins. Enzymatic proteolysis of a human liver lysate pool was monitored during a 96-hours' time course. Results were normalized to the highest quantified amount. The first six measurements were performed in triplicates (2-42h), all others as single measurements (66-96h) Means are given, if available. Proteins determined by more than one surrogate peptide, are specified by the TXP-epitope of the peptides. The best compromise is indicated as gray box.

39 out of 58 tested peptides could be quantified in liver lysate of a PB-treated rat (Figure 14). The miscleaved variants of four peptides could be detected either at early time points or only in one replicate (Table 69). Another four were detected at all time points in at least two replicates. The last peptides were excluded from further method development. Three proteins were analyzed by two different peptides: Cyp3a9 was determined by means of a FTNR peptide which peaked at 6 hours followed by a strong decrease. The LPNK peptide on the other hand reached a plateau after 16 hours. The

EIQK peptide for Cyp4b1 quantification peaked already after 2 hours, while the ESTR peptide reached a plateau between 6 and 24 hours. At the same time signals for the endogenous ESTR peptide were often in the background and therefore not quantifiable, whereas the EIQK peptide was quantifiable in all replicates during the first 24 hours. The third protein analyzed by two different peptides was NTCP. Both peptides reached a plateau, but while the GDLK peptide amount increased for 42 hours, the ALEK peptide was already at plateau level at the first measured time point. As for the human assays, the best compromise was between 16 and 18 hours to be able to quantify all targets with the same sample preparation.

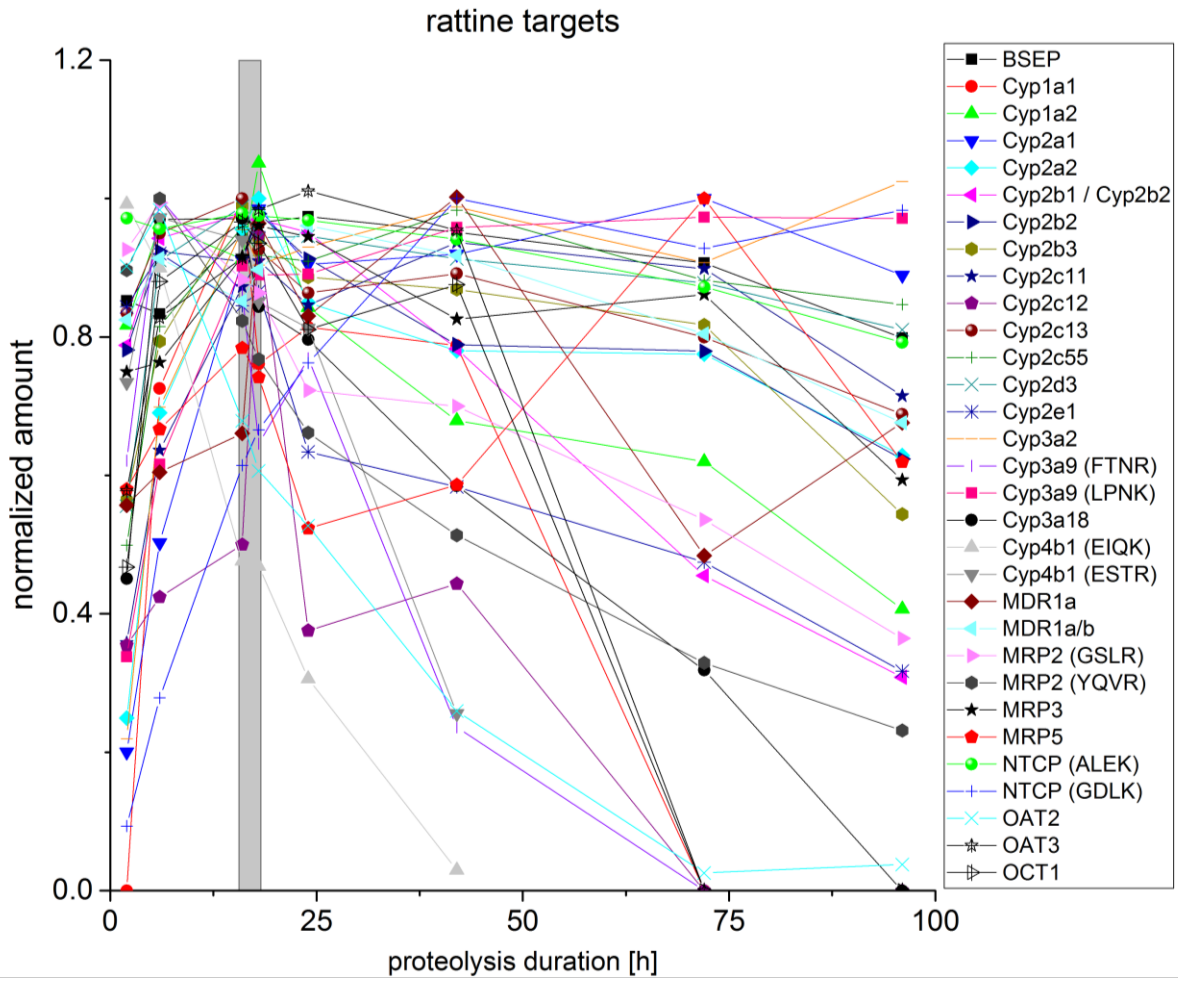


Figure 14 Kinetics of tryptic proteolysis for rattine target proteins. Enzymatic proteolysis of PB-treated rat liver lysate was monitored during a 96-hours' time course. Results were normalized to the highest quantified amount. The first six measurements were performed in triplicates (2-42h), all others as single measurements (72-96h) Means are given, if available. Proteins determined by more than one surrogate peptide, are indicated by the TXP-epitope of the peptides. The best compromise is indicated as gray box.

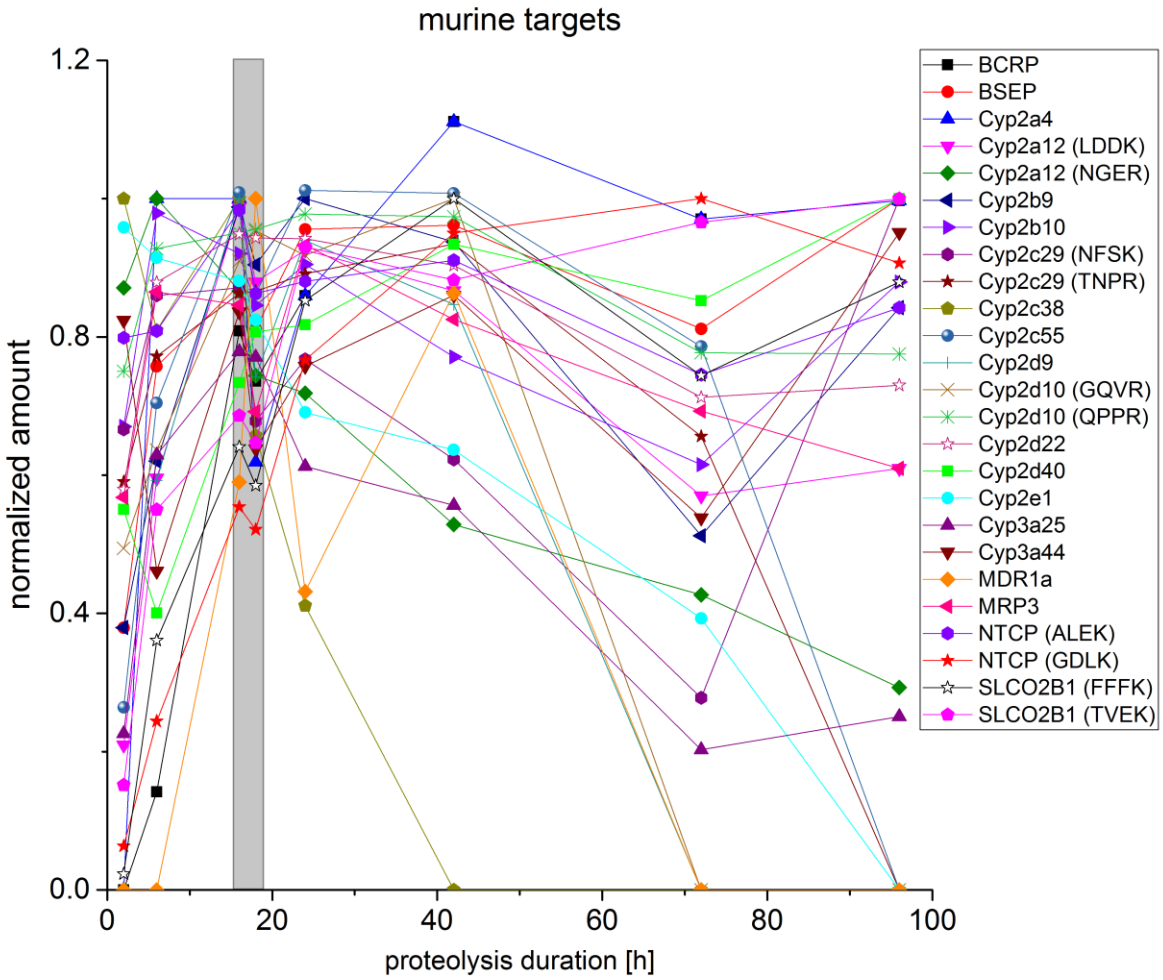


Figure 15 Kinetics of tryptic proteolysis for murine target proteins Enzymatic proteolysis of PB-treated mouse liver lysate was monitored during a 96-hours' time course. Results were normalized to the highest quantified amount. The first six measurements were performed in triplicates (2-42h), all others as single measurements (72-96h) Means are given, if available. Proteins determined by more than one surrogate peptide, are indicated by the TXP-epitope of the peptides. The best compromise is indicated as gray box.

In liver lysates of PB-treated mice, 36 out of 52 tested peptides could be quantified. Two according N-terminal missed cleavage peptides were detected at early time points or only in one replicate. In addition, further nine missed cleavage peptides were detected at all time points and in all replicates (Table 70). The latter were removed from further method development. Four proteins were determined by two independent peptides each. For Cyp2c29, the TNPR peptide reached a plateau after 16 hours, the NFSK peptide on the other hand, reached a maximum between 6 and

16 hours. Both peptides which were used for Cyp2d10 analysis leveled out between 16 and 42 hours. The peptides of SLCO2B1 also showed the same kinetic: they reached a plateau after 24 hours. The rattine and murine assays for NTCP used the same peptide and showed the same kinetics, too. As for the human and rattine assays, the best compromise of the proteolysis duration was between 16 and 18 hours for a standardized sample preparation which allows to quantify all target proteins. Further experiments were performed with all antibodies which were able to enrich their target peptides for quantification.

4.2.8 Determination of the precise and accurate range of the assays

In the previous chapters, it was shown, that the developed assays are sensitive and stable enough to quantify the target peptides in test samples. Since the expression level of the target proteins can vary strongly between sample types, treatments or species, it is important to know the range in which the assays are precise and accurate. Therefor three serial dilutions of all EN peptides of a multiplexed assay were prepared and quantified. The recovery of the EN peptides was determined in percent of the spiked-in amount. An assay was considered precise and accurate enough, when the recovery rate was between 80 and 120 % and, at the same time, the standard deviation was below 20 %. This was defined as measuring range, and the limits as lower and upper limits of quantification (LLOQ and ULOQ).

Recovery was determined for 58 peptides. For 54 of these, a measuring range between one and four orders of magnitude was observed. For four peptides, the assays were either not precise or not accurate enough. Two of the respective proteins could still be quantified with another peptide of the set.

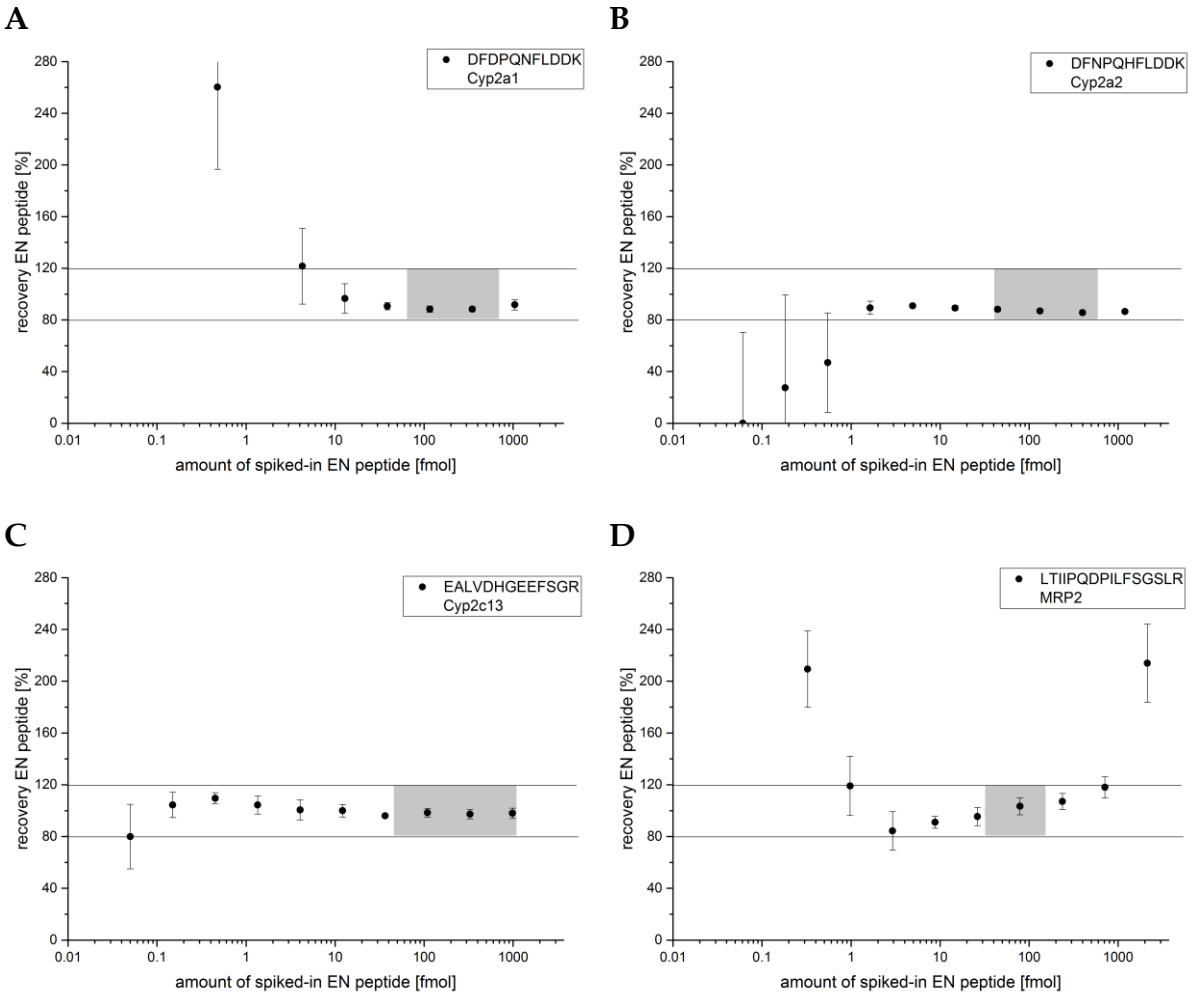
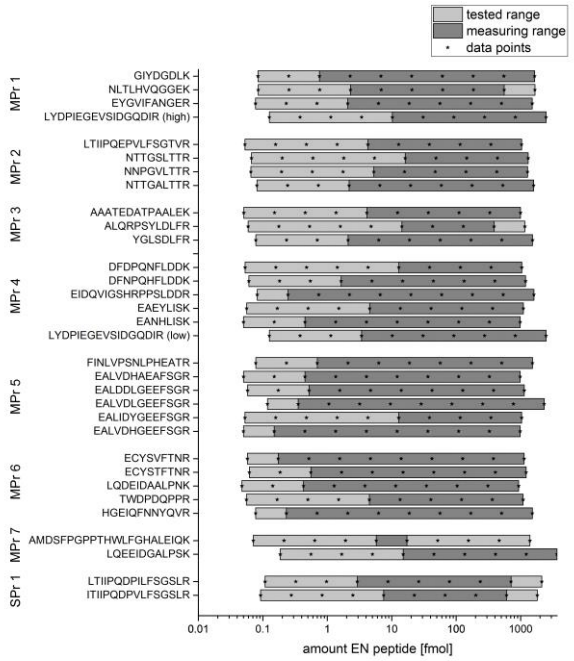


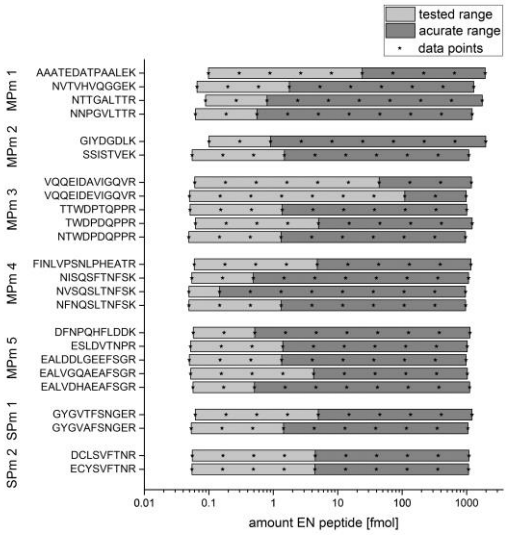
Figure 16: Recovery plots of selected peptides. A serial dilution of EN peptides in blocking buffer and a constant amount of IS peptides was prepared and quantified. Recovery of EN peptide as well as RSD were determined in percent. The range between 80 and 120 % is indicated by lines. The range in which peptides were quantified in rat samples (4.3.4) are indicated as gray boxes. (n=3)

Representative recovery plots of four peptides are depicted in Figure 16. Most assays were precise and accurate for high amounts, but over- (A) or underestimated (B) small amounts of EN peptide as well as becoming less precise. The best assays were stable within four orders of magnitude (C). In some cases, the assays had also an upper limit becoming inaccurate and imprecise for high amounts of spiked-in EN peptide (D). Regardless of the characteristics, all assays were suitable for the intended samples: The amount quantified in liver tissue of azole treated rats was within the measuring range and is indicated in grey (4.3.4).

A rattine assays



B murine assays



C human assays

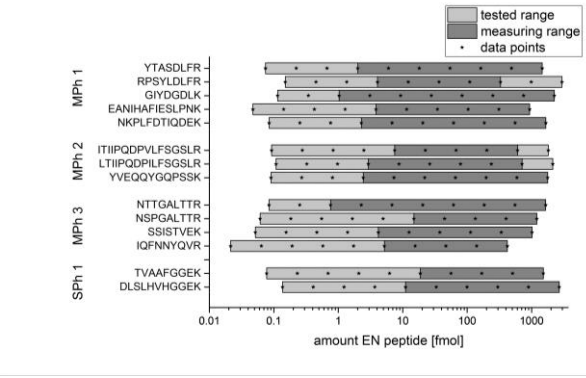


Figure 17: Measuring range of all assays. All EN peptides of a multiplex assay were diluted 1 : 3 in blocking buffer comprising constant amounts of the respective IS peptides. The tested range is depicted in light gray. Each measuring point is indicated by a star. The measuring range is highlighted in dark gray. Here, recovery was between 80 and 120 % and RSD less than 20 %. (n=3)

The recovery plots of all peptides were summarized in Figure 17. Here, the range of the serial dilution is indicated in light gray, while the measuring range is highlighted in darker gray. In case a peptide was part of several multiplexed assays, a recovery plot was produced for each assay. Most of these peptides were in assays for different species but LYDPIEGEVSIDGQDIR, which was part of two rattine assays: The assay MPr3 was based on less spiked-in IS peptide, to lower the measuring range in comparison to MPr1.

4.2.9 Reproducibility of TXP-assays

To investigate the reproducibility of the developed assays, the intraday and interday variation were determined. Due to sample availability, for each species a different starting point was chosen: For the rat assays, three pieces of liver from the same phenobarbital-treated animal were lysed and aliquots were processed individually with the TXP method for each intraday experiment. For the murine assays, nine pieces of liver from the same phenobarbital-treated animal were lysed and processed individually with the TXP method, three for each intraday experiment. Due to low sample quantity, an already existing liver lysate was processed thrice to test the human assays. Three intraday experiments were merged to obtain the interday variation. All data points below LLOQ were set to half LLOQ for following analysis and statistics. The LLOQs determined in 4.2.8 were not applicable for the human assays MDR1 (QDIR), NTCP and OAT7 because they were measured with preliminary multiplexed methods. Here, 1 % of the spiked-in IS peptide was set as lower limit.

The results are depicted in Figure 18. Since the expression level of the target proteins varied between 0.02 and 75 fmol per µg extracted protein, standard deviation of intra- and interday variation is expressed in percent in the text for better comparability.

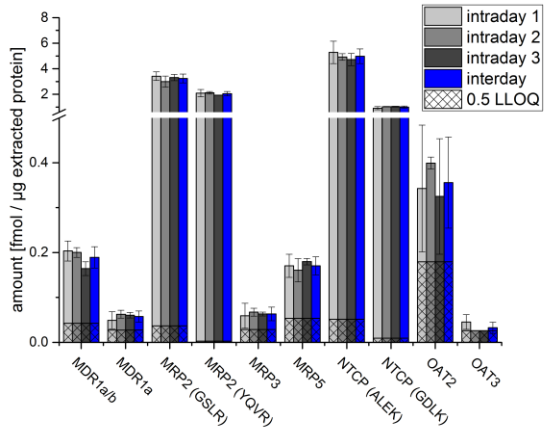
Fourteen human peptides were tested, two of these were below LLOQ. Nine assays showed intraday variation of the quantified targets below 20 %. For two assays, MDR1 (QDIR) and SLCO1B1, one intraday experiment resulted in RSDs greater than 20 %. MRP2 (GSLR) was the only assay which produced always RSDs greater than 20 %. Eight assays also had an interday variation below 20 %.

For the rattine assays, 26 out of 31 tested targets could be quantified. Sixteen of these assays resulted in an intraday variation of less than 15 % in all experiments, three additional ones in less than 20 %. The interday variations of nineteen were also below 20 %. Only the OAT3 assay never revealed an RSD below 20 %, because one or more data points were always below LLOQ.

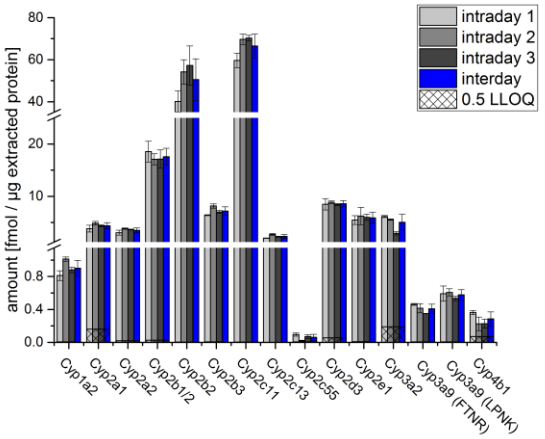
Out of 24 murine assays, six quantified the target with intraday variations below 20 %. Another four assays, had always intraday variations greater than 20 % and five targets

were constantly below LLOQ. The remaining nine assays resulted in at least one replicate below 20 % RSD but also at least one with up to 35 % intraday variation. Ten of these showed interday variation below 30 %.

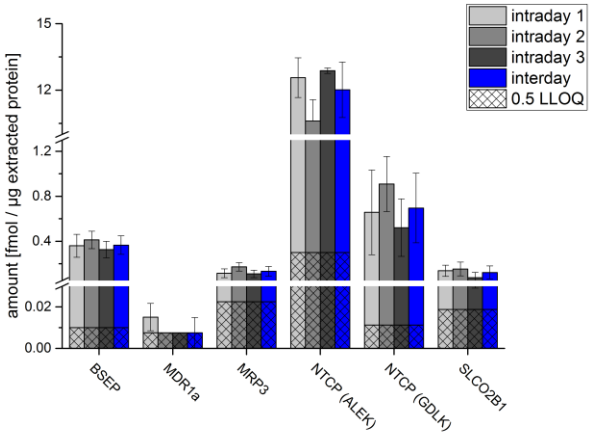
A rattine transporter assays



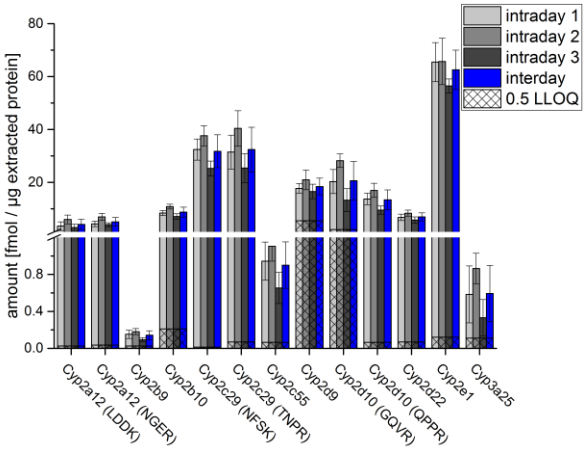
B rattine CYP assays



C murine transporter assays



D murine CYP assays



E human transporter assays

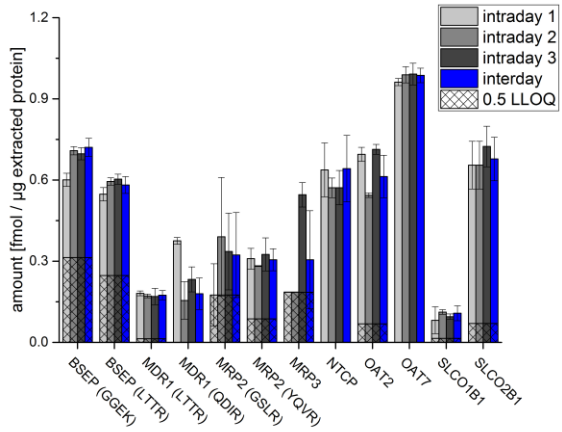


Figure 18: Intra- and interday variation Liver tissue of phenobarbital-treated animals was used to determine intraday variation of rat and mouse TXP assays (A-D). Human assays were tested with one liver lysate (E) (n=3). All intraday experiments were merged to calculate interday variation (n=9). Data points below LLOQ were set to 0.5 LLOQ. Mean and SD are given.

4.3 Application of the developed assays

4.3.1 Study of transporter amount in healthy human liver tissue

Transporter expression in healthy liver tissue which was removed in the course of carcinoma therapy was analyzed. Tissue was processed according to TXP method and immunoprecipitation was performed with 30 µg proteolyzed protein. Nine out of ten transporters could be quantified in the samples (Figure 19). The expression of the transporters was between 0.1 and 1.1 fmol per µg protein. MRP1 was below detection limit in all samples. Most transporters were expressed homogenously, but OAT2 and OAT7 showed a greater variety. The differences could not be linked to the medication of the patients.

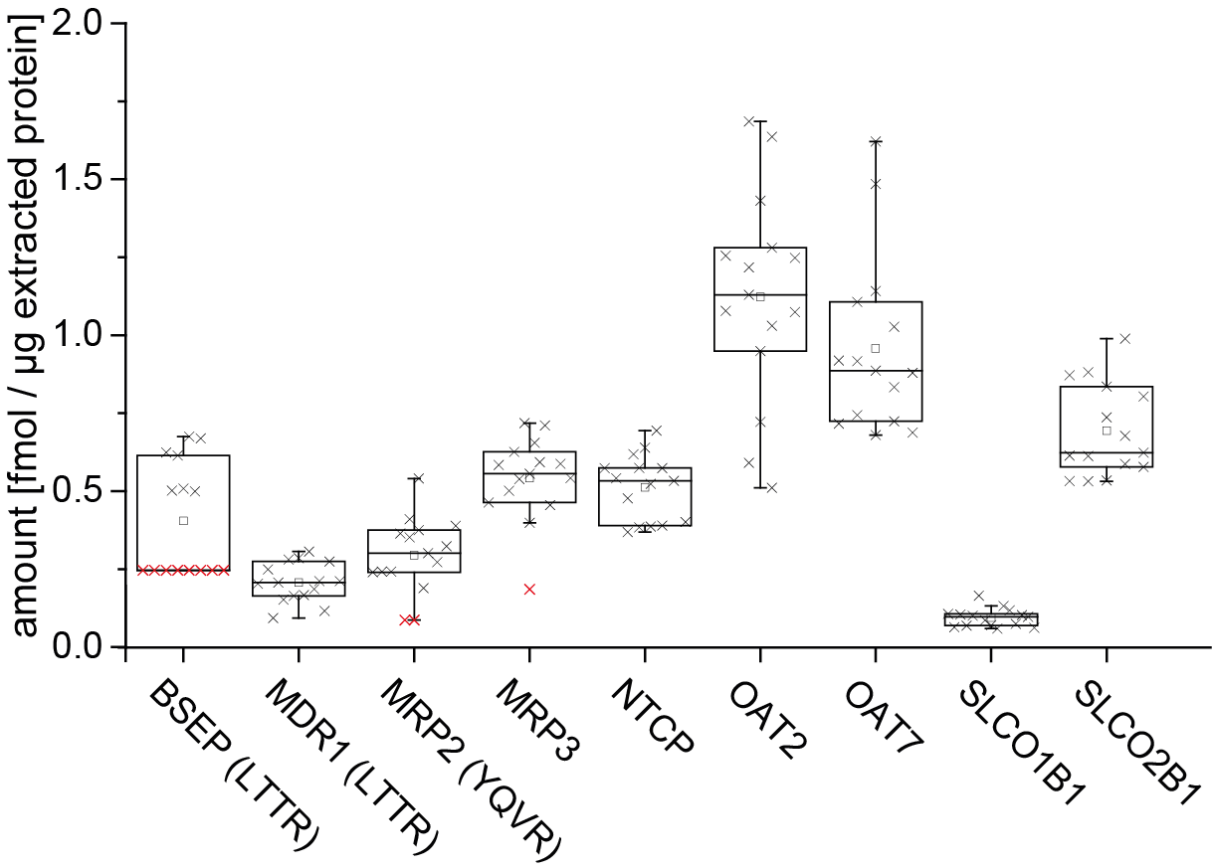


Figure 19: Transporter expression in healthy human liver tissue. Ten analytes were quantified in biopsies of healthy liver tissue. Data points below LLOQ were set to 0.5 LLOQ for further analysis and are indicated in red. MRP1 was below LLOQ in all samples and is not depicted. n=15

4.3.2 Study of transporter amount in paired kidney samples

Kidney biopsies from eleven patients were tested for differences of protein expression in normal tissue and tumor tissue. Protein expression profiles were compared to expressed sequence tag (EST) profiles of UniGene (Table 52).

