CYP450 Protein Assay – Human Induction Kit

Quantitation of Cytochrome P450 Protein Isoforms using an AB SCIEX Triple Quad™ or QTRAP® System

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Cytochrome P450 proteins (CYPs) are major drug metabolizing enzymes responsible for phase I biotransformations (Figure 1). For pharmaceutical companies, measurement of CYP induction in response to a drug candidate is a fundamental aspect of assessing drug-drug interactions, and evaluating drug safety and efficacy. There are currently three main methodologies for assessing CYP450 induction: mRNA measurements, enzymatic activity measurements, and Western blot analysis. Although all three are widely used throughout the pharmaceutical industry, there are drawbacks to each.

For example, while CYP induction is normally the result of an increase in gene transcription, some non-transcriptional mechanisms, such as prevention of CYP degradation, can also have a measurable effect on enzyme concentration. Thus, mRNA studies alone can be misleading for understanding increases in protein expression.

Enzyme activity assays measure induction by quantifying the metabolite of a CYP-specific model substrate generated from drug treated hepatocytes. CYP induction from the drug of interest leads to higher enzyme activity and thus more metabolites are generated from the model substrate. Because the method relies on the specificity of enzymatic conversion of model compounds, a different probe compound is required for each CYP isozyme which is not always possible.

Western blotting techniques measure the actual protein levels of CYP450 enzymes. However the high sequence homology of this family of proteins makes generating isoform-specific antibodies difficult. Currently, there are only a few good isoform-specific antibodies available. As a result, multi-bands are routinely detected in Western blotting because of the high cross-reactivity of CYP isoforms.

To overcome these shortfalls and improve the understanding of CYP induction from drug candidates, it is advantageous to directly measure protein expression changes of individual CYP450 isoforms with high specificity, sensitivity, and accuracy. As described here, a kit has been developed for using LC-MS to quantify the protein levels of key CYP450 protein isoforms for induction studies.

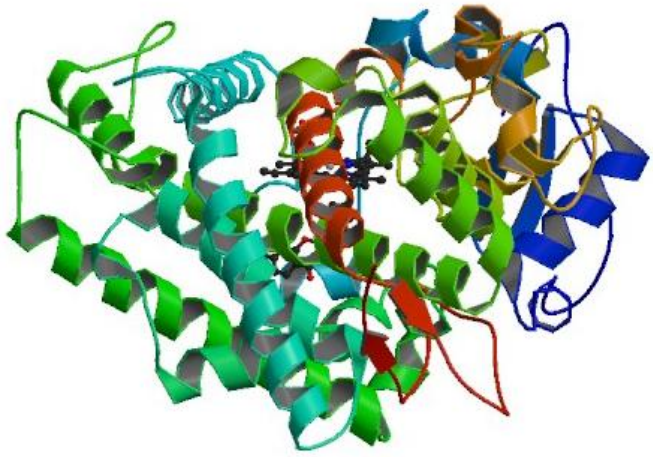

Figure1. Cytochrome P450 1A2 Protein Structure. The cytochrome P450 protein superfamily of mono-oxygenases is the major phase I enzyme system responsible for metabolism of most drugs and other foreign chemicals frequently resulting in the formation of toxic drug metabolites. In higher eukaryotes, there are over 70 families of P450s involved in drug metabolism, comprising over 200 different isoforms, with different substrate specificities and inducible by different drugs or chemicals.

Key Features of the CYP450 Protein Assay – Human Induction Kit

- Enables measurement of CYP 1A2, 2B6, 3A4, and 3A5 isoforms at the protein level using multiple isoform-specific peptides per protein and LC-MS analysis
- Stable isotope labeled peptides are supplied as internal standards for quantitation of each protein isoform
- The assay can be performed using microsomal or S9 fractions from human hepatocytes or cell lines
- Works with current LC-MS/MS instrumentation already used for enzyme activity assays and inhibition studies
- Multiple Reaction Monitoring combined with liquid chromatography separation (LC-MRM) is used in this protein assay to provide very specific detection and quantitation of isoform-specific peptides in a complex cell matrix.

Kit Contents

Starter Kit Content

- Stable isotope labeled peptide standards for four CYP450 isoforms: 1A2, 2B6, 3A4, and 3A5 (several peptides for each isoform)
- Peptide C18 column (2.1 mm x 5 cm)
- All sample preparation buffers and reagents (for denaturation, reduction, alkylation, trypsin digestion and dilution)
- Sample preparation protocol
- Acquisition methods
- Data processing and report templates

User Supplied Content

- MultiQuant™ Software1.2
- Microsoft Excel Software 2007
- AB SCIEX Triple Quad™ 5500 System, QTRAP® 5500 System, API 5000™ System, API 4000™ System, or 4000 QTRAP® System
- Analyst® Software (version 1.5 or above preferred)
- LC System (kit designed to be used with high flow chromatography, but method can be adapted for lower flow)

Protocol

The kit was designed to work with standard workflows typically employed for induction studies. For example, following enzyme activity assays or mRNA assays from a 96 well or 24 well plate, the same cells can be prepared for LC-MRM protein analysis (Figure 2).

