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Title Page

Title: Enrichment-free high throughput LC-MRM quantification of cytochrome P450 proteins in plated human hepatocytes direct from 96-well plates enables routine protein induction measurements

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Running Title: Enrichment-free CYP protein quantification by LC-MRM

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List of abbreviations used: ABC, ammonium bicarbonate; CYP, Cytochrome P450; MRM, Multiple Reaction Monitoring; CITCO, 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime; PXR, pregnane X receptor; CAR, constitutive androstane receptor; hPXR, human pregnane X receptor; AhR, aryl hydrocarbon receptor

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Abstract

Despite the availability of LC-MS methods for quantifying cytochrome P450 (CYP) proteins, incorporation of CYP protein quantification into induction study workflows has not been widely adopted. To more readily enable CYP protein quantification in induction study workflows, DMPK research groups need a simple, robust, cost-effective, high-throughput method compatible with 96-well plated human hepatocyte formats. Here, we provide such a methodology. Our method bypasses both microsomal enrichment and antibody-based enrichment to go directly from the plate to LC-MS/MS analysis. We use this “plate-to-peaks” approach for quantifying CYP3A4, CYP2B6, and CYP1A2 – the major inducible hepatic CYPs representative of PXR, CAR and AhR mediated induction, respectively. We leveraged our induction study-aligned assay format to assess induction across mRNA, protein, and enzyme activity using known induction control compounds. As expected, results from the 3 methods using model inducers were broadly concordant, but the magnitude of the induction response differed. Induction of CYP3A4 using 10 μ M rifampicin was 12-fold for RNA, 8-fold for protein, and 3-fold for activity; for CYP1A2 with 50 μ M omeprazole induction was 30-fold for RNA, 13-fold for protein, and 17-fold for activity; for CYP2B6 with 50 μ M phenytoin induction was 23-fold for RNA, 2-fold for protein, and 5-fold for activity. Most importantly, we anticipate the relative ease of this method will enable researchers to routinely adopt CYP protein quantification as part of non-clinical evaluation of CYP induction.

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Significance Statement:

Current methodologies for quantifying CYP proteins by LC-MS/MS are either cumbersome, too costly, or both, to be widely adopted into induction study workflows by the ADME research community. We present a simplified LC-MS/MS methodology for quantifying CYP proteins directly from human hepatocytes, without any form of enrichment, in 96-well induction assay plate format that should be readily adoptable by any ADME lab with LC-MRM capabilities.

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Introduction

Drug-induced changes in cytochrome P450 expression levels can alter elimination rates of drugs that are P450 substrates. Both suppression and induction of P450 proteins may occur, but the latter is more commonly observed. Strong inducers of CYP3A4 include rifampicin, enzalutamide and ivosidenib (Wochenschr 1973, Gibbons, de Vries et al. 2015, Anonymous 2018). Narrow therapeutic index drugs that are sensitive substrates of the induced P450 isoforms may lose efficacy and can therefore be contraindicated when given with inducing agents.

Plated human hepatocytes have long been used as an *in vitro* model to evaluate CYP induction. Early versions of the assay involved measuring increases in probe substrate turnover for catalytic activity or higher levels of immunoreactive protein following a 2-3-day exposure to a test compound (Pichard, Fabre et al. 1990). Over time, it became apparent that enzyme activity assays could be misleading in the event that the test compound was an inhibitor of the induced enzyme, as it could mask the induction response. While presence of masking could be discerned by orthogonal Western blot analysis of the target protein, this technique is time-consuming and generally regarded as semi-quantitative.

In the past decade, mRNA quantification by qPCR has emerged as the preferred method to evaluate *in vitro* induction as the measured response is unaffected by enzyme inhibition and is generally regarded to have better analytical sensitivity (Fahmi, Kish et al. 2010, FDA 2017). However, mRNA remains a surrogate measurement for the induced protein. In addition, inherent assumptions are that increases in mRNA directly correlate with an increase in functional enzyme and that processes affecting enzyme expression levels occurring post-transcription (e.g. protein stabilization, alterations in translation efficiency, rates of protein degradation) are absent.

In recent years, proteomic quantification by LC-MS/MS has gained wide popularity, including as part of CYP induction testing (MacLean, Weiss et al. 2017). A key need for

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proteomic analysis in hepatocyte CYP induction studies is the measurement of an accurate baseline to serve as the denominator of a fold-induction calculation. To date, successful evaluations of induction by proteomic quantification has required a CYP protein enrichment step, typically by preparation of microsomes. While most labs pursuing this strategy have invoked ultracentrifugation methods to prepare microsomes (Jenkins, Kitteringham et al. 2006, Langenfeld, Zanger et al. 2009, Kawakami, Ohtsuki et al. 2011, Sakamoto, Matsumaru et al. 2011, Williamson, Purkayastha et al. 2011), Xu et al. demonstrated LC-MS/MS quantification of CYP3A4 from hepatocyte lysate using CaCl_2 /methanol-based microsomal enrichment (Xu, Ma et al. 2014). In 2015, Weiss et al. circumvented the need for microsomal preparation by utilizing peptide group antibodies to enrich digested peptides for LC-MS/MS quantification of CYP3A subfamily member proteins from human hepatocytes (Weiss, Schnabel et al. 2015). This technology was expanded to also quantify CYP1A2, CYP2B6, and CYP2C8 (MacLean, Weiss et al. 2017) and then again to include 14 CYP proteins in liver tissue and cultured hepatocytes in an induction experiment (Weiss, Hammer et al. 2018). While peptide group antibody-mediated enrichment is an innovative solution to avoid tedious microsomal enrichment, the dependence on antibodies, their associated cost and time to produce, and the complex nature of antibody-mediated enrichment sample preparation may be prohibitive for most researchers.

We report a novel sample preparation strategy for LC-MS/MS quantification of CYP proteins in plated human hepatocytes that avoids enrichment steps. The key elements of the process are minimizing sample handling, avoiding transfer steps and eliminating the use of detergents. To quantify protein, we simply replace the culture media with a solution of ammonium bicarbonate, reducing agent (DTT), and the alkylating agent iodoacetamide. We then heat the cells to 90°C, cool, add trypsin, digest for 3 h, quench the reaction, and inject the supernatant for LC-MS/MS. We applied this new approach (which we term “plate-to-peaks” owing to its direct culture plate-to-LC-MS/MS peaks) to protein quantification of CYP3A4, CYP1A2, and CYP2B6 in hepatocyte induction studies. In this manuscript, we compare

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induction response from several model inducing agents across 3 measurements: LC-MS/MS quantified protein, mRNA and enzyme endpoints. In addition, we highlight two options for easing the adoption of protein quantification into routine DMPK lab drug-drug interaction (or other) assay formats, and also assess the protein levels of CYP3A4 upon treatment with AZD1208, a compound where *in vitro* induction assay results using conventional mRNA/enzyme activity assays and cut-offs largely failed to predict *in vivo* induction response (Jones, Rollison et al. 2017).

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Materials and Methods

Cell Culture and Drug Treatment

Cryopreserved primary human hepatocytes (donor ACB, sex: male) were purchased from BioIVT, USA. Cells were thawed and plated according to the vendor's protocol. Hepatocytes were thawed in a 37°C water bath and decanted into pre-warmed 5 mL/vial InVitroGro CP medium (BioIVT). Cells were plated at a density of ~55,000 viable cells per well on collagen-coated 96 well culture plates. Cells were then incubated in a 5% CO₂ incubator, and the plating medium was replaced by incubation Medium (Williams' Medium E (1x, no phenol red)) containing Hepatocyte Maintenance Supplements (Serum-free, ThermoFisher) and GelTrex (ThermoFisher) 0.35 mg/mL after 4-6 hours.

After overnight stabilization, hepatocytes were treated with solvent vehicle (0.1% DMSO) and test compounds at various concentrations with 0.1% DMSO in incubation medium for 2 days (nominal 48 hours of treatment) with media change and replenishment after 1 day. Rifampin (µM), phenytoin (0.1-200 µM) and omeprazole (0.1-200 µM), CITCO (0.1 µM), probenecid (10 µM) were purchase from Sigma-Aldrich.

RNA Isolation

The RNA was isolated using MagMAX™ Express 96 RNA Isolation System from ThermoFisher with RNA extraction kits (ThermoFisher). Briefly, hepatocytes were washed once with 1X PBS. 140 µL of RNA lysis buffer was added to each well and mixed with the samples. The lysate was transferred to a well of the 96-well binding plate and samples were mixed with 20 µL of a solution containing magnetic beads. The RNA was bound to beads and the beads were captured on a 96-well magnetic tip manifold. The RNA sample beads were washed with wash solution. Then the samples were treated with TURBO™ DNase. The RNA was re-bound with beads after DNase treatment and washed twice with buffer. The beads were dried and RNA was eluted with 50 µL elution buffer.

cDNA Synthesis and RT-PCR

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cDNA synthesis followed ThermoFisher's protocol for SuperScript VILO Master Mix (ThermoFisher). Briefly, 4 μ L of SuperScript VILO Master Mix was mixed with 16 μ L of RNA followed by incubation: 25°C for 10 minutes; 42 °C for 60 minutes and terminated at 85°C for 5 minutes on a thermocycler.

RT-PCR was performed on the ABI QuantStudio, with the following parameters: 2.5 μ L of each cDNA sample was pipetted into a 96-well optical reaction plate (ThermoFisher/Applied Biosystems). Reagent mix is made using the TaqMan Fast Advanced Master Mix RT-PCR kit (ThermoFisher/Applied Biosystems). 17.5 μ L of each reagent mix is added to each well with 40 cycles of RT-PCR using the primer and probes from ThermoFisher listed below:

Human CYP1A2: TaqMan® Gene Expression Assay: ThermoFisher Assay ID: Hs00167927_m1

Human CYP2B6: TaqMan® Gene Expression Assay: ThermoFisher Assay ID: Hs03044634_m1

Human CYP3A4: TaqMan® Gene Expression Assay: ThermoFisher Assay ID: Hs00604506_m1

GAPDH (control to account for any variability in RNA levels): TaqMan® Gene Expression Assay: ThermoFisher Assay ID: Hs02758991_g1

CYP Enzyme Activity Assay

After 2-day treatment with test compounds (with media change and replenishment after 1 day), the hepatocytes were washed with incubation medium, then incubated with probe substrates as follows: 100 μ M phenacetin for 60 minutes, 250 μ M bupropion for 30 minutes, and 30 μ M midazolam (Sigma) for 30 minutes. The enzyme activity was determined by the production of the appropriate metabolites, acetamidophenol for CYP1A2, hydroxybupropion for CYP2B6, and 1'-hydroxymidazolam for CYP3A4 with stable labeled internal standards (all from Corning Life Sciences, Tewksbury, MA), ¹⁵N-acetaminophen, d6-hydroxy bupropion, and ¹³C₃-1'-hydroxy midazolam for CYP3A4 using area-ratio method by LCMS.

