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

Abstract #P91

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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 (Fahmi et al., 2010)1 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)2 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 isozymes. 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), rifampicin (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 78.4 vs 82.8, phenobarbital 40.7 vs. 34.0, and rifampicin 45.9 vs. 36.3 by manual and automation methods, respectively. The throughput of the automated assay drastically improved when compared with the manual method. The results from this study demonstrate the robustness and reliability of an automated, high throughput method for evaluating CYP induction liability.

Methods

- Day 1: Cryopreserved human primary hepatocytes from 3 donors (Bioreclamation IVT, Westbury, NY) were thawed and plated onto collagen I-coated 96-well plates at a density of 0.7 x 106 viable cells/mL. The cells were incubated overnight before compound addition.
- Day 2-4: The medium was replaced with each test compound testing 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 for RT-qPCR. 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)3 method, in which the 0.1% DMSO is 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 <= 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

Dose Dependent CYP Induction in Two Hepatocytes Lots

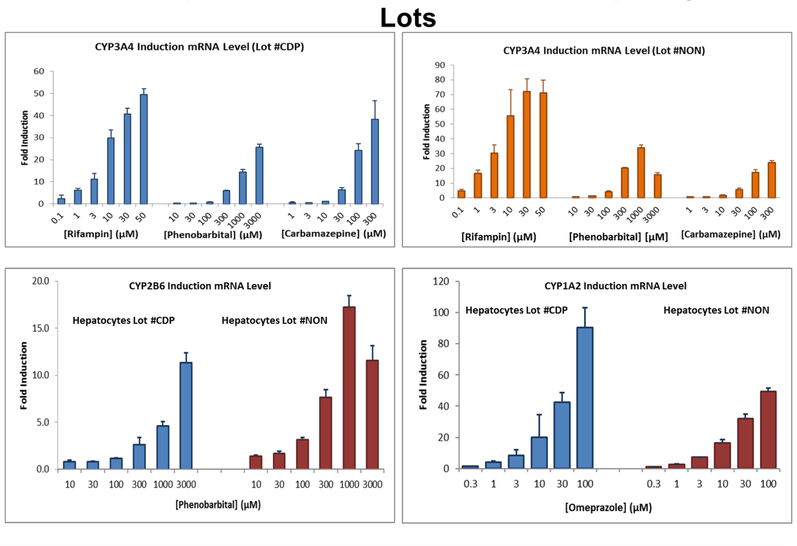


Figure 1. Dose response effect of known inducers of each CYP enzyme on two lots of hepatocytes. The reference compounds: Omeprazole-CYP1A2, Phenobarbital-CYP2B6, and Rifampin-CYP3A4.

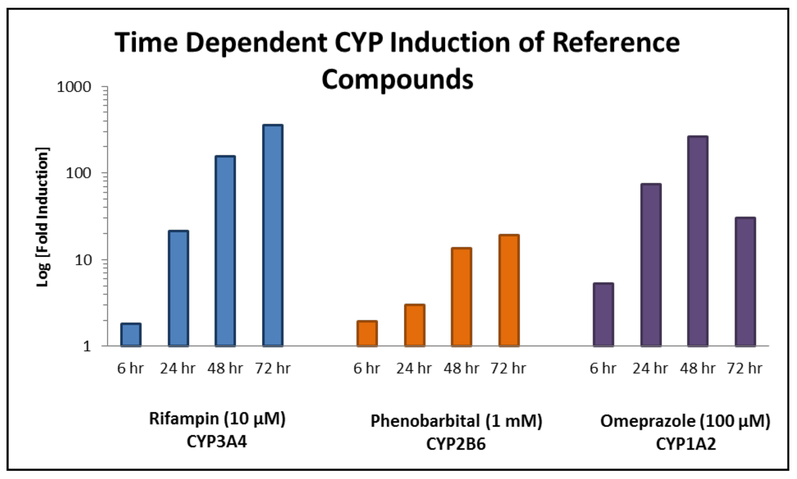


Figure 2. Time dependent response of the reference compounds for each enzyme. The compounds are freshly added once a day for 3 days.