- S9 or microsomal subcellular fractions are prepared by lysing the cells and performing one or more steps of centrifugation.
- For best results, 2 x 10e6 cells per analysis are recommended (250 ug total protein). Samples may be pooled.
- Each sample is reduced, alkylated, and digested.
- Time of sample prep is 2 hours plus over night for tryptic digestion.
- Samples are analyzed using the QQQ or QTRAP® System.
- Time of LCMS analysis is 15 minutes per sample using conventional HPLC analysis.
- Data is processed with MultiQuant™ Software and Microsoft Excel.
- Protein expression changes automatically computed and graphically displayed.

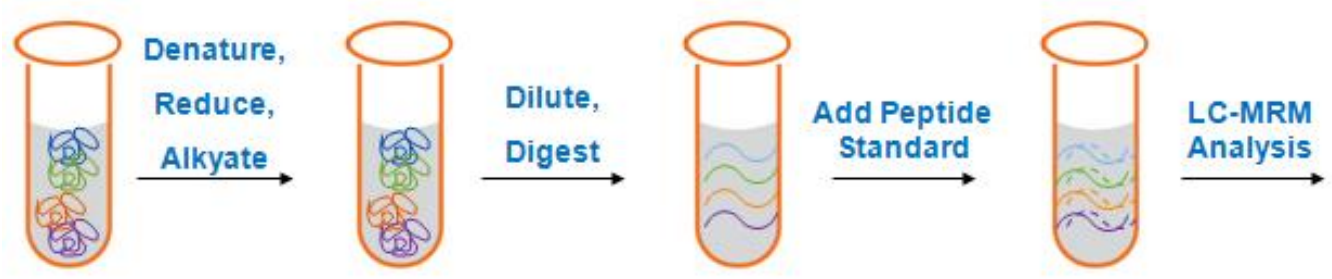

Figure 2. Simple Sample Preparation Protocol. Protein fraction is denatured, then disulfide bridges are reduced and alkylated. Solution is diluted to reduce the denaturant concentration before protein digestion. Trypsin is added and the proteins are digested overnight. The stable isotope labeled peptide internal standards (heavy peptide solution) are added and sample is submitted for LC-MRM analysis.

CYP450 Peptide Standards

The kit contains several peptide standards for each protein, synthesized using isotopically enriched amino acids, lysine and arginine. Peptides and MRM transitions where chosen based upon several criteria:

- Uniqueness of peptide sequence to a specific isoform
- Consistent and reproducible digestion behavior with no multiple K and R in series (which can create ragged ends)
- Minimum number of residues that are prone to chemical and post-translational modification
- Good MS ionization and gas phase fragmentation
- Chromatographic reproducibility
- No interferences in MRM transitions

An example of a typical LC-MRM analysis of the chosen peptide standards from CYP isoforms 1A2, 2B6, 3A4 and 3A5 is shown in Figure 3.

Microsomal preparations from incubations of hepatocytes with prototypical inducers were analyzed using the CYP450 Protein Assay – Human Induction Kit. Figure 4 shows the MRM response for Peptide 1 from CYP 1A2 for the analysis of control (a) and 3-MC induced (b) samples . As is shown, a large increase in the peptide can be observed clearly indicating induction of this isoform by 3-MC. The protein level results for all CYP450 proteins are summarized in Figure 5.

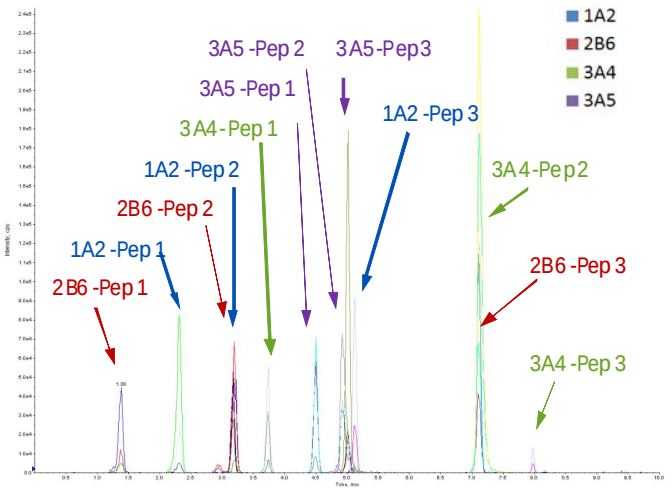

Figure 3. Typical LC-MRM Analysis of CYP450 Peptide Standards. Several peptides per protein are analyzed using acquisition methods created with the Scheduled MRM™ Algorithm3 to maintain quantitative robustness. Good chromatographic resolution and peak shape were obtained for all peptides.