For LCMS, samples were quenched with at least two volume equivalents of organic solvent containing internal standard, followed by centrifugation and injection of the supernatant

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for LC-MS/MS. Sciex Analyst™ 1.5 (or higher) software was used to determine peak areas for the compounds of interest. Additional LCMS method details can be found in Table 1.

Data Analysis (mRNA and Enzyme Activity)

The fold induction in CYP isoform mRNA caused by the test compounds was determined using quantitative real-time polymerase chain reaction (RT-PCR) and the $\Delta\Delta CT$ method with the equation: Fold change = $2^{\Delta\Delta CT}$, where ΔCT is the difference in threshold cycle between the target and reference genes and $\Delta\Delta CT = \Delta CT(\text{treated sample}) - \Delta CT(\text{vehicle})$. The fold induction in enzyme activities is determined by the metabolite production with the testing compound compared to treatment by the vehicle.

LC-MRM Method Development

LC-MRM was performed on an AB SCIEX QTRAP® 6500 coupled to an Agilent Technologies 1290 Infinity pump and Gerstel MultiPurpose Sampler MPS 3C autosampler. The LC column used was an Acquity UPLC® CSH™ C18 1.7 μm 2.1 x 150 mm. The flow rate was 250 $\mu\text{L}/\text{min}$. The column was heated to 60° C. Mobile Phase A was 0.1% Formic Acid in water; Mobile Phase B was 0.1% Formic Acid in Acetonitrile. An optimized LC gradient was as follows: from 0 – 8 min ramp Mobile Phase B from 2% to 50%; from 8 – 8.1 min ramp Mobile Phase B from 50% to 95%; from 8.1 – 9.1 min hold Mobile Phase B at 95%; from 9.1 – 9.2 min decrease Mobile Phase B from 95% to 2%; from 9.2 – 11.2 min hold Mobile Phase B at 2%. It should be noted that some data reported in this work were generated using slight modifications of the gradient.

Surrogate peptides were selected using a combination of *in silico* prediction and empirical analysis of recombinant protein digests. Skyline software was used to generate multiple reaction monitoring (MRM) methods for theoretical tryptic peptides after *in silico* digestion of each CYP protein sequence. We applied little constraint *a priori* on peptide selection beyond the general default Skyline parameters, opting rather to empirically test for those tryptic peptides having 2+ or 3+ precursors and yielding robust ionization signals. The AB

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SCIEX default Skyline settings for declustering potential (DP) and collisional energy (CE) were used and transition lists were exported for empirical testing. Successive injections of recombinant protein digest were made to run the set of MRM methods covering the theoretical tryptic peptides generated from *in silico* digest. The MRM data were then imported into Skyline and peptides were analyzed for their fit for use based on peak heights, peak shapes, and amount and relative abundance of fragment ions per precursor. The *in silico* list of theoretical tryptic peptides were evaluated for uniqueness against a tryptic digest of the human proteome. The final peptides chosen for use in analysis of biological samples are described in Table 2. Representative electrospray source conditions are also included in Table 2.

Protein Quantification Assay

Sandwich cultured cryopreserved human hepatocytes (lot ACB, sex: male) at a density of ~55,000 viable cells per well in collagen-coated 96-well plates were treated with model inducers, experimental compound AZD1208, or other assay controls. After 48 h incubation with compounds in serum-free maintenance media, cell plates were washed twice with ice cold PBS. Subsequently, 60 μ L of 50 mM ammonium bicarbonate containing 5 mM of DTT and 10 mM of IAA were added to each well. The plate was covered tightly using adhesive PCR plate seals (clear film, heat-resistant) and heated at 90°C with shaking for 15 min and then removed and allowed to cool to room temperature. The mixture was then digested directly in the 96-well plate by adding trypsin (Pierce, MS Grade) at an enzyme to protein ratio of 1:30 (w:w) to each well and incubated at 37°C for 3 hours. Protein concentration per well was determined initially during assay development by BCA assay after the heating step; it was omitted in subsequent studies because we always used the same fixed amount of hepatocytes (55,000 per well) and cells were always cultured for the same time-frame (48 h). The reaction was stopped by adding quench solution to a final concentration of 5% acetonitrile and 0.5% TFA per well. Samples were then transferred to injection plates for analysis by LC-MS/MS. A representative step-by-step protocol is described in Table 3.

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LC-MRM analysis was performed using the surrogate peptides and instrument configuration/settings described above. Relative protein quantification was performed using Skyline software. Data were imported into Skyline and peak areas for each surrogate peptide were compared to the same surrogate peptide between treated and vehicle control samples. Data for each surrogate peptide were normalized to the peak area in vehicle control samples and are presented as fold-change relative to vehicle control throughout the manuscript. We did not include an internal standard to normalize for injection-to-injection variability or protein extraction efficiency. However, cell plating efficiency in hepatocytes induction assays is typically quite uniform and within-well sample digestion ensures efficient recovery. This was demonstrated by the use of triplicate wells for each treatment condition that showed acceptable precision.

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Results

Enrichment-Free CYP Protein Quantification from Plated Human Hepatocytes in 96-well Format by LC-MS/MS

Compared to previous LC-MS/MS methods for CYP protein quantification, our new method simplifies the process significantly (Fig. 1). Early methods quantified CYP proteins from liver microsomes (Alterman, Kornilayev et al. 2005, Jenkins, Kitteringham et al. 2006, Langenfeld, Zanger et al. 2009, Kawakami, Ohtsuki et al. 2011, Sakamoto, Matsumaru et al. 2011), a process which was commercialized in a kit-based format by AB SCIEX™ (Williamson, Purkayastha et al. 2011). However, two factors impede the wide-spread adoption of such methodology: 1) Microsomes need to be prepared from hepatocytes, meaning they must be cultured in low-well density format (e.g. 6-well) or even dishes to ensure adequate yield of material following cell scraping; 2) Microsomal enrichment requires ultra-centrifugation, which is inherently a laborious and time-consuming process (Fig. 1A). More recent efforts have achieved CYP protein quantification in a form more amenable to hepatocyte induction assays by using antibody-mediated enrichment of tryptic peptides (Weiss, Schnabel et al. 2015, MacLean, Weiss et al. 2017, Weiss, Hammer et al. 2018) (Fig. 1B). However, this technique is relatively costly, time-consuming, and carries the added complexities of generation and use of multiple affinity reagents. To simplify the process, we tested whether CYP protein surrogate peptides could be detected by LC-MS/MS from plated human hepatocytes without the use of antibody-mediated peptide enrichment or microsomal enrichment (Fig. 1C). Because we complete all the sample processing in the plate and inject the supernatant directly onto LC-MS/MS (to generate peaks), we informally name our new methodology “plate-to-peaks.”

Using our plate-to-peaks method we show that basal levels of CYP3A4, CYP1A2, and CYP2B6 protein are readily measured from plated human hepatocytes in 96-well format without antibody-mediated enrichment or microsomal enrichment (Fig. 2). To empirically determine which surrogate peptides have favorable LC-MS/MS responses in MRM mode, we used Skyline

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software and recombinant protein digests to monitor for peak heights and LC elution profiles of all possible tryptic peptides within pre-set criteria (see Methods section). We pruned the transition lists per peptide based on the MRM responses and monitored for basal protein levels from plated hepatocytes that had been cultured for 48 hours. As shown in Fig. 2, we successfully detected endogenous surrogate peptides for CYP3A4 (Fig. 2A), CYP1A2 (Fig. 2B), and CYP2B6 (Fig. 2C) as evidenced by matching transition patterns compared to the positive control.

We next tested if our plate-to-peaks method was capable of quantifying CYP protein induction following incubation with known inducers in 96-well format. We monitored for CYP3A4, CYP1A2, and CYP2B6 protein using four surrogate peptides per protein to identify which of the four gave the maximal analytical response. We quantified induction by comparing the LC-MRM peak areas of each surrogate peptide between control and treated cells and represent the data as fold-change compared to control cells (Fig. 3). Incubation with probenecid, a negative control, did not alter CYP3A4 protein levels, whereas incubation with CITCO, a CAR pathway weak inducer of CYP3A4, caused up to a 3-fold increase in CYP3A4 protein levels (Fig. 3A). Incubation with omeprazole, an AhR pathway activator, caused an up to 5-fold increase in CYP3A4 protein levels (Fig. 3A). As expected, incubation with increasing concentrations of rifampin, a PXR pathway activator and strong inducer of CYP3A4, produced a dose-dependent increase in CYP3A4 protein levels up to 12-fold (Fig. 3B). For CYP1A2, incubation with omeprazole, an AhR pathway activator and strong inducer of CYP1A2, caused up to a 13-fold increase in CYP1A2 protein levels, whereas incubation with rifampin gave no increase (Fig. 3C). For CYP2B6, neither incubation with rifampin nor CITCO produced appreciable protein level changes (only a modest ~2-fold maximum for CITCO treatment), which was unexpected as both compounds have been shown to readily induce CYP2B6 message *in vitro* (Faucette, Zhang et al. 2007). Overall induction response profiles for these compounds across the 3 CYP isoforms

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evaluated are consistent with previous findings (Curi-Pedrosa, Daujat et al. 1994, Rae, Johnson et al. 2001).

