A multiplex approach to investigate drug-induced changes in p450 enzyme gene expression

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Introduction

Drug metabolizing enzymes and transporter induction can result in clinically meaningful drug interactions. Therefore, it is important to identify potential drug interactions early in the drug development process. Both p450 gene and enzyme induction studies are helpful in identifying p450-inducing compounds. Measuring gene expression has traditionally relied on RNA extraction from treated hepatocytes followed by RT-qPCR. An alternative, potentially more efficient method for measuring gene induction in this setting is the automated multiplex HTG Edge system and HTG Edge chemistry, quantitative nuclease protection assay (qNPA).

To measure E_{max} and EC_{50} values, as well as fold changes in response to treatment, a single donor lot of primary human hepatocytes was dosed for 48 hours with ten (10) different compounds at varying concentrations. Expression levels of p450 enzymes were measured using the HTG Edge system and HTG Edge chemistry, as well as traditional RT-qPCR. Dose response curves for each compound and testing method were generated using fold changes of treated cells vs. vehicle control treated cells. Concentration-dependent changes in gene induction were assessed for CYP1A2, CYP2B6, CYP3A4, CYP2C8 and CYP2C9 using both methods. Correlation of the dose response curves between the two methods support the use of the HTG Edge multiplex assay for measuring p450 enzyme induction in hepatocytes. The multiplex capabilities of the HTG Edge chemistry support the use of the HTG Edge system as a more efficient alternative to RT-qPCR.

Cell Culture & Treatment

Primary hepatocytes, donor lot DQB, (BioreclamationIVT) were thawed in pre-warmed InVitroGro CP medium (BioreclamationIVT) completed with antibiotics and plated into 96 well plates at a density of 50,000 cells per well. Plates were washed 4 and 24 hours later with InVitroGro CP medium (BioreclamationIVT). Dosing of each compound (Sigma-Aldrich) occurred for 2 days starting 48 hours after plating. On day 5, the medium was aspirated, the appropriate lysis buffer was added RLT buffer (Qiagen) for