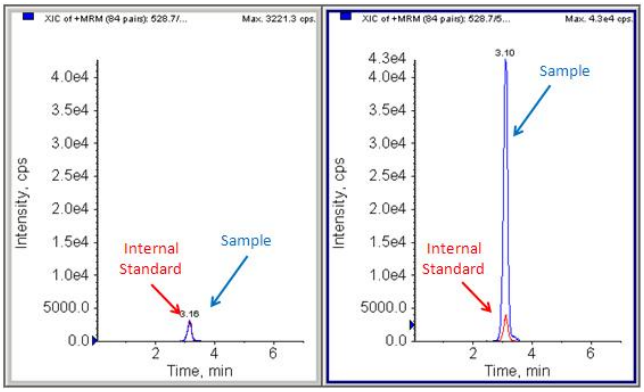

Figure 4. Example Raw MRM Data from Peptide 1 from CYP 1A2 from Microsomes Induced with 3-Methylcholanthrene. The MRM ratio to the internal standard heavy peptide is computed and this ratio of increase in expression to the negative control (Left) is compared between samples (Right).

Software for Data Processing

Once the acquisition is complete, data is processed with MultiQuant™ Software. The software automatically integrates all the MRM data and computes area ratios to the added peptide internal standards for all samples. Processed area ratio results are exported and imported into a Microsoft Excel template for computation of protein level data. Results can be plotted in a simple bar chart to compare the increase in expression of each CYP450 isoform in each sample as compared to the negative controls (Figure 5).

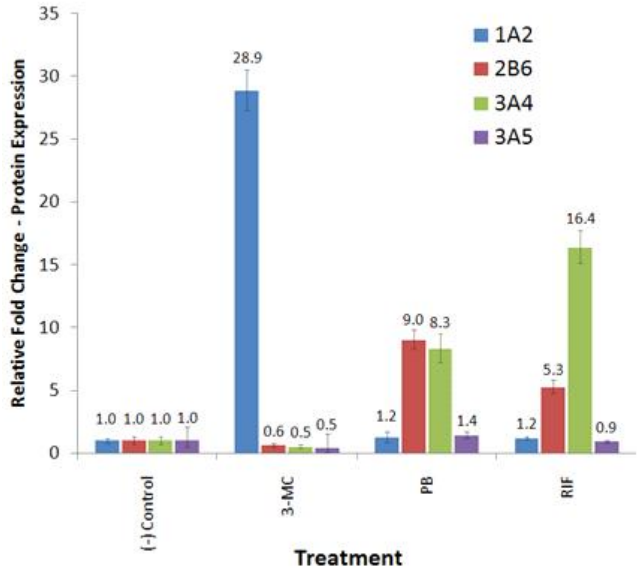

Figure 5. Expression Changes of the Four CYP Isoforms Measured by LCMS. 3-MC (2 uM) is the prototypical inducer of CYP1A2 by AhR nuclear receptor activation. PB (1000 uM) is the prototypical inducer of CYP 2B6 through activation of CAR nuclear receptor but also shows significant induction of 3A4. RIF (10 uM) is the prototypical inducer of CYP 3A4 by PXR nuclear receptor activation but is also known to induce 2B6.

Comparison with Other Assays

The correlation between enzymatic activity, mRNA expression and the protein expression measured by LC-MS/MS was analyzed in a seperate induction study (different donor) using the same prototypical inducers of the CYP proteins (Figure 6: 2 uM 3-MC, 1000 uM PB, 10 uM RIF). The protein levels in both the baseline and induced human hepatocytes were easily detected by the LC-MRM protein quantitation method. The observed changes agreed with expected results for incubation of hepatocytes with these known inducers. Overall, good correlation between total protein increase, enzyme activity and mRNA levels were observed. Further induction studies are planned to elucidate the source of any differences in the degree of induction observed between the different methods.

The results obtained with LC-MRM analysis agree with previous studies using Western blot analysis to study induction of these CYP isoforms in human hepatocytes (Figure 7).