Two proteins, MDR1 and MRP1, could be quantified in both samples types. For MDR1, this was in accordance with the EST profiles, while MRP1 expression is not predicted for tumor tissue. OAT2 and OAT3 were detected only in normal tissue as predicted by the EST profiles. MRP2 showed a similar pattern, even though it should be expressed in both tissue types according to the EST profile. As predicted by the EST profiles, three proteins could not be detected in any sample: BSEP, OAT7 and SLCO1B1. Additionally, SLCO2B1, MRP3 and NTCP were also below LLOQ in most samples.

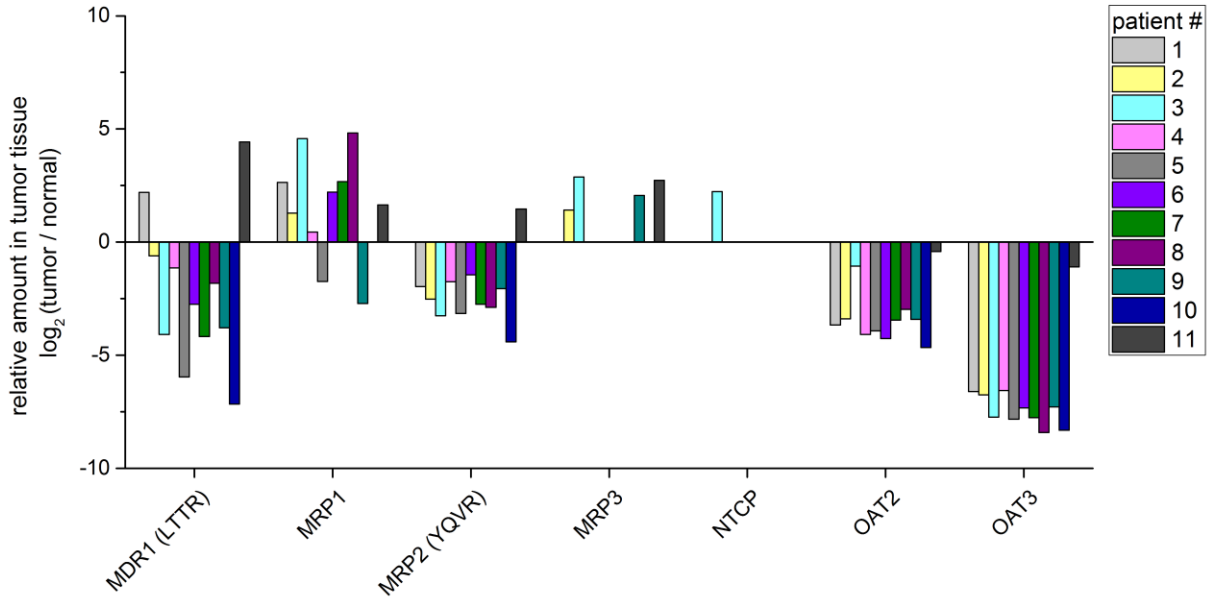


Figure 20: Comparison of transporter expression in paired kidney samples. Expression in tumor and normal tissue samples of the same patient was quantified. Values below LLOQ were set to 0.5 LLOQ for further analysis. Results are expressed as logarithmized fold change. Positive values indicate upregulation in the tumor tissue.

The protein expression of four transporters was downregulated in the tumor, while two transporters were upregulated (Figure 20). The expression profile of patient eleven differed with respect to the downregulated transporters: MDR1, MRP2, OAT2 and OAT3 were expressed less in normal tissue in comparison to the other patients. At

the same time, MDR1 and OAT2 were stronger expressed in the tumor tissue. This led to an upregulation of MDR1 and MRP2 and only a slight downregulation of OAT2 and OAT3 in the tumor tissue compared to the normal tissue sample.

Table 52: Transporter quantification in malign and benign kidney tissue. The protein expression of eleven transporters was investigated in normal (N) kidney tissue and tumor (T) tissue by TXP quantification. Immunoprecipitation was performed with 50 µg protein. Results are expressed as fmol per µg extracted protein. Gray fields indicate measurements below LLOQ. The last row indicates whether the EST profile of UniGene predicts mRNA expression in healthy kidney (N) and kidney tumors (T).

patient #	tissue type	BSEP	MDR1 (LTTR)	MRP1	MRP2 (YQVR)	MRP3	NTCP	OAT2	OAT3	OAT7	SLCO1B1	SLCO2B1
1	N		1.21	0.04	0.20			0.35	1.95			
	T		5.58	0.23								
2	N		1.36	0.07	0.30			0.29	2.16			
	T		0.89	0.16		0.30						
3	N		1.54	0.03	0.50			0.06	4.28			
	T		0.09	0.70		0.82	0.05					
4	N		1.07	0.09				0.47	1.88			
	T		0.48	0.12								
5	N		1.77	0.03	0.46			0.42	4.54			
	T		0.03									
6	N		1.58	0.03	0.35			0.53	3.22			
	T		0.24	0.12	0.13							
7	N		1.27		0.35			0.30	4.33			
	T		0.07	0.05								
8	N		1.19		0.38			0.22	6.84			
	T		0.34	0.22								
9	N		0.98	0.05	0.22			0.29	3.11			
	T		0.07			0.47	0.01					
10	N		3.54		1.11			0.70	6.36			
	T		0.02									
11	N		0.31	0.08				0.12	0.04			
	T		6.66	0.25	0.14	0.74		0.09				
kidney EST profile	N		yes	yes	yes	yes	yes	yes	yes			yes
	T		yes		yes	yes	yes					yes

4.3.3 Protein expression in periportal and pericentral liver cells

Periportal and pericentral liver cells were isolated selectively by digitonin/collagenase perfusion from murine liver. Immunoprecipitation of the processed samples was performed with 5 µg extracted protein because of the small sample amount. Four transporters and nine CYPs could be quantified. The transporters showed no different expression level, but three CYPs, Cyp2c29, Cyp2c55 and Cyp2e1, were expressed higher in pericentral cells.

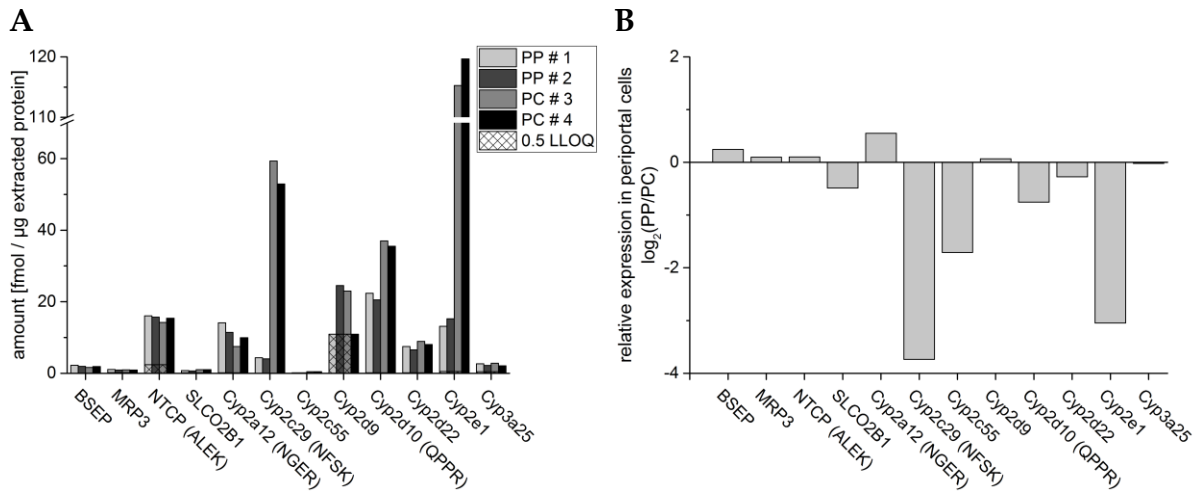


Figure 21: Protein expression in periportal and pericentral cells. Periportal (PP) and pericentral (PC) cells were isolated by perfusion from two mice each. The amount is given for each mouse individually (A) as well as the logarithmized ratio of the means (B). Results below LLOQ were set to 0.5 LLOQ for further analysis.

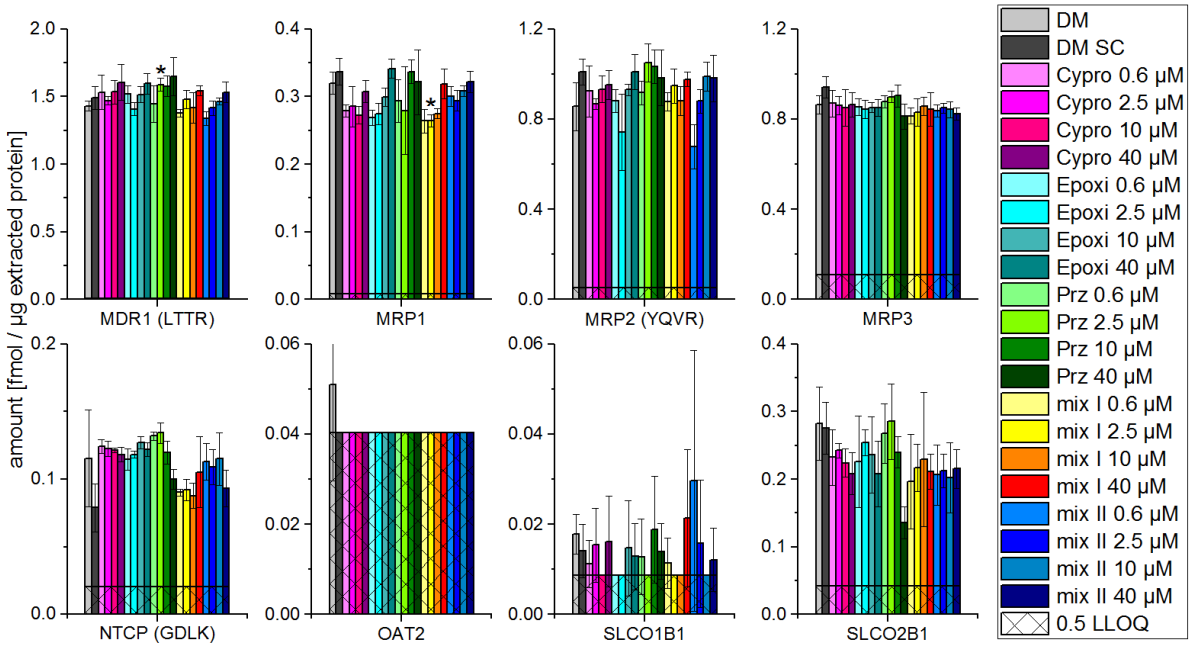
4.3.4 Cross species study of fungicides affecting protein expression

The effects of three azole fungicides, cyproconazole, epoxiconazole and prochloraz, as well as mixtures thereof were investigated in four different models: rats, mice, humanized mice and human cell culture. Frozen liver tissue and lysed cell pellets were processed according to the TXP – protocol.

For the cell culture experiment, the human liver cell line HepaRG was used. Cells were treated for 24 h with the four concentrations of each substance and mixture except for the positive control Citco, which was applied in three doses and with two different media. The differentiation medium (DM) contained more DMSO and fetal calf serum than the work medium (WM). In general, the protein expression of the tested transporters did not change much as response to the different treatments (Figure 22).

MDR1 increased slightly with increasing concentration of mix II but not the single substances. Citco with differentiation medium, on the other hand, led to a slight decrease in comparison to the solvent control. MRP1 amounts increased with epoxiconazole concentration and less pronounced with increasing concentration of mix I. MRP2 was induced slightly with increasing epoxiconazole and mix II concentration but not by mix I. Transporter MRP3 expression was not affected by any treatment but by the change of media for the positive control. The reduction of DMSO and fetal calf serum resulted in higher MRP3 expression regardless of the addition of Citco. NTCP amounts decreased with increasing prochloraz concentration. In addition, treatment with mix I resulted in lower NTCP expression than treatment with the single substances and mix II. SLCO2B1 decreased strongly after treatment with 40 µM prochloraz but was not affected by the other conditions. OAT2 and SLCO1B1 amounts were close to and below the LLOQ of the respective TXP assay. This resulted in high SDs and it was not possible to draw a conclusion whether the expression was affected by the test substances. However, OAT2 did react to the different media used for the positive control. Reduction of DMSO and fetal calf serum led to increased OAT2 amounts regardless of the addition of Citco.

A



B

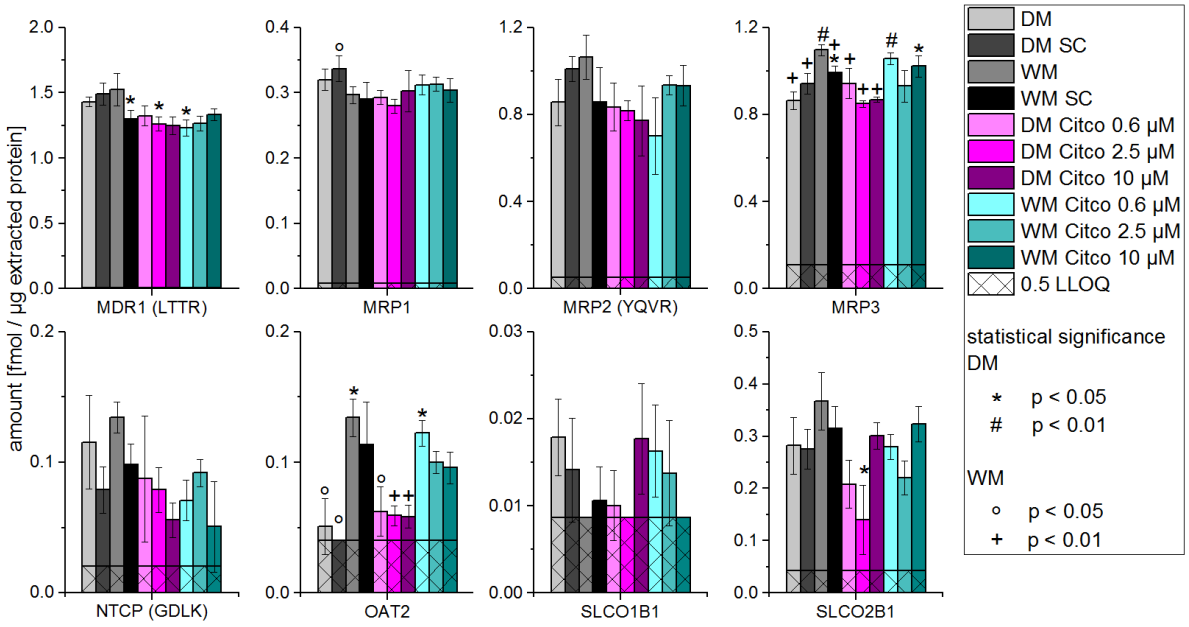


Figure 22: Protein expression of HepaRG cells treated with fungicides. HepaRG cells were treated for 24 h with cyproconazole (Cypro), epoxiconazole (Epoxi), prochloraz (Prz) or combinations thereof (mix I: Cypro + Epoxi and mix II: Cypro +Epoxi+Prz) (A). Additionally, Citco was used as control substance with two different media (DM and WM) (B). Solvent controls are indicated with SC. Values below LLOQ were set to 0.5 LLOQ for further analysis. Mean and SD are given. Significant differences to control (DM) are indicated (Two sample t-test for unequal variances with Bonferroni correction). n=4, solvent control n=8

The rats were treated with the same substances as used in the cell culture experiment but only two concentrations were tested (Figure 23). Additionally, Citco was replaced by phenobarbital as positive control. Cyp1a1 and Cyp1a2 were induced significantly by the high doses of epoxiconazole and mix II. Cyp3a9, Cyp3a18, MRP2 and MRP3 were induced by the high doses of cyproconazole, epoxiconazole and both mixes. The transporters were also induced by phenobarbital and were affected stronger by the mixes than by the single substances. All fungicides and mixes increased the amounts of Cyp2a2, Cyp2b1/2, Cyp2b2, Cyp2c13 and Cyp2c55 as well as the transporters BSEP, MDR1a and MDR1a/b. The induction of these proteins was mainly dose dependent and Cyp2b1/2, Cyp2b2, MDR1a and MDR1a/b were affected stronger by the mixes than by the single substances. Cyp2b1/2, Cyp2b2, MDR1a and MDR1a/b were also induced by phenobarbital. Cyp2b3 amounts were slightly increased after treatment with cyproconazole and mix II. Cyp2c12, Cyp2e1 and NTCP were not affected by the test substances. The amount of Cyp2d3 was decreased by treatment with high doses of cyproconazole and both mixes as well as phenobarbital. OAT2 amounts were reduced by phenobarbital, cyproconazole, epoxiconazole and both mixes. The expression of OAT3 was reduced by all substances in the high dose as well as both mixes in a dose dependent manner. Cyp2c11 amounts were reduced by treatment with the high doses of the mixes. All proteins were reduced stronger by the mixes than by the single substances. MDR1b, MRP1 and MRP5 were below LLOQ in all samples.

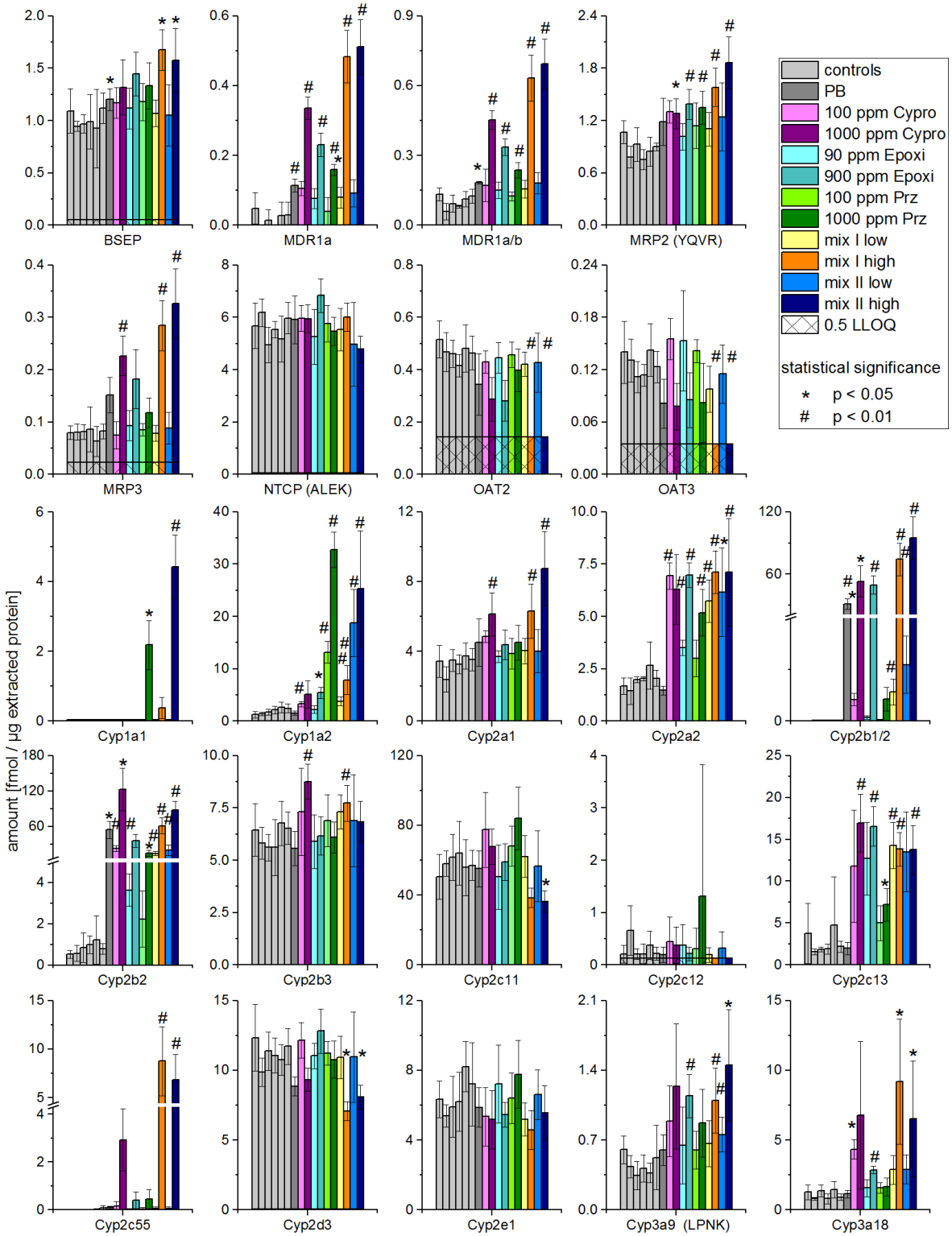


Figure 23: CYP and transporter protein expression in livers of fungicide treated rats. Animals were treated for 28 days with cyproconazole (Cypro), epoxiconazole (Epoxi), prochloraz (Prz) or combinations thereof (mix I: Cypro + Epoxi and mix II: Cypro + Epoxi + Prz) as well as PB as positive control. CYP and transporter expression in liver tissue was quantified. Values below LLOQ were set to 0.5 LLOQ for further analysis. Mean and SD are given. Significant differences to respective control are indicated (Two sample t-test for unequal variances with Bonferroni correction). Control groups did not differ significantly. n=5, mixes n=10

The mouse experiment was performed to compare how wildtype and humanized mice are affected by the fungicides. The humanized mice express only the human forms of the nuclear receptors CAR and PXR. Two concentrations of cyproconazole and prochloraz as well as phenobarbital as positive control were tested. In general, it could be observed, that the protein expression was induced by all test substances and that humanized animals were affected less strongly (Figure 24). Cyp2c38 was only induced by cyproconazole, while Cyp2b9 was induced by cyproconazole and phenobarbital but not by epoxiconazole. Cyp2e1 amounts were increased slightly by phenobarbital and epoxiconazole in wildtype mice. The high dose of cyproconazole, on the other hand, decreased the amount of Cyp2e1. In the humanized mice, Cyp2e1 was decreased slightly after cyproconazole and low epoxiconazole treatment. NTCP and SLCO2B1 amounts were hardly affected by the treatments short of the high cyproconazole dose. It led to decreased expression of these transporters. BSEP and MDR1a were hardly affected and Cyp2a22, Cyp2d40 as well as Cyp3a44 were below LLOQ of the respective assay in all samples.

Additionally, it was compared how the azole fungicides affected ortholog proteins in the different models. Cyproconazole and prochloraz were administered in all experimental set ups. For the comparison, only two concentrations of the cell culture experiment were considered. They correspond to the amounts quantified in rat liver tissue after cyproconazole and prochloraz treatment [91].

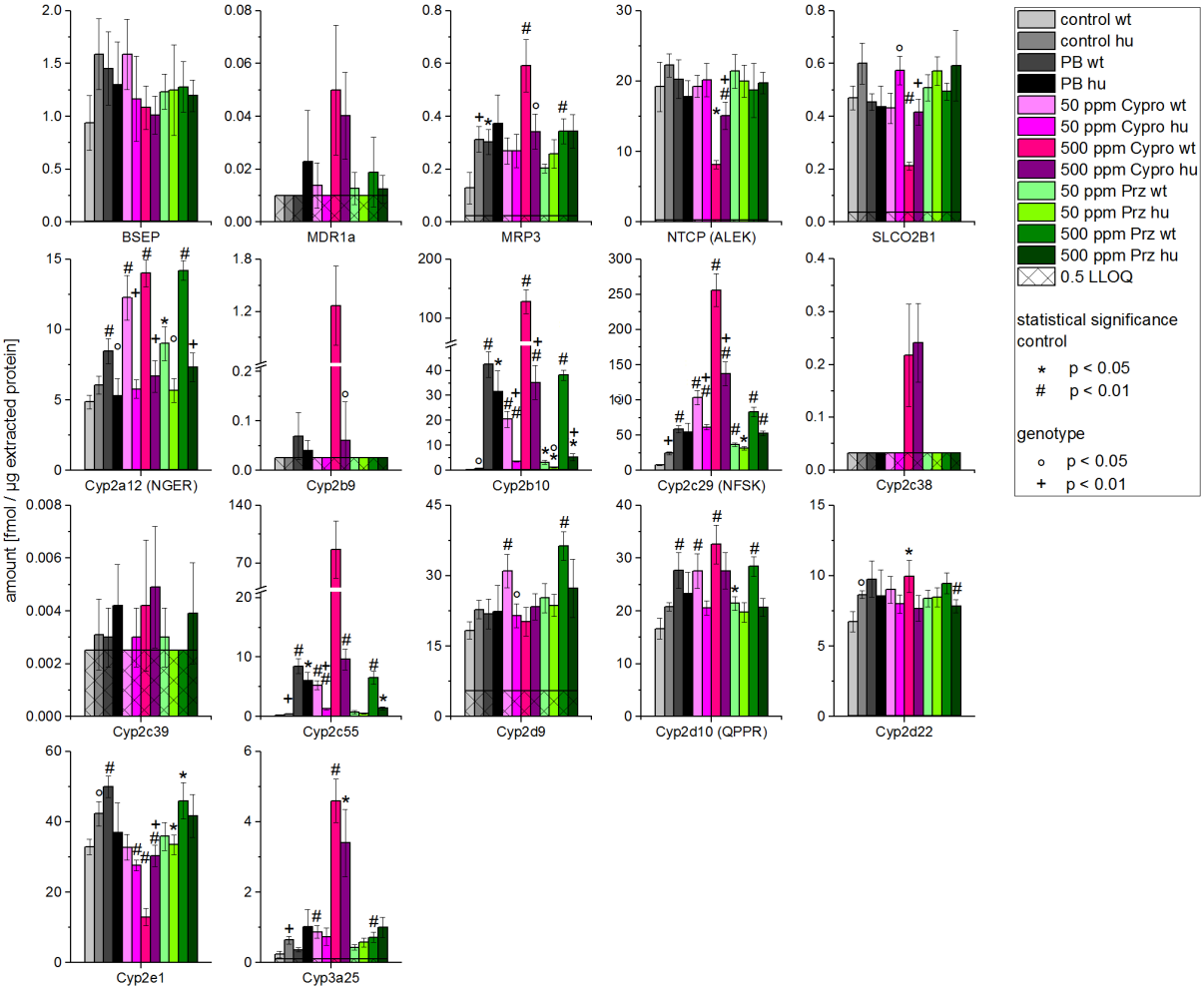


Figure 24: Protein expression in wildtype and humanized mice after fungicide treatment. Mice which express the human forms of the nuclear receptors CAR and PXR were compared to wildtype mice. Animals were treated for 28 days with cyproconazole (Cypro), prochloraz (Prz) or phenobarbital (PB). CYP and transporter expression in liver tissue was quantified. Values below LLOQ were set to 0.5 LLOQ for further analysis. Mean and SD are given. Significant differences to respective control are indicated (* $p<0.05$ and # $p<0.01$) as well as differences to the wildtype group with same treatment (° $p<0.05$ and + $p<0.01$). (Two sample t-test for unequal variances with Bonferroni correction) n=5

MDR1/a, MRP3 and NTCP were quantified with the same TXP assay in all models (Figure 25). MDR1a was induced by cyproconazole and to a lesser extent by epoxiconazole in the rodent models. In HepaRG cells, the transporter was not affected by the treatments. MRP3 amounts were increased by the fungicides in rats and wildtype mice. This effect was not observed in humanized mice and HepaRG. The expression of NTCP was only decreased by the high dose of cyproconazole in both

murine models. All other conditions did not affect its expression level. In general, MDR1 and MRP3 were expressed higher in HepaRG than in the rodent models. NTCP amounts, on the other hand, were much lower in the cell culture samples than in the rodent tissue samples. For the humanized mouse models, the expression levels were similar to the other rodent models, but the reaction to the treatment matched better with the HepaRG than the other rodent models did.

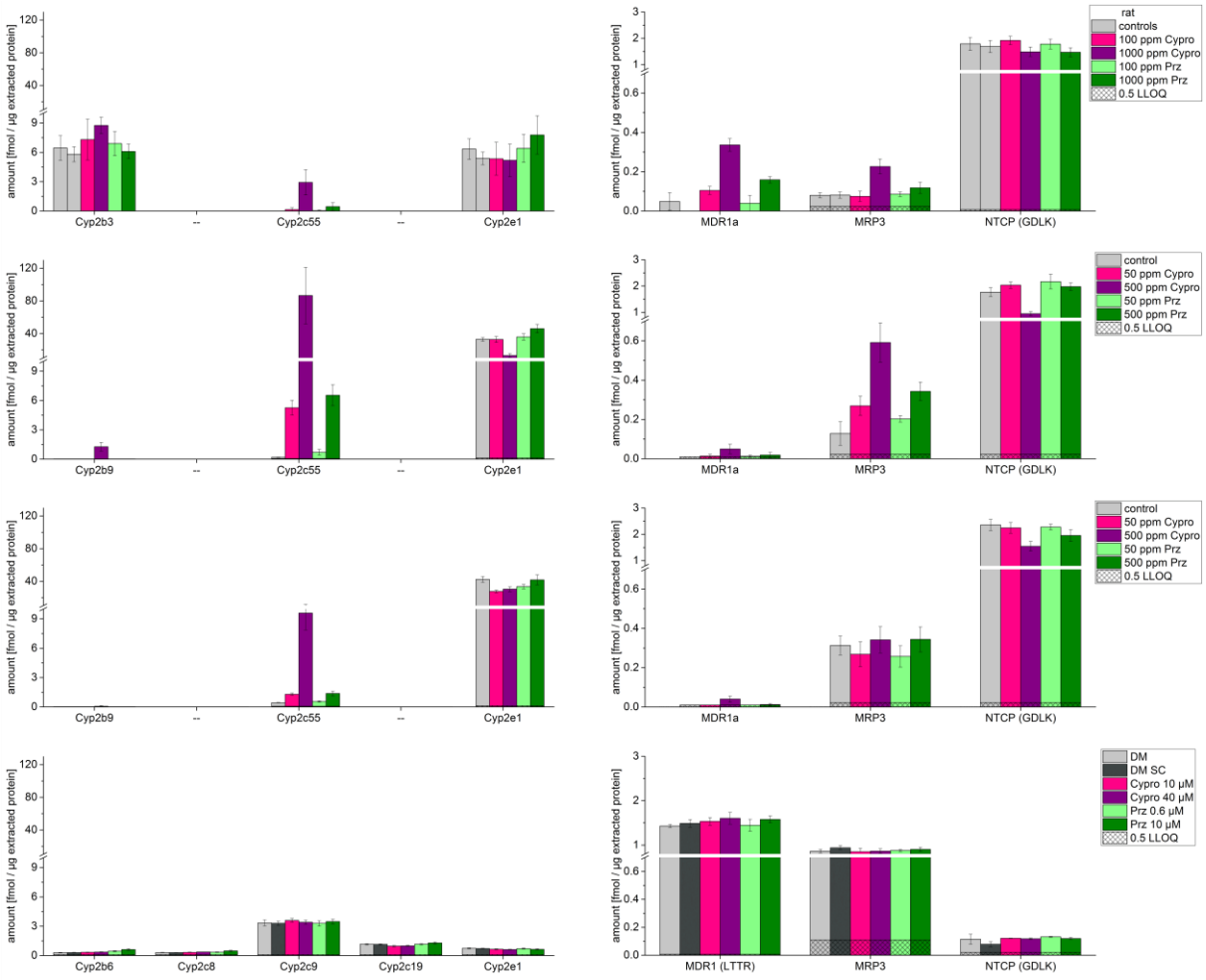


Figure 25: Comparison of fungicide treatment in different experimental models. MDR1(a), MRP3 and NTCP were measured in all models with the same TXP assay while the CYPs were measured only in the rodent models with the same TXP assays. The HepaRG CYP data was kindly provided by Frederik Weiß and thus analyzed with different TXP assays. Orthologous proteins are arranged in columns. The rodent Cyp2c55 is orthologous to the human Cyp2c8, Cyp2c9 and Cyp2c19. Mean and SD are given. Rodent models n=5, HepaRG n=4.