Application of Plate-to-Peaks Protein Quantification Method to Induction Analysis of CYP3A4, CYP1A2, and CYP2B6 by RNA, Protein, and Activity

Having demonstrated the ability to monitor for CYP3A4, CYP1A2, and CYP2B6 protein levels by LC-MRM in 96-well assay format, we next compared the induction profiles of these CYPs across mRNA, protein, and enzyme activity. We incubated hepatocytes with increasing concentrations of rifampin to evaluate CYP3A4, omeprazole for CYP1A2, and phenytoin for CYP2B6 induction, and used a single surrogate peptide per CYP protein for quantification, the choice of which was based on our results described above and in Figure 3. In each case, we included CITCO and probenecid as additional controls. CYP3A4 showed a dose-dependent increase in mRNA, protein, and activity compared to vehicle treated control cells with maximum effect for mRNA of 12-fold by 10 μ M, for protein of 9-fold by 30 μ M, and for activity of 3-fold by 1 μ M (Fig. 4A). There was no induction of CYP3A4 by probenecid and modest induction by CITCO, varying in magnitude between RNA (4-fold), protein (2-fold), and activity (2-fold) (Fig. 4A). CYP1A2 showed a dose-dependent increase in mRNA, protein, and activity compared to control cells out to 50 μ M omeprazole treatment, after which RNA started to decline in magnitude of induction while protein and activity continued to rise out to 200 μ M, the highest concentration tested (Fig. 4B). The maximum effect for CYP1A2 mRNA was 30-fold at 50 μ M, 23-fold for protein at 200 μ M, and 28-fold for activity at 200 μ M omeprazole (Fig. 4B). There was no induction of CYP1A2 mRNA, protein, or activity by CITCO or probenecid (Fig. 4B). CYP2B6 mRNA was induced to a maximum effect of 23-fold by 30 μ M phenytoin, whereas with higher concentrations the fold-induction was either the same or decreased slightly (Fig. 4C); CYP2B6 protein was induced to a maximum effect of 2-fold by 1 μ M and maintained through the higher concentrations (Fig. 4C); CYP2B6 activity was induced to a maximum effect of 5-fold by 10 μ M and was maintained through the higher concentrations (Fig. 4C). CITCO induced CYP2B6

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mRNA 15-fold, protein 2-fold, and activity 5-fold (Fig. 4C); there was no CYP2B6 induction by probenecid (Fig. 4C). Taken together, these data show the utility of our plate-to-peaks processing for protein analysis in comparing induction profiles across mRNA, protein, and enzymatic activity. They also show that the induction response profile for a given CYP can vary across mRNA, protein, and activity, which is consistent with previous observations from MacLean *et al.* (MacLean, Weiss *et al.* 2017).

Features of Plate-to-Peaks Protein Quantification Method Complimentary to Routine Induction Assay Formats: Direct Analysis from Fresh, Frozen and Assay Plates

To explore options for how plate-to-peaks protein quantification could be integrated into routine CYP induction assay workflows, we tested whether target protein induction is readily quantifiable in the same plate which had been used for enzyme activity assessment just prior. Additionally, we examined whether storing plates at -80°C prior to sample processing would affect protein quantification. Both options would offer benefits in the form of improved efficiency/quality (activity and protein quantification from the same plate) or introduction of a stopping point (freezing the plate for later analysis) in what otherwise could be a long process. We incubated hepatocytes with increasing concentrations of rifampin and monitored protein induction of CYP3A4 across “fresh” plates, “assay” plates (where target protein quantification was assessed following enzyme activity assessment), and “frozen” plates, which were stored at -80°C prior to thaw and extraction. All three plates showed similar induction profiles, with maximum effects of 10-fold at the maximum rifampin concentration tested ($30\ \mu\text{M}$) (Fig. 5). Worth noting is that the “fresh plate” data is the average \pm standard deviation of six replicate measures across two independent users (three replicates each). The relatively small error speaks to the robustness of this assay (Fig. 5). These data suggest that CYP protein quantification using a plate-to-peaks processing could be readily introduced into conventional induction screening workflows that typically accommodate mRNA and/or enzyme activity endpoints. When conducted, enzyme activity is assessed within wells at the end of the

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induction treatment. This can then be followed by cell harvest for mRNA analysis. Under this scenario, cells would no longer be available for target protein quantification, forcing the analyst to choose between using cells for mRNA or protein analysis. Therefore, we foresee replicate treatment plates being needed to accommodate all three endpoints. One could imagine scenarios where all three endpoints could be generated within a single plate – for example a split level insert that could be removed for one workstream and plated housing cells proceed through another workstream. The desire and effort to multiplex this assay as much as possible has been ongoing for a long time (Halladay, Wong et al. 2012).

Application of Plate-to-Peaks Method for Quantifying CYP3A4 Protein Induction by AZD1208

With the ability to quantify CYP protein induction, we evaluated AZD1208 for CYP3A4 protein induction potential in plated hepatocytes. This compound had been identified as an *in vivo* inducer of CYP3A4, but was not predicted as such based on *in vitro* data generated with HepaRG cells and human hepatocytes using enzyme activity and mRNA as endpoints (Jones, Rollison et al. 2017). Therefore, we felt this compound would be interesting to evaluate using our approach. We treated plated human hepatocytes with increasing concentrations of AZD1208 ranging from 0.03 – 50 μ M, covering the maximum concentrations tested by Jones, Rollison et al., and monitored CYP3A4 protein induction. CYP3A4 was increased to a maximum effect of 3-fold at 10 μ M AZD1208 treatment compared to control cells (Fig. 6); at lower concentrations the increases were less in magnitude but a clear dose-dependent increase starting at 1 μ M could be seen (Fig. 6); fold-changes decreased at concentrations higher than 10 μ M (Fig. 6). The 3-fold CYP3A4 protein induction *in vitro* by AZD1208 shown here may be consistent with the clinical findings (Jones, Rollison et al. 2017).

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Discussion

We have demonstrated the ability to monitor for CYP3A4, CYP1A2, and CYP2B6 protein induction by surrogate peptide LC-MRM directly from 96-well plated human hepatocytes without the use of antibody-mediated or microsomal enrichment steps. We also compared induction across mRNA, protein, and enzymatic activity for CYP3A4, CYP1A2, and CYP2B6 using known inducers for each isoform. We elected to use a single exposure time for inducers (e.g. 2 X 24h treatments) for all 3 endpoints. This is because it is the format most researchers use, at least for mRNA and enzyme activity measurements. Since mRNA temporally precedes protein expression and maximal responses are not always concurrent (Zhang, Ho et al. 2010), it is an important consideration when comparing endpoint responses. Notwithstanding, induction results were broadly concordant across the three CYPs analyzed and highlight the potential for facile analysis of all 3 major endpoints (mRNA, protein, and activity measurements) within a single experimental setting (Fig. 4A – C, compare relative responses of mRNA, protein, and activity per CYP). We anticipate such analysis will enable interrogation beyond mRNA/enzyme activity only, endpoints favored by industrial scientists and regulatory agencies since 2012.

It has been proposed that induction of CYP2E1 (reviewed in (Gonzalez 2007)) and more recently CYP2C19 (Hariparsad, Ramsden et al. 2017) may occur through a mechanism other than an increase in transcription, such as protein stabilization. Although CYP2E1 and CYP2C19 have important, but limited roles in drug metabolism, it is tempting to speculate that major drug metabolizing enzymes such as CYP3A4 may also exhibit induction by mechanisms in addition to transcriptional. In addition, it is conceivable that mRNA induction may be accompanied by no induction of protein, a finding which would certainly be valuable for de-risking a perceived induction liability. Our method should enable rapid expansion of CYP protein induction analysis, which may include testing CYP proteins beyond those we quantified here. While a global proteomics approach by nanoLC-MS/MS can readily monitor additional CYP proteins, the advantage of the method we report is simplicity of process enabling quantification with analytical

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flow LC and conventional bioanalytical lab triple quadrupole MS. Over time, and as a collective body of knowledge begins to amass, we will gain a robust understanding of the diversity in mRNA, protein, and activity CYP induction profiles across a wide array of hepatocyte donors and compounds. We hope that our simplified methodology for CYP protein quantification in *in vitro* induction studies described here will enable such exploration. For example, we have very recently observed rifampin-mediated induction of CYP3A4 protein in a second human hepatocyte donor consistent with the findings reported here (data not shown).

A common question in the field of LC-MS/MS surrogate peptide quantification is which peptide(s) should be used to represent the protein. Our data for CYP3A4 protein induction are a case in point: across four surrogate peptides for the same protein there is up to a 2-fold difference in induction response depending on the peptide (Fig. 3A & B). So, which response is closer to the true induction value at the CYP3A4 protein level? At 30 μ M rifampin treatment, was CYP3A4 induced 6-fold or was it induced 12-fold (Fig. 3B)? Potential explanations for these differences include non-uniform digestion to peptide analyte over the range of target encountered, or differences in ionization efficiencies of each peptide in relation to increased concentrations. In situations of discrepancy between surrogate peptide quantitative results, we recommend taking the highest responding peptide as closest to the true quantitative result of the protein, under the assumption that lower response peptides may be less robust in their signals due to increased susceptibility to ion suppression, variable digestion, or other phenomenon. Use of an isotopically labelled intact protein standard expressed within the biological system of interest would test this assumption.

Our analysis raises some interesting questions. For example, why do CYP3A4 protein induction levels steadily increase to 9-fold at 30 μ M rifampin while enzyme activity levels plateau at 3-fold beginning at 1 μ M rifampin (Fig. 4A). One explanation for this observation is that necessary components for catalytic activity (e.g. heme incorporation, expression of NADPH cytochrome P450 reductase, cytochrome b5) fail to track with levels of CYP protein expression.

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Since substrate concentration is well in excess of the K_m for midazolam 1'-hydroxylation in our activity assay, we do not believe the observed plateau is due to substrate depletion. By contrast, CYP1A2 and CYP2B6 induction of enzyme activity is generally higher than protein levels, up to 3-fold for CYP2B6 (Fig. 4B & C). This might be explained by choice of sub-optimal peptide for quantification, yielding lower-fold induction (*vide supra*). Another explanation is that there is induction of enzymes in addition to CYP1A2 or CYP2B6 catalyzing probe substrate turnover.