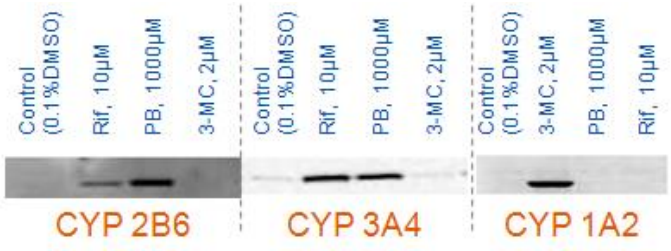

Figure 7. Typical Western Blot Data from an Induction Study. Typical Western Blot induction data illustrates the induction of: CYP 2B6 via RIF and PB, CYP 3A4 via RIF and PB and CYP 1A2 via 3MC.

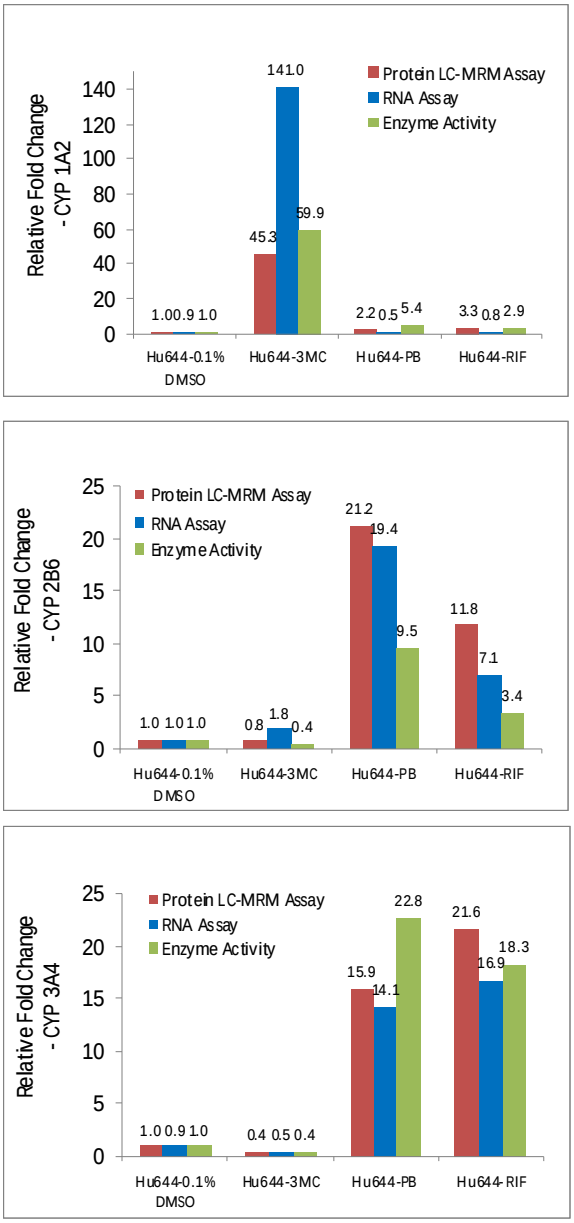

Figure 6. Comparing Protein, mRNA and Enzyme Activity in the Induction Controls. Protein expression changes measured by LC-MRM analysis agree with the changes observed in mRNA assays and enzyme activity assays. (Top) CYP 1A2 showed induction by 3-MC as expected . (Middle) CYP 2B6 was induced by both PB and RIF. (Bottom) CYP 3A4 was also induced by both PB and RIF.

Conclusions

A Cytochrome P450 protein assay kit for human CYP induction analysis is now available for measuring induction on human samples. The kit contains peptide standards, sample preparation reagents and buffers, a column, data acquisition methods, and processing methods and templates for performing protein quantitation using LC-MRM analysis on an AB SCIEX Triple Quad™ or QTRAP® system. Correlation between enzymatic activity, mRNA expression, and protein expression measured by LC-MRM was observed following treatment of human hepatocytes with the prototypical CYP inducers. Both the baseline and inducible levels of the CYP isoforms were easily detected by the LC-MRM protein quantitation method. This method allows for a direct measurement of CYP protein levels on the same analytical platform as the enzymatic assays are measured, replacing the need for Western blot analysis.

References

1. MultiQuant™ Software for Protein / Peptide Quantitation: the Gold Standard for Quantitation. AB SCIEX Technical Note, 2010, Publication 0921210-01.
2. To download a trial version of MultiQuant Software please visit: http://licensing.absciex.com/download/MultiQuant/2.0/
3. Scheduled MRM™ Algorithm Enables Intelligent Use of Retention Times During Multiple Reaction Monitoring. AB SCIEX Technical Note, Publication 0921010-01.

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