The rodent CYP data is complemented by data from HepaRG, which were kindly analyzed and provided by Frederik Weiß. Cyp2e1 was quantified with the same TXP

assay in rodents. For the human HepaRGs the peptide DEFSGR was used. The expression level of Cyp2e1 was higher in rodents than in HepaRG. Treatment with cyproconazole or prochloraz did not affect the expression in HepaRG and in rats. In the murine models cyproconazole reduced the Cyp2e1 expression. This was more pronounced in the wildtype mice. The human Cyp2b6 has several orthologs in rodents. Cyp2b9 in mice and Cyp2b3 in rats are two of them [47, 48]. In contrast to Cyp2b3 and Cyp2b9, Cyp2b6 was quantified using the peptide AEAFSGR. While the Cyp2b3 and Cyp2b6 were not affected by the treatment, the amount of murine Cyp2b9 was increased by cyproconazole. Humanized mice were affected to a lesser extent than the wildtype animals. Furthermore, the basal expression level was highest in rats and lowest in the murine models. The rodent Cyp2c55 has several orthologs in humans: Cyp2c8 (EALIDNGEEFSGR), Cyp2c9 (GIFPLAER), Cyp2c18 (EALIDHGEEFSGR) and Cyp2c19 (GHFPLAER) [22, 47, 48]. Cyp2c55 was induced in rats and mice by all tested substances, but by far the most with the high dose of cyproconazole. At the same time, none of the human orthologous proteins was induced by the treatment. Cyp2c18 was below the LOQ of the respective TXP assay.

Additionally, three analytes were quantified in two species: MRP2 was quantified in HepaRG and rat. In both models the expression was affected by epoxiconazole and the mixes. SLCO2B1 was not affected by the treatment in HepaRG and humanized mice, but was decreased by cyproconazole in wildtype mice. Cyp3a25 in mice and Cyp3a18 in rats were analyzed with the same assay: Both analytes were induced by cyproconazole and epoxiconazole. Induction by the high cyproconazole dose had by far the greatest effect in all models.

5 Discussion

5.1 Selection of TXP epitopes

The TXP epitopes and proteotypic peptide standards for new TXP assays are chosen by bioinformatical means and peptides and epitopes with undesirable characteristics are excluded. Nonetheless, it is not possible to predict for which epitope a sensitive assay can be developed, because a multitude of factors influence the production of TXP antibodies. Retrospectively, two aspects which have been discussed to be included in the selection process have been analyzed: The number of proteins which are addressed by a TXP epitope and whether or not a high abundant protein is addressed [22].

To investigate the effect of the number of proteins covered by a TXP epitope, the ratio of proteins covered and all UniProtKB reference proteome entries for this species were determined. The ratio was used to correct the bias of using a database which is still under progress. Subsequently, TXP epitopes were sorted into groups for successful and non-successful assay development for each species separately. These groups could not be discriminated by the protein ratios.

To test the effect of high abundant proteins sharing the TXP epitope on the success rate of the assay development, fifteen high abundant liver proteins were chosen per species (Table 45). 13 % of the chosen TXP epitopes target one or more high abundant proteins. Excluding these epitopes from the assay development would not improve the success rate. In fact, 13 % of the assays in the final set use an antibody which addresses also a high abundant protein. The presence in high abundant proteins of representative epitopes was compared to the success of assay development in Table 53. The complete list is given in the supplemental information (Table 60). This selection of epitopes shows that the epitope presence in these high abundant proteins could not be linked to the success rate of assay development: The epitopes DLVR, LTTR, QDEK and YQVR did not address one of the high abundant proteins, but it was still not possible to establish a TXP assay for DLVR. On the other hand, it was possible to develop assays with the TXP epitope EATR even though it is present in a high abundant protein. This

becomes even more apparent, when the different species are taken into account. While LDDK and NGER addressed only high abundant proteins of one species, it was possible to establish a functional assay in all tested species. Vice versa, even though LSGK addressed only human phosphatidylethanolamine-binding protein 1, it was not possible to establish a functional assay in any of the tested species.

Table 53: Comparison of epitope presence and successful assay development. The presence of a TXP epitope in one or two of the high abundant proteins (HAP) is indicated by light red (1) or dark red (2) respectively. Green fields ( ) indicate successful assay development, gray fields ( ) indicate either no presence in high abundant proteins or no successful method development and white fields ( ) no measurement at all.

epitope	epitope present in HAP			successful assay development		
	human	rat	mouse	human	rat	mouse
DLFR			2			
DLVR						
EATR		1	1			
EVLR		1				
LDDK		1				
LSGK	1					
LTTR						
NGER			1			
QDEK						
YQVR						

Therefore, the amount of addressed proteins and the epitope presence in high abundant proteins should not be considered in the selection process. It is likely that they influence the sensitivity of the TXP epitope but the effects are very small in comparison to other factors, for example the difference between the sera of two animals. Adding these criteria to the selection process would result in rejecting also epitopes which are suitable for TXP assays without improving the success rate of the assay development.

5.2 TXP assay development

The sequence logos were created with a standardized matrix produced from lysates of three different human cell lines. It has been shown that sequence logos generated for

different human cell lines vary only slightly [35]. To ensure that the sequence logos can also be applied for other species and tissues, three antibodies were analyzed with mouse and rat liver as well as rattine heart and kidney. All samples resulted in similar sequence logos. Restrictive positions of the epitope as well as preferences for certain amino acids were conserved in all sample types. This showed that the binding motif determined with the standardized matrix can be transferred between sample types, organs and species. The sequence logos served as quality control of the immunization process as well as reference for the selection process of new TXP assays. They could not be used as decision criterion whether to pursue assay development or not, because enrichment of the target peptide did not correlate well. Eight purified antibody sera enriched the spiked-in target peptide even though no statistically significant sequence logo could be determined.

Several antibodies enriched spiked-in peptides well, but no endogen peptide could be detected in the samples used for the assay development. Two explanations could be that either the target proteins were not expressed in the sample or that the assays were not sensitive enough. To examine the latter, ten transporters were quantified in human liver tissue samples and preparations thereof: membrane enriched fractions, microsomes and cytosol as negative control. All analytes could be quantified either in all tissue samples and enriched sample preparations or none except for BSEP. It could be quantified in all membrane enriched fractions, but was below LLOQ for some tissue and microsomal samples. This was resolved by using a more sensitive assay (BSEP (LTTR)) which utilizes a different surrogate peptide. The analytes were enriched two- to fivefold in the membrane enriched fraction and up to twofold in the microsomal fractions. This can also be achieved by using more proteolyzed protein for the immunoprecipitation. Thus, there is no benefit in additional sample prefractionation like ultracentrifugation to enrich the target proteins. Furthermore, the quantified amounts correlated better between tissue and membrane enriched fractions which had been prepared by one additional step than between tissue and microsomal preparations. This is may be caused by sample loss during additional work steps of

the microsome preparation work flow. Additionally, fractionation of samples results in loss of the analytes, especially when an analyte is distributed over more than one fraction [101, 102]. Each step of the sample preparation is time consuming and an additional source of errors. Therefore, it was resigned from additional subcellular fractionation to enrich the analytes.

Another important issue was after which steps of the preparation the analysis can be interrupted and the samples be stored. The stability experiments showed that the analytes were more stable at protein level than on the peptide level. Therefor it was preferred to store samples as lysates instead of proteolyzed solutions. Due to limited sample amounts and time, it was not possible to also test the stability of the frozen tissue samples. However, the sample set used to compare different forms of sample preparation was collected between December 1999 and August 2001. The samples used to study transporter expression in healthy human liver, on the other hand, were collected between August 2013 and March 2015. The samples had nothing in common but being human liver tissue samples. Nevertheless, the quantified amounts were in the same range for each target protein (Table 54 in the supplemental data). Therefor it can be assumed that storage of frozen tissue is not problematic for this assay.

Following the functionality test of the antibodies, it was estimated how much antibody and proteolyzed protein was needed to ensure a stable quantification. This experiments was also used to choose the better antibody in case more than one passed the first threshold. The design of the experiment made it difficult to decide whether an assay could be set up only with 5 µg antibody and 40 µg proteolyzed protein because this decision had to be based on a single measurement. Hence RSD as criterion was not applicable. Therefore, MS signal quality was taken into account. In case the peptides were detectable under two other conditions but with high RSD, it was estimated as likely that the assay could be used with 5 µg antibody and 40 µg proteolyzed protein. Four antibodies were tested further with reservation: pAB_TXP_LPSK_rbt2 and pAB_TXP_GTVR_rbt1 as well as pAB_TXP_ESTR_rbt1 and pAB_TXP_KPHR_rbt1. The first two turned out to be suitable assays, while the

development for the latter two had to be terminated. Assays which reliably enriched the IS peptide but no endogenous peptide were excluded from further assay development unless the antibody was part of another assay which fulfilled the criteria. This was done in case they could be measured in other sample types. This was the case for MRP1 (GSLR) and ABCB1b (LLTR). While MRP1 could be quantified in human kidney and HepaRG, ABCB1b was below detection limit in all tested samples. Based on this experiment multiplexed assays were arranged to reduce the required sample amount and time. The limiting factor was the amount of bead solution, which could be transferred completely during the immunoprecipitation with the magnetic particle processor. Remnants in the eluate could clog the chromatography system and damage the columns. Four multiplexed assays for human samples, eight for rattine and seven for murine samples were established.

The multiplexed assays were used to examine the kinetics of tryptic proteolysis which can vary strongly between different proteins [100] and even different cleavage points of the same protein. Three proteins with very differing kinetics are for example human MRP3, OAT3 and SLCO1B1. While the quantified amount of MRP3 decreased from the first measuring point after two hours, SLCO1B1 showed the other extreme: The quantified amount increased for 66 hours before it reached a plateau. OAT3, on the other hand, stayed on a plateau for 72 hours. The human transporter MDR1 was quantified by means of three peptides, while two peaked within 16 hours followed by a decrease, the third peptide stayed on a plateau for 96 hours. Therefore, it is very important to determine the proteolysis kinetics of every analyte. Overnight proteolysis was not optimal for several analytes, but it was the best compromise which allowed to quantify all analytes subsequently to the same sample preparation. This was observed for all three species independently.

LLOQ and ULOQ of the assays were estimated by quantifying a dilution series of synthetic EN peptide in blocking buffer. It would have been more accurate to use forward and reverse curves as described for example by Razavi et al. [103] but this requires large amount of sample which is especially difficult for human tissue.

Therefore, the proteolyzed sample was imitated by digested fish gelatin. This had the additional advantage, that there were no endogenous levels of the analytes. The tested range was based on earlier experiments with one additional order of magnitude at the lower end [22]. The TXP assays covered a range of one to four orders of magnitude in which the recovery was precise and accurate. The characteristics of the recovery plots depend on the peptide as well as on the used purified antibody serum. Cyp2a1 and Cyp2a2 for example were both quantified by using the antibody pAB_TXP_LLDK_rbt1. Below the LLOQ Cyp2a1 amount was overestimated while Cyp2a2 was underestimated. Low analyte amounts resulted in imprecise and inaccurate measurements. For all assays the LLOQ lay within the tested range. It is very likely that the assays also become imprecise or inaccurate for very high amounts of EN peptide, but for most assays this was not the case within the tested range. The tested range was not extended, because it already spanned the range expected in the samples.

Last but not least, the intra- and interday variation of the developed assays was determined. Due to sample availability, a different starting point was chosen for each species. For the human assay set, one lysate was processed nine times. For the rat assay set one tissue sample was split into three pieces and resulting lysates were processed for each intra assay experiment. For the murine assays, the tissue sample was split into nine pieces, three for each intraday experiment. This influenced also the RSDs of the intra- and interday experiments. While the vast majority of all intraday and interday variations were below 20 % in human and rattine samples, only four analytes of the murine sets achieved an interday variation below 20 %. This was especially apparent for NTCP which was quantified in every species with the GDLK peptide. While the RSD of all intraday and the interday variations were below 20 % in human and rat samples, they were between 26 and 57 % for the murine samples. This suggested, that the lysis of very small tissue pieces, which had been used for the murine experiment, was still the greatest source of imprecision. Therefore, the lysis of very small tissue

samples should be either further improved or it should be refrained from using tissue samples smaller than 15 mg.

Measuring a target protein with more than one TXP assay is a good quality control. For the human MDR1, three TXP assays were tested: MDR1 (QDIR) and MDR1 (LTTR) as well as MDR1 (LPNK) which was developed by Frederik Weiss [22]. The set of healthy liver tissue samples (4.3.1) was analyzed with all MDR1 TXP assays (Figure 26). While the results of the LPNK and the LTTR assay matched very well, the quantified amount of the QDIR assay differed tremendously. Since the QDIR antibody worked well with the rat peptide, this was probably due to the peptide of the human assay. According to UniProtKB, none of the peptides contains a known position for amino acid modification or natural sequence variants, which could explain the differences (Table 71). However, only the human QDIR peptide contained a methionine which is prone to oxidation. Therefore, the MDR1 (QDIR) assay was not used further. MDR1 (LTTR) was the assay of choice, because it had the lowest LLOQ and a lower intra- and interday variation.

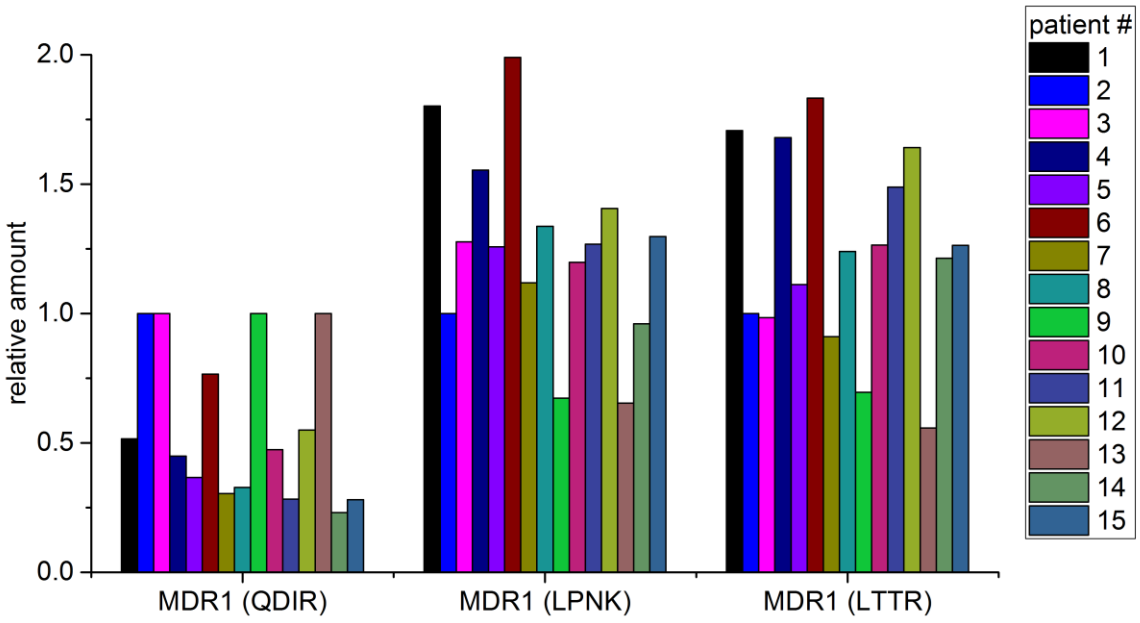


Figure 26: Comparison of three TXP assays for MDR1 quantification. The human liver biopsies described in 4.3.1 were analyzed with three independent TXP assays for MDR1 quantification. Fold change is given with respect to patient # 2.

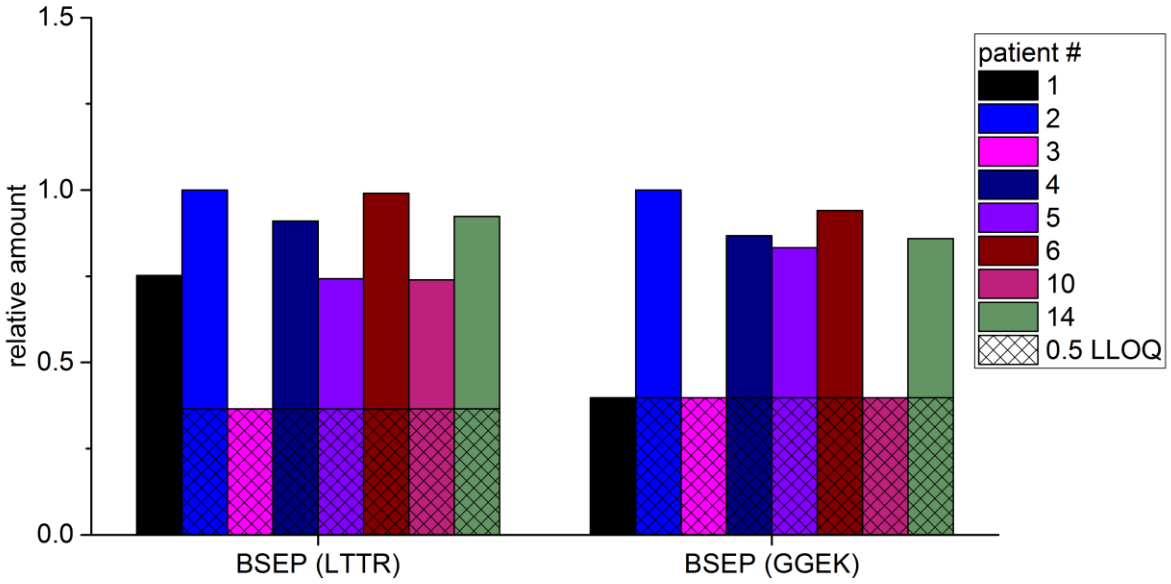


Figure 27: Comparison of two TXP assays for human BSEP quantification. The human liver biopsies described in 4.3.1 were analyzed with two independent TXP assays for BSEP quantification. The samples of patients 7-9, 11-13 and 15 were below the LLOQ of both assays. Fold change is given with respect to patient # 2.

Human BSEP could also be analyzed with two antibodies (Figure 27). The results were in line, but the LTTR assay had a lower LLOQ and the peptide a slightly better proteolysis kinetics. Since both antibodies were needed to cover other targets and natural sequence variants were reported for both peptides (Table 71), BSEP was quantified with the LTTR assay and the GGEK assay was used as quality control. The results gained with MRP2 (YQVR) and MRP2 (GSLR) were comparable (Figure 28) but the YQVR assay was favored because its peptide was more stable during proteolysis and the assay showed a lower LLOQ and inter- and intraday variation. Additionally, no modified amino acid or natural sequence variant was reported for the YQVR peptide while there were reported two for the GSLR peptide in patients with Dubin – Johnson – Syndrome (Table 71) [104].

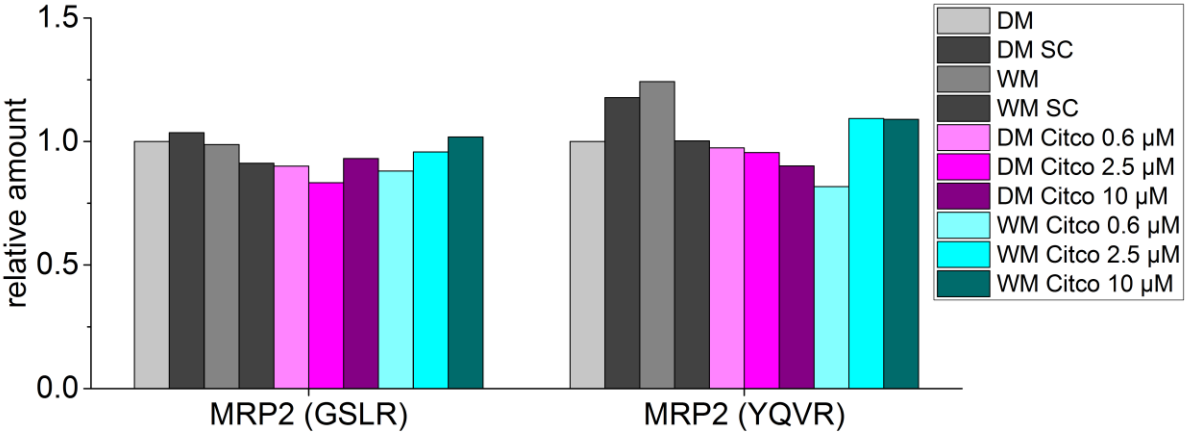


Figure 28: Comparison of two TXP assays for human MRP2 quantification. MRP2 was quantified in HepaRG (4.3.4) with two independent TXP assays. Fold change to control is given. n=4

While there was only one assay to quantify NTCP in human, an alternative assay was established for rodents. The assays demonstrated good comparability in the cross species approach even though only for the ALEK peptides a site of amino acid modification was reported (Table 71, Figure 29 and Figure 25). Both assays had similar measuring ranges and inter- and intraday variation. Since the ALEK peptide was released much faster during proteolysis than the GDLK peptide and was also stable, this assay was preferred for projects without human samples.

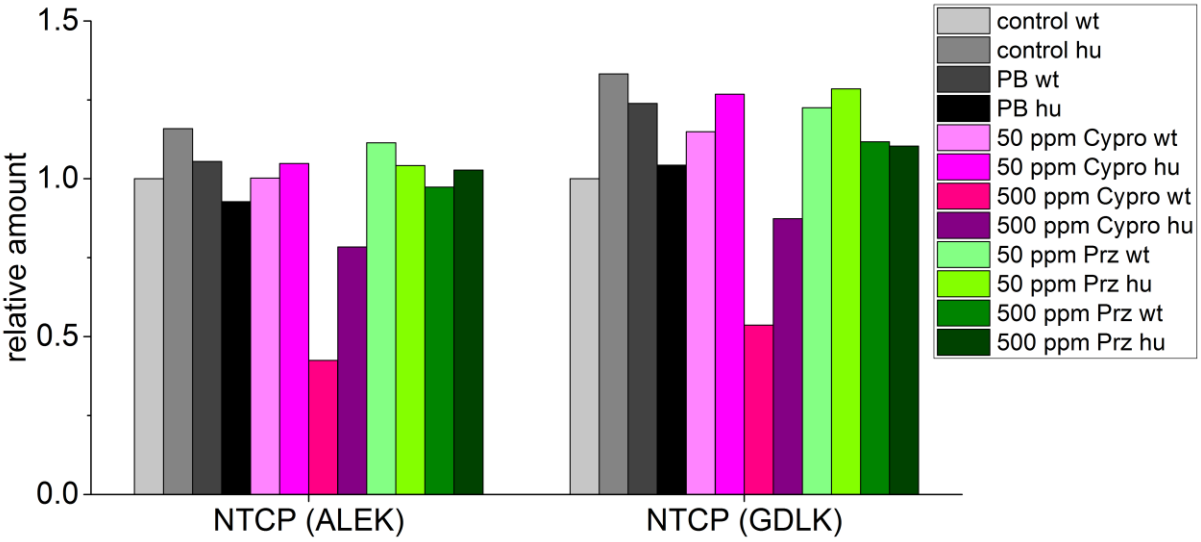


Figure 29: Comparison of two TXP assays for rodent NTCP quantification. NTCP was quantified in wild type mice and humanized mice (4.3.4) with two independent TXP assays. Fold change to wildtype control is given. n=5

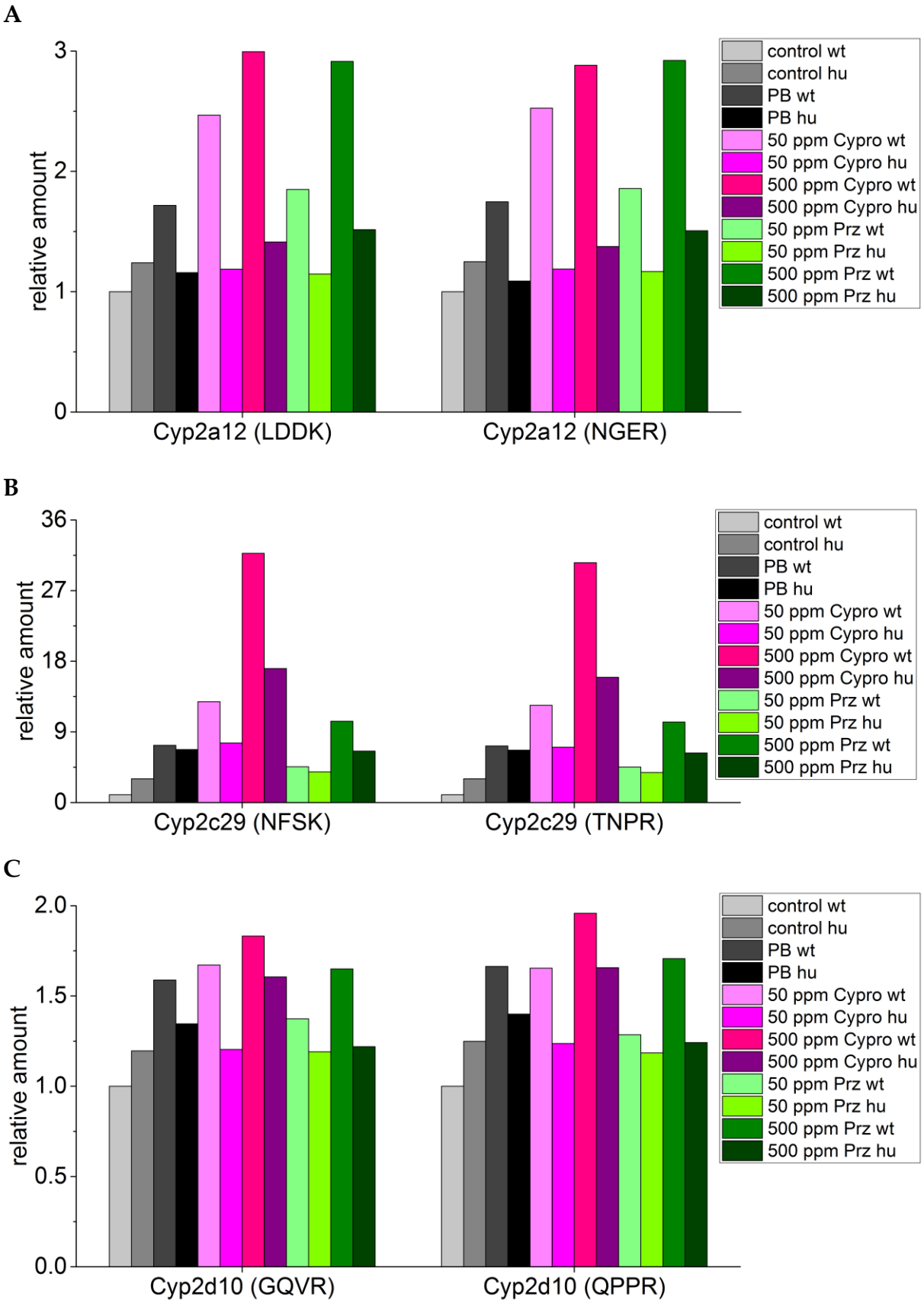
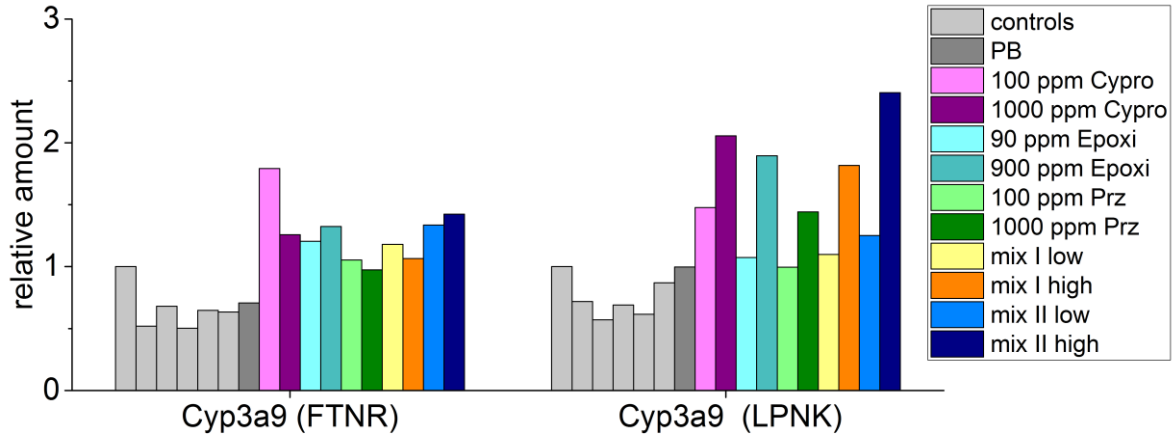


Figure 30: CYP quantification by two independent TXP assays. Cyp2a12 (A), Cyp2c29 (B) and Cyp2d10 (C) were quantified in wild type mice and humanized mice (4.3.4) with two independent TXP assays each. Fold change to wildtype control is given. n=5

For murine Cyp2a12, Cyp2c29 and Cyp2d10, two independent TXP assays were developed, too. The CYP expression in the fungicide-treated mice was analyzed with two assays each (Figure 30). As for NTCP, the results matched very well. Cyp2c29 (NFSK) and Cyp2d10 (QPPR) were the preferred assays, because they had lower intra- and interday variations as well as lower LLOQs. Additionally, the QPPR peptide was more stable during proteolysis. For Cyp2a12 the LDDK peptide was slightly more stable during proteolysis and had the lower LLOQ. However, the NGER assay was preferred, because it showed less intra- and interday variation and was sensitive enough for the expected expression levels in the samples.