The ultimate question for the ADME community is which measure – mRNA, protein, or activity – assessed for induction *in vitro* best predicts induction *in vivo*? One could argue protein has always been the intended target for *in vitro* DDI induction assays, but since the field did not have a practical, high-throughput assay, we collectively settled for mRNA and activity as surrogate measures. As reported here, protein analysis *in vitro* has been made possible with acceptable cost in a high-throughput format, and we therefore anticipate a growing adoption of this technique. Thus, as more labs quantify CYP protein in *in vitro* induction studies, it will be interesting to learn whether a single endpoint or combinations of the three will yield best predictions. Evaluation of all three endpoints may also yield insight into compound-dependent mechanism of induction, for example relative roles of transcriptional regulation and protein stabilization.

To speed the adoption of our plate-to-peaks methodology into routine *in vitro* workflows across the industry, we showed how it could be integrated into current workflows in one of three ways: use a fresh plate and process right away, use a fresh plate and freeze it for later processing, or use the activity assay plate after taking the supernatant for activity assessment. In our hands each of these approaches yielded the same induction results (Fig. 5). The value of having options like this for CYP protein quantification is that it allows for integration of protein quantification into current workflows for *in vitro* induction by ADME labs without an overhaul to their current process. Thus, induction assays can carry on as usual while the same labs begin to explore CYP protein quantification in addition. If confronted with an already long day for an

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mRNA-based induction assay, now the analytical scientist can simply incubate an extra plate of hepatocytes with compound and freeze it for protein quantification later – the mRNA assay carries on unimpeded. If workflows cannot readily accommodate additional plates for compound incubation, the same plate used for assessing enzyme activity can be also utilized for protein quantification – two assays from the same plate. As more ADME labs amass their own results for CYP induction by protein analysis we might collectively decide that CYP protein is the better endpoint for *in vitro* assays and thus experience a shift toward protein quantification as the primary assay.

Upon evaluating AZD1208 induction potential for CYP3A4 protein, we observed two results of note: 1) CYP3A4 protein induction reached 3-fold at a similar concentration of compound to that which produced 3-fold mRNA induction as previously reported (Fig. 6) (Jones, Rollison et al. 2017); 2) at higher concentrations of compound, CYP3A4 protein induction started to decrease (Fig. 6). It would be interesting to see how consistent the 3-fold CYP3A4 protein induction is across multiple hepatocyte donors. The decrease in the magnitude of CYP3A4 induction at concentrations higher than 10 μ M could be explained by cell death and the dead cells being washed away during the early steps of the process. If that were true, accounting directly for cell death in the protein quantification assay would be needed for accurate interpretation of induction on a per cell basis. We suggest the inclusion of a panel of common housekeeping genes in MRM assays to serve as internal controls to which CYP protein induction can be normalized to – a solution that could be accommodated within our plate-to-peaks process with no negative impact owing to fast LC-MS/MS duty cycles of triple quadrupole mass spectrometers and their inherent ability to multiplex and monitor multiple peptides per injection. A final thought is in the power of orthogonal methodology to increase the confidence in results seen. In the case of AZD1208, *in vitro* mRNA results did pick up a 3-fold induction, but without any other method to confirm this relatively low response, perhaps this was disregarded as not significant enough or too low. With the studies described in this manuscript,

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addition of protein measures of induction can now be performed to inform on induction potential and to highlight potential disconnects for further interrogation on the potentially complex mechanisms behind CYP induction.

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Acknowledgments

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Authorship Contributions

Participated in research design: Jenkins, Liu, Savaryn, Stresser

Conducted experiments: Liu, Ma, Sun

Contributed new reagents or analytic tools: Liu

Performed data analysis: Liu, Ma, Savaryn, Sun

Wrote or contributed to the writing of the manuscript: Jenkins, Liu, Savaryn, Stresser

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References

- Alterman, M. A., B. Kornilayev, T. Duzhak and D. Yakovlev (2005). "Quantitative analysis of cytochrome p450 isozymes by means of unique isozyme-specific tryptic peptides: a proteomic approach." Drug Metab Dispos **33**(9): 1399-1407.
- Anonymous (2018). "<https://didb.druginteractioninfo.org/drug/monograph/15107/#ddi-summary>."
- Curi-Pedrosa, R., M. Daujat, L. Pichard, J. C. Ourlin, P. Clair, L. Gervot, P. Lesca, J. Domergue, H. Joyeux, G. Fournanier and et al. (1994). "Omeprazole and lansoprazole are mixed inducers of CYP1A and CYP3A in human hepatocytes in primary culture." J Pharmacol Exp Ther **269**(1): 384-392.
- Fahmi, O. A., M. Kish, S. Boldt and R. S. Obach (2010). "Cytochrome P450 3A4 mRNA is a more reliable marker than CYP3A4 activity for detecting pregnane X receptor-activated induction of drug-metabolizing enzymes." Drug Metab Dispos **38**(9): 1605-1611.
- Faucette, S. R., T. C. Zhang, R. Moore, T. Sueyoshi, C. J. Omiecinski, E. L. LeCluyse, M. Negishi and H. Wang (2007). "Relative activation of human pregnane X receptor versus constitutive androstane receptor defines distinct classes of CYP2B6 and CYP3A4 inducers." J Pharmacol Exp Ther **320**(1): 72-80.
- FDA (2017). "In Vitro Metabolism- and Transporter- Mediated Drug-Drug Interaction Studies Guidance for Industry - DRAFT GUIDANCE."
- Gibbons, J. A., M. de Vries, W. Krauwinkel, Y. Ohtsu, J. Noukens, J. S. van der Walt, R. Mol, J. Mordenti and T. Ouatas (2015). "Pharmacokinetic Drug Interaction Studies with Enzalutamide." Clin Pharmacokinet **54**(10): 1057-1069.
- Gonzalez, F. J. (2007). "The 2006 Bernard B. Brodie Award Lecture. Cyp2e1." Drug Metab Dispos **35**(1): 1-8.
- Halladay, J. S., S. Wong, S. C. Khojasteh and S. Grepper (2012). "An 'all-inclusive' 96-well cytochrome P450 induction method: measuring enzyme activity, mRNA levels, protein levels,

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and cytotoxicity from one well using cryopreserved human hepatocytes." J Pharmacol Toxicol Methods **66**(3): 270-275.

Hariparsad, N., D. Ramsden, J. Palamanda, J. G. Dekeyser, O. A. Fahmi, J. R. Kenny, H. Einolf, M. Mohutsky, M. Pardon, Y. A. Siu, L. Chen, M. Sinz, B. Jones, R. Walsky, S. Dallas, S. K. Balani, G. Zhang, D. Buckley and D. Tweedie (2017). "Considerations from the IQ Induction Working Group in Response to Drug-Drug Interaction Guidance from Regulatory Agencies: Focus on Downregulation, CYP2C Induction, and CYP2B6 Positive Control." Drug Metab Dispos **45**(10): 1049-1059.

Jenkins, R. E., N. R. Kitteringham, C. L. Hunter, S. Webb, T. J. Hunt, R. Elsby, R. B. Watson, D. Williams, S. R. Pennington and B. K. Park (2006). "Relative and absolute quantitative expression profiling of cytochromes P450 using isotope-coded affinity tags." Proteomics **6**(6): 1934-1947.

Jones, B. C., H. Rollison, S. Johansson, K. P. Kanebratt, C. Lambert, K. Vishwanathan and T. B. Andersson (2017). "Managing the Risk of CYP3A Induction in Drug Development: A Strategic Approach." Drug Metab Dispos **45**(1): 35-41.

Kawakami, H., S. Ohtsuki, J. Kamiie, T. Suzuki, T. Abe and T. Terasaki (2011). "Simultaneous absolute quantification of 11 cytochrome P450 isoforms in human liver microsomes by liquid chromatography tandem mass spectrometry with in silico target peptide selection." J Pharm Sci **100**(1): 341-352.

Langenfeld, E., U. M. Zanger, K. Jung, H. E. Meyer and K. Marcus (2009). "Mass spectrometry-based absolute quantification of microsomal cytochrome P450 2D6 in human liver." Proteomics **9**(9): 2313-2323.

MacLean, C., F. Weiss, O. Poetz and T. Ebner (2017). "Concept: The Use of Targeted Immunoaffinity Proteomics for Routine Assessment of In Vitro Enzyme Induction." J Pharm Sci **106**(12): 3453-3457.

DMD # 90480

Pichard, L., I. Fabre, G. Fabre, J. Domergue, B. Saint Aubert, G. Mourad and P. Maurel (1990).

"Cyclosporin A drug interactions. Screening for inducers and inhibitors of cytochrome P-450 (cyclosporin A oxidase) in primary cultures of human hepatocytes and in liver microsomes."

Drug Metab Dispos **18**(5): 595-606.

Rae, J. M., M. D. Johnson, M. E. Lippman and D. A. Flockhart (2001). "Rifampin is a selective, pleiotropic inducer of drug metabolism genes in human hepatocytes: studies with cDNA and oligonucleotide expression arrays." J Pharmacol Exp Ther **299**(3): 849-857.

Sakamoto, A., T. Matsumaru, N. Ishiguro, O. Schaefer, S. Ohtsuki, T. Inoue, H. Kawakami and T. Terasaki (2011). "Reliability and robustness of simultaneous absolute quantification of drug transporters, cytochrome P450 enzymes, and Udp-glucuronosyltransferases in human liver tissue by multiplexed MRM/selected reaction monitoring mode tandem mass spectrometry with nano-liquid chromatography." J Pharm Sci **100**(9): 4037-4043.

Weiss, F., H. S. Hammer, K. Klein, H. Planatscher, U. M. Zanger, A. Noren, C. Wegler, P. Artursson, T. O. Joos and O. Poetz (2018). "Direct Quantification of Cytochromes P450 and Drug Transporters-A Rapid, Targeted Mass Spectrometry-Based Immunoassay Panel for Tissues and Cell Culture Lysates." Drug Metab Dispos **46**(4): 387-396.