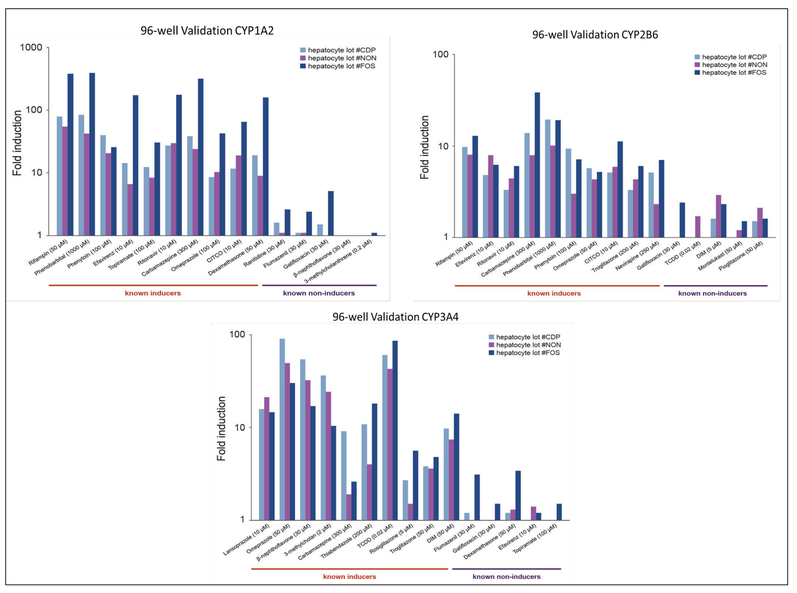
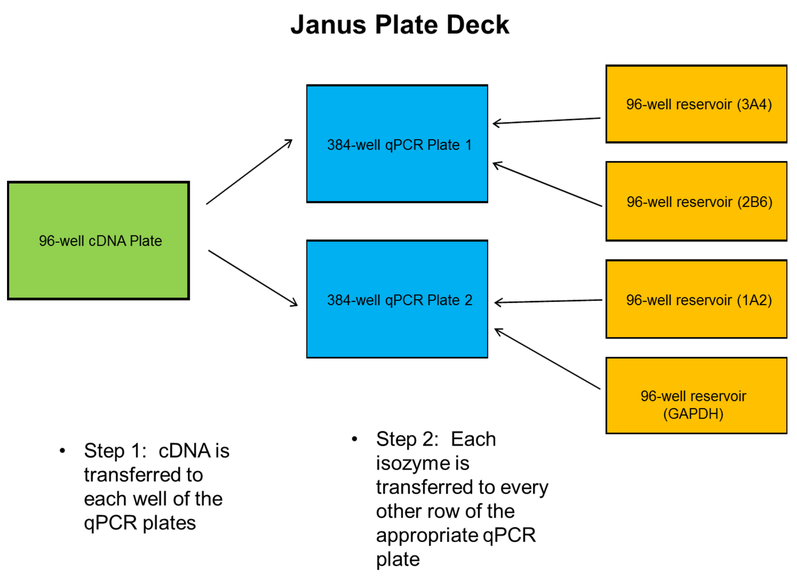


Figure 3. 96-well format was validated of each lot of hepatocytes using 10 known inducers and 5 known non-inducers for each enzyme. The cut off values for each enzyme of was determined based on the fold induction of the reference compounds.

Automated 384-Well Transfer Method



- Step 1: cDNA is transferred to each well of the qPCR plates
- Step 2: Each isozyme is transferred to every other row of the appropriate qPCR plate

Figure 4. The Janus liquid handling system is used to transfer cDNA and SYBR green assay mix into two 384-well qPCR plates. Step 1: cDNA is transferred to each well of the two 384-well plates. Step 2: each isozyme is transferred into every other row of one of the 384-well plates.