Four analytes were covered twice with the rattine TXP assay set. The quantified amounts of Cyp3a9 (FTNR) and Cyp3a9 (LPNK) did not agree as well as for the other assays (Figure 31, A). While the FTNR assays varied less in the intra- and interday experiment, the LPNK assay had a lower LLOQ and the peptide stayed on a plateau during proteolysis. Therefore, Cyp3a9 (LPNK) was chosen for the analysis of the fungicide treated rats. For Cyp4b1 quantification, two peptides were tested. The EIQK peptide peaked already after 2 hours proteolysis, while the ESTR peptide reached a plateau. At the same time, only the EIQK peptides was quantifiable in all replicates during the first 24 hours. The problematic reproducibility of the ESTR peptide was also reflected in the recovery plot. For this assay, no measuring range could be estimated because it was too imprecise. Therefore, only the Cyp4b1 (EIQK) assay was established. As for the human MRP2, the rattine MRP2 was best measured with the YQVR assay. The results matched very well (Figure 31), but inter- and intraday variation as well the LLOQ were lower.

A



B

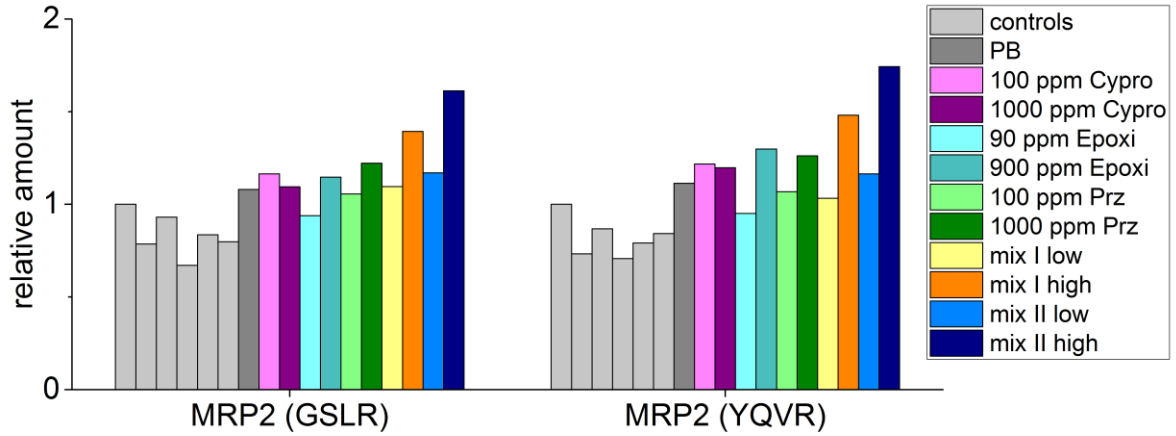


Figure 31: Quantification of rat Cyp3a9 and MRP2 by two independent TXP assays each. Cyp3a9 (A) and MRP2 (B) were quantified in fungicide-treated rats (4.3.4) with two independent TXP assays each. Fold change to first control group is given. n=5, mixes: n=10

5.3 Thresholds for effective assay development

During this thesis, TXP assays for 71 proteins and three species should be developed. This included testing 151 purified antibodies and 144 peptides for their usability. The choice of thresholds was critical to reduce required sample amount, costs and time by sorting out unsuitable sera and peptides as early as possible. The first criterion was that each antibody had to enrich at least one spiked-in target peptide and each surrogate peptide had to be enriched by at least one antibody. The threshold was a total file area greater than 10^4 of the tSIM signal. This led to a reduction of the tested antibodies and peptides in the following experiments of 38 % and 29 % respectively.

By increasing the threshold to 10^5 the number of purified antibody sera could have been reduced by 53 %. Then again, four antibody sera and five peptides which are part of the final TXP assay set would have been sorted out by this threshold. This amounts to 10 % of the final assay set. The second experiment, which was used to select the most promising antibodies and peptides, was the adjustment of the required antibody and protein amount. With two experiments, the number of antibodies and peptides was reduced to 75 assays for which successful assay development was very likely. 23 assays were quit even though they enriched spiked-in peptides well but no endogenous peptide could be detected. They can be continued with samples expressing the target proteins. The study of the enzymatic proteolysis was used to double-check the antibodies which were chosen with reservation during the adjustment or discarded because no endogenous peptide could be detected. None of the endogenous surrogate peptides of the discarded assays could be detected. Thereby the decision to quit these assays was confirmed. Additionally, surrogate peptides were sorted out for which missed cleavage peptide variants were detectable. Of the remaining 58 assays, only four had to be discarded because they did not meet the required precision and accuracy criteria. This shows that these three experiments and the respective thresholds were well suited to select the antibodies and peptides for which successful assay development was very likely.

During this thesis, 54 single TXP assays which could be combined to 19 multiplexed assays have been developed. Even though the development already examined some aspects which are tested during assay validation, this has not been done as extensively as it is needed for a full validation. The accuracy of the quantification could be further improved by determining the amount of the peptide standards by means of amino acid analysis. This would also further improve the comparability of quantification with two independent TXP assays on the level of total amounts. Quantifying the standards by amino acid analysis was not necessary for the assay development, but will be done for the assay validation and future application of these assays.

5.4 Comparison to literature

For most targets, it is difficult or impossible to find comparable references of the expressed amount in liver. Most publications used western blots, and immunohistochemistry on protein level or fold change of mRNA levels. If the protein amount was determined, it was not directly comparable because normally membrane enriched fractions were used for quantification. Nevertheless, the transporter amounts quantified in human liver (4.2.3 and 4.3.1) were compared to published values keeping the different sample preparations in mind (Table 54). Five transporters were quantified in membrane enriched fractions of human liver in the same range as published by others. Only SLCO1B1 was quantified less than published by Wang et al. [98]. This might be due to the different donors, small sample sets and intra-tumor heterogeneity. Another reason could be, that the protocol for membrane enrichment was not suitable for SLCO1B1 enrichment. Nevertheless, this comparison shows that protein quantification by means of the developed TXP assays is well in line with published data. This also suggests, that the other targets, which could not be compared to published data, were quantified in a reasonable range.

Table 54: Protein amount in human liver determined by TXP compared to literature. Total transporter amounts quantified in human liver tissue (4.2.3 and 4.3.1) and membrane enriched fractions thereof (4.2.3) are listed together with values reported for membrane fractions. All values are expressed as fmol per µg extracted protein.

protein	tissue (4.3.1)	tissue (4.2.3)	membrane enriched fraction (4.2.3)	literature	reference
BSEP	0.2-0.6	0.4-0.6	1.2-2.5	3	[95]
MDR1	0.1-0.3	0.1-0.4	0.2-1.3	0.5-0.7	[97, 99]
MRP2	0.1-0.5	0.3-1	0.9-2.4	0.5-3	[94, 96, 99]
MRP3	0.1-0.3	0.3-0.9	0.5-2	0.5	[98]
NTCP	0.15-0.3	0.3-0.5	0.6-1.3	2	[98]
SLCO1B1	0.06-0.1	0.2-0.4	0.2-0.5	2.5	[98]

Comparing the different quantification methods (Table 54), the main advantage of the TXP protocol was the speed. The immunoprecipitation in the TXP protocol allowed the LC-MS analysis with a 2.75 min gradient for peptide separation. The other protocols, on the other hand, relied on separation with 15 – 30 min gradients [94-98], resulting in 5 – to 10 -fold longer LC-MS occupancy.

The results of the transporter expression in normal kidney tissue and kidney tumors, on the other hand, was compared to the according EST profiles. For six transporters, protein expression and EST profiles matched well. Four transporters were below LLOQ in some or even all samples even though the EST profile predicted expression. MRP1, on the other hand, was also detected in some tumor samples even though the EST profile predicted the expression only in normal tissue. Whether the transporters were upregulated or downregulated in the tumor tissue could neither be linked to the tumor type nor the medication. To do so, a greater sample set would have been necessary. Apart from basic research, the TXP approach could also be used to customize cancer treatment, by evaluating the transporter expression in the tumor. This knowledge of the transporter expression would allow a more specific selection of drugs.

The differential expression of CYPs and transporters was examined in primary murine hepatocytes. Pericentral and periportal cells were isolated separately for analysis. This resulted in very low sample amount. Nevertheless, thirteen proteins of the murine set could be quantified from 5 µg extracted protein, which is between one fourth and one eighth of the normal amount. The results were in accordance with published PCR and immunohistochemistry data (Table 55). BSEP and NTCP were expressed equally in periportal and pericentral cells, while the three CYPs were predominately expressed in pericentral cells.

Table 55: Protein expression in periportal and pericentral hepatocytes

protein	localization	literature	method
BSEP	not zoned	not zoned	protein: immunofluorescence[105]
NTCP	not zoned	not zoned	protein: immunofluorescence[105]
Cyp2c29	mainly pericentral	pericentral	mRNA: microarray [106] protein immunohistochemistry [69]
Cyp2c55	only pericentral	pericentral	mRNA: microarray [106] protein immunohistochemistry [69]
Cyp2e1	mainly pericentral	pericentral	mRNA: RT-PCR /microarray [68, 69, 106] protein: immunohistochemistry [69]

The rattine sample set of the cross species study had been analyzed before. Heise et al. investigated the hepatotoxic effects of single fungicide administration. Liver weights and apoptosis rates were recorded as well as gene expression and activity of three CYP isoforms. The doses indicated as NOAEL and NOAELx10 correspond to 90 / 100 ppm and 900 / 1000 ppm fungicide respectively [73]. The protein amounts of Cyp1a2 and Cyp2b1/2 determined via the TXP assays were compared to the published mRNA induction (Figure 32). The TXP assay cannot discriminate between Cyp2b1 and Cyp2b2 but their regulation is closely related [107, 108]. Therefore, Cyp2b1 mRNA induction was compared to the combined induction of Cyp2b1 and Cyp2b2. Nevertheless, the data correlated well. For Cyp1a2 however, the fold change in the protein expression was much more pronounced than in the mRNA expression. Additionally, a correlation could only be observed because of the high inductive effect of 1000 ppm prochloraz. For Cyp1a1 it was possible to compare the TXP generated protein data to gene expression data as well enzyme activity. Cyp1a1 was induced massively by high dosed prochloraz on all levels. The strongest response could be observed on mRNA level, while the fold change of protein expression and activity was less pronounced.

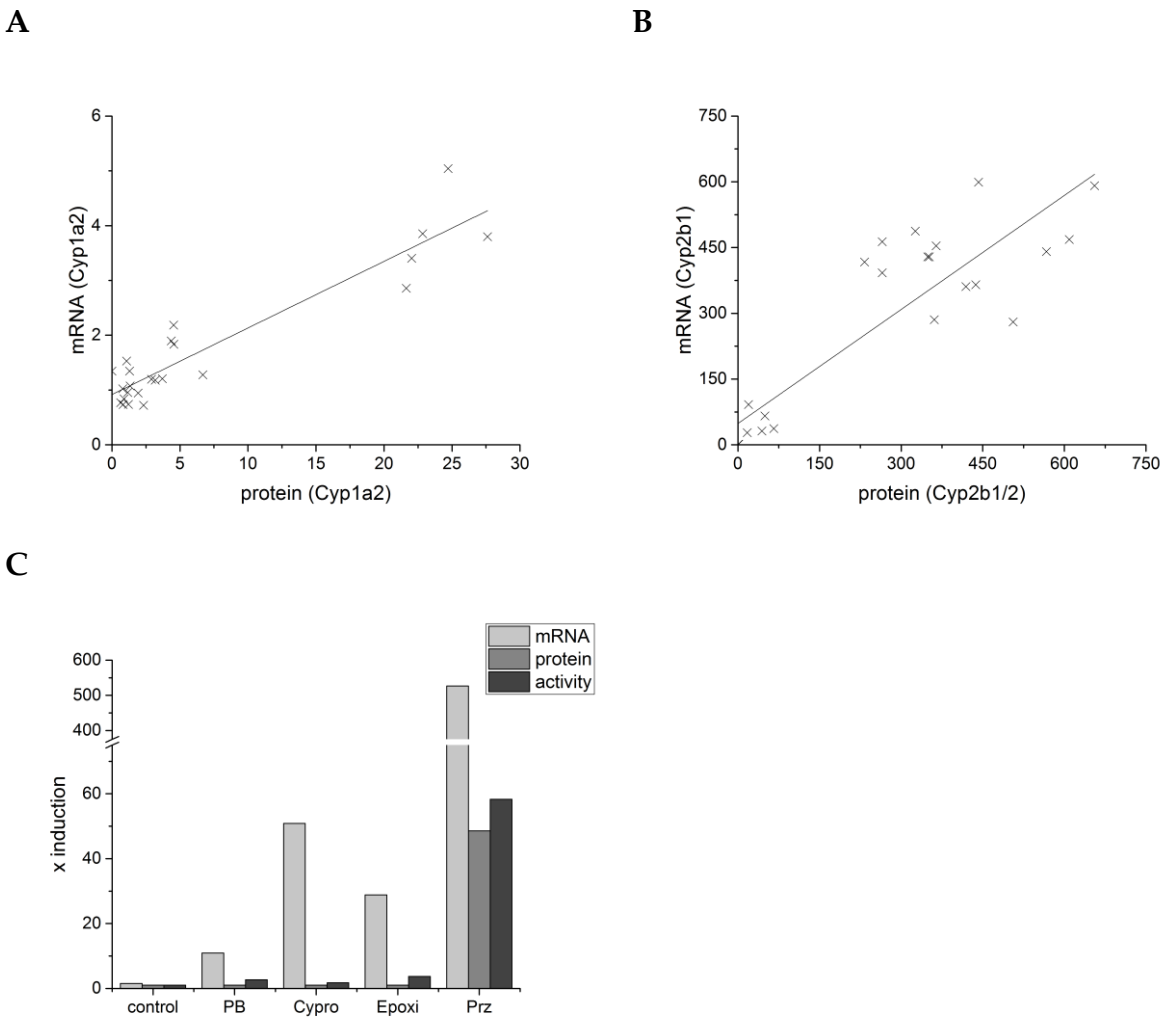


Figure 32: TXP protein quantification compared to mRNA and activity data. The protein expression in liver tissue of rats treated with fungicides was compared to mRNA expression determined by qPCR and CYP activity determined by EROD dealkylation published by Heise et al. [73]. Following treatment groups were used: control, PB, 1000 ppm cyproconazole, 900 ppm epoxiconazole and 1000 ppm prochloraz. Protein induction of Cyp1a2 (A) and Cyp2b1/2 (B) was correlated to mRNA expression. The data correlates well with Pearson R equaling 0.93 and 0.88 respectively. The Cyp1a1 amount quantified by means of TXP is expressed as fold change to control to compare it to mRNA expression and enzyme activity (C). Cyp1a1 was mainly induced by prochloraz on all levels. The induction of mRNA was the strongest. n=5

Two analytes of murine sample set, MRP3 and Cyp2b10, were also compared to mRNA expression data [109]. Both methods showed that the MRP3 and Cyp2b10 were induced to a greater extent in the wildtype mice than in the humanized mice. However, while the mRNA was induced 20'00-fold in wildtype mice by the high

cyproconazole dose, the protein expression was induced 600-fold. MRP3, on the other hand, was induced 3-fold on the mRNA level and 5-fold on the protein level under the same conditions. [109]

Overall, mRNA and protein expression both identified the inductive potential of the tested fungicides and qualitatively the results correlated well. However, the magnitude of the inductive effect differed strongly for some analytes. The results of Cyp1a1 suggest that the protein expression level may be a better surrogate for the protein activity than the mRNA expression level.

5.5 Cross species analysis of the inductive potential of fungicides

There are several *in vitro* and *in vivo* models which are used to predict toxicity and pharmacokinetics in man. Cell culture experiments are used to examine specific aspects, such as protein induction or substrate transport across membranes. For more complex questions, animal models are utilized, of which rat and mice are the most common [85]. Additionally, humanized animal models, in which one or more proteins were replaced by the human variants, gain importance [85, 89]. A great challenge of these studies is to compare the results of the different models and to draw the right conclusion for man [88]. Even though CYP enzymes are classified according to their homology, expression levels and isoforms can vary strongly between species. One amino acid exchange may alter the specificity of the enzyme [43]. In the cross species study, the human cell line HepaRG, rats and mice as well as humanized mice were treated with azole fungicides and combinations thereof. All three fungicides induced the expression of CAR and PXR targets, such as Cyp2b1, Cyp2b2 and MDR1 in rat and Cyp2b10 in mouse [43, 73]. Additionally, the high cyproconazole dose reduced NTCP expression in mice and OAT2 expression in rats, which is also typical for CAR and PXR activation [74]. Only prochloraz treatment led to a strong induction of Cyp1a1 and Cyp1a2 which are targets of AhR. This is in accordance with cyproconazole and epoxiconazole being described as CAR/PXR agonists and prochloraz as agonist of all three nuclear receptors [73, 109].

The quantitative comparison of the inductive potential of substances can be made easier by TXP assays, which can be applied for several species. MDR1/a, MRP3 and NTCP were quantified with the same TXP assay in all models. The basal expression levels differed between the models. Qualitatively, the target proteins were affected similarly in all rodent models, e.g. induction of Cyp2c55 and MDR1a by cyproconazole, but the magnitude of the effects varied. In contrast to that, the target proteins were only slightly affected in HepaRG and significance criteria were not met. This might be either due to species differences or due to the fact, that cultured cells react differently than hepatocytes in a *in vivo* experiment. For the humanized mouse models, the basal expression levels were like the other rodent models, but the reaction to the treatment was less pronounced. Therefore, it matched better with the HepaRG than the other rodent models did even though the effects were more distinct in the humanized mouse model than in HepaRG. This might be due to the fact, that human receptors are addressed in the context of a murine liver. Species differences of the affinity and ligand specificity of CAR and PXR have been described for several substances [110]. Human PXR for examples leads to stronger induction of a reporter gene after Mevastatin treatment than murine PXR [110]. TCPOBOP and CITCO, on the other hand, selectively bind to murine CAR and human CAR, respectively [75].

5.6 Potential areas of application for TXP assays

TXP assays are fast and sensitive MS-based immunoassays. They are well suitable for low sample amounts and low abundant proteins as well as highly inducible proteins because the accurate and precise measuring range covers up to four orders of magnitude. Furthermore, the workflow is also applicable for hydrophobic proteins, because the use of surrogate peptides avoids solubility issues.

The assays could be used for the molecular characterization of samples for scientific or medical purposes. As an example, the assays could be used to guide therapeutic strategies based on the transporter expression profile of tumor tissue to avoid or evade multiple drug resistance.

Another area of application could be the characterization of the inductive potential of chemicals within the framework of adverse outcome pathways (AOP). They are conceptual constructs which describe a causal linkage between molecular initiating events, key events and a biologically relevant adverse outcome on the level of the organism [111]. The molecular initiating event is defined as a specialized key event during which a chemical directly interacts with a biomolecule thereby disturbing the cellular homeostasis. Key events are measurable biological changes which are crucial for the progression from the molecular initiating event to the adverse outcome. [111] AOPs are based on the assumption that any chemical which triggers the initiating event sufficiently severe, will lead to the adverse outcome. The goal is to predict the outcome for regulatory purposes by measuring key events such as protein dysregulation [111]. The initiating event of the AOP for cholestatic liver injury is the inhibition of BSEP [112]. Key events which could be used to predict the outcome are the downregulation of NTCP and SLCO1B1 as well as the upregulation of Cyp2b10, MRP2 and MRP3 [112]. The developed TXP assays could be a valuable tool to assess whether an uncharacterized chemical triggers this AOP by monitoring the expression level of these proteins.

6 Summary

CYPs and transporters are important for the turnover of xenobiotic compounds. Their expression levels and activity influence bioavailability and convey drug-drug interactions. Moreover, transporters mediate barrier maintenance of several organs such as the blood – brain - barrier and the placenta-barrier. Overexpression of export transporters in tumors can lead to multiple drug resistance. Therefore, they are investigated thoroughly during drug development. However, it is still difficult to establish conventional assays such as sandwich immunoassays to quantify these proteins because CYP and transporter families are homologous and additionally transporters are very hydrophobic.

The objective of this thesis was to develop MS – based immunoassays for ABC and SLC transporters as well as CYPs in up to three species which are relevant for research and drug development.

During this thesis, nineteen TXP – multiplex assays were successfully developed which allow the quantification of up to 14 transporters in human, rat and mouse as well as up to 18 CYPs in rat and mouse. In total this corresponds to 61 different proteins. Additionally, it was shown that the TXP methodology is sensitive enough to quantify the low abundant target proteins from whole cell lysates without further enrichment such as membrane enrichment. The developed TXP -assays were used to analyze several independent studies: The amounts of transporters quantified in healthy human liver tissue was well line with published data. The protein expression profile of pericentral and periportal murine hepatocytes was analyzed and correlated with published immunohistochemistry as well as RNA expression data. Furthermore, a cross species study investigating the effects of azole fungicides in human cell culture, rat and wildtype as well as humanized mouse models was analyzed. It could be shown that high dosed fungicide treatment induced CAR, PXR and AhR target proteins as well as that the extent of the response differed between the models.

7 Zusammenfassung

CYPs und Transporter sind wichtige Bestandteile des Fremdstoffmetabolismus, deren Expression und Aktivität durch Nahrungsbestandteile und Medikamente moduliert werden. Sie beeinflussen die Bioverfügbarkeit von Medikamenten und vermitteln Wechselwirkungen zwischen verschiedenen Medikamenten oder Nahrungsbestandteilen. Des Weiteren sind Transporter an der Aufrechterhaltung von Blutschranken verschiedener Organe beteiligt wie zum Beispiel der Blut – Hirn – Schranke. Das Phänomen der *multiple drug resistance* wird unter anderem durch die Überexpression von Exportpumpen vermittelt. Da CYPs und Transporter an so vielen Prozessen beteiligt sind, werden sie während der Medikamentenentwicklung ausführlich untersucht. Allerdings ist es immer noch schwierig sie mit konventionellen Methoden wie zum Beispiel Sandwich - Assays zu quantifizieren, weil sie zu sehr homologen Proteinfamilien gehören und Transporter des Weiteren auch noch sehr hydrophob sind.

Aus diesem Grund, war das Ziel dieser Arbeit MS-basierte Immunoassays zu entwickeln um ABC und SLC Transporter sowie CYPs in bis zu drei für die Forschung und Medikamentenentwicklung wichtigen Spezies zu quantifizieren.

Es wurden neunzehn TXP-Assays entwickelt mit denen bis zu 14 Transporter in Mensch, Ratte und Maus sowie bis zu 18 verschiedene CYPs in Ratte und Maus gemessen werden können. Das entspricht insgesamt 61 verschiedenen Proteinen. Zusätzlich konnte gezeigt werden, dass die TXP – Methode sensitiv genug ist, sodass auf zusätzlich Anreicherungsverfahren wie verzichtet werden kann. Die entwickelten TXP – Assays wurden verwendet um bei vier verschiedenen Studien CYPs und Transporter zu quantifizieren. Die in humanem Normalgewebe aus der Leber bestimmten Mengen sowie die Zonierung in Mausleber, stimmten gut mit den Literaturwerten überein. Des Weiteren wurde eine speziesübergreifende Studie zu den Effekten von Pyrrolfungiziden analysiert. Humane Zellkultur, Ratten und Wildtyp - sowie humanisierte Mäuse wurden als Modelle verwendet. Es konnte gezeigt werden, dass die Fungizide in hohen Dosen die Expression von CAR, PXR und

AhR Zielproteinen in den vier Modellen beeinflussen. Das Ausmaß der Antwort hängt jedoch von dem verwendeten Modell ab.

8 Curriculum Vitae

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References

10 Supplemental Information

Table 56: Proteins covered in this thesis. All proteins covered in this thesis are listed together with the used acronym. The recommended name (UniProtKB), gene name and UniProt entry ID are given for clear identification, too.

Protein name	Acronym	recommended name (UniProtKB)	Gene name	UniProt entry ID human	rat	mouse
Neutral amino acid transporter B(0)	ATB(0)	Neutral amino acid transporter B(0)	Slc1a5	Q15758		
Neutral amino acid transporter B(0)	ATB(0)	Neutral amino acid transporter ASCT2	Slc1a5			Q9ESU7
Breast cancer resistance protein	BCRP	ATP-binding cassette sub-family G member 2	Abcg2	Q9UNQ0	Q80W57	Q7TMS5
Bile salt export pump	BSEP	Bile salt export pump	Abcb11	O95342	O70127	Q9QY30
Cytochrome P450 1A1	Cyp1a1	Cytochrome P450 1A1	Cyp1a1		P00185	P00184
Cytochrome P450 1A2	Cyp1a2	Cytochrome P450 1A2	Cyp1a2		P04799	P00186
Cytochrome P450 1B1	Cyp1b1	Cytochrome P450 1B1	Cyp1b1		Q64678	Q64429
Cytochrome P450 2A1	Cyp2a1	Cytochrome P450 2A1	Cyp2a1		P11711	
Cytochrome P450 2A2	Cyp2a2	Cytochrome P450 2A2	Cyp2a2		P15149	
Cytochrome P450 2A4	Cyp2a4	Cytochrome P450 2A4	Cyp2a4		P20812	P15392
Cytochrome P450 2A5	Cyp2a5	Cytochrome P450 2A5	Cyp2a5			P20852
Cytochrome P450 2A12	Cyp2a12	Cytochrome P450 2A12	Cyp2a12			P56593
Cytochrome P450 2A22	Cyp2a22	Cyp2a22 protein	Cyp2a22			B2RXZ2
Cytochrome P450 2B1	Cyp2b1	Cytochrome P450 2B1	Cyp2b1		P00176	
Cytochrome P450 2B2	Cyp2b2	Cytochrome P450 2B2	Cyp2b2		P04167	
Cytochrome P450 2B3	Cyp2b3	Cytochrome P450 2B3	Cyp2b3		P13107	
Cytochrome P450 2B9	Cyp2b9	Cytochrome P450 2B9	Cyp2b9			P12790
Cytochrome P450 2B10	Cyp2b10	Cytochrome P450 2B10	Cyp2b10			P12791
Cytochrome P450 2C6	Cyp2c6	Cytochrome P450 2C6	Cyp2c6		P05178	
Cytochrome P450 2C7	Cyp2c7	Cytochrome P450 2C7	Cyp2c7		P05179	
Cytochrome P450 2C11	Cyp2c11	Cytochrome P450 2C11	Cyp2c11		P08683	
Cytochrome P450 2C12	Cyp2c12	Cytochrome P450 2C12	Cyp2c12		P11510	
Cytochrome P450 2C13	Cyp2c13	Cytochrome P450 2C13	Cyp2c13		P20814	
Cytochrome P450 2C29	Cyp2c29	Cytochrome P450 2C29	Cyp2c29			Q64458
Cytochrome P450 2C37	Cyp2c37	Cytochrome P450 2C37	Cyp2c37			P56654

Protein name	Acronym	recommended name (UniProtKB)	Gene name	UniProt entry ID human	rat	mouse
Cytochrome P450 2C38	Cyp2c38	Cytochrome P450 2C38	Cyp2c38			P56655
Cytochrome P450 2C39	Cyp2c39	Cytochrome P450 2C39	Cyp2c39			P56656
Cytochrome P450 2C55	Cyp2c55	Cytochrome P450 2C55	Cyp2c55		P33273	Q9D816
Cytochrome P450 2D1	Cyp2d1	Cytochrome P450 2D1	Cyp2d1		P10633	
Cytochrome P450 2D3	Cyp2d3	Cytochrome P450 2D3	Cyp2d3		P12938	
Cytochrome P450 2D9	Cyp2d9	Cytochrome P450 2D9	Cyp2d9			P11714
Cytochrome P450 2D10	Cyp2d10	Cytochrome P450 2D10	Cyp2d10			P24456
Cytochrome P450 2D11	Cyp2d11	Cytochrome P450 2D11	Cyp2d11			P24457
Cytochrome P450 CYP2D22	Cyp2d22	Cytochrome P450 CYP2D22	Cyp2d22			Q9JKY7
Cytochrome P450 2D40	Cyp2d40	Cytochrome P450, family 2, subfamily d, polypeptide 40	Cyp2d40			Q6P8N9
Cytochrome P450 2E1	Cyp2e1	Cytochrome P450 2E1	Cyp2e1		P05182	Q05421
Cytochrome P450 3A1	Cyp3a1	Cytochrome P450 3A1	Cyp3a1		P04800	
Cytochrome P450 3A2	Cyp3a2	Cytochrome P450 3A2	Cyp3a2		P05183	
Cytochrome P450 3A9	Cyp3a9	Cytochrome P450 3A9	Cyp3a9		P51538	
Cytochrome P450 3A11	Cyp3a11	Cytochrome P450 3A11	Cyp3a11			Q64459
Cytochrome P450 3A13	Cyp3a13	Cytochrome P450 3A13	Cyp3a13			Q64464
Cytochrome P450 3A18	Cyp3a18	Cytochrome P450 3A18	Cyp3a18		Q64581	
Cytochrome P450 3A25	Cyp3a25	Cytochrome P450 3A25	Cyp3a25			O09158
Cytochrome P450 3A41	Cyp3a41	Cytochrome P450 3A41	Cyp3a41a			Q9JMA7
Cytochrome P450 3A44	Cyp3a44	Cytochrome P450, CYP3A	Cyp3a44			Q9EQW4
Cytochrome P450 4B1	Cyp4b1	Cytochrome P450 4B1	Cyp4b1		P15129	Q64462
Cytochrome P450 7B1	Cyp7b1	25-hydroxycholesterol 7-alpha-hydroxylase	Cyp7b1		Q63688	
Cytochrome P450 19A1	Cyp19a1	Aromatase	Cyp19a1		P22443	P28649
Multidrug and toxin extrusion protein 1	MATE1	Multidrug and toxin extrusion protein 1	Slc47a1	Q96FL8		Q8K0H1
Multidrug and toxin extrusion protein 2	MATE2	Multidrug and toxin extrusion protein 2	Slc47a2	Q86VL8		
Multidrug resistance protein 1	MDR1	Multidrug resistance protein 1	Abcb1	P08183	P43245	
Multidrug resistance protein 1A	MDR1a	Multidrug resistance protein 1A	Abcb1a		Q9JK64	P21447

Protein name	Acronym	recommended name (UniProtKB)	Gene name	UniProt entry ID human	rat	mouse
Multidrug resistance protein 1B	MDR1b	Multidrug resistance protein 1B	Abcb1b			P06795
Multidrug resistance-associated protein 1	MRP1	Multidrug resistance-associated protein 1	Abcc1	P33527	Q8CG09	O35379
Multidrug resistance-associated protein 2	MRP2	Canalicular multispecific organic anion transporter 1	Abcc2	Q92887	Q63120	Q8VI47
Multidrug resistance-associated protein 3	MRP3	Canalicular multispecific organic anion transporter 2	Abcc3	O15438	O88563	B2RX12
Multidrug resistance-associated protein 5	MRP5	Multidrug resistance-associated protein 5	Abcc5	O15440	Q9QYM0	Q9R1X5
Multidrug resistance-associated protein 8	MRP8	ATP-binding cassette sub-family C member 11	Abcc11	Q96J66		
Sodium/bile acid cotransporter	NTCP	Sodium/bile acid cotransporter	Slc10a1	Q14973	O08705	P26435
Organic anion transporter 2	OAT2	Solute carrier family 22 member 7	Slc22a7	Q9Y694	Q5RLM2	Q91WU2
Organic anion transporter 3	OAT3	Solute carrier family 22 member 8	Slc22a8	Q8TCC7	Q9R1U7	O88909
Organic anion transporter 7	OAT7	Solute carrier family 22 member 9	Slc22a9	Q8IVM8		
Solute carrier organic anion transporter family member 1B1	SLCO1B1	Solute carrier organic anion transporter family member 1B1	Slco1b1	Q9Y6L6		
Solute carrier organic anion transporter family member 1B3	SLCO1B3	Solute carrier organic anion transporter family member 1B3	Slco1b3	Q9NPD5		
Solute carrier organic anion transporter family member 2B1	SLCO2B1	Solute carrier organic anion transporter family member 2B1	Slco2b1	O94956	Q9JHI3	Q8BXB6
Organic cation transporter 1	OCT1	Solute carrier family 22 member 1	Slc22a1	O15245	Q63089	O08966
Organic cation transporter 2	OCT2	Solute carrier family 22 member 2	Slc22a2	O15244	Q9R0W2	O70577
Organic cation transporter 3	OCT3	Solute carrier family 22 member 3	Slc22a3	O75751		
Solute carrier family 22 member 5	SLC22A5	Solute carrier family 22 member 5	Slc22a5	O76082	O70594	Q9Z0E8
Solute carrier family 28 member 3	SLC28A3	Solute carrier family 28 member 3	Slc28a3	Q9HAS3	Q8VIH3	Q9ERH8

Table 57: Peptide sequences and corresponding targets in three species. All peptide sequences which were chosen for method development, are listed below as well as corresponding proteins for each species.