Weiss, F., A. Schnabel, H. Planatscher, B. H. van den Berg, B. Serschnitzki, A. K. Nuessler, W. E. Thasler, T. S. Weiss, M. Reuss, D. Stoll, M. F. Templin, T. O. Joos, K. Marcus and O. Poetz (2015). "Indirect protein quantification of drug-transforming enzymes using peptide group-specific immunoaffinity enrichment and mass spectrometry." Sci Rep **5**: 8759.

Williamson, B. L., S. Purkayastha, C. L. Hunter, L. Nuwaysir, J. Hill, L. Easterwood and J. Hill (2011). "Quantitative protein determination for CYP induction via LC-MS/MS." Proteomics **11**(1): 33-41.

Wochenschr, D. m. (1973). "Wirkung von Rifampicin auf den menstruationszyklus und die ostrogenausscheidung bei Einnahme oraler Kontrazeptiva (Effect of rifampicin on the menstrual cycle and the estrogen secretion when taking oral contraceptives)." **98**: 1521-1523.

DMD # 90480

Xu, L., B. Ma, S. Yu, C. Xia and J. T. Wu (2014). "The use of a rapid MS-based method for the quantification of the CYP 3A4 protein directly from hepatocyte cell lysate for CYP induction studies." Bioanalysis **6**(24): 3271-3282.

Zhang, J. G., T. Ho, A. L. Callendrello, C. L. Crespi and D. M. Stresser (2010). "A multi-endpoint evaluation of cytochrome P450 1A2, 2B6 and 3A4 induction response in human hepatocyte cultures after treatment with beta-naphthoflavone, phenobarbital and rifampicin." Drug Metab Lett **4**(4): 185-194.

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Footnotes

Disclosure: All authors are or were employees of AbbVie at the time the work was conducted and may own AbbVie stock. NL is currently retired. AbbVie sponsored and funded the study; contributed to the design; participated in the collection, analysis, and interpretation of data, and in writing, reviewing, and approval of the final publication.

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Figure Legends

Figure 1. Comparison of previous methods for CYP protein quantification to new Plate-to-Peaks method.

Figure 2. LC-MRM chromatograms from plated human hepatocytes after 48 h in culture (Basal) are compared to digestion of recombinant protein as a positive control (Pos Control) for: A) CYP3A4 surrogate peptides; B) CYP1A2 surrogate peptides; C) CYP2B6 surrogate peptides. Each color represents a precursor/product ion transition belonging to the respective surrogate tryptic peptides (abbreviated as the first four N-terminal amino acids).

Figure 3. CYP protein induction in plated human hepatocytes quantified by LC-MRM of surrogate peptides. Quantification was performed by comparing LC-MRM peak areas per surrogate peptide to peak areas observed in DMSO treated cells. For each graph the first four N-terminal amino acids per surrogate peptide are listed. Data were collected in triplicate and presented as average +/- standard deviation. Values represent the average of triplicate samples and error bars represent the standard deviation. A) CYP3A4 protein fold-change after treatment with probenecid (10 μ M), CITCO (0.1 μ M), or omeprazole (50 μ M). B) CYP3A4 protein fold-change after treatment with increasing concentrations of rifampin (μ M). C) CYP1A2 protein fold-change after treatment with rifampin (10 μ M), or omeprazole (50 μ M). D) CP2B6 protein fold-change after treatment with rifampin (30 μ M) or CITCO (0.1 μ M).

Figure 4. Comparison of RNA, protein, and activity data for A) CYP3A4, B) CYP1A2, and C) CYP2B6. In all cases data are represented as fold change compared to levels in vehicle (0.1% DMSO) treated controls. Data were collected in triplicate and presented as average +/- standard deviation. A) Increasing concentrations of Rifampin (μ M). CITCO = 0.1 μ M; Probenecid (Prob.) = 10 μ M. Midazolam (30 μ M) was used as the substrate for activity measurements. B) Increasing concentrations of Omeprazole (μ M). CITCO = 0.1 μ M; Probenecid (Prob.) = 10 μ M. Phenacetin (100 μ M) was used as the substrate for activity measurements. C) Increasing

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concentrations of Phenytoin (μM). CITCO = 0.1 μM ; Probenecid (Prob.) = 10 μM . Bupropion (250 μM) was used as the substrate for activity measurements.

Figure 5. Practical applications of new LC-MS/MS method for CYP protein quantification.

Consistency of quantitative results, in terms of fold change compared to vehicle (0.1% DMSO) treated cells, is compared between fresh plates, plates previously used in activity assays (Assay Plate), and plates frozen after incubation with rifampin but prior to sample processing (Frozen Plate) for CYP3A4. Note that in the 0.1 μM Rifampin treated assay plate data point, one of the three replicate wells was a low outlier causing high error. Fresh plate data is the average of six replicates across two experiments run on different days and by different users (three replicates per experiment). Assay Plate and Frozen Plate data is the average of three replicates from one experiment each. In all cases data are presented as average \pm standard deviation.

Figure 6. Levels of CP3A4 in plated human hepatocytes following treatment with AZD1208.

Quantification was performed by comparing LCMS peak areas of the LSLG surrogate peptide between cells treated with AZD1208 at increasing concentrations and cells treated with DMSO as a control. Data were collected in triplicate and presented as average \pm standard deviation.

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Tables

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Table 1. Enzyme Activity Assay LCMS Method Details

Autosampler: CTC PAL
Mass Spectrometer: API 5500 (Sciex)
Injection Volume: 5 μ L
Column: Fortis Pace C18, 30 x 2.1 mm, 5 micron
Gradient: Gradient Elution (see example below)
Solvent A: 0.1% formic acid in water
Solvent B: 0.1% formic acid in acetonitrile

Mass Spec Parameters

Isoform	Metabolite	Metabolite MRM	DP	CE	Ionization Mode
CYP1A2	acetamidophenol	152 \rightarrow 110	66	23	Positive
CYP2B6	Hydroxybupropion	256 \rightarrow 139	26	41	Positive
CYP3A4	1'-hydroxymidazolam	342 \rightarrow 203	106	45	Positive

Example Gradient for 1'-hydroxymidazolam

Time (min)	Flow Rate (μL/min)	A (%)	B (%)
0.00	600	95	5
0.10	600	70	30
0.40	600	70	30
0.50	600	40	60
0.80	600	40	60
0.90	600	0	100
1.20	600	0	100
1.30	600	95	5
1.50	600	95	5

Table 2. Surrogate Peptides and MRM Transitions Used For CYP3A4, CYP1A2, and CYP2B6

Protein	Peptide*	Uniqueness	Q1	Q3
CYP3A4	GVVVMIPSYALHR	Unique	481.27	843.45, 746.39, 659.36, 422.23
	LSLGLLQPEKPVVLK	Unique	564.35	909.58, 683.48, 555.39, 455.29
	<u>E</u> CYSVFTNR	Also present in CYP3A5 and CYP3A7	588.26	723.38, 636.35, 537.28, 390.21
	SLLSPTFTSGK	Also present in CYP3A5 and CYP3A7	569.31	737.38, 640.33, 539.28, 369.20
CYP1A2	IGSTPVLVLSR	Unique	571.35	783.51, 587.39, 392.26, 686.46
	YLPNPALQR	Unique	536.3	795.45, 698.39, 584.35, 398.23
	DITGALFK	Unique	432.74	636.37, 535.32, 478.30
	ASGNLIPQEK	Unique	528.79	727.43, 614.35, 501.27
CYP2B6	IAMVDPFFR	Unique	548.29	780.40, 681.34, 566.31
	<u>I</u> CLGEGIAR	Unique	494.77	602.33, 545.30, 416.26, 246.16
	GYGVIFANGNR	Unique	584.3	678.33, 531.26, 460.23
	ETLDPSAPK	Unique	479.25	614.31, 499.29, 244.17, 315.20

*Peptides are abbreviated to the first four N-terminal amino acids throughout the manuscript

Note: Bold underlined "C" indicates carbamidomethyl Cys

Representative Electrospray Ionization Source Conditions (AB SCIEX QTRAP® 6500)

Polarity: Positive
 CUR: 40
 IS: 5500
 TEM: 450
 GS1: 60
 GS2: 70
 CAD: Medium
 EP: 10
 CXP: 8
 CE: Used Skyline predicted values
 DP: Used Skyline predicted values

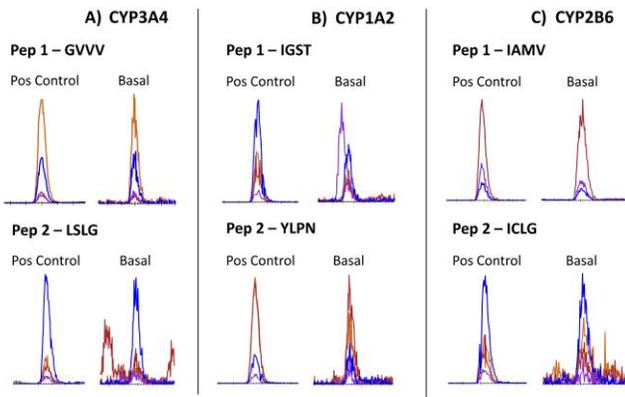
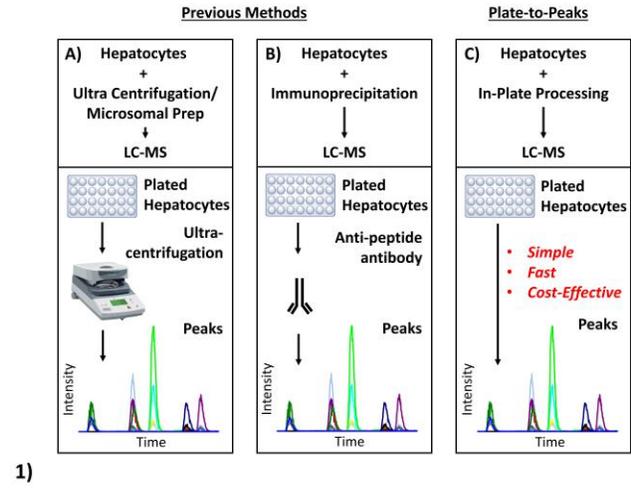
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Table 3. CYP Protein Quantification Sample Preparation Method*