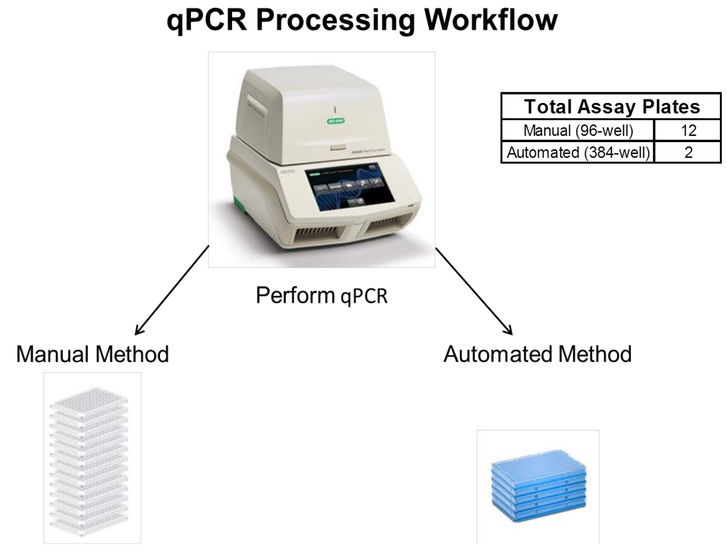
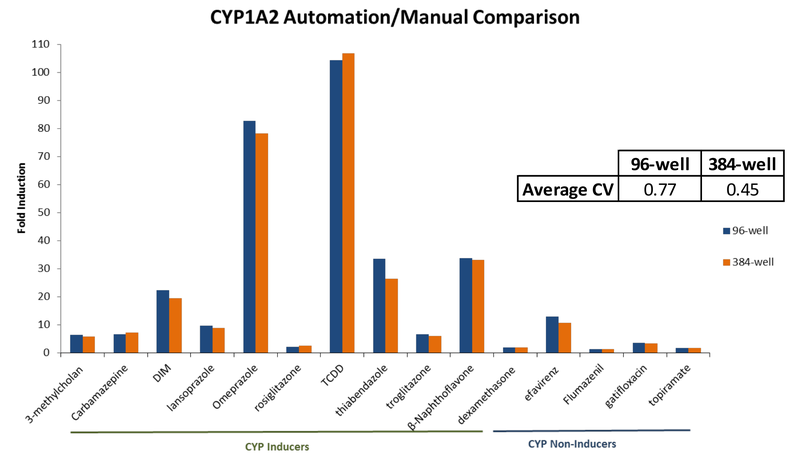
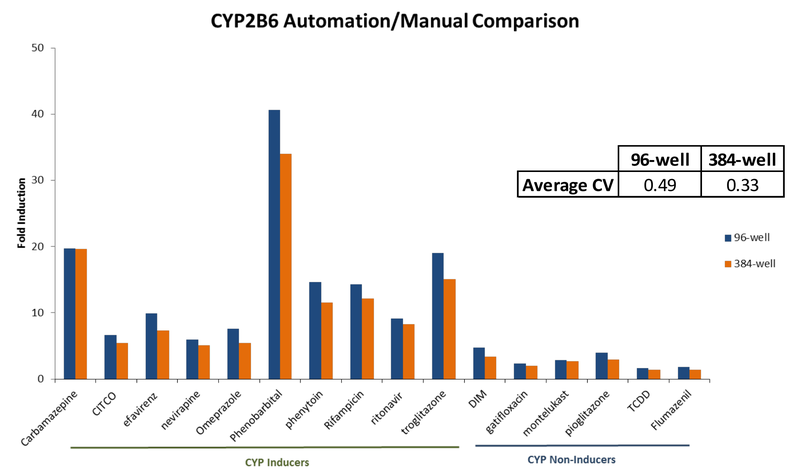


Figure 5. The manual method of qPCR on 96-well plates will require 12 total plates per donor, while the automated method only requires a total of 2 plates.

Results





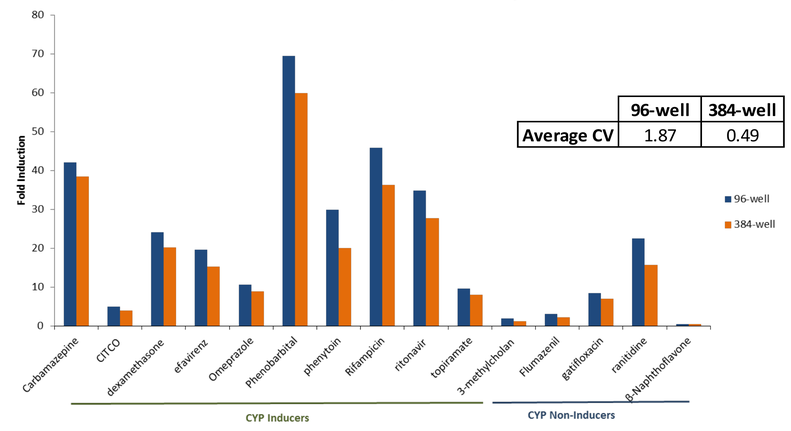


Figure 6. The results of CYP Induction were compared from 1 donor for all 3 enzymes. The qPCR for the manual and automated method where prepared from the same cDNA samples. The fold induction was calculated and compared between each method. There is no significant difference in the results between each method.

Time Savings (1 donor)

# of Compounds	# of qPCR plates		Time (hrs)		Total Savings (hrs)
	96-well	384-well	96-well	384-well	
1	4	2	8	4	4
2	4	2	8	4	4
3	8	2	16	4	12
4	8	2	16	4	12
5	8	2	16	4	12
6	8	2	16	4	12
7	12	2	24	4	20
8	12	2	24	4	20

- Using automation gives a maximum throughput of 40 compounds/week (3 donors) with a turn around time of 14 days per FTE
- This is compared to the manual method which would have a turn around time of 29 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 qPCR plates
- The turn around time is drastically reduced by running the assay using automation in 384-well qPCR plates
- The 384-well automated qPCR assay is a robust and high throughput method to assess CYP induction in drug-drug interaction studies

References

1. Fahmi OA, Kish M, Boldt S, and Oback RS, "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, 2010.

2. FDA (2012) Guidance for Industry: Drug Interaction Studies-Study Design, Data Analysis, Implications for Dosing, and Labeling Recommendations.

3. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta)Ct methods. Methods 25: 402–408, 2001.

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