Peptide	human	rat	mouse
EVLDSFLDLVR			ATB(0)
MVADPPR	ATB(0)		
LFIHEYISGYYR	BCRP	BCRP	BCRP
GLSGDVLINGAPQPANFK		BCRP	
DPSGLSGDVLINGAPRPANFK	BCRP		
AELHQLSGGEK	BCRP		
NSPGALTTR	BSEP		
NNPGVLTTR		BSEP	BSEP
TVAAFGGEK	BSEP		
MSDSVILR	BSEP	BSEP	BSEP
ANIYGLCFAFSQGIAFLANSAAYR		BSEP	
LSDRPQLPYLEAFILETFR		Cyp1a1	Cyp1a1
EAEYLISK		Cyp1a1	
ELWGDPNEFRPER			Cyp1a1
EANHLISK		Cyp1a2	
DPFVFRPER		Cyp1a2	Cyp1a2
CIGEELSK		Cyp1b1	Cyp1b1
LQTEEGCK			Cyp1b1
NAAFLPFSTGK		Cyp2a1	
DFDPQNFLDDK		Cyp2a1	
DFNPQHFLDDK		Cyp2a2	Cyp2a12
SDAFVPFSIGK		Cyp2a4	Cyp2a4
IVVLCGQEAVK			Cyp2a5
NDAFVPFSIGK			Cyp2a5
EALVDHAEEFSGR			Cyp2a12
GYGVAFSNGER			Cyp2a12
EALEDNAEEFSGR			Cyp2a22
GYGVTFSNGER			Cyp2a22
SEAFMPFSTGK		Cyp2b1 / Cyp2b2	
EYGVIFANGER		Cyp2b1 / Cyp2b2	Cyp2b10
EALVGQAEAFSGR			Cyp2b10
EIDQVIGSHRPPSLDDR		Cyp2b2	
EALVDHAEAFSGR		Cyp2b3	Cyp2b9
CEAFLPFSTGK			Cyp2b9
EHQESLDVTNPR		Cyp2c6	
IEEHQESLDVTNPR		Cyp2c7	
EALVDLGEEFSGR		Cyp2c11	
EALIDYGEEFSGR		Cyp2c12	
TYGPVFTLYFGSQPTVVLHGYEAVK		Cyp2c12	
EALVDHGEEFSGR		Cyp2c13	
AYGPVFTLYLGSKPTVILHGYEAVK			Cyp2c29
NISQSFTNFSK			Cyp2c29
ESLDVTNPR			Cyp2c29

Peptide	human	rat	mouse
SDYFIPFSTGK			Cyp2C37
NFNQSLTNFSK			Cyp2c38
AYGPVFTLYLGSRPTVVLHGYEAVK			Cyp2c39
NVSQSLTNFSK			Cyp2c39
EALDDLGEEFSGR		Cyp2c55	Cyp2c55
VYGPVFTLYFGSKPTVVVHGYEAVK			Cyp2c55
DETVWEKPHR		Cyp2d1	
VQQEIDEVIGQVR			Cyp2d9
VQQEIDAVIGQVR			Cyp2d10
NTWDPDQPPR			Cyp2d10
TTWDPDQPPR			Cyp2d11
TTWDPTQPPR			Cyp2d22
TWDPDQPPR		Cyp2d3	Cyp2d40
NEFSGR		Cyp2e1	Cyp2e1
FINLVPSNLPHEATR		Cyp2e1	Cyp2e1
DPQHWPEPEEFRPER		Cyp3a1	
LQEEIDGALPSK		Cyp3a2	
LQDEIDEALPNK			Cyp3a11
YWPEPEEFRPER			Cyp3a13
LQDEIDAALPNK		Cyp3a9	Cyp3a13
ECYSTFTNR		Cyp3a9	Cyp3a13
DPHYWPEPEEFRPER		Cyp3a9	
ECYSVFTNR		Cyp3a18	Cyp3a25
LQEEIDETLPNK			Cyp3a41
DCLSVFTNR			Cyp3a44
LLTPGFHYDVLKPYVAIFAESTR		Cyp4b1	Cyp4b1
AMDSFPGPPTHWLFGHALEIQK		Cyp4b1	
AALQDEK		Cyp4b1	Cyp4b1
GDFVAVFPPMIHNDPEVFDAPK		Cyp7b1	
EQLDSLVCLESAILEVLR		Cyp7b1	
IQGYFNAWQALLIKPNIFFK		Cyp19a1	Cyp19a1
MVEVCVESIK		Cyp19a1	Cyp19a1
DLVGYIFTTDR			MATE1
DHVGYIFTTDR	MATE1		
GGPEATLEVR	MATE1		
SECHVDFFR	MATE2		
LAAEEAK	MATE2		
EANIHAFIESLPNK	MDR1		
LYDPLEGVVSIDGQDIR			MDR1b
LYDPTEGMVSVDGQDIR	MDR1		
LYDPIEGEVSIDGQDIR		MDR1a/b	
NTTGALTTR	MDR1	MDR1a	MDR1a
NTTGSLTTR		MDR1b	
QPLEGSDLWSLNK	MRP1		
SSDLWSLNK		MRP1	
QPLESSDLWSLNK			MRP1

Peptide	human	rat	mouse
ITIIPQDPVLFSGSLR	MRP1	MRP1	MRP1
LTIIPQDPILFSGSLR	MRP2	MRP2	
LAHDILLFLNPQLLK			MRP2
LIHDLLVFLNPQLLK		MRP2	
LVNDIFTFVSPQLLK	MRP2		
HGEIQFNNYQVR		MRP2	
IQFNNYQVR	MRP2		
GEIQFNNYQVR			MRP2
YLGGDDLDTSAIR	MRP2	MRP2	
LEQYLGSDDLDLSAIR			MRP2
LAILGYR		MRP3	MRP3
SPQSFFDTTPSGR	MRP3		MRP3
APQSFFDTTPSGR		MRP3	
DLSLHVHGGEK	MRP3		
NLTLHVQGGEK		MRP3	
NVTVHVQGGEK			MRP3
LTIIPQEPVLFSGTVR		MRP5	
LAIIPQEPVLFSGTVR			MRP5
LSIIPQEPVLFSGTVR	MRP5		
SLSEASVAVDR	MRP5	MRP5	MRP5
TLSLEAPAR	MRP5	MRP5	MRP5
TYTLQDGPWSQQER	MRP8		
DNTPTVLHGINLTIR	MRP8		
AAVPPWGK	MRP8		
AAATEDATPAALEK		NTCP	NTCP
GIYDGDLK	NTCP	NTCP	NTCP
RPSYLDLFR	OAT2		
ALQRPSYLDLFR		OAT2	
VSQRPSYLDLFR			OAT2
NLVLLALPR			OAT2
NVALLALPR	OAT2		
NLVLMALPR		OAT2	
WLVLSGK	OAT3	OAT3	OAT3
LSLEELK	OAT3		
LTIEELK		OAT3	
YGLSDLFR		OAT3	OAT3
YTASDLFR	OAT3		
FVHPPNASLPNDTQR	OAT3		
NKPLFDTIQDEK	OAT7		
LSPSFADLFR	OCT1		
SPSFADLFR		OCT1	OCT1
LNPSFLDLVR	OCT2	OCT2	OCT2
SLPASLQR	OCT2		
GPSAAALAER	OCT3		
LGSILSPYFVYLGAYDR	SLC22A5	SLC22A5	
DYDEVTAFLGEWGPFQR	SLC22A5	SLC22A5	SLC22A5

Peptide	human	rat	mouse
YTDHFFAFK		SLC28A3	SLC28A3
DHFFAFK	SLC28A3		
HWFWLK		SLC28A3	SLC28A3
LLNSHWFWLK	SLC28A3		
YDTVCGFCR	SLC28A3		
YVEQQYGQPSSK	SLCO1B1		
TLGGILAPIYFGALIDK	SLCO1B3		
FIGLQFFFK	SLCO2B1	SLCO2B1	SLCO2B1
SSISTVEK	SLCO2B1	SLCO2B1	SLCO2B1

Table 58: Number of proteins covered by TXP antibody epitope. Number of target proteins in human, rat and mouse covered by each TXP tag and the sum thereof.

TXP tag	human	rat	mouse	sum
AAYR		1		**1**
ALEK		1	1	**2**
ALPR	1	1	1	**3**
ANFK	1	1		**2**
APAR	1	1	1	**3**
AVDR	1	1	1	**3**
AYDR	1	1		**2**
DAPK		1		**1**
DFFR	1			**1**
DLFR	2	3	3	**8**
DLVR	1	1	2	**4**
DPPR	1			**1**
DTQR	1			**1**
EATR		1	1	**2**
EAVK		1	4	**5**
EEAK	1			**1**
EELK	1	1		**2**
EGCK			1	**1**
EIQK		1		**1**
ELSK		1	1	**2**
ESIK		1	1	**2**
ESTR		1	1	**2**
ETFR		1	1	**2**
EVLR		1		**1**
FAFK	1	1	1	**3**
FFFK	1	1	1	**3**
FSGR		6	6	**12**
FTNR		2	3	**5**
FWLK	1	1	1	**3**
GDLK	1	1	1	**3**

TXP tag	human	rat	mouse	sum
GFCR	1			**1**
GGEK	3	1	1	**5**
GQVR			2	**2**
GSLR	2	2	1	**5**
GTVR	1	1	1	**3**
GYYR	1	1	1	**3**
IFFK		1	1	**2**
KPHR		1		**1**
LAER	1			**1**
LDDK		2	1	**3**
LDDR		1		**1**
LEVR	1			**1**
LGYR		1	1	**2**
LIDK	1			**1**
LISK		2		**2**
LPNK	1	1	3	**5**
LPSK		1		**1**
LSGK	1	1	1	**3**
LTIR	1			**1**
LTTR	2	3	2	**7**
NFSK			3	**3**
NGER		2	2	**4**
PFQR	1	1	1	**3**
PSGR	1	1	1	**3**
PSSK	1			**1**
PWGK	1			**1**
QDEK	1	1	1	**3**
QDIR	1	1	1	**3**
QLLK	1	1	1	**3**
QPPR		1	4	**5**
QQER	1			**1**
RPER		3	3	**6**
SAIR	1	1	1	**3**
SIGK		1	2	**3**
SLNK	1	1	1	**3**
SLQR	1			**1**
STGK		2	2	**4**
TNPR		2	1	**3**
TTDR	1		1	**2**
TVEK	1	1	1	**3**
VILR	1	1	1	**3**
YQVR	1	1	1	**3**

Table 59: UniProt IDs of high abundant proteins in human, rat and mouse. The fifteen most abundant proteins for each species are listed according to the PaxDb entries for human liver (integrated), mouse liver (integrated) and rat whole organism. Only proteins with Swiss-Prot entries were included. The canonical sequences as well as all isoforms were checked whether they include any of the chosen TXP epitopes.

recommended name	human	rat	mouse
10 kDa heat shock protein, mitochondrial		P26772	
3-ketoacyl-CoA thiolase, mitochondrial	P42765		
60S ribosomal protein L19			P84099
60S ribosomal protein L21			Q9CQM8
60S ribosomal protein L3			P27659
60S ribosomal protein L39		P62893	
78 kDa glucose-regulated protein			P20029
Actin, cytoplasmic 1		P60711	
Alcohol dehydrogenase 1			P00329
Alcohol dehydrogenase 1A	P07327		
Alcohol dehydrogenase 1B	P00325		
Alcohol dehydrogenase 4	P08319		
Argininosuccinate synthase		P09034	
ATP synthase subunit beta, mitochondrial		P10719	
Carbamoyl-phosphate synthase [ammonia], mitochondrial	P31327	P07756	Q8C196
Carbonic anhydrase 3			P16015
Cytochrome b5		P00173	
Elongation factor 1-alpha 1			P10126
Endoplasmin			P08113
Fatty acid-binding protein, liver	P07148	P02692	
Ferritin light chain	P02792		
Fructose-bisphosphate aldolase B	P05062	P00884	
Glutathione S-transferase A1	P08263		
Glutathione S-transferase A3			P30115
Heat shock cognate 71 kDa protein			P63017
Hemoglobin subunit alpha	P69905		P01942
Hemoglobin subunit beta	P68871		
Hemoglobin subunit beta-1		P02091	
Hemoglobin subunit delta	P02042		
Myelin basic protein		P02688	
Peroxiredoxin-1		Q63716	P35700
Phosphatidylethanolamine-binding protein 1	P30086		
Protein disulfide-isomerase			P09103
Serum albumin	P02768	P02770	P07724
Superoxide dismutase [Cu-Zn]	P00441	P07632	
Thymosin beta-4		P62329	
Ubiquitin-60S ribosomal protein L40		P62986	

Table 60: Comparison of epitope presence and successful assay development (complete list). The presence of a TXP epitope in one or two of the high abundant proteins is indicated by light red (1) or dark red (2) respectively. Green fields ( ) indicate successful assay development, gray fields ( ) indicate either no presence in high abundant proteins or no successful method development and white fields ( ) no measurement at all.

epitope	epitope present in protein			Successful assay development		
	human	rat	mouse	human	rat	mouse
AAYR						
ALEK						
ALPR						
ANFK						
APAR						
AVDR						
AYDR						
DAPK						
DFFR						
DLFR			2			
DLVR						
DPPR						
DTQR						
EATR		1	1			
EAVK			1			
EEAK						
EELK						
EGCK						
EIQK		1				
ELSK						
ESIK						
ESTR						
ETFR						
EVLR		1				
FAFK						
FFFK						
FSGR						
FTNR						
FWLK						
GFCR						
GGEK						
GQVR						
GSLR						
GTVR						
GYYR						
IFFK						
KPHR						
LAER						
LDDK		1				
LDDR						

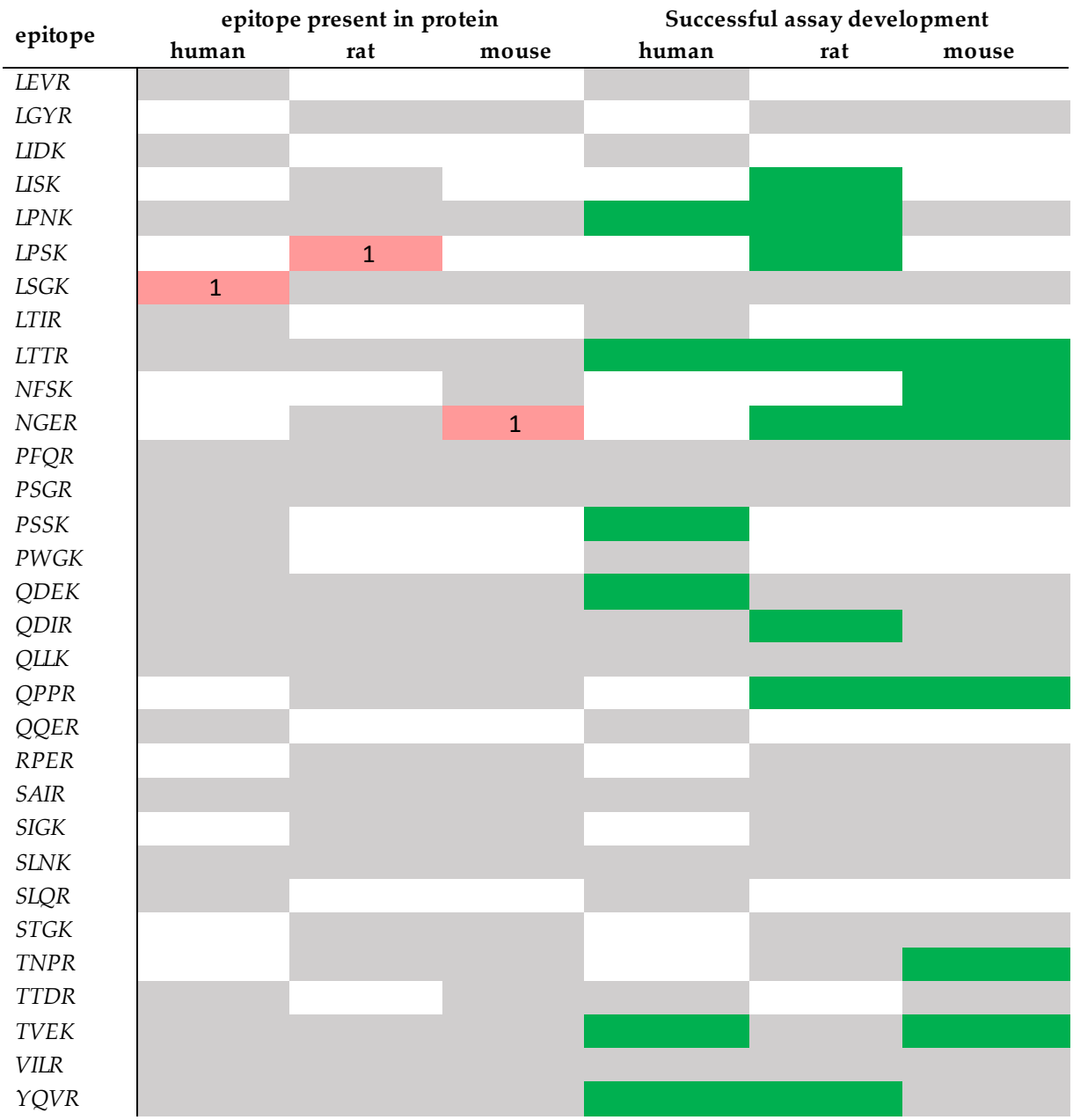

epitope
epitope present in protein
human
rat
mouse
Successful assay development
human
rat
mouse
LEVR
LGYR
LIDK
LISK
LPNK
LPSK
LSGK
LTIR
LTTR
NFSK
NGER
PFQR
PSGR
PSSK
PWGK
QDEK
QDIR
QLLK
QPPR
QQER
RPER
SAIR
SIGK
SLNK
SLQR
STGK
TNPR
TTDR
TVEK
VILR
YQVR
1
1
1

Table 61: Overview of lysis buffers used for each experiment. Used buffers are indicated by x. In case samples of more than one species were used in an experiment, the species are specified by single letters (human: h, rat: r, mouse: m).

experiment	lysis buffer 1 -/-	lysis buffer 1 +/-	lysis buffer 1 +/+	lysis buffer 2 -/-	lysis buffer 2 +/-	lysis buffer 3
antibody functionality		x				
optimization of lysis conditions		x		x	x	x
alternative sample preparations				x (h)		
stability on protein and peptide level				x		
antibody and lysate amount	x (h)			x (r, m)		
kinetic of proteolysis	x (h)			x (r)		
intra and interday variation	x (h)			x (r, m)		
Human liver panel	x (h)					
paired human kidney samples				x (h)		
cross species				x (h, r, m)		

Table 62: Overview of antibodies used in each experiment. Used antibodies are indicated by x. In case samples of more than one species were used in an experiment, the species are specified by single letters (human: h, rat: r, mouse: m).

Antibody ID	AB functionality	lysis optimization	alternative sample preparations	analyte stability	AB and lysate amount	kinetic of proteolysis	recover plots	intra and interday variation	human liver panel	paired kidney samples	PP and PC murine cells	cross species
mAB_cMyc_ms1	x											
pAB_GIYDGDLK_rbt1	x											
pAB_GIYDGDLK_rbt2	x	x	x	x	x (h, m)	x (h, r, m)	x (h, r, m)	x (h, r, m)	x	x		x (h, r, m)
pAB_TXP_AAYR_rbt1	x											
pAB_TXP_AAYR_rbt2	x				x (r)	x (r)						
pAB_TXP_ALEK_rbt1	x	x		x	x (r, m)	x (r, m)	x (r, m)	x (r, m)			x	x (r, m)
pAB_TXP_ALEK_rbt2												
pAB_TXP_ALPR_rbt1	x											

Antibody ID	AB functionality	lysis optimization	alternative sample preparations	analyte stability	AB and lysate amount	kinetic of proteolysis	recover plots	intra and interday variation	human liver panel	paired kidney samples	PP and PC murine cells	cross species
pAB_TXP_ALPR_rbt2	x											
pAB_TXP_ANFK_rbt1												
pAB_TXP_ANFK_rbt2												
pAB_TXP_APAR_rbt1												
pAB_TXP_APAR_rbt2												
pAB_TXP_AVDR_rbt1					x (m)							
pAB_TXP_AVDR_rbt2	x				x (m)	x (h, r, m)						
pAB_TXP_AYDR_rbt1	x					x (r)						
pAB_TXP_AYDR_rbt2	x					x (r)						
pAB_TXP_DAPK_rbt1	x					x (r)						
pAB_TXP_DAPK_rbt2	x											
pAB_TXP_DFFR_rbt1	x											
pAB_TXP_DLFR_rbt1	x											
pAB_TXP_DLFR_rbt2	x				x (m)							
pAB_TXP_DLFR_rbt3	x	x	x	x	x (h, r)	x (h, r, m)	x (h, r)	x (h, r)	x	x		x (h, r)
pAB_TXP_DLFR_rbt4	x											
pAB_TXP_DLVR_rbt1	x				x (m)	x (h, r, m)						
pAB_TXP_DLVR_rbt2	x				x (m)	x (r)						
pAB_TXP_DLVR_rbt3	x				x (m)	x (r)						
pAB_TXP_DLVR_rbt4	x				x (m)	x (r)						
pAB_TXP_DPPR_rbt1	x											
pAB_TXP_DPPR_rbt2	x											
pAB_TXP_DTQR_rbt1	x											
pAB_TXP_DTQR_rbt2	x											
pAB_TXP_EATR_rbt1	x				x (r, m)	x (r, m)	x (r, m)	x (r, m)			x	x (r, m)
pAB_TXP_EATR_rbt2	x				x (r, m)							
pAB_TXP_EAVK_rbt1	x				x (m)							
pAB_TXP_EAVK_rbt2	x				x (m)							
pAB_TXP_EEAK_rbt1	x											
pAB_TXP_EELK_rbt1	x											
pAB_TXP_EELK_rbt2	x											
pAB_TXP_EGCK_rbt1	x											
pAB_TXP_EGCK_rbt2	x											
pAB_TXP_EIQK_rbt1	x			x	x (r)	x (r)	x (r)	x (r)				x (r)
pAB_TXP_ELSK_rbt1	x											
pAB_TXP_ESIK_rbt1	x				x (m)	x (r, m)						

Antibody ID	AB functionality	lysis optimization	alternative sample preparations	analyte stability	AB and lysate amount	kinetic of proteolysis	recover plots	intra and interday variation	human liver panel	paired kidney samples	PP and PC murine cells	cross species
pAB_TXP_ESIK_rbt2	x			x	x (r, m)	x (r)						
pAB_TXP_ESTR_rbt1	x			x	x (r)	x (r)	x (r)					
pAB_TXP_ETFR_rbt1	x					x (r)						
pAB_TXP_ETFR_rbt2	x					x (r)						
pAB_TXP_EVLR_rbt1	x					x (r)						
pAB_TXP_EVLR_rbt2	x					x (r)						
pAB_TXP_FAFK_rbt1	x					x (r)						
pAB_TXP_FAFK_rbt2	x					x (r)						
pAB_TXP_FFFK_rbt1	x											
pAB_TXP_FFFK_rbt2	x				x (h, m)	x (h, r, m)	x (m)					
pAB_TXP_FSGR_rbt1	x				x (r, m)	x (r, m)	x (r, m)	x (r, m)			x	x (r, m)
pAB_TXP_FSGR_rbt2	x				x (r, m)							
pAB_TXP_FTNR_rbt1	x				x (r, m)							
pAB_TXP_FTNR_rbt2	x				x (r, m)	x (r, m)	x (r, m)	x (r, m)			x	x (r, m)
pAB_TXP_FWLK_rbt1	x				x (r, m)							
pAB_TXP_FWLK_rbt2	x				x (r, m)	x (r, m)						
pAB_TXP_GFCR_rbt1	x											
pAB_TXP_GFCR_rbt2	x											
pAB_TXP_GGEK_rbt1	x	x	x	x	x (r, m)	x (h, r, m)	x (h, r, m)	x (h, r, m)	x	x	x	x (h, r, m)
pAB_TXP_GGEK_rbt2	x				x (m)							
pAB_TXP_GQVR_rbt1	x				x (m)	x (m)	x (m)	x (m)			x	x (m)
pAB_TXP_GQVR_rbt2	x				x (m)							
pAB_TXP_GSLR_rbt1	x	x	x	x	x (h, r, m)	x (h, r, m)	x (h, r)	x (h)	x	x		x (h, r)
pAB_TXP_GSLR_rbt2	x											
pAB_TXP_GTVR_rbt1	x	x		x	x (r, m)	x (h, r, m)	x (r)	x (r)				x (r)
pAB_TXP_GTVR_rbt2	x				x (r, m)							
pAB_TXP_GYYR_rbt1	x				x (m)	x (r)						
pAB_TXP_GYYR_rbt2	x				x (m)	x (h, r, m)	x (m)					
pAB_TXP_IFFK_rbt1	x				x (r, m)	x (r, m)						
pAB_TXP_IFFK_rbt2	x				x (r, m)							
pAB_TXP_KPHR_rbt1	x				x (r)	x (r)						
pAB_TXP_KPHR_rbt2	x				x (r)							
pAB_TXP_LAER_rbt1	x											
pAB_TXP_LAER_rbt2	x											
pAB_TXP_LAER_rbt3	x											

Antibody ID	AB functionality	lysis optimization	alternative sample preparations	analyte stability	AB and lysate amount	kinetic of proteolysis	recover plots	intra and interday variation	human liver panel	paired kidney samples	PP and PC murine cells	cross species
pAB_TXP_LDDK_rbt1	x				x (r, m)	x (r, m)	x (r, m)	x (r, m)			x	x (r, m)
pAB_TXP_LDDR_rbt1	x				x (r)	x (r)	x (r)	x (r)				x (r)
pAB_TXP_LDDR_rbt2	x				x (r)							
pAB_TXP_LEVR_rbt1	x				x (h)	x (h)						
pAB_TXP_LFSK_rbt1	x											
pAB_TXP_LFSK_rbt2	x											
pAB_TXP_LGYR_rbt1	x											
pAB_TXP_LIDK_rbt1	x											
pAB_TXP_LIDK_rbt2	x				x (h)	x (h)						
pAB_TXP_LISK_rbt1	x				x (r)							
pAB_TXP_LISK_rbt2	x				x (r)	x (r)	x (r)	x (r)				x (r)
pAB_TXP_LPNK_rbt1			x			x (h)	x (h)		x			x (h)
pAB_TXP_LPNK_rbt2	x				x (r, m)	x (r, m)	x (r)	x (r)				x (r)
pAB_TXP_LPNK_rbt3	x				x (r, m)							
pAB_TXP_LPSK_rbt1	x				x (r)							
pAB_TXP_LPSK_rbt2	x				x (r)	x (r)	x (r)	x (r)				x (r)
pAB_TXP_LSGK_rbt1	x				x (r, m)	x (r, m)						
pAB_TXP_LSQR_rbt1	x											
pAB_TXP_LSQR_rbt2	x											
pAB_TXP_LTIR_rbt1												
pAB_TXP_LTIR_rbt2	x											
pAB_TXP_LTTR_rbt1	x											
pAB_TXP_LTTR_rbt2	x			x	x (h, r, m)	x (h, r, m)	x (h, r, m)	x (h, r, m)	x	x	x	x (h, r, m)
pAB_TXP_NFSK_rbt1	x				x (m)	x (m)	x (m)	x (m)			x	x (m)
pAB_TXP_NFSK_rbt2	x				x (m)							
pAB_TXP_NGER_rbt1	x	x		x	x (r, m)	x (r, m)	x (r, m)	x (r, m)			x	x (r, m)
pAB_TXP_NGER_rbt2	x				x (r, m)							
pAB_TXP_PFQR_rbt1	x				x (r, m)							
pAB_TXP_PFQR_rbt2	x				x (r, m)							
pAB_TXP_PSGR_rbt1												
pAB_TXP_PSGR_rbt2												
pAB_TXP_PSSK_rbt1	x				x (h)							
pAB_TXP_PSSK_rbt2	x		x		x (h)	x (h)	x (h)	x (h)	x	x		x (h)
pAB_TXP_PTVK_rbt1	x											
pAB_TXP_PTVK_rbt2	x											
pAB_TXP_PWGK_rbt1	x											
pAB_TXP_PWGK_rbt2	x											
pAB_TXP_QDEK_rbt1	x											

Antibody ID	AB functionality	lysis optimization	alternative sample preparations	analyte stability	AB and lysate amount	kinetic of proteolysis	recover plots	intra and interday variation	human liver panel	paired kidney samples	PP and PC murine cells	cross species
pAB_TXP_QDEK_rbt2	x		x		x (h)	x (h, r)	x (h)	x (h)	x	x		x (h)
pAB_TXP_QDIR_rbt1	x	x		x	x (h, r, m)	x (h, r, m)	x (r)	x (h, r)				x (r)
pAB_TXP_QDIR_rbt2					x (m)							
pAB_TXP_QLLK_rbt1	x				x (m)							
pAB_TXP_QLLK_rbt2	x				x (m)	x (m)						
pAB_TXP_QLLK_rbt3												
pAB_TXP_QLLK_rbt4	x				x (m)	x (r)						
pAB_TXP_QPPR_rbt1	x				x (r, m)	x (r, m)	x (r, m)	x (r, m)			x	x (r, m)
pAB_TXP_QPPR_rbt2	x				x (r, m)							
pAB_TXP_QQER_rbt1	x											
pAB_TXP_QQER_rbt2	x											
pAB_TXP_RPER_rbt1	x				x (r, m)							
pAB_TXP_RPER_rbt2	x				x (r, m)	x (r)						
pAB_TXP_SAIR_rbt1												
pAB_TXP_SAIR_rbt2												
pAB_TXP_SIGK_rbt1	x				x (r, m)							
pAB_TXP_SIGK_rbt2	x				x (r, m)	x (r, m)						
pAB_TXP_SLNK_rbt1												
pAB_TXP_SLNK_rbt2	x				x (m)	x (r)						
pAB_TXP_SLNK_rbt3												
pAB_TXP_SLNK_rbt4	x				x (m)	x (r, m)						
pAB_TXP_STGK_rbt1	x											
pAB_TXP_STGK_rbt2	x				x (r, m)	x (m)						
pAB_TXP_TNPR_rbt1	x				x (r, m)							
pAB_TXP_TNPR_rbt2	x				x (r, m)	x (r, m)	x (m)	x (m)				x (m)
pAB_TXP_TTDR_rbt1	x											
pAB_TXP_TTDR_rbt2	x											
pAB_TXP_TVEK_rbt1	x				x (h, r, m)	x (h, r, m)	x (h, m)	x (h, m)	x	x	x	x (h, m)
pAB_TXP_TVEK_rbt2	x				x (m)							
pAB_TXP_VILR_rbt1	x				x (r, m)	x (r, m)						
pAB_TXP_VILR_rbt2	x				x (r, m)							
pAB_TXP_YQVR_rbt1	x				x (h, r, m)	x (h, r, m)	x (h, r)	x (h, r)	x	x		x (h, r)
pAB_TXP_YQVR_rbt2	x				x (r, m)							

Table 63: Overview of peptide standards used in each experiment. Used peptides are indicated by x. In case samples of more than one species were used in an experiment, the species are specified by single letters (human: h, rat: r, mouse: m). Peptides are sorted according to their TXP-tag.