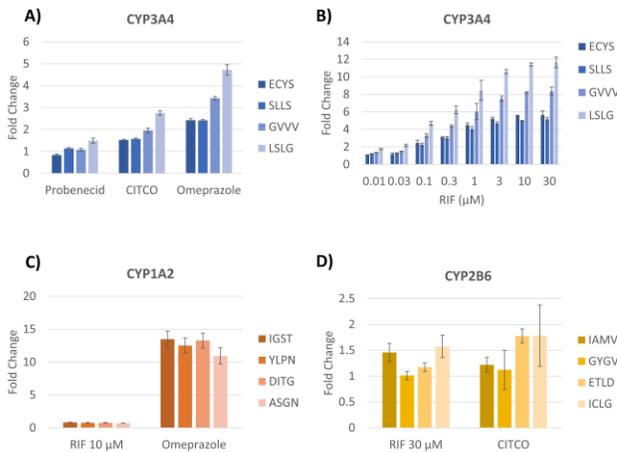
Step	Procedure	μL per well
1	Add 50 mM ABC	50
2	Add 100 mM DTT	3
3	Add 100 mM IAA	7
4	Incubate covered plate at 90°C in dark for 15 min with 400rpm shaking	
5	Allow plate to cool down to room temperature	
6	Withdraw 10 μL /well for BCA protein concentration assay	NA
7	Add trypsin (0.1 $\mu\text{g}/\mu\text{L}$ stock concentration)	15
8	Incubate covered plate at 37°C for 3 h with 400rpm shaking	
9	Add 10% TFA	7
10	Add Acetonitrile	3
11	Centrifuge at 8,000xg	
12	Add 0.1% FA in water	25
13	Inject 40 μL per sample to LC/MS	

** Slight variations on this method have also been used throughout the manuscript; the critical features of the method are represented here.*

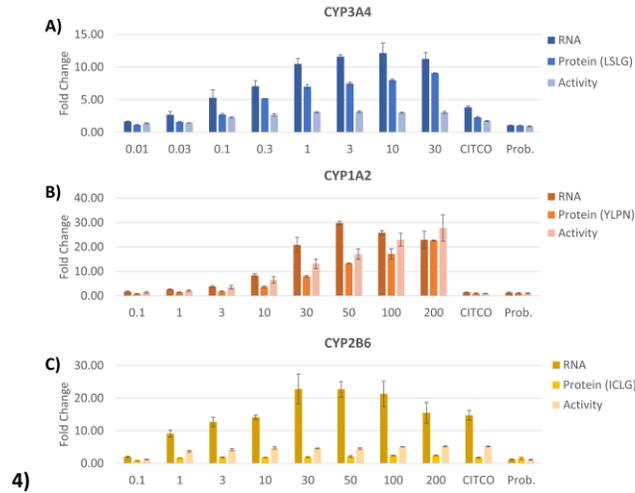
Figures



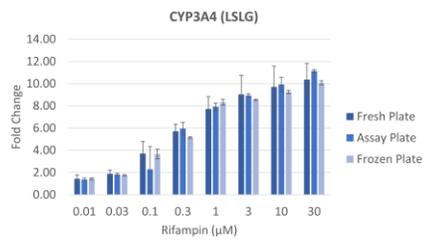
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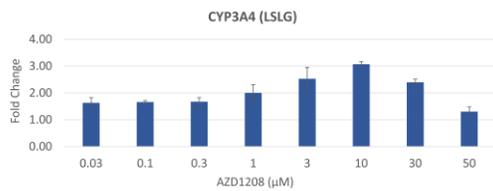
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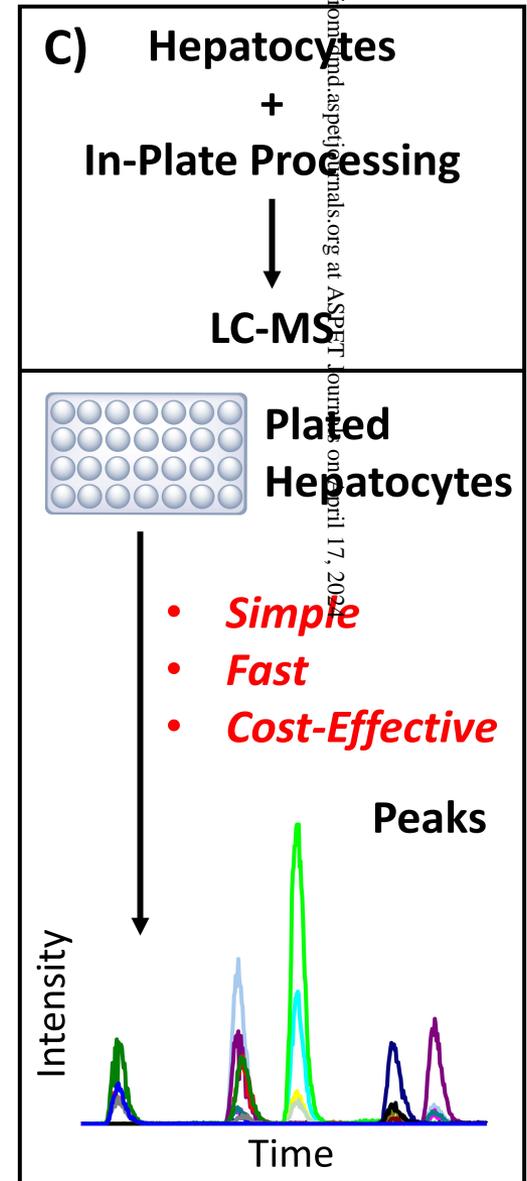
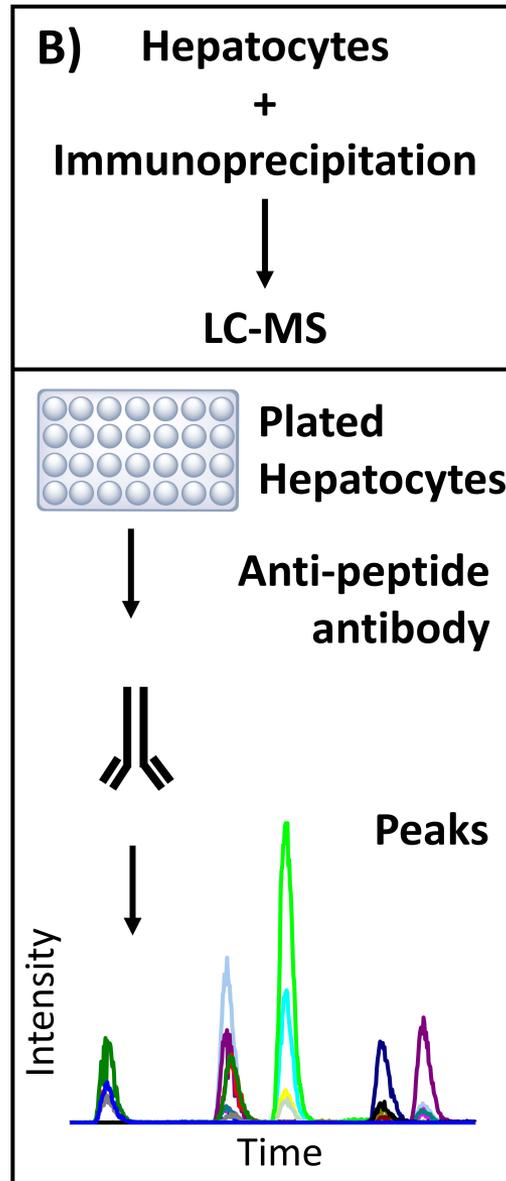
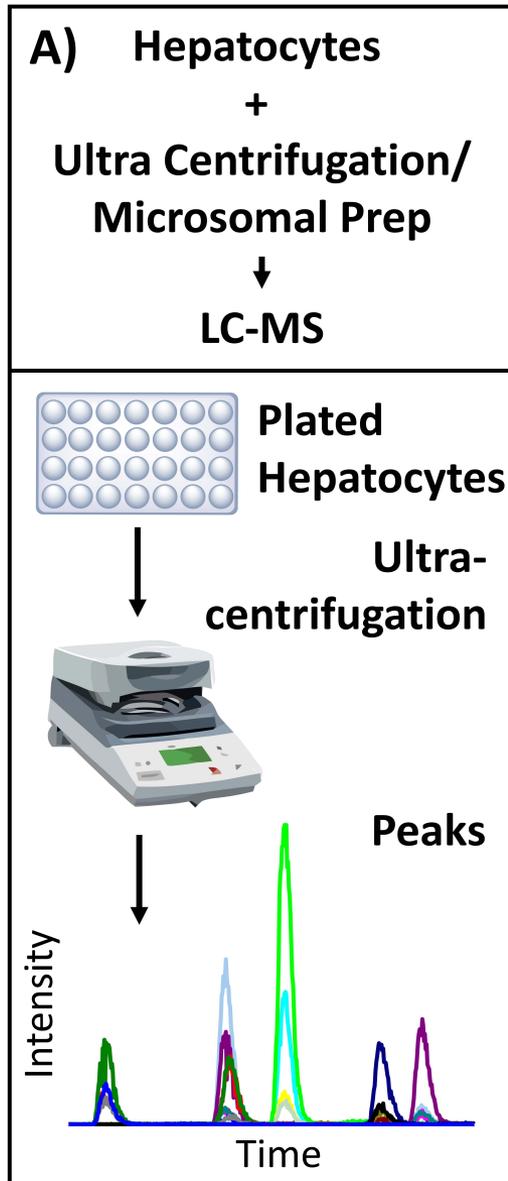
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6)

Previous Methods

Plate-to-Peaks



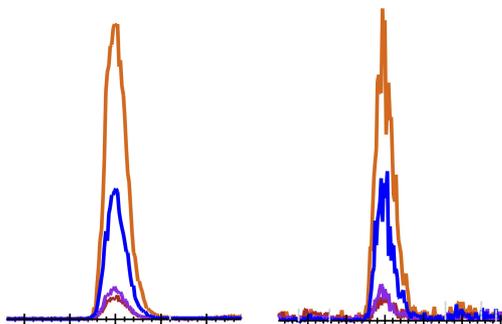
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A) CYP3A4

