Development of a High-Throughput CYP Induction Assay by mRNA Measurement in Human Primary Hepatocytes

Abstract
Cytochrome P450 (CYP) enzymes play a major role in the metabolism of drugs and can be induced by many xenobiotics. To minimize drug interaction liability, understanding CYP induction potential of a new molecular entity (NME) is critical. Recent research (Fahn S, et al., 2010) demonstrates that measurement of CYP isoform mRNA levels offers greater sensitivity and reliability in detection of CYP induction compared to enzyme activity. The FDA guidance (2012) recommends that the potential of CYP1A2, CYP2B6 and CYP3A4 induction by a NME should be evaluated by assessing the mRNA fold induction of the respective CYP isoforms. In this study we validated a CYP induction assay which measures CYP1A2, CYP2B6, and CYP3A4 mRNA levels in a high-throughput format using 24 drugs with known CYP induction properties in primary human hepatocytes. The standard reference compounds, omeprazole (CYP1A2), phenobarbital (CYP2B6), rifampin (CYP3A4), and flumazenil (negative control) were used in this study. The CYP fold induction generated by reference compounds was not significantly different between the two methods: omeprazole 19.8 vs 20.5, phenobarbital 40.7 vs 34.0, and rifampin 64.2 vs 36.3.

Methods
- Day 1: Cryopreserved human primary hepatocytes from 3 donors (Bioloreclamation IVT, Westbury, NY) were thawed and plated onto collagen I-coated 96-well plates at a density of 0.7 x 10^6 viable cells/mL. The cells were incubated overnight before compound addition.
- Day 2-4: The medium was replaced with each test compound solution in triplicates. The incubation was continued for 3 days with the replacement of freshly made testing solutions daily.
- Day 5: The medium was removed and the hepatocytes were washed with PBS. The hepatocytes were lysed and the cell lysate was used as the mRNA template and was reverse transcribed into cDNA. The relative expression (fold difference over vehicle control) of each isoform is determined by singleplex (SYBR Green) two-step RT-qPCR using CYP1A2, CYP2B6 and CYP3A4 sequence specified primers, respectively, and GAPDH as the reference gene. The qPCR reactions were assembled by two methods:
  - Manual Method: Manually transfer cDNA and qPCR reagents to 96-well qPCR plates (n=2)
  - Automated Method: Automation using the Janus® Automated Liquid Handler (Perkin-Elmer, Waltham, MA) to transfer cDNA along with SYBR green primer assay mix to 384-well qPCR plates (n=2)
- The threshold cycle (CT) is measured for each isoform cDNA by real time qPCR. The mRNA level of each isoform is assessed by the relative quantification method with reference gene (GAPDH) as the normalizer. The normalized fold induction is calculated by 2-ΔΔCT (Livak method, 2001) with the 1.0% DMSO used as the reference (vehicle control).
- Assay acceptance criteria:
  - CT >=35 from the No Template Control (NTC, no cDNA template to indicate false positive)
  - CT >=35 from no-RT (no DNA polymerase to control genomic DNA contamination)
  - The precision between the Ct values for the two qPCR replicates of the same cDNA sample is ± 1Ct
  - Each amplicon should have a single peak in the melt curve and the melting temperature should be same as theoretical melt temperature.

Validation of 96-Well CYP Induction mRNA Assay

Figure 1. Dose response effect of known inducers of each CYP enzyme on two lots of hepatocytes. The reference compounds: Omeprazole-CYP1A2, Phenobarbital-CYP2B6, and Rifampin-CYP3A4.

Figure 2. Time dependent response of the reference compounds for each enzyme. The compounds are freshly added once a day for 3 days.

Table 1. The maximum amount of compound one full time employee (FTE) can run in one week is 40. The results show that using the automated method cuts the turn around time in half when compared to the manual method.

Conclusion
- The study demonstrates that there is equivalence between manual preparation of 96-well qPCR plates and automation preparation of 384-well plates.
- The turn around time is drastically reduced by running the assay using automation in 384-well qPCR plates.
- The 384-well automated assay has a robust and high throughput method to assess CYP induction in drug-drug interaction studies.

References