Peptide sequence		AB functionality	Lysis optimization	alternative sample preparations	analyte stability	AB and lysate amount	kinetic of proteolysis	recovery plots	intra and interday variation	Human liver panel	paired kidney samples	PP and PC murine cells	Cross species
ANIYGLCFAFSQGIAFLANS	AAYR	x				x (r)	x (r)						
AAATEDATPA	ALEK	x	x		x	x (r, m)	x (r, m)	x (r, m)	x (r, m)			x	x (r, m)
NLVLL	ALPR	x											
NVALL	ALPR	x											
NLVLM	ALPR	x											
GLSGDVLINGAPQP	ANFK												
DPSGLSGDVLINGAPRP	ANFK												
TLSLE	APAR												
SLSEASV	AVDR	x				x (m)	x (h, r, m)						
LGSILSPYFVYLG	AYDR	x					x (r)						
GDFVAVFPPMIHNDPEVF	DAPK	x					x (r)						
SECHV	DFFR	x											
LSPSFA	DLFR	x				x (h)	x (h)						
SPSFA	DLFR	x			x	x (r)	x (r)	x (r)					x (r)
RPSYL	DLFR	x		x		x (h)	x (h)	x (h)	x (h)	x	x		x (h)
ALQRPSYL	DLFR	x	x		x	x (r)	x (r)	x (r)	x (r)				x (r)
VSQRPSYL	DLFR	x											
YGLS	DLFR	x	x		x	x (r, m)	x (r, m)	x (r)	x (r)				x (r)
YTAS	DLFR	x		x		x (h)	x (h)	x (h)	x (h)	x	x		x (h)
EVLDSFL	DLVR	x				x (m)	x (m)						
LNPSFL	DLVR	x					x (h, r)						
MVA	DPPR	x											
FVHPPNASLPN	DTQR	x											
FINLVPSNLPH	EATR	x				x (r, m)	x (r, m)	x (r, m)	x (r, m)			x	x (r, m)
IVVLCGQ	EAVK	x				x (m)							
TYGPVFTLYFGSQPTVVLHGY	EAVK	x											
AYGPVFTLYLGSKPTVILHGY	EAVK	x				x (m)							
AYGPVFTLYLGSRPTVVLHGY	EAVK	x				x (m)							

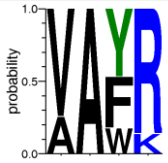
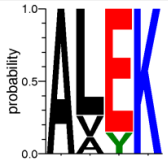
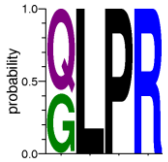
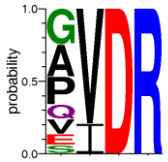
Peptide sequence		AB functionality	Lysis optimization	alternative sample preparations	analyte stability	AB and lysate amount	kinetic of proteolysis	recovery plots	intra and interday variation	Human liver panel	paired kidney samples	PP and PC murine cells	Cross species
VYGPVFTLYFGSKPTVVVHGY	EAVK	x				x (m)							
LAA	EEAK	x											
LSL	EELK	x											
LTI	EELK	x											
LQTE	EGCK	x											
AMDSFPGPPTHWLFGHAL	EIQK	x			x		x (r)	x (r)	x (r)				x (r)
CIGE	ELSK	x											
MVEVCV	ESIK	x			x	x (r, m)	x (r, m)						
LLTPGFHYDVLKPYVAIFA	ESTR	x			x	x (r)	x (r)	x (r)					
LSDRPQLPYLEAFIL	ETFR	x					x (r)						
EQLDSLVCLESAIL	EVLR						x (r)						
YTDHF	FAFK	x					x (r)						
DHF	FAFK	x											
FIGLQ	FFFK	x				x (h, m)	x (h, r, m)	x (m)					
EALVDHAEE	FSGR	x				x (m)	x (m)						
EALEDNAEE	FSGR	x				x (m)	x (m)						
EALVGQAEA	FSGR	x				x (m)	x (m)	x (m)	x (m)			x	x (m)
EALVDHAEA	FSGR	x				x (r, m)	x (r, m)	x (r, m)	x (r, m)			x	x (r, m)
EALVDLGEE	FSGR	x				x (r)	x (r)	x (r)	x (r)				x (r)
EALIDYGEE	FSGR	x				x (r)	x (r)	x (r)	x (r)				x (r)
EALVDHGEE	FSGR	x				x (r)	x (r)	x (r)	x (r)				x (r)
EALDDLGEE	FSGR	x				x (r, m)	x (r, m)	x (r, m)	x (r, m)			x	x (r, m)
NE	FSGR	x				x (r, m)	x (r, m)						
ECYST	FTNR	x				x (r, m)	x (r, m)	x (r)	x (r)				x (r)
ECYSV	FTNR	x				x (r, m)	x (r, m)	x (r, m)	x (r, m)			x	x (r, m)
DCLSV	FTNR	x				x (m)	x (m)	x (m)	x (m)			x	x (m)
HW	FWLK	x				x (r, m)	x (r, m)						
LLNSHW	FWLK	x											
GIYD	GDLK	x	x	x	x	x (h, m)	x (h, r, m)	x (h, r, m)	x (h, r, m)	x	x		x (h, r, m)
YDTVC	GFCR	x											
TVAAF	GGEK	x		x			x (h)	x (h)	x (h)				x (h)
DLSLHVH	GGEK	x		x			x (h)	x (h)	x (h)	x	x		x (h)
NLTLHVQ	GGEK	x	x		x	x (r)	x (r)	x (r)	x (r)				x (r)

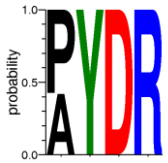
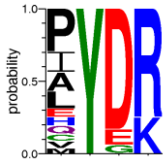
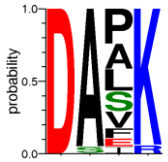
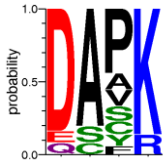
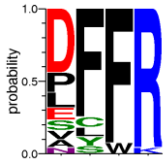
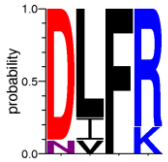
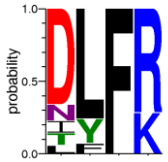
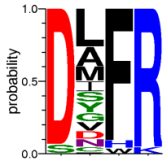
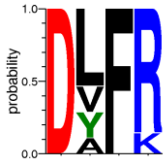
Peptide sequence		AB functionality	Lysis optimization	alternative sample preparations	analyte stability	AB and lysate amount	kinetic of proteolysis	recovery plots	intra and interday variation	Human liver panel	paired kidney samples	PP and PC murine cells	Cross species
NVTVHVQ	GGEK	x				x (m)	x (m)	x (m)	x (m)			x	x (m)
AELHQLS	GGEK	x											
VQQEIDAVI	GQVR	x				x (m)	x (m)	x (m)	x (m)				x (m)
VQQEIDEVI	GQVR	x				x (m)	x (m)	x (m)	x (m)			x	x (m)
ITIIPQDPVLFS	GSLR	x	x	x	x	x (h, r, m)	x (h, r, m)	x (h, r)	x (h, r)	x	x		x (h, r)
LTIIPQDPILFS	GSLR	x	x	x	x	x (h, r)	x (h, r)	x (h, r)	x (h, r)				x (h, r)
LTIIPQEPVLFS	GTVR	x	x		x	x (r)	x (r)	x (r)	x (r)				x (r)
LAIIPQEPVLFS	GTVR	x				x (m)	x (m)						
LSIIPQEPVLFS	GTVR	x					x (h)						
LFIHEYIS	GYYR	x				x (m)	x (h, r, m)	x (m)					
IQGYFNAWQALLIKPN	IFFK	x				x (r, m)	x (r, m)						
DETVWE	KPHR	x				x (r)	x (r)						
GPSAAA	LAER	x											
DFDPQNF	LDDK	x				x (r)	x (r)	x (r)	x (r)				x (r)
DFNPQHF	LDDK	x				x (r, m)	x (r, m)	x (r, m)	x (r, m)			x	x (r, m)
EIDQVIGSHRPPS	LDDR	x				x (r)	x (r)	x (r)	x (r)				x (r)
GGPEAT	LEVR	x				x (h)	x (h)						
LAI	LGYR	x											
TLGGILAPIYFGA	LIDK	x				x (h)	x (h)						
EAEY	LISK	x				x (r)	x (r)	x (r)	x (r)				x (r)
EANH	LISK	x				x (r)	x (r)	x (r)	x (r)				x (r)
EANIHAFIES	LPNK			x			x (h)	x (h)		x			x (h)
LQDEIDEA	LPNK	x				x (m)	x (m)						
LQDEIDAA	LPNK	x				x (r, m)	x (r, m)	x (r)	x (r)				x (r)
LQEEIDET	LPNK	x				x (m)	x (m)						
LQEEIDGA	LPSK	x				x (r)	x (r)	x (r)	x (r)				x (r)
WLV	LSGK	x				x (r, m)	x (r, m)						
DNTPTVLHGIN	LTIR	x											
NTTGA	LTTR	x			x	x (h, r, m)	x (h, r, m)	x (h, r, m)	x (h, r, m)	x	x	x	x (h, r, m)
NTTGS	LTTR	x			x	x (r)	x (r)	x (r)	x (r)				x (r)
NSPGA	LTTR	x				x (h)	x (h)	x (h)	x (h)	x	x		x (h)
NNPGV	LTTR	x			x	x (r, m)	x (r, m)	x (r, m)	x (r, m)			x	x (r, m)
NISQSFT	NFSK	x				x (m)	x (m)	x (m)	x (m)			x	x (m)
NFNQSLT	NFSK	x				x (m)	x (m)	x (m)	x (m)			x	x (m)

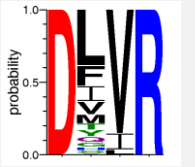
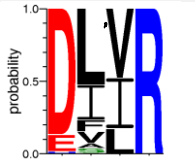
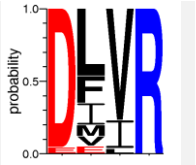
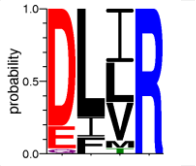
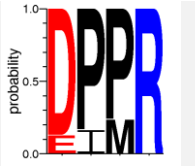
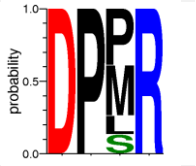
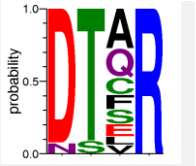
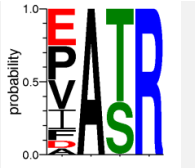
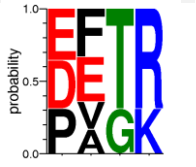
Peptide sequence		AB functionality	Lysis optimization	alternative sample preparations	analyte stability	AB and lysate amount	kinetic of proteolysis	recovery plots	intra and interday variation	Human liver panel	paired kidney samples	PP and PC murine cells	Cross species
NVSQSLT	NFSK	x				x (m)	x (m)	x (m)	x (m)			x	x (m)
GYGVTFS	NGER	x				x (m)	x (m)	x (m)	x (m)			x	x (m)
EYGVIFA	NGER	x	x		x	x (r, m)	x (r, m)	x (r)	x (r)				x (r)
GYGVAFS	NGER	x				x (m)	x (m)	x (m)	x (m)			x	x (m)
DYDEVTAFLGEWG	PFQR					x (r, m)							
SPQSFFDTT	PSGR												
APQSFFDTT	PSGR												
YVEQQYGQ	PSSK	x		x		x (h)	x (h)	x (h)			x		x (h)
AAVP	PWGK	x											
AAL	QDEK	x					x (r)						
NKPLFDTI	QDEK	x		x		x (h)	x (h)	x (h)	x (h)	x	x		x (h)
LYDPLEGVVSIDG	QDIR	x				x (m)	x (m)						
LYDPTEGMVSVDG	QDIR	x				x (h)	x (h)		x (h)				
LYDPIEGEVSIDG	QDIR	x	x		x	x (r)	x (r)	x (r)	x (r)				x (r)
LAHDILLFLNP	QLLK	x				x (m)	x (m)						
LIHDLLVFLNP	QLLK	x					x (r)						
LVNDIFTFVSP	QLLK	x											
NTWDPD	QPPR	x				x (m)	x (m)	x (m)	x (m)			x	x (m)
TTWDPD	QPPR	x				x (m)	x (m)						
TTWDPT	QPPR	x				x (m)	x (m)	x (m)	x (m)			x	x (m)
TWDPD	QPPR	x				x (r, m)	x (r, m)	x (r, m)	x (r, m)			x	x (r, m)
TYTLQDGPWS	QQER	x											
ELWGDPNEF	RPER	x				x (m)							
DPFVF	RPER	x				x (r, m)	x (r)						
DPQHWPEPEEF	RPER	x				x (r)							
YWPEPEEF	RPER	x				x (m)							
DPHYWPEPEEF	RPER	x				x (r)							
YLGGDDLDT	SAIR												
LEQYLGSDDLDL	SAIR												
SDAFVPF	SIGK	x				x (r, m)	x (r, m)						
NDAFVPF	SIGK	x				x (m)	x (m)						
QPLEGSDLW	SLNK	x											
SSDLW	SLNK	x					x (r)						
QPLESSDLW	SLNK	x				x (m)	x (m)						
SLPA	SLQR												

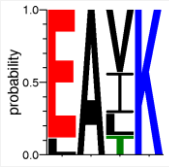
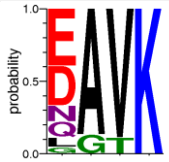
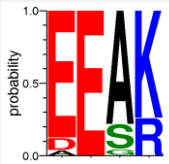
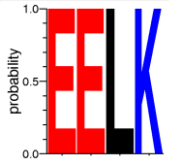
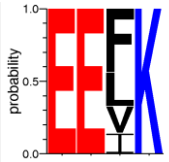
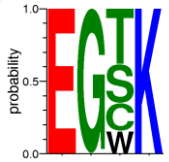
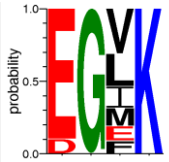
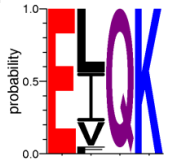
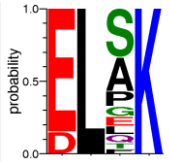
Peptide sequence		AB functionality	Lysis optimization	alternative sample preparations	analyte stability	AB and lysate amount	kinetic of proteolysis	recovery plots	intra and interday variation	Human liver panel	paired kidney samples	PP and PC murine cells	Cross species
NAAFLPF	STGK	x				x (r)							
SEAFMPF	STGK												
CEAFLPF	STGK	x											
SDYFIPF	STGK	x				x (m)	x (m)						
ESLDV	TNPR	x				x (m)	x (m)	x (m)	x (m)				x (m)
EHQESLDV	TNPR	x				x (r)	x (r)						
IEEHQESLDV	TNPR	x				x (r)	x (r)						
DLVGYIF	TTDR	x											
DHVGYIF	TTDR	x											
SSIS	TVEK	x				x (h, r, m)	x (h, r, m)	x (h, m)	x (h, m)	x	x	x	x (h, m)
MSDS	VILR	x				x (r, m)	x (r, m)						
HGEIQFNN	YQVR	x				x (r)	x (r)	x (r)	x (r)				x (r)
IQFNN	YQVR	x				x (h)	x (h)	x (h)	x (h)	x	x		x (h)
GEIQFNN	YQVR	x				x (m)	x (m)						

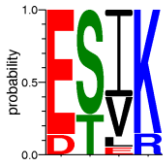
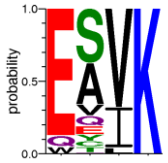
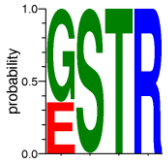
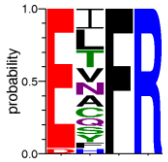
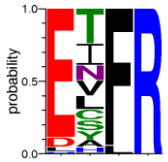
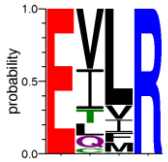
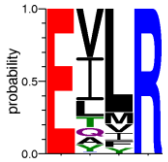
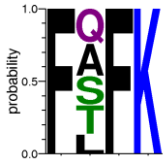
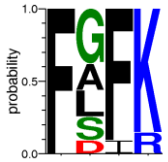
Table 64: Detailed information of Sequence logos of all used antibodies. The antibody ID is unique for each serum and contains following information: clonality (monoclonal mAB / polyclonal pAB), antigen, host species and a consecutive number. The ratio of enriched peptides is obtained by referring the number of enriched peptides with the expected TXP epitope to the total number of peptides present in the proteome sharing this epitope (UniProtKB reference proteome June2014). Additionally, the sequence logo as well as the number of peptides and tags it is based on are listed. Some antibodies were excluded from this experiment, because they either failed a preliminary test (*) or are peptide specific (**). A monoclonal anti-cMyc antibody was used as negative control (***). pAB_TXP_LPNK_rbt1 has already been characterized (+) [22].

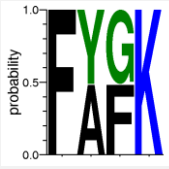
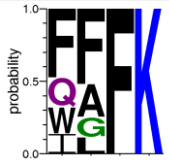
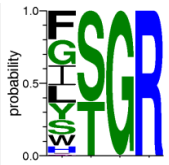
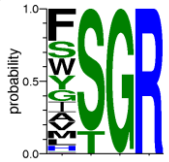
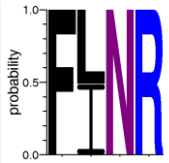
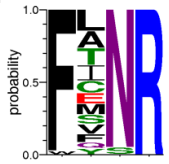
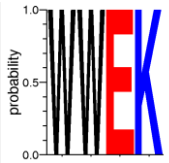
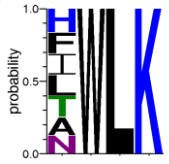
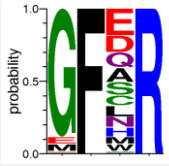
Antibody ID	enriched at least 1 spiked in peptide	enriched target peptides	total number of target peptides	ratio of enriched peptide	included tags	peptide sequences logo is based on	Sequence logo
mAB_cMyc_ms1	***	***	***	***	***	***	***
pAB_GIYDGDLK_rbt1	yes	**	**	**	**	**	**
pAB_GIYDGDLK_rbt2	yes	**	**	**	**	**	**
pAB_TXP_AAYR_rbt1	no	5	68	7.4 %	6	26	
pAB_TXP_AAYR_rbt2	yes	0	68	0.0 %	0	0	
pAB_TXP_ALEK_rbt1	yes	24	280	8.6 %	4	36	
pAB_TXP_ALEK_rbt2	*	*	*	*	*	*	*
pAB_TXP_ALPR_rbt1	yes	0	165	0.0 %	0	0	
pAB_TXP_ALPR_rbt2	yes	0	165	0.0 %	2	4	
pAB_TXP_ANFK_rbt1	*	*	*	*	*	*	*
pAB_TXP_ANFK_rbt2	*	*	*	*	*	*	*
pAB_TXP_APAR_rbt1	*	*	*	*	*	*	*
pAB_TXP_APAR_rbt2	*	*	*	*	*	*	*
pAB_TXP_AVDR_rbt1	*	*	*	*	*	*	*
pAB_TXP_AVDR_rbt2	yes	7	109	6.4 %	9	44	

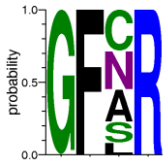
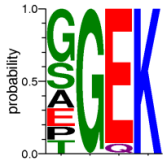
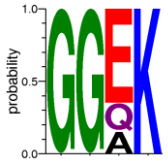
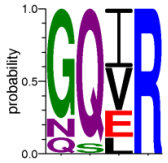
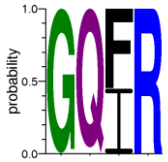
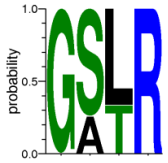
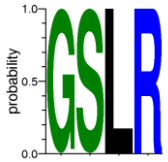
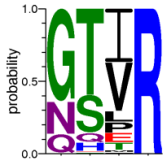
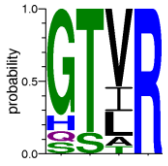
Antibody ID	enriched at least 1 spiked in peptide	enriched target peptides	total number of target peptides	ratio of enriched peptide	included tags	peptide sequences logo is based on	Sequence logo
pAB_TXP_AYDR_rbt1	yes	10	221	4.5 %	2	13	
pAB_TXP_AYDR_rbt2	yes	11	221	5.0 %	15	66	
pAB_TXP_DAPK_rbt1	yes	7	43	16.3 %	10	62	
pAB_TXP_DAPK_rbt2	yes	12	43	27.9 %	10	64	
pAB_TXP_DFFR_rbt1	yes	11	42	26.2 %	13	69	
pAB_TXP_DLFR_rbt1	yes	21	98	21.4 %	5	39	
pAB_TXP_DLFR_rbt2	yes	16	98	16.3 %	9	46	
pAB_TXP_DLFR_rbt3	yes	20	98	20.4 %	15	90	
pAB_TXP_DLFR_rbt4	yes	15	98	15.3 %	5	32	

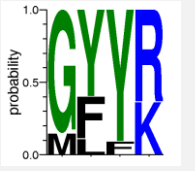
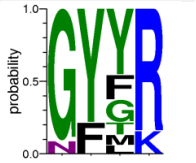
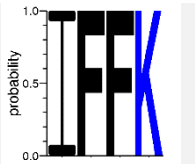
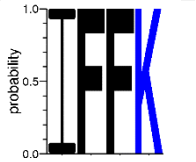
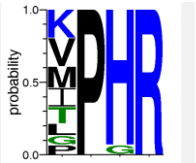
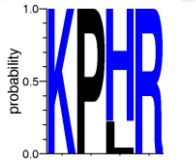
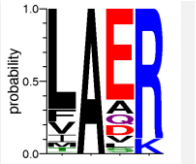
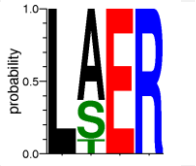
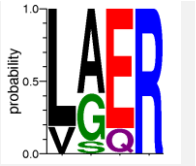
Antibody ID	enriched at least 1 spiked in peptide	enriched target peptides	total number of target peptides	ratio of enriched peptide	included tags	peptide sequences logo is based on	Sequence logo
pAB_TXP_DLVR_rbt1	yes	39	110	35.5 %	14	157	
pAB_TXP_DLVR_rbt2	yes	18	110	16.4 %	14	123	
pAB_TXP_DLVR_rbt3	yes	26	110	23.6 %	10	94	
pAB_TXP_DLVR_rbt4	yes	17	110	15.5 %	11	134	
pAB_TXP_DPPR_rbt1	yes	17	79	21.5 %	4	29	
pAB_TXP_DPPR_rbt2	yes	11	79	13.9 %	4	21	
pAB_TXP_DTQR_rbt1	yes	4	35	11.4 %	10	42	
pAB_TXP_DTQR_rbt2	yes	0	35	0.0 %	0	0	
pAB_TXP_EATR_rbt1	yes	12	102	11.8 %	11	69	
pAB_TXP_EATR_rbt2	yes	3	102	2.9 %	4	16	

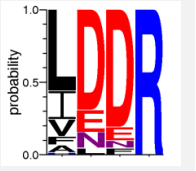
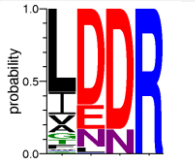
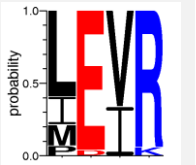
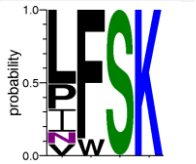
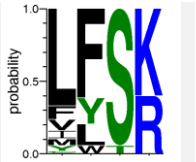
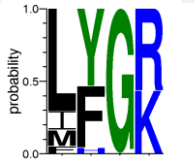
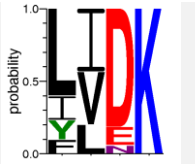
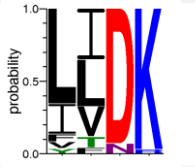
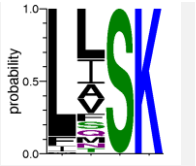
Antibody ID	enriched at least 1 spiked in peptide	enriched target peptides	total number of target peptides	ratio of enriched peptide	included tags	peptide sequences logo is based on	Sequence logo
pAB_TXP_EAVK_rbt1	yes	0	167	0.0 %	20	28	
pAB_TXP_EAVK_rbt2	yes	21	167	12.6 %	8	66	
pAB_TXP_EEAK_rbt1	no	53	266	19.9 %	9	115	
pAB_TXP_EELK_rbt1	no	3	394	0.8 %	1	3	
pAB_TXP_EELK_rbt2	yes	6	394	1.5 %	4	19	
pAB_TXP_EGCK_rbt1	no	4	38	10.5 %	4	27	
pAB_TXP_EGCK_rbt2	no	0	38	0.0 %	7	55	
pAB_TXP_EIQK_rbt1	yes	23	114	20.2 %	4	73	
pAB_TXP_ELSK_rbt1	yes	33	206	16.0 %	11	129	

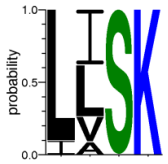
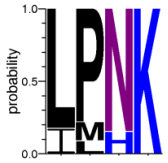
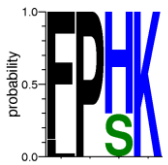
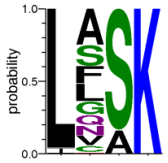
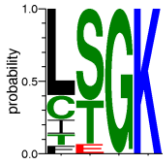
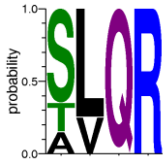
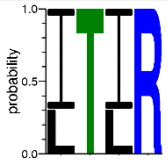
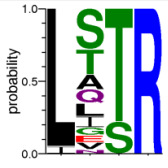
Antibody ID	enriched at least 1 spiked in peptide	enriched target peptides	total number of target peptides	ratio of enriched peptide	included tags	peptide sequences logo is based on	Sequence logo
pAB_TXP_ESIK_rbt1	yes	21	93	22.6 %	8	91	
pAB_TXP_ESIK_rbt2	yes	15	93	16.1 %	17	144	
pAB_TXP_ESTR_rbt1	yes	2	82	2.4 %	2	6	
pAB_TXP_ETFR_rbt1	yes	11	61	18.0 %	14	127	
pAB_TXP_ETFR_rbt2	yes	10	61	16.4 %	15	142	
pAB_TXP_EVLR_rbt1	yes	75	215	34.9 %	19	425	
pAB_TXP_EVLR_rbt2	yes	69	215	32.1 %	31	495	
pAB_TXP_FAFK_rbt1	yes	4	26	15.4 %	5	20	
pAB_TXP_FAFK_rbt2	yes	5	26	19.2 %	7	30	

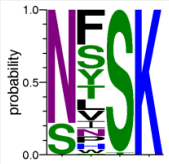
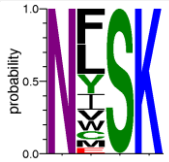
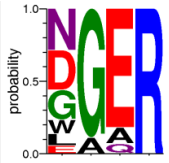
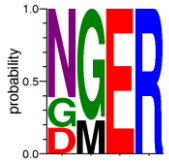
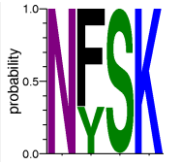
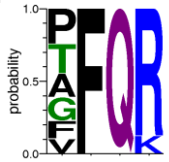
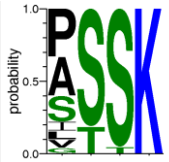
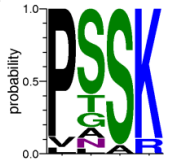
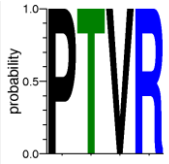
Antibody ID	enriched at least 1 spiked in peptide	enriched target peptides	total number of target peptides	ratio of enriched peptide	included tags	peptide sequences logo is based on	Sequence logo
pAB_TXP_FFFK_rbt1	no	0	28	0.0 %	2	4	
pAB_TXP_FFFK_rbt2	yes	0	28	0.0 %	6	20	
pAB_TXP_FSGR_rbt1	yes	18	85	21.2 %	21	195	
pAB_TXP_FSGR_rbt2	yes	14	85	16.5 %	15	109	
pAB_TXP_FTNR_rbt1	yes	0	36	0.0 %	2	8	
pAB_TXP_FTNR_rbt2	yes	7	36	19.4 %	14	97	
pAB_TXP_FWLK_rbt1	yes	0	15	0.0 %	1	2	
pAB_TXP_FWLK_rbt2	yes	3	15	20.0 %	7	23	
pAB_TXP_GFCR_rbt1	yes	7	19	36.8 %	19	118	

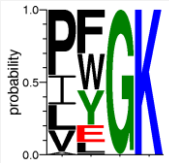
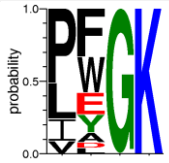
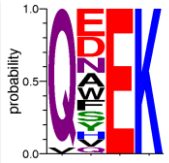
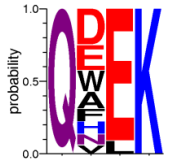
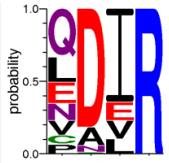
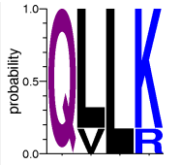
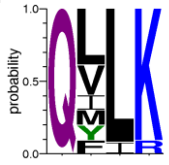
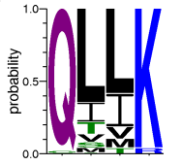
Antibody ID	enriched at least 1 spiked in peptide	enriched target peptides	total number of target peptides	ratio of enriched peptide	included tags	peptide sequences logo is based on	Sequence logo
pAB_TXP_GFCR_rbt2	yes	5	19	26.3 %	5	24	
pAB_TXP_GGEK_rbt1	yes	17	99	17.2 %	7	61	
pAB_TXP_GGEK_rbt2	yes	14	99	14.1 %	3	21	
pAB_TXP_GQVR_rbt1	yes	3	46	6.5 %	7	29	
pAB_TXP_GQVR_rbt2	yes	0	46	0.0 %	2	4	
pAB_TXP_GSLR_rbt1	yes	7	233	3.0 %	3	14	
pAB_TXP_GSLR_rbt2	yes	3	233	1.3 %	1	3	
pAB_TXP_GTVR_rbt1	yes	9	95	9.5 %	22	149	
pAB_TXP_GTVR_rbt2	yes	10	95	10.5 %	9	51	

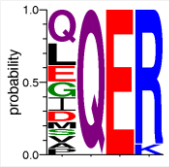
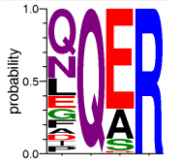
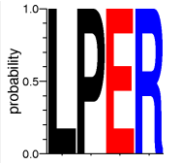
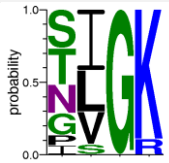
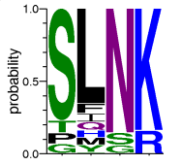
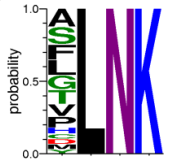
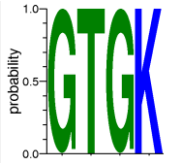
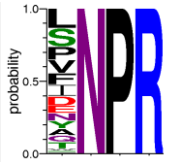
Antibody ID	enriched at least 1 spiked in peptide	enriched target peptides	total number of target peptides	ratio of enriched peptide	included tags	peptide sequences logo is based on	Sequence logo
pAB_TXP_GYYR_rbt1	yes	7	42	16.7 %	7	30	
pAB_TXP_GYYR_rbt2	yes	6	42	14.3 %	10	45	
pAB_TXP_IFFK_rbt1	yes	2	27	7.4 %	1	2	
pAB_TXP_IFFK_rbt2	yes	4	27	14.8 %	1	4	
pAB_TXP_KPHR_rbt1	yes	5	29	17.2 %	9	36	
pAB_TXP_KPHR_rbt2	yes	7	29	24.1 %	2	11	
pAB_TXP_LAER_rbt1	yes	51	201	25.4 %	18	233	
pAB_TXP_LAER_rbt2	yes	23	201	11.4 %	3	35	
pAB_TXP_LAER_rbt3	yes	17	201	8.5 %	6	45	
pAB_TXP_LDDK_rbt1	yes	0	69	0.0 %	0	0	

Antibody ID	enriched at least 1 spiked in peptide	enriched target peptides	total number of target peptides	ratio of enriched peptide	included tags	peptide sequences logo is based on	Sequence logo
pAB_TXP_LDDR_rbt1	yes	20	60	33.3 %	17	126	
pAB_TXP_LDDR_rbt2	yes	23	60	38.3 %	20	161	
pAB_TXP_LEVR_rbt1	yes	34	108	31.5 %	9	79	
pAB_TXP_LFSK_rbt1	no	28	124	22.6 %	6	62	
pAB_TXP_LFSK_rbt2	no	22	124	17.7 %	18	123	
pAB_TXP_LGYR_rbt1	no	0	70	0.0 %	10	73	
pAB_TXP_LIDK_rbt1	yes	14	71	19.7 %	13	102	
pAB_TXP_LIDK_rbt2	yes	16	71	22.5 %	5	153	
pAB_TXP_LISK_rbt1	yes	37	146	25.3 %	18	287	

Antibody ID	enriched at least 1 spiked in peptide	enriched target peptides	total number of target peptides	ratio of enriched peptide	included tags	peptide sequences logo is based on	Sequence logo
pAB_TXP_LISK_rbt2	yes	33	146	22.6 %	5	90	
pAB_TXP_LPNK_rbt1	+	+	+	+	+	+	
pAB_TXP_LPNK_rbt2	yes	10	50	20.0 %	5	21	
pAB_TXP_LPNK_rbt3	yes	0	50	0.0 %	0	0	
pAB_TXP_LPSK_rbt1	yes	3	156	1.9 %	3	9	
pAB_TXP_LPSK_rbt2	yes	0	156	0.0 %	14	110	
pAB_TXP_LSGK_rbt1	yes	25	207	12.1 %	7	64	
pAB_TXP_LSQR_rbt1	yes	0	145	0.0 %	4	16	
pAB_TXP_LSQR_rbt2	yes	0	145	0.0 %	0	0	
pAB_TXP_LTIR_rbt1	*	*	*	*	*	*	
pAB_TXP_LTIR_rbt2	yes	0	73	0.0 %	2	8	
pAB_TXP_LTTR_rbt1	no	0	65	0.0 %	0	0	
pAB_TXP_LTTR_rbt2	yes	7	65	10.8 %	13	122	

Antibody ID	enriched at least 1 spiked in peptide	enriched target peptides	total number of target peptides	ratio of enriched peptide	included tags	peptide sequences logo is based on	Sequence logo
pAB_TXP_NFSK_rbt1	yes	20	64	31.3 %	19	167	
pAB_TXP_NFSK_rbt2	yes	19	64	29.7 %	9	72	
pAB_TXP_NGER_rbt1	yes	5	55	9.1 %	9	49	
pAB_TXP_NGER_rbt2	yes	5	55	9.1 %	4	14	
pAB_TXP_PFQR_rbt1	yes	0	36	0.0 %	2	9	
pAB_TXP_PFQR_rbt2	yes	3	36	8.3 %	7	25	
pAB_TXP_PSGR_rbt1	*	*	*	*	*	*	*
pAB_TXP_PSGR_rbt2	*	*	*	*	*	*	*
pAB_TXP_PSSK_rbt1	yes	32	210	15.2 %	11	130	
pAB_TXP_PSSK_rbt2	yes	45	210	21.4 %	10	136	
pAB_TXP_PTVK_rbt1	no	0	73	0.0 %	1	2	
pAB_TXP_PTVK_rbt2	no	0	73	0.0 %	0	0	

Antibody ID	enriched at least 1 spiked in peptide	enriched target peptides	total number of target peptides	ratio of enriched peptide	included tags	peptide sequences logo is based on	Sequence logo
pAB_TXP_PWGK_rbt1	yes	5	15	33.3 %	16	91	
pAB_TXP_PWGK_rbt2	yes	4	15	26.7 %	14	84	
pAB_TXP_QDEK_rbt1	yes	6	53	11.3 %	12	65	
pAB_TXP_QDEK_rbt2	yes	7	53	13.2 %	9	44	
pAB_TXP_QDIR_rbt1	yes	9	45	20.0 %	16	97	
pAB_TXP_QDIR_rbt2	*	*	*	*	*	*	*
pAB_TXP_QLLK_rbt1	yes	14	233	6.0 %	3	21	
pAB_TXP_QLLK_rbt2	yes	18	233	7.7 %	8	51	
pAB_TXP_QLLK_rbt3	*	*	*	*	*	*	*
pAB_TXP_QLLK_rbt4	yes	48	233	20.6 %	18	173	
pAB_TXP_QPPR_rbt1	yes	0	130	0.0 %	0	0	
pAB_TXP_QPPR_rbt2	yes	0	130	0.0 %	0	0	

Antibody ID	enriched at least 1 spiked in peptide	enriched target peptides	total number of target peptides	ratio of enriched peptide	included tags	peptide sequences logo is based on	Sequence logo
pAB_TXP_QQER_rbt1	yes	15	69	21.7 %	12	99	
pAB_TXP_QQER_rbt2	yes	23	69	33.3 %	24	241	
pAB_TXP_RPER_rbt1	yes	0	70	0.0 %	1	2	
pAB_TXP_RPER_rbt2	yes	0	70	0.0 %	0	0	
pAB_TXP_SAIR_rbt1	*	*	*	*	*	*	*
pAB_TXP_SAIR_rbt2	*	*	*	*	*	*	*
pAB_TXP_SIGK_rbt1	yes	0	67	0.0 %	0	0	
pAB_TXP_SIGK_rbt2	yes	10	67	14.9 %	12	87	
pAB_TXP_SLNK_rbt1	*	*	*	*	*	*	*
pAB_TXP_SLNK_rbt2	yes	18	114	15.8 %	14	95	
pAB_TXP_SLNK_rbt3	*	*	*	*	*	*	*
pAB_TXP_SLNK_rbt4	yes	15	114	13.2 %	13	116	
pAB_TXP_STGK_rbt1	no	0	101	0.0 %	1	3	
pAB_TXP_STGK_rbt2	yes	0	101	0.0 %	0	0	
pAB_TXP_TNPR_rbt1	yes	6	32	18.8 %	17	143	

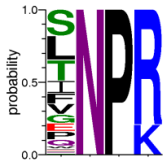
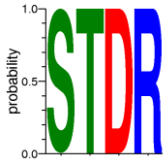
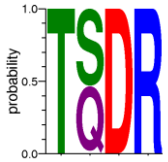
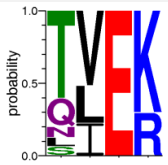
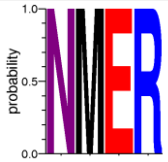
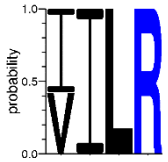
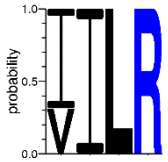
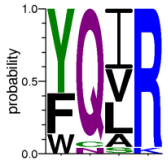
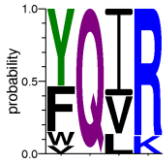
Antibody ID	enriched at least 1 spiked in peptide	enriched target peptides	total number of target peptides	ratio of enriched peptide	included tags	peptide sequences logo is based on	Sequence logo
pAB_TXP_TNPR_rbt2	yes	7	32	21.9 %	19	124	
pAB_TXP_TTDR_rbt1	yes	0	42	0.0 %	1	2	
pAB_TXP_TTDR_rbt2	yes	0	42	0.0 %	1	5	
pAB_TXP_TVEK_rbt1	yes	16	101	15.8 %	11	56	
pAB_TXP_TVEK_rbt2	yes	0	101	0.0 %	1	2	
pAB_TXP_VILR_rbt1	yes	3	95	3.2 %	2	6	
pAB_TXP_VILR_rbt2	yes	6	95	6.3 %	2	17	
pAB_TXP_YQVR_rbt1	yes	7	24	29.2 %	13	71	
pAB_TXP_YQVR_rbt2	yes	5	24	20.8 %	9	46	

Table 65: AB functionality-results with respect to peptides. For further assay development, peptides had to be enriched by at least one purified antibody serum. The criterion for successful enrichment was a total file area greater than 10^4 after tSIM analysis. Some peptides were excluded from this experiment, because they failed a preliminary test (*) or were already tested successfully (**).

Peptide sequence		enrichment
ANIYGLCFAFSQGIAFLANS	AAYR	yes
AAATEDATPA	ALEK	yes
NLVLL	ALPR	no
NVALL	ALPR	no
NLVLM	ALPR	no
GLSGDVLINGAPQP	ANFK	no*
DPSGLSGDVLINGAPRP	ANFK	no*
TLSLE	APAR	no*
SLSEASV	AVDR	yes
LGSILSPYFVYLG	AYDR	yes
GDFVAVFPPMIHNDPEVF	DAPK	yes
SECHV	DFFR	yes
LSPSFA	DLFR	yes
SPSFA	DLFR	yes
RPSYL	DLFR	yes
ALQRPSYL	DLFR	yes
VSQRPSYL	DLFR	no
YGLS	DLFR	yes
YTAS	DLFR	yes
EVLDSFL	DLVR	yes
LNPSFL	DLVR	yes
MVA	DPPR	yes
FVHPPNASLPN	DTQR	yes
FINLVPSNLPH	EATR	yes
IVVLCGQ	EAVK	yes
TYGPVFTLYFGSQPTVVLHGY	EAVK	no
AYGPVFTLYLGSKPTVILHGY	EAVK	yes
AYGPVFTLYLGSRPTVVLHGY	EAVK	yes
VYGPVFTLYFGSKPTVVVHGY	EAVK	yes
LAA	EEAK	no
LSL	EELK	no
LTI	EELK	yes
LQTE	EGCK	no
AMDSFPGPPTHWLFGHAL	EIQK	yes
CIGE	ELSK	yes
MVEVCV	ESIK	yes
LLTPGFHYDVLKPYVAIFA	ESTR	yes
LSDRPQLPYLEAFIL	ETFR	yes
EQLDSLVCLESAIL	EVLR	no*

Peptide sequence		enrichment
YTDHF	FAFK	yes
DHF	FAFK	yes
FIGLQ	FFFK	yes
EALVDHAEE	FSGR	yes
EALEDNAEE	FSGR	yes
EALVGQAEA	FSGR	yes
EALVDHAEA	FSGR	yes
EALVDLGEE	FSGR	yes
EALIDYGEE	FSGR	yes
EALVDHGEE	FSGR	yes
EALDDLGEE	FSGR	yes
NE	FSGR	yes
ECYST	FTNR	yes
ECYSV	FTNR	yes
DCLSV	FTNR	yes
HW	FWLK	yes
LLNSHW	FWLK	yes
GIYD	GDLK	yes
YDTVC	GFCR	yes
TVAAF	GGEK	yes
DLSLHVH	GGEK	yes
NLTLHVQ	GGEK	yes
NVTVHVQ	GGEK	yes
AELHQLS	GGEK	yes
VQQEIDAVI	GQVR	yes
VQQEIDEVI	GQVR	yes
ITIIPQDPVLFS	GSLR	yes
LTIIPQDPILFS	GSLR	yes
LTIIPQEPVLFS	GTVR	yes
LAIIPQEPVLFS	GTVR	yes
LSIIPQEPVLFS	GTVR	yes
LFIHEYIS	GYYR	yes
IQGYFNAWQALLIKPN	IFFK	yes
DETVWE	KPHR	yes
GPSAAA	LAER	yes
DFDPQNF	LDDK	yes
DFNPQHF	LDDK	yes
EIDQVIGSHRPPS	LDDR	yes
GGPEAT	LEVR	yes
LAI	LGYR	no
TLGGILAPIYFGA	LIDK	yes
EAEY	LISK	yes
EANH	LISK	yes
EANIHAFIES	LPNK	no**

Peptide sequence		enrichment
LQDEIDEA	LPNK	yes
LQDEIDAA	LPNK	yes
LQEEIDET	LPNK	yes
LQEEIDGA	LPSK	yes
WLV	LSGK	yes
DNTPTVLHGIN	LTIR	yes
NTTGA	LTTR	yes
NTTGS	LTTR	yes
NSPGA	LTTR	yes
NNPGV	LTTR	yes
NISQSFT	NFSK	yes
NFNQSLT	NFSK	yes
NVSQSLT	NFSK	yes
GYGVTFS	NGER	yes
EYGVIFA	NGER	yes
GYGVAFS	NGER	yes
DYDEVTAFLGEWG	PFQR	no*
SPQSFFDTT	PSGR	no*
APQSFFDTT	PSGR	no*
YVEQQYGQ	PSSK	yes
AAVP	PWGK	yes
AAL	QDEK	yes
NKPLFDTI	QDEK	yes
LYDPLEGVVSIDG	QDIR	yes
LYDPTEGMVSVDG	QDIR	yes
LYDPIEGEVSIDG	QDIR	yes
LAHDILLFLNP	QLLK	yes
LIHDLLVFLNP	QLLK	no
LVNDIFTFVSP	QLLK	yes
NTWDPD	QPPR	yes
TTWDPD	QPPR	yes
TTWDPT	QPPR	yes
TWDPD	QPPR	yes
TYTLQDGPWS	QQER	yes
ELWGDPNEF	RPER	yes
DPFVF	RPER	yes
DPQHWPEPEEF	RPER	yes
YWPEPEEF	RPER	yes
DPHYWPEPEEF	RPER	yes
YLGGDDLDT	SAIR	no*
LEQYLGSDDLDL	SAIR	no*
SDAFVPF	SIGK	yes
NDAFVPF	SIGK	yes
QPLEGSDLW	SLNK	yes

Peptide sequence		enrichment
SSDLW	SLNK	yes
QPLESSDLW	SLNK	yes
SLPA	SLQR	no*
NAAFLPF	STGK	yes
SEAFMPF	STGK	no*
CEAFLPF	STGK	yes
SDYFIPF	STGK	yes
ESLDV	TNPR	yes
EHQESLDV	TNPR	yes
IEEHQESLDV	TNPR	yes
DLVGYIF	TTDR	no
DHVGYIF	TTDR	yes
SSIS	TVEK	yes
MSDS	VILR	yes
HGEIQFNN	YQVR	yes
IQFNN	YQVR	yes
GEIQFNN	YQVR	yes

Table 66: Adjustment of antibody and proteolyzed protein amount for rat samples. Three different antibody and proteins amounts were tested whether they are sufficient for reproducible analyte quantification. For each antibody serum 1, 2 and 5 µg were tested with every protein amount. Results are given as % RSD. If not stated otherwise, the conclusion column gives the minimal amount of antibody and protein necessary, but more is also possible. Antibodies which did not enrich EN or IS peptide sufficiently for quantification were not used further (n.u.f.). Antibodies which lead to suitable IS signals and should be tested again when a sample containing this target is available are additionally indicated with (#). Decisions which were made with reservations are marked with *.

antibody	peptide	10-40 µg protein AB: 1 –	2 -	5µg	20-40 µg protein AB: 1 –	2 -	5µg	1-5 µg AB protein: 10 -	20 -	40 µg	2-5 µg AB protein: 10 -	20 -	40 µg	conclusion protein / AB
...AAYR_rbt2	ANIYGLCFAFSQGIAFLANSAAYR	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f. (#)
...ALEK_rbt1	AAATEDATPAALEK	15	20	17	11	1.6	0.4	29	8.5	2.6	42	1.6	3.5	20 µg / 2µg
...DLFR_rbt3	ALQRPSYLDLFR	8.7	6.9	8.8	9.1	5.8	12	1.7	4.5	12	2.1	3.2	15	40 µg / 5 µg
	SPSFADLFR	—	—	—	—	—	—	—	—	—	—	—	—	
	YGLSDLFR	—	—	—	—	—	—	—	—	—	—	—	—	
...EATR_rbt1	FINLVPSNLPHEATR	16	36	3.6	0.4	23	5.1	25	31	33	12	17	35	10 µg / 5 µg
...EATR_rbt2	FINLVPSNLPHEATR	—	—	—	—	—	—	—	—	—	—	—	—	use other AB
...EIQK_rbt1	AMDSFPGP PTHWLFGHALEIQK	12	7.2	12	9.5	9.5	3.7	9.5	11	6.7	3.7	9.5	3.7	20 µg / 1-2 µg

antibody	peptide	10-40 µg protein AB: 1 –	2 -	5µg	20-40 µg protein AB: 1 –	2 -	5µg	1-5 µg AB protein: 10 -	20 -	40 µg	2-5 µg AB protein: 10 -	20 -	40 µg	conclusion protein / AB
...ESIK_rbt2	MVEVCVESIK	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f. (#)
...ESTR_rbt1	LLTPGFHYDVLKPYVAIFAESTR	173	60	21	—	22	16	99	88	101	88	12	49	40 µg / 5 µg *
...FSGR_rbt1	NEFSGR	28	9.4	7.8	8.7	11	9.1	17	2.0	15	0.5	2.8	1.2	20 µg / 2µg
	EALVDHAEAFSGR	5.9	5.1	9.9	6.7	7.1	9.8	1.6	4.7	2.6	2.3	6.3	3.6	
	EALVDLGEEFSGR	15	16	2.5	18	20	0.8	4.8	21	18	2.9	6.0	25	
	EALIDYGEEFSGR	6.1	5.2	13	2.7	6.5	7.9	6.3	3.2	2.7	8.7	3.9	2.5	
	EALDDLGEEFSGR	48	7.7	131	15	4.4	1.7	136	30	19	113	2.8	0.1	
	EALVDHGEEFSGR	64	6.2	10	92	6.6	10.9	1.6	53	27	2.3	2.5	1.8	
...FSGR_rbt2	NEFSGR	—	173	18	—	—	2.6	98	173	173	43	141	141	use other AB
	EALVDHAEAFSGR	15	8.7	5.8	16	4.5	0.1	2.8	3.7	12	1.9	3.1	1.3	
	EALVDLGEEFSGR	—	—	—	—	—	—	—	—	—	—	—	—	
	EALIDYGEEFSGR	119	103	49	77	141	5.8	87	23	107	0.6	24	141	
	EALDDLGEEFSGR	—	—	—	—	—	—	—	—	—	—	—	—	
	EALVDHGEEFSGR	101	22	129	141	25	3.8	139	11	87	119	13	8.3	
...FTNR_rbt1	ECYSVFTNR	4.4	14	3.8	6.1	11	1.6	9.8	2.5	11	14	1.3	11	use other AB
	ECYSTFTNR	1.5	7.5	5.2	0.8	3.2	4.9	8.9	4.0	2.3	12	0.9	2.6	
...FTNR_rbt2	ECYSVFTNR	5.1	4.3	3.3	4.8	1.6	4.1	2.1	3.1	0.8	1.0	3.3	0.9	10 µg / 1 µg
	ECYSTFTNR	5.3	5.6	1.9	7.3	6.9	1.1	3.6	2.3	2.8	4.9	2.2	3.6	
...FWLK_rbt1	HWFWLK	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f.
...FWLK_rbt2	HWFWLK	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f. (#)
...GGEK_rbt1	NLTLHVQGGEK	—	31	45	—	17	28	87	87	89	3.9	6.6	18	20 µg / 2 µg
...GSLR_rbt1	LTIIPQDPILFSGSLR	—	95	13	—	36	3.8	173	87	93	141	0.2	32	40 µg / 5 µg
	ITIIPQDPVLFSGSLR	—	—	—	—	—	—	—	—	—	—	—	—	
...GTVR_rbt1	LTIIPQEPVLFSGTVR	—	50	51	—	33	26	90	88	90	21	17	23	40µg / 5 µg *
...GTVR_rbt2	LTIIPQEPVLFSGTVR	—	—	58	—	—	29	173	173	173	141	141	141	use other AB
...IFFK_rbt1	IQGYFNAWQALLIKPNIFFK	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f. (#)
...IFFK_rbt2	IQGYFNAWQALLIKPNIFFK	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f. (#)
...KPHR_rbt1	DETVWEKPHR	87	173	51	141	—	25	19	89	173	27	141	141	10 µg / 1-2 µg 10-40 µg / 5 µg *
...KPHR_rbt2	DETVWEKPHR	173	173	59	—	—	65	17	173	173	23	141	141	use other AB
...LDDK_rbt1	DFNPQHFLDDK	3.8	5.5	3.1	3.6	7.5	2.5	5.1	5.6	5.9	3.4	0.2	5.2	10 µg / 1 µg
	DFDPQNFLDDK	2.0	2.9	0.5	0.5	3.6	0.2	6.1	5.0	3.7	3.4	0.7	2.8	
...LDDR_rbt1	EIDQVIGSHRPPSLDDR	3.4	7.5	2.4	4.8	3.8	3.2	5.3	1.6	8.5	7.3	0.4	0.3	10 µg / 1 µg
...LDDR_rbt2	EIDQVIGSHRPPSLDDR	2.5	3.1	16.6	3.5	3.9	18.4	2.2	0.9	17.9	3.0	1.2	21.0	use other AB
...LISK_rbt1	EAEYLISK	—	—	—	—	—	—	—	—	—	—	—	—	use other AB
	EANHLISK	87	87	12	141	141	12	1.7	1.1	173	2.4	0.1	141	
...LISK_rbt2	EAEYLISK	—	—	—	—	—	—	—	—	—	—	—	—	20 µg / 2 µg
	EANHLISK	173	4.3	3.7	141	5.9	2.5	87	3.0	87	3.6	3.2	5.1	
...LPNK_rbt2	LQDEIDAALPNK	11	15	7.4	0.5	3.9	1.4	9.7	3.0	2.6	13	4.2	1.7	20 µg / 1 µg
...LPNK_rbt3	LQDEIDAALPNK	32	99	56	12	75	71	22	38	122	29	56	130	use other AB
...LPSK_rbt1	LQEEIDGALPSK	—	—	—	—	—	—	—	—	—	—	—	—	use other AB
...LPSK_rbt2	LQEEIDGALPSK	—	—	—	—	—	—	—	—	—	—	—	—	40 µg / 5 µg *
...LSGK_rbt1	WLVLSGK	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f.

antibody	peptide	10-40 µg protein AB: 1 –	2 -	5µg	20-40 µg protein AB: 1 –	2 -	5µg	1-5 µg AB protein: 10 -	20 -40 µg		2-5 µg AB protein: 10 -	20 -40 µg		conclusion protein / AB
...LTTR_rbt2	NTTGSLTTR	—	—	—	—	—	—	—	—	—	—	—	—	20 µg / 2 µg
	NNPGVLTTR	8.6	8.5	3.7	11	2.2	5.1	6.7	3.1	3.4	9.3	1.3	1.6	
	NTTGALTTR	10	9.6	1.1	13	4.1	1.4	13	10	3.7	8.9	1.2	3.9	
...NGER_rbt1	EYGVIFANGER	2.4	3.6	1.4	3.2	0.1	1.5	4.9	5.6	2.3	3.6	2.7	1.1	10 µg / 1 µg
...NGER_rbt2	EYGVIFANGER	11	5.9	1.9	15	8.4	2.4	5.0	2.9	7.5	0.9	4.2	1.8	use other AB
...PFQR_rbt1	DYDEVTAFLGEWGPFQR	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f.
...PFQR_rbt2	DYDEVTAFLGEWGPFQR	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f.
...QDIR_rbt1	LYDPIEGEVSIDGQDIR	173	87	7.0	141	9.0	9.9	173	87	7.1	141	2.1	1.2	20 µg / 2 µg
...QPPR_rbt1	TWDPDQPPR	3.3	34	87	3.2	46	1.6	87	3.1	35	141	3.1	47	10 µg / 1 µg
...QPPR_rbt2	TWDPDQPPR	11	29	87	15	39	4.1	87	4.5	28	141	5.1	40	use other AB
...RPER_rbt1	DPFVFRPER	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f.
	DPQHWPEPEEFRPER	—	—	—	—	—	—	—	—	—	—	—	—	
	DPHYWPEPEEFRPER	—	—	—	—	—	—	—	—	—	—	—	—	
...RPER_rbt2	DPFVFRPER	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f. (#)
	DPQHWPEPEEFRPER	—	—	—	—	—	—	—	—	—	—	—	—	
	DPHYWPEPEEFRPER	—	—	—	—	—	—	—	—	—	—	—	—	
...SIGK_rbt1	SDAFVPFSIGK	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f.
...SIGK_rbt2	SDAFVPFSIGK	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f. (#)
...STGK_rbt2	NAAFLPFSTGK	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f.
...TNPR_rbt1	EHQESLDVTNPR	4.4	3.7	3.5	3.0	4.6	1.3	1.4	1.7	1.4	1.9	2.1	1.3	use other AB
	IEEHQESLDVTNPR	88	15	4.7	116	18	2.7	32	74	22	2.1	11	26	
...TNPR_rbt2	EHQESLDVTNPR	4.4	0.6	0.9	2.1	0.0	0.9	4.0	3.3	4.6	4.5	4.0	4.9	40 µg / 2 µg 10-40 µg / 5 µg
	IEEHQESLDVTNPR	28	7.2	0.7	32	5.6	0.5	12	13	12	9.3	4.4	1.7	
...TVEK_rbt1	SSISTVEK	20	6.3	4.7	28	0.6	5.3	19	9.0	31	13	5.4	11	20 µg / 2 µg
...VILR_rbt1	MSDSVILR	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f. (#)
...VILR_rbt2	MSDSVILR	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f.
...YQVR_rbt1	HGEIQFNNYQVR	48	50	36	30	32	26	5.6	14	18	0.3	13	19	10 µg / 1 µg
...YQVR_rbt2	HGEIQFNNYQVR	49	51	44	42	40	37	3.3	6.6	10	1.7	8.4	12	use other AB

Table 67: Adjustment of antibody and proteolyzed protein amount for mouse samples. Three different antibody and proteins amounts were tested whether they are sufficient for reproducible analyte quantification. For each antibody serum 1, 2 and 5 µg were tested with every protein amount. Results are given as % RSD. If not stated otherwise, the conclusion column gives the minimal amount of antibody and protein necessary, but more is also possible. Antibodies which did not enrich EN or IS peptide sufficiently for quantification were not used further (n.u.f.). Antibodies which lead to suitable IS signals and should be tested again when a sample containing this target is available are additionally indicated with (#). Decisions which were made with reservations are marked with *.