Pep 1 – GVVV

Pos Control

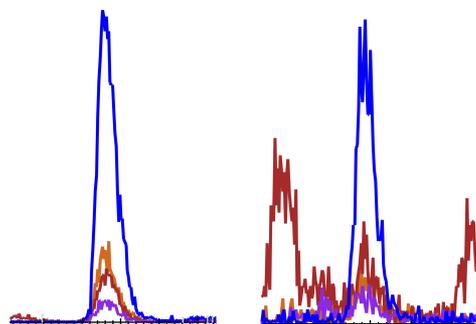
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Pep 2 – LSLG

Pos Control

Basal

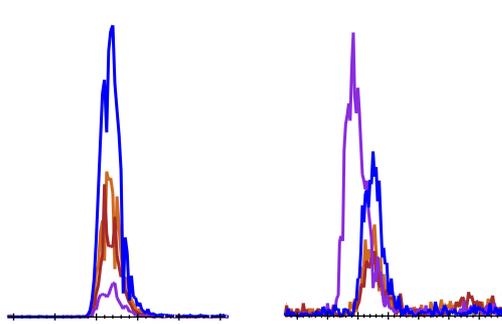


B) CYP1A2

Pep 1 – IGST

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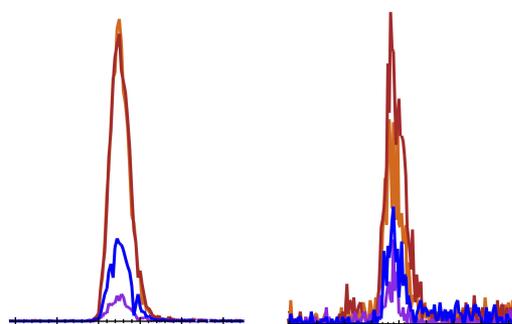
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Pep 2 – YLPN

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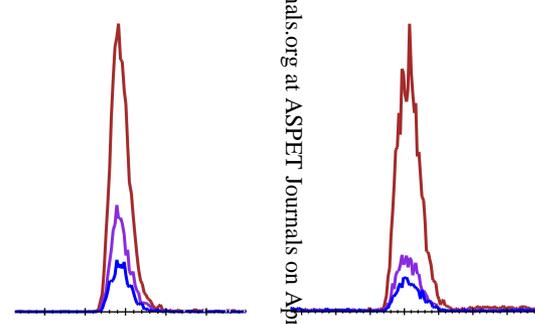


C) CYP2B6

Pep 1 – IAMV

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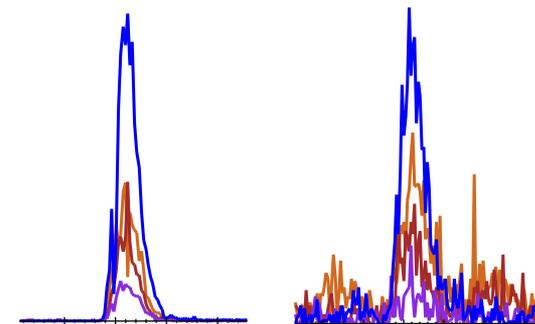
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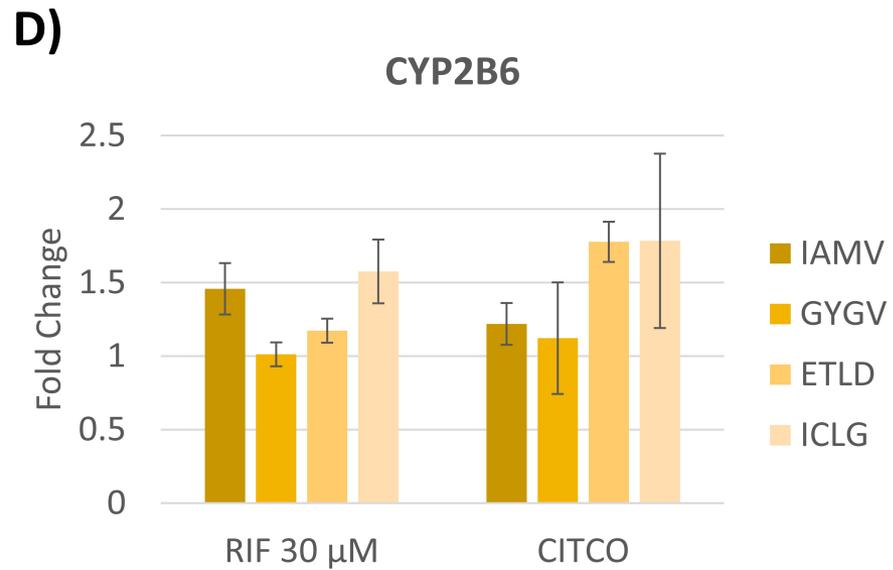
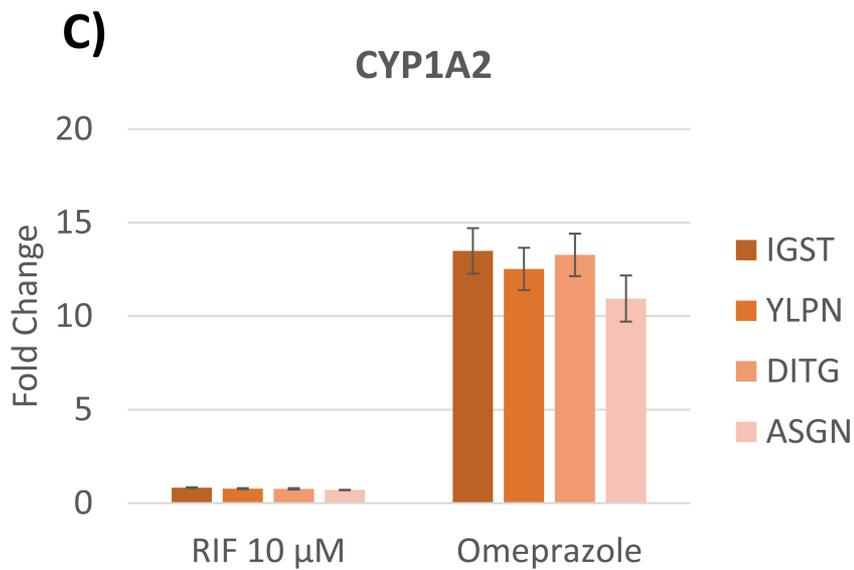
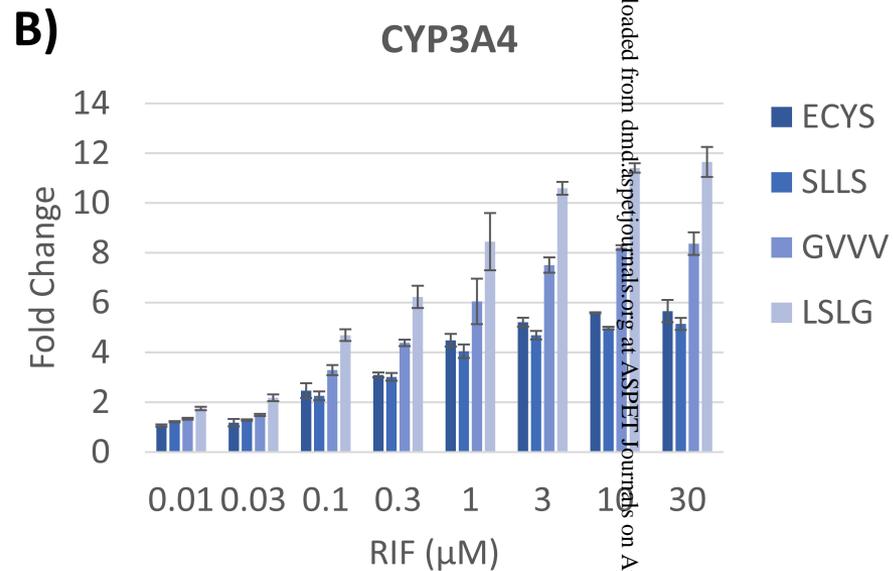
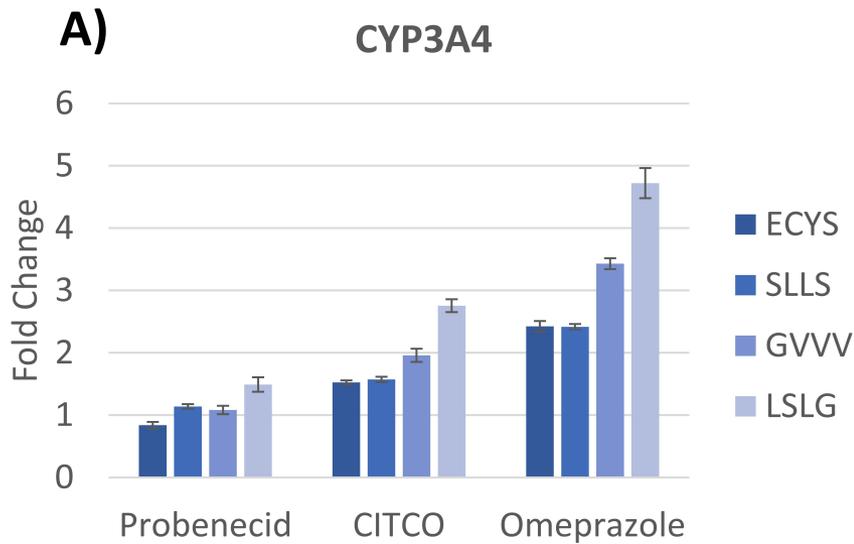