antibody	peptide	10-40 µg protein AB: 1 –	10-40 µg protein AB: 2 -	10-40 µg protein AB: 5µg	20-40 µg protein AB: 1 –	20-40 µg protein AB: 2 -	20-40 µg protein AB: 5µg	1-5 µg AB protein: 10 -	1-5 µg AB protein: 20 -	1-5 µg AB protein: 40 µg	2-5 µg AB protein: 10 -	2-5 µg AB protein: 20 -	2-5 µg AB protein: 40 µg	conclusion protein / AB
...ALEK_rbt1	AAATEDATPAALEK	15	4.9	3.2	7.7	4.6	1.6	18	7.9	5.6	17	11	7.7	20 µg / 2 µg
...AVDR_rbt1	SLSEASVAVDR	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f.
...AVDR_rbt2	SLSEASVAVDR	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f. (#)
...DLFR_rbt3	YGLSDLFR	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f. (#)
...DLVR_rbt1	EVLDSFLDLVR	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f. (#)
...DLVR_rbt2	EVLDSFLDLVR	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f.
...DLVR_rbt3	EVLDSFLDLVR	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f.
...DLVR_rbt4	EVLDSFLDLVR	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f.
...EATR_rbt1	FINLVPSNLPHEATR	159	30	3.9	141	37	4.4	83	100	39	5.3	47	15	10 µg / 5 µg
...EATR_rbt2	FINLVPSNLPHEATR	—	—	—	—	—	—	—	—	—	—	—	—	use other AB
...EAVK_rbt1	AYGPVFTLYLGSRPTVVLHGYEAVK	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f.
	AYGPVFTLYLGSKPTVILHGYEAVK	—	—	—	—	—	—	—	—	—	—	—	—	
	VYGPVFTLYFGSKPTVVVHGYEAVK	—	—	—	—	—	—	—	—	—	—	—	—	
	IVVLCGQEAVK	—	—	—	—	—	—	—	—	—	—	—	—	
...EAVK_rbt2	AYGPVFTLYLGSRPTVVLHGYEAVK	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f.
	AYGPVFTLYLGSKPTVILHGYEAVK	—	—	—	—	—	—	—	—	—	—	—	—	
	VYGPVFTLYFGSKPTVVVHGYEAVK	—	—	—	—	—	—	—	—	—	—	—	—	
	IVVLCGQEAVK	—	—	—	—	—	—	—	—	—	—	—	—	
...ESIK_rbt1	MVEVCVESIK	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f. (#)
...ESIK_rbt2	MVEVCVESIK	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f.
...FFFK_rbt2	FIGLQFFFK	—	173	6.8	—	141	7.1	173	173	104	141	141	54	20 µg / 2 µg
...FSGR_rbt1	NEFSGR	7.2	173	8.8	1.3	—	8.2	6.2	87	87	3.5	141	141	20 µg / 2 µg
	EALVDHAEAFSGR	5.6	2.7	1.9	5.4	1.7	2.6	6.7	5.0	4.5	8.7	6.8	6.0	
	EALVGQAEAFSGR	4.9	1.3	2.4	6.4	1.6	3.4	1.6	3.2	5.0	2.0	1.7	3.6	
	EALVDHAEEFSGR	11	3.8	2.1	15	4.0	0.3	3.9	0.8	11	2.0	0.8	4.5	
	EALDDLGEEFSGR	1.2	2.0	4.3	1.7	2.8	5.5	1.9	3.1	2.1	2.7	1.2	1.4	
	EALEDNAEEFSGR	—	—	173	—	—	141	—	—	173	—	—	141	
...FSGR_rbt2	NEFSGR	—	117	39	—	141	34	92	173	120	28	141	78	use other AB
	EALVDHAEAFSGR	173	11	1.7	141	15	2.3	87	38	87	1.7	8.5	8.8	
	EALVGQAEAFSGR	3.3	2.5	8.5	4.7	2.4	12	3.1	6.9	6.1	1.7	6.6	7.6	
	EALVDHAEEFSGR	—	20	5.2	—	24	7.0	87	87	90	1.2	5.8	23	
	EALDDLGEEFSGR	—	—	15	—	—	20	173	173	173	141	141	141	
	EALEDNAEEFSGR	—	—	—	—	—	—	—	—	—	—	—	—	
...FTNR_rbt1	ECYSVFTNR	91	18	3.7	141	25	3.3	9.6	17	89	6.5	9.7	19	use other AB
	ECYSTFTNR	27	7.5	11	39	9.5	12	8.9	6.0	28	7.7	6.1	15	
	DCLSVFTNR	—	—	—	—	—	—	—	—	—	—	—	—	

antibody	peptide	10-40 µg protein AB: 1 –	10-40 µg protein AB: 2 -	10-40 µg protein AB: 5µg	20-40 µg protein AB: 1 –	20-40 µg protein AB: 2 -	20-40 µg protein AB: 5µg	1-5 µg AB protein: 10 -	1-5 µg AB protein: 20 -	1-5 µg AB protein: 40 µg	2-5 µg AB protein: 10 -	2-5 µg AB protein: 20 -	2-5 µg AB protein: 40 µg	conclusion protein / AB
...FTNR_rbt2	ECYSVFTNR	48	16	6.3	62	20	8.9	22	39	17	3.2	5.5	24	10 µg / 5 µg
	ECYSTFTNR	14	5.7	6.3	17	7.3	8.1	13	12	24	1.3	6.1	9.3	
	DCLSVFTNR	—	—	—	—	—	—	—	—	—	—	—	—	
...FWLK_rbt1	HWFWLK	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f.
...FWLK_rbt2	HWFWLK	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f. (#)
...GDLK_rbt2	GIYDGDLK	2.8	3.4	15	1.2	2.2	10	7.8	2.5	6.2	9.3	1.3	6.8	10 µg / 1 µg
...GGEK_rbt1	NVTVHVQGGEK	20	25	6.4	7.4	1.3	8.1	42	0.8	7.9	35	0.5	9.9	20 µg / 2 µg
...GGEK_rbt2	NVTVHVQGGEK	32	11	6.1	3.0	14	8.2	38	16	4.1	1.9	6.7	0.5	use other AB
...GQVR_rbt1	VQQEIDEVIGQVR	18	16	3.9	21	9.8	3.3	2.3	15	14	2.4	14	8.0	10-20 µg / 2 µg
	VQQEIDAVIGQVR	173	17	4.4	—	24	5.9	7.1	87	90	10	3.7	22	
...GQVR_rbt2	VQQEIDEVIGQVR	—	—	—	—	—	—	—	—	—	—	—	—	use other AB
	VQQEIDAVIGQVR	—	—	—	—	—	—	—	—	—	—	—	—	
...GSLR_rbt1	ITIIPQDPVLFSGSLR	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f. (#)
...GTVR_rbt1	LAIIPQEPVLFSGTVR	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f. (#)
...GTVR_rbt2	LAIIPQEPVLFSGTVR	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f.
...GYYR_rbt1	LFIHEYISGYYR	89	87	8.2	20	11	10	173	22	28	141	0.5	0.3	use other AB
...GYYR_rbt2	LFIHEYISGYYR	15	5.0	6.1	—	4.9	6.3	7.8	4.6	0.0	8.8	1.4	0.0	10 µg / 1 µg
...IFFK_rbt1	IQGYFNAWQALLIKPNIFFK	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f.
...IFFK_rbt2	IQGYFNAWQALLIKPNIFFK	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f.
...LDDK_rbt1	DFNPQHFLDDK	89	4.9	3.3	141	3.6	4.6	12	87	1.2	3.2	1.6	0.7	10 µg / 2 µg
...LPNK_rbt2	LQDEIDEALPNK	6.2	8.9	18	0.1	13	19	4.7	5.0	19	4.6	6.6	25	10 µg / 1 µg
	LQDEIDAALPNK	6.0	9.2	5.7	0.9	13	0.1	5.2	4.9	6.1	7.2	6.9	6.1	
	LQEEIDETLPNK	173	173	173	141	141	141	—	—	11	—	—	8.7	
...LPNK_rbt3	LQDEIDEALPNK	—	25	14	—	4.6	19	91	89	87	27	19	4.4	use other AB
	LQDEIDAALPNK	—	146	87	—	122	141	91	160	173	25	127	141	
	LQEEIDETLPNK	—	173	130	—	—	100	156	173	173	122	141	141	
...LSGK_rbt1	WLVLSGK	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f.
...LTTR_rbt2	NTTGALTTR	87	4.3	87	2.9	2.6	7.0	173	6.2	7.4	141	3.9	5.7	10 µg / 1 µg
	NNPGVLTTR	3.2	2.9	3.3	4.5	3.5	1.2	1.2	4.8	2.6	0.3	3.0	0.7	
...NFSK_rbt1	NFNQSLTNFSK	105	91	88	55	27	16	—	10	62	—	7.0	49	40 µg / 2 µg
	NVSQSLTNFSK	—	—	—	—	—	—	—	—	—	—	—	—	
	NISQSFTNFSK	11	1.9	2.1	15	0.8	1.9	4.8	1.1	9.8	0.7	1.0	0.1	
...NFSK_rbt2	NFNQSLTNFSK	91	39	92	141	51	141	22	173	41	29	141	49	use other AB
	NVSQSLTNFSK	—	—	—	—	—	—	—	—	—	—	—	—	
	NISQSFTNFSK	7.5	2.6	5.8	4.9	3.6	8.1	2.0	5.9	8.6	1.8	7.5	4.3	
...NGER_rbt1	EYGVIFANGER	9.6	7.1	7.5	14	4.8	10	8.1	3.6	6.7	2.5	4.4	1.0	40 µg / 5 µg
	GYGVAFSNGER	98	43	2.5	57	50	0.9	8.8	138	124	5.6	52	1.9	
	GYGVTFSNGER	—	—	—	—	—	—	—	—	—	—	—	—	
...NGER_rbt2	EYGVIFANGER	—	107	70	—	59	90	173	120	98	141	78	43	use other AB
	GYGVAFSNGER	—	—	—	—	—	—	—	—	—	—	—	—	
	GYGVTFSNGER	—	—	—	—	—	—	—	—	—	—	—	—	
...PFQR_rbt1	DYDEVTAFLGEWGPFQR	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f.
...PFQR_rbt2	DYDEVTAFLGEWGPFQR	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f.
...QDIR_rbt1	LYDPLEGVVSIDGQDIR	—	—	173	—	—	141	—	—	173	—	—	141	n.u.f. (#)
...QDIR_rbt2	LYDPLEGVVSIDGQDIR	—	—	173	—	—	141	—	—	173	—	—	141	n.u.f.

antibody	peptide	10-40 µg protein AB: 1 –	2 -	5µg	20-40 µg protein AB: 1 –	2 -	5µg	1-5 µg AB protein: 10 -	20 -	40 µg	2-5 µg AB protein: 10 -	20 -	40 µg	conclusion protein / AB
...QLLK_rbt1	LAHDILLFLNPQLLK	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f.
...QLLK_rbt2	LAHDILLFLNPQLLK	73	173	173	95	141	141	173	107	105	—	141	141	n.u.f. (#)
...QLLK_rbt4	LAHDILLFLNPQLLK	—	173	—	—	141	—	—	173	—	—	141	—	n.u.f.
...QPPR_rbt1	NTWDPDQPPR	4.8	9.7	7.8	1.4	5.1	9.8	4.7	3.7	4.2	6.2	2.4	2.4	10-20 µg / 5 µg
	TTWDPDQPPR	—	—	—	—	—	—	—	—	—	—	—	—	
	TWDPDQPPR	56	23	23	65	5.0	32	10	7.3	62	14	8.0	30	
	TTWDPTQPPR	—	173	119	—	—	141	111	—	173	66	—	141	
...QPPR_rbt2	NTWDPDQPPR	—	—	30	—	—	20	173	173	173	141	141	141	use other AB
	TTWDPDQPPR	—	—	—	—	—	—	—	—	—	—	—	—	
	TWDPDQPPR	—	—	—	—	—	—	—	—	—	—	—	—	
	TTWDPTQPPR	121	87	4.2	107	141	4.6	25	11	136	10	1.2	141	
...RPER_rbt1	ELWGDPNEFRPER	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f.
	DPFVFRPER	98	93	62	44	31	79	173	64	40	141	53	1.7	
	YWPEPEEFRPER	—	173	57	—	141	59	173	87	173	141	4.1	141	
...RPER_rbt2	ELWGDPNEFRPER	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f. (#)
	DPFVFRPER	—	145	121	—	109	62	173	87	109	141	12	62	
	YWPEPEEFRPER	—	173	87	—	141	141	173	92	—	141	30	—	
...SIGK_rbt1	SDAFVPFSIGK	—	—	—	—	—	—	—	—	—	—	—	—	use other AB
	NDAFVPFSIGK	—	—	—	—	—	—	—	—	—	—	—	—	
...SIGK_rbt2	SDAFVPFSIGK	173	57	87	141	27	6.9	173	108	107	141	84	59	40 µg / 5 µg
	NDAFVPFSIGK	—	8.7	16.8	—	5.8	12	87	87	87	10	5.1	1.3	
...SLNK_rbt2	QPLESSDLWSLNK	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f. (#)
...SLNK_rbt4	QPLESSDLWSLNK	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f. (#)
...STGK_rbt2	SDYFIPFSTGK	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f.
...TNPR_rbt1	ESLDVTNPR	1.5	9.0	2.3	0.0	11	3.0	6.4	6.7	11	2.1	0.8	14	use other AB
...TNPR_rbt2	ESLDVTNPR	6.7	1.6	2.4	2.0	0.2	1.9	4.7	1.5	0.4	0.5	2.0	0.1	10 µg / 2 µg
...TVEK_rbt1	SSISTVEK	28	9.1	6.2	10	11	8.1	7.7	25	26	11	0.9	3.7	20 µg / 5 µg
...TVEK_rbt2	SSISTVEK	—	—	—	—	—	—	—	—	—	—	—	—	use other AB
...VILR_rbt1	MSDSVILR	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f. (#)
...VILR_rbt2	MSDSVILR	—	—	—	—	—	—	—	—	—	—	—	—	n.u.f. (#)
...YQVR_rbt1	GEIQFNNYQVR	1.2	2.8	5.4	1.7	3.4	0.7	5.2	2.6	3.0	7.3	0.2	2.6	10 µg / 1 µg
...YQVR_rbt2	GEIQFNNYQVR	3.1	3.9	11	2.7	0.5	6.2	4.1	1.9	7.8	5.7	0.2	5.5	use other AB

Table 68: Results of peptides with N-terminal missed cleavage in human. During the proteolysis kinetics, proteotypic peptides were quantified at each time point. At the same time, the according peptides with one N-terminal missed cleavage site were monitored. Sequences of the missed cleavage variants and the results of monitoring them are given.

proteotypic peptide		peptide with one N-terminal missed cleavage site	peptide detected
SLSEASV	AVDR	VTPFSVKSLSEASVAVDR	no
RPSYL	DLFR	VVRRPSYLDLFR	no
YTAS	DLFR	AKYTASDLFR	no
LSPSFA	DLFR	MLSLEEDVTEKLSPSFADLFR	no
LNPSFL	DLVR	KLNPSFLDLVR	no
FIGLQ	FFFK	NRFIGLQFFFK	no
GIYD	GDLK	GDMNLSIVMTTCSTFCALGMMPLLLYIYSRGIYDGDLK	too long
DLSLHVH	GGEK	NYSVRYRPGLDLVLRDLSLHVHGGEK	no
TVAAF	GGEK	AGVVADEVISSMRTVAAFGGEK	no
LTIIPQDPILFS	GSLR	EKLTIIPQDPILFSGSLR	no
ITIIPQDPVLFS	GSLR	FKITIIPQDPVLFSGSLR	no
LSIIPQEPVLFS	GTVR	SKLSIIPQEPVLFSGTVR	no
LFIHEYIS	GYYR	KLFIHEYISGYYR	no
GGPEAT	LEVR	EAPEEPAPVRGGPEATLEVR	no
TLGGILAPIYFGA	LIDK	ALAMGFQSMVIRTLGGILAPIYFGALIDK	too long
EANIHAFIES	LPNK	AAKEANIHAFIESLPNK	no
NSPGA	LTTR	AMLGQDIAWFDDLRNSPGALTTR	no
NTTGA	LTTR	QDVSWFDDPKNTTGALTTR	no
YVEQQYGQ	PSSK	SILTNPLYVMFVLLTLLQVSSYIGAFTYVFKYVEQQYGQPSSK	too long
NKPLFDTI	QDEK	AMGINATFANIAGALAPLMMILSVYSPPLPWIIYGVFPFISGFAFLLLPETRNKPLFDTIQDEK	too long
LYDPTEGMVSVDG	QDIR	STTVQLMQRLYDPTEGMVSVDGQDIR	no
SSIS	TVEK	LFVLCHSLLQLAQLMISGYLKSSISTVEK	too long
IQFNN	YQVR	GKIQFNNYQVR	no

Table 69: Results of peptides with N-terminal missed cleavage in rat. During the proteolysis kinetics, proteotypic peptides were quantified at each time point. At the same time, the according peptides with one N-terminal missed cleavage site were monitored. Sequences of the missed cleavage variants and the results of monitoring them are given.

peptide		peptide with N-terminal missed cleavage	peptide detected
ANIYGLCFAFSQGIAFLANS	AAYR	KANIYGLCFAFSQGIAFLANSAAYR	no
AAATEDATPA	ALEK	ITYKAAATEDATPAALEK	no
SLSEASV	AVDR	VTPFSVKSLSEASVAVDR	no
LGSILSPYFVYLG	AYDR	NMGVGVSSTASRLGSILSPYFVYLGAYDR	no
GDFVAVFPPMIHNDPEVF	DAPK	KGDFVAVFPPMIHNDPEVFDAPK	no
ALQRPSYL	DLFR	PVGEGSLSQEALNNVVTMERALQRPSYLDLFR	too long
SPSFA	DLFR	RSPSFADLFR	after 2h and 6h
YGLS	DLFR	VKYGLSDLFR	no
LNPSFL	DLVR	KLNPSFLDLVR	no
FINLVPSNLPH	EATR	LDMPYMDAVVHEIQRFINLVPSNLPHEATR	too long
AMDSFPGPPTHWLFGHAL	EIQK	LARAMDSFPGPPTHWLFGHALEIQK	no
MVEVCV	ESIK	ALTGPGLIRMVEVCVESIK	no
LLTPGFHYDVLKPYVAIFA	ESTR	KLLTPGFHYDVLKPYVAIFAESTR	no
LSDRPQLPYLEAFIL	ETFR	QPRLSDRPQLPYLEAFILETFR	not determined
EQLDSLVCLESAIL	EVLR	GPGISVHFTREQLDSLVCLESAILEVLR	no
YTDHF	FAFK	QVQTFLGYTDAGAQFVFGEKYTDHFFAFK	too long
FIGLQ	FFFK	NRFIGLQFFFK	no
NE	FSGR	EVLLNHKNEFSGR	yes
EALVDHAEA	FSGR	HGDVFTVYFGPRPVVMLCGTQTIREALVDHAEAFSGR	too long
EALDDLGEE	FSGR	IYGPVFTLYFGPKPTVVVHGYEAVKEALDDLGEEFSGR	too long
EALVDLGEE	FSGR	VYGPIFTLYLGMKPFVVLHGYEAVKEALVDLGEEFSG	too long
EALIDYGEE	FSGR	TYGPVFTLYFGSQPTVVLHGYEAVKEALIDYGEEFSGR	too long
EALVDHGEE	FSGR	TYGPVYTLYVGSQPTVVLHGYEALKEALVDHGEEFSGR	too long
ECYSV	FTNR	MVLVKECYSVFTNR	no
ECYST	FTNR	TVLVKECYSTFTNR	no
HW	FWLK	LLERHWFWLK	no
GIYD	GDLK	GDMNLSIVMTTCSSFSALGMMPLLLYVYSKGIYDGDLK	too long
NLTLHVQ	GGEK	PGLELVLKNLTLHVQGGEK	no
LTIIPQDPILFS	GSLR	ERLTIIPQDPILFSGSLR	no
ITIIPQDPVLFS	GSLR	FKITIIPQDPVLFSGSLR	no
LTIIPQEPVLFS	GTVR	SKLTIIPQEPVLFSGTVR	no

peptide		peptide with N-terminal missed cleavage	peptide detected
LFIHEYIS	GYYR	KLFIHEYISGYYR	no
IQGYFNAWQALLIKPN	IFFK	KIQGYFNAWQALLIKPNIFFK	no
DETVWE	KPHR	GTTLIINLSSVLKDETVWEKPHR	in 2 replicates
DFDPQNF	LDDK	FFPSPKDFDPQNFLDDK	no
DFNPQHF	LDDK	FFPNHKDFNPQHFLDDK	no
EIDQVIGSHRPPS	LDDR	VQKEIDQVIGSHRPPSLDDR	no
EAEY	LISK	SFSIASDPTLASSCYLEEHVSKEAEYLI SK	too long
EANH	LISK	SFSIASDPTSVSSCYLEEHVSKEANHL ISK	too long
LQDEIDAA	LPNK	KLQDEIDAALPNK	in 1 replicate
LQEEIDGA	LPSK	KLQEEIDGALPSK	no
WLV	LSGK	WLQLTSSAPFFIFSLLSWWVPESIRW LVLSGK	too long
DETVWE	KPHR	GTTLIINLSSVLKDETVWEKPHR	in 2 replicates
NTTGS	LTTR	QDISWFDDHKNTTGSLTTR	no
NNPGV	LTTR	AMLGQDIGWFDDLRNNPGVLTTR	no
NTTGA	LTTR	QDISWFDDPKNTTGALTTR	no
EYGVIFA	NGER	GTIAVIEPIFKEYGVIFANGER	no
AAL	QDEK	KAALQDEK	between 2 – 16h
LYDPIEGEVSIDG	QDIR	STTVQLLQRLYDPIEGEVSIDGQDIR	too long
LIHDLLVFLNP	QLLK	SFILKLIHDLLVFLNPQLLK	no
TWDPD	QPPR	KTWDPDQPPR	in 1 replicate
DPFVF	RPER	QWKDPFVFRPER	no
SDAFVPF	SIGK	KSDAFVPFSIGK	no
SSDLW	SLNK	QPLKSSDLWSLNK	no
EHQESLDV	TNPR	IKEHQESLDVTNPR	yes
IEEHQESLDV	TNPR	KIEEHQESLDVTNPR	yes
SSIS	TVEK	FFVLCHSILQLAQLMISGYLKSSISTV EK	too long
MSDS	VILR	N-terminus	N-terminus
HGEIQFNN	YQVR	RPPADWPRHGEIQFNNYQVR	no

Table 70: Results of peptides with N-terminal missed cleavage in mouse. During the proteolysis kinetics, proteotypic peptides were quantified at each time point. At the same time, the according peptides with one N-terminal missed cleavage site were monitored. Sequences of the missed cleavage variants and the results of monitoring them are given.

peptide		peptide with N-terminal missed cleavage	peptide detected
AAATEDATPA	ALEK	ITYKAAATEDATPAALEK	1 replicate
SLSEASV	AVDR	VTPFSVKSLSEASVAVDR	no
YGLS	DLFR	VKYGLSDLFR	no
FINLVPSNLPH	EATR	MNMPYMDAVVHEIQRFINLVPSNLPHEATR	too long
MVEVCV	ESIK	ALTGPGLVRMVEVCVESIK	no
FIGLQ	FFFK	NRFIGLQFFFK	no
NE	FSGR	EVLLNHKNEFSGR	in 2 replicates
EALVDHAEA	FSGR	HGDVFTVHLGPRPVVVLCGTQTIREALVDHAEAFSGR	too long
EALVGQAEA	FSGR	YGDVFTVHLGPRPVVMLCGTDTIREALVGQAEAFSGR	too long
EALVDHAEE	FSGR	VVVLYGYDAVKEALVDHAEEFSGR	yes
EALDDLGEE	FSGR	VYGPVFTLYFGSKPTVVVHGYEAVKEALDDLGEEFSGR	too long
EALEDNAEE	FSGR	VVVLYGYDAVKEALEDNAEEFSGR	no
ECYSV	FTNR	IVLVKECYSVFTNR	no
ECYST	FTNR	TVLVKECYSTFTNR	yes
DCLSV	FTNR	NVLVKDCLSVFTNR	no
HW	FWLK	LLDRHWFWLK	no
GIYD	GDLK	GDMNLSIVMTTCSSFTALGMMPLLLYIYSKGIYDGDLK	too long
NVTVHVQ	GGEK	YRPGLELVLKNVTVHVQGGEK	no
VQQEIDEVI	GQVR	RVQQEIDEVIGQVR	no
VQQEIDAVI	GQVR	RVQQEIDAVIGQVR	no
ITIIPQDPVLFS	GSLR	FKITIIPQDPVLFSGSLR	no
LAIIPQEPVLFS	GTVR	SKLAIIPQEPVLFSGTVR	no
LFIHEYIS	GYYR	KLFIHEYISGYYR	no
IQGYFNAWQALLIKPN	IFFK	KIQGYFNAWQALLIKPNIFFK	no
DFNPQHF	LDDK	FFSSPKDFNPQHFLDDK	no
LQDEIDEA	LPNK	KLQDEIDEALPNK	yes
LQDEIDAA	LPNK	KLQDEIDAALPNK	yes
LQEEIDET	LPNK	KLQEEIDETLPNK	no
WLV	LSGK	WLQLSVSAAFFIFSLLSWWVPESIRWLVLSGK	no
NNPGV	LTTR	QDIGWFDDLKNNPGVLTTR	after 2 and 6h
NTTGA	LTTR	QDVSWFDDPKNTTGALTTR	no
EVLDSFLD	LVR	SAPTKEVLDSFLDLVR	no
NFNQSLT	NFSK	LPPGPTPFPIIGNFLQIDVKNFNQSLTNFSK	too long
NVSQSLT	NFSK	GSLPPGPTPFPIIGNFLQIDIKNVSQSLTNFSK	too long
NISQSFT	NFSK	LPPGPTPLPIIGNFLQIDVKNISQSFTNFSK	too long

peptide		peptide with N-terminal missed cleavage	peptide detected
EYGVIFA	NGER	GTVAVVEPTFKEYGVIFANGER	yes
GYGVAFS	NGER	GEQATFNTLFKGYGVAFSNGER	no
GYGVTFS	NGER	GEQATFNTLFKGYGVTFSNGER	no
LYDPLEGVVSIDG	QDIR	STTVQLMQRLYDPLEGVVSIDGQDIR	no
LAHDILLFLNP	QLLK	SFILKLAHDILLFLNPQLLK	no
NTWDPD	QPPR	SLLAIVENLLTENRNTWDPDQPPR	no
TTWDPD	QPPR	SFMAILDNLLTENRTTWDPDQPPR	no
TWDPD	QPPR	RTWDPDQPPR	no
TTWDPT	QPPR	AFVTMLDELLAEHKTTWDPTQPPR	no
SDAFVPF	SIGK	KSDAFVPFSIGK	no
NDAFVPF	SIGK	KNDAFVPFSIGK	yes
QPLESSDLW	SLNK	ITFWWITGMMVHGYRQPLESSDLWSLNK	no
SDYFIPF	STGK	KSDYFIPFSTGK	no
ESLDV	TNPR	EHKESLDVTNPR	no
SSIS	TVEK	FFVLCHSLLQLTQLMISGYLKSSISTVEK	too long
MSDS	VILR	SDSVILR	yes
GEIQFNN	YQVR	KGEIQFNNYQVR	yes

Table 71: Sites of natural sequence variants and posttranslational modification. The peptides are listed in the order of the multiplexes. Sites of natural sequence variants and posttranslational modification listed by UniProtKB (December 2016) are given.

	peptide	species	natural sequence variants	sites of posttranslational modification
MPh1	GIYDGDLK	human	no	no
	NKPLFDTIQDEK	human	N --> K in cancer	no
	YTASDLFR	human	no	no
	EANIHAFIESLPNK	human	no	no
	RPSYLDLFR	human	no	no
MPh2	YVEQQYGQPSSK	human	no	no
	ITIIPQDPVLFSGSLR	human	no	no
	LTIIPQDPILFSGSLR	human	Q --> R and R missing in DJS	no
MPh3	SSISTVEK	human	E --> K in breast cancer samples	no
	NTTGALTTR	human	no	no
	NSPGALTTR	human	R --> Q in cholestasis	no
	IQFNNYQVR	human	no	no
SPh1	DLSLHVHGGEK	human	no	no
	TVAAFGGEK	human	E--> G	no
MPr1	NLTLHVQGGEK	rat	no	no
	GIYDGDLK	rat	no	no
	EYGVIFANGER	rat	no	no
	LYDPIEGEVSIDGQDIR	rat	no	no
MPr2	NTTGSLTTR	rat	no	no
	NTTGALTTR	rat	no	no
	NNPGVLTTR	rat	no	no
	LTIIPQEPVLFSGTVR	rat	no	no
MPr3	AAATEDATPAALEK	rat	no	T phosphorylation
	ALQRPSYLDLFR	rat	no	no
	YGLSDLFR	rat	no	no
MPr4	EANHLISK	rat	no	no
	EAEYLISK	rat	no	no
	EIDQVIGSHRPPSLDDR	rat	no	no
	DFNPQHFLDDK	rat	no	no
	LYDPIEGEVSIDGQDIR	rat	no	no
	DFDPQNFLDDK	rat	no	no
MPr5	EALVDHGEEFSGR	rat	no	no
	EALVDHAEAFSGR	rat	no	no
	FINLVPSNLPHEATR	rat	no	no
	EALDDLGEEFSGR	rat	no	no

	peptide	species	natural sequence variants	sites of posttranslational modification
	EALIDYGEEFSGR	rat	no	no
	EALVDLGEEFSGR	rat	no	no
MPr6	ECYSTFTNR	rat	no	no
	TWDPDQPPR	rat	no	no
	HGEIQFNNYQVR	rat	no	no
	ECYSVFTNR	rat	no	no
	LQDEIDAALPNK	rat	no	no
MPr7	LQEEIDGALPSK	rat	no	no
	AMDSFPGPPTHWLFGHALEIQK	rat	no	no
SPr1	ITIIPQDPVLFSGSLR	rat	no	no
	LTIIPQDPILFSGSLR	rat	no	no
MPm1	NVTVHVQGGEK	mouse	no	no
	NTTGALTTR	mouse	no	no
	NNPGVLTTR	mouse	no	no
	AAATEDATPAALEK	mouse	no	T phosphorylation
MPm2	SSISTVEK	mouse	no	no
	GIYDGDLK	mouse	no	no
MPm3	TWDPDQPPR	mouse	no	no
	NTWDPDQPPR	mouse	no	no
	TTWDPTQPPR	mouse	no	no
	VQQEIDEVIGQVR	mouse	no	no
	VQQEIDAVIGQVR	mouse	no	no
MPm4	NVSQSLTNFSK	mouse	no	no
	NFNQSLTNFSK	mouse	no	no
	NISQSFTNFSK	mouse	no	no
	FINLVPSNLPHEATR	mouse	no	no
MPm5	ESLDVTNPR	mouse	no	no
	EALVDHAEAFSGR	mouse	no	no
	EALVGQAEAFSGR	mouse	no	no
	DFNPQHFLDDK	mouse	no	no
	EALDDLGEEFSGR	mouse	no	no
SPm1	GYGVAFSNGER	mouse	no	no
	GYGVTFSNGER	mouse	no	no
SPm2	ECYSVFTNR	mouse	no	no
	DCLSVFTNR	mouse	no	no