Pep 2 – ICLG

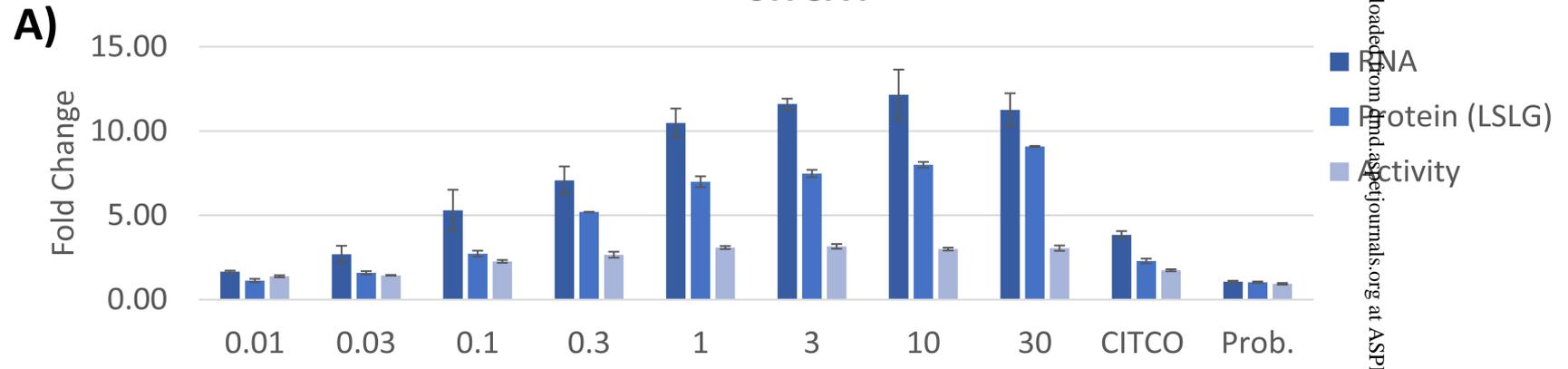
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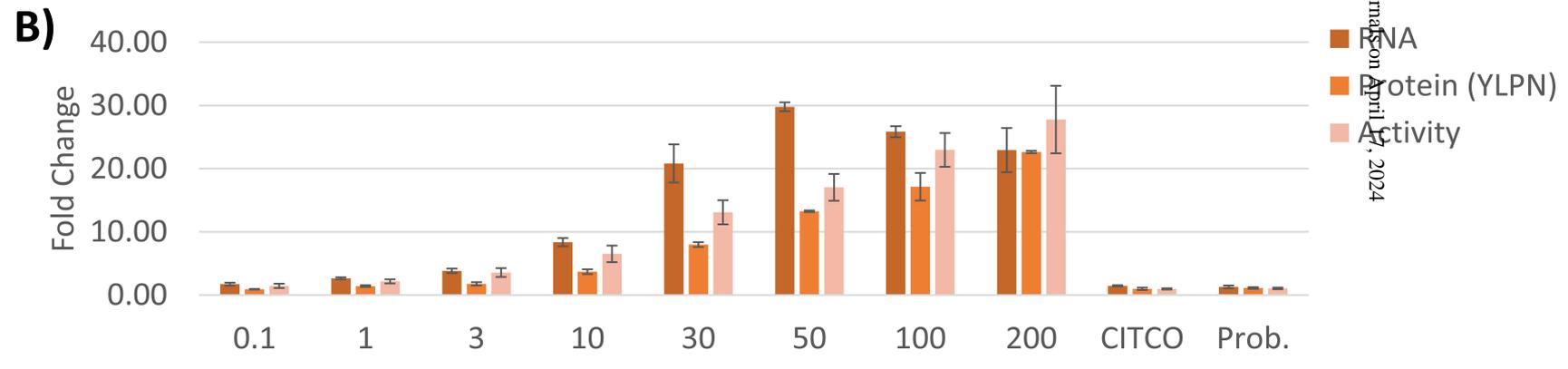




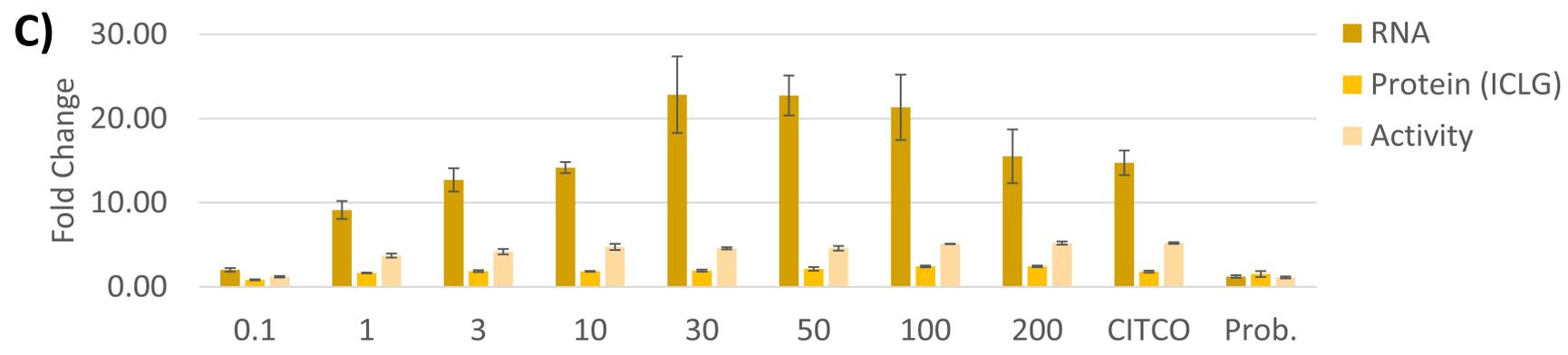
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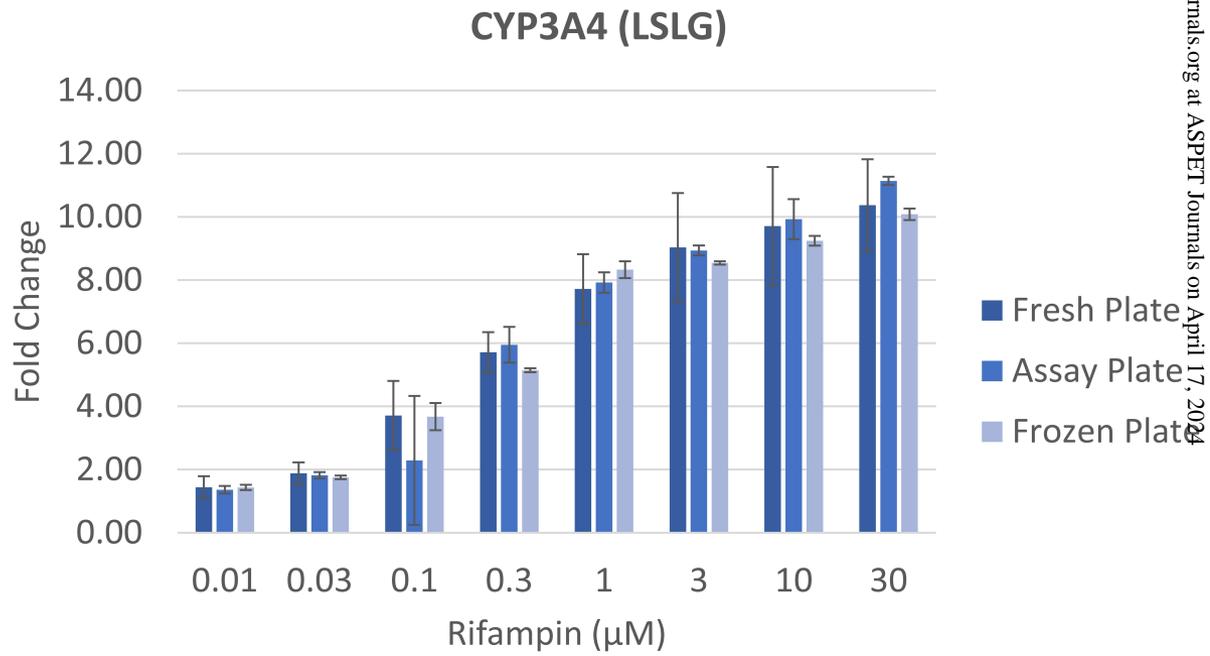
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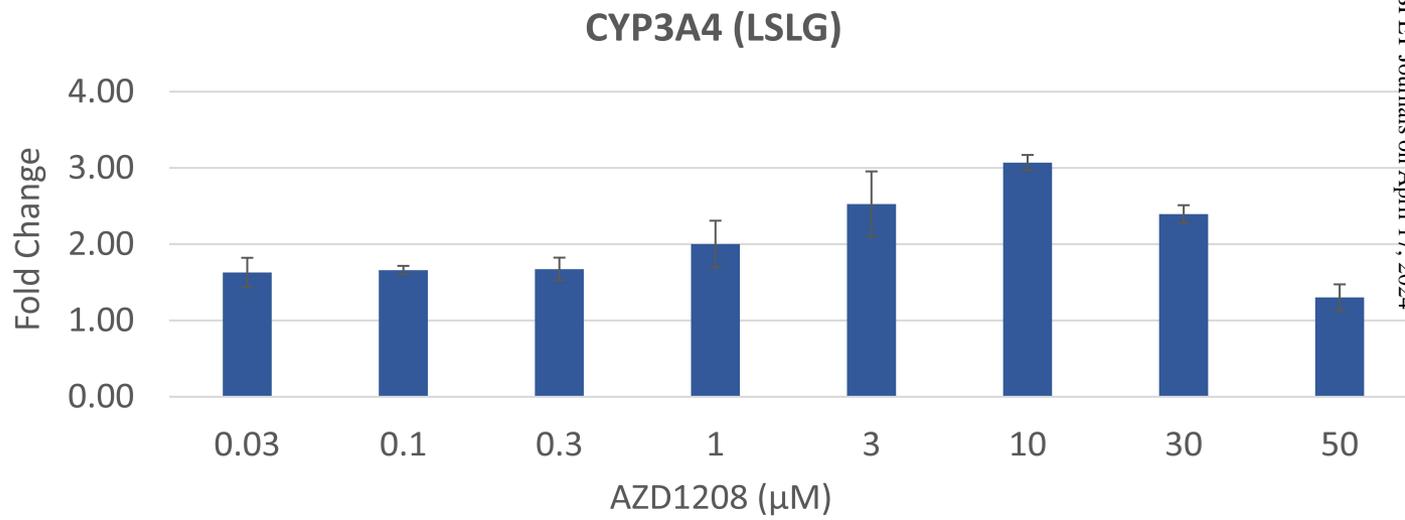
CYP2B6



4)



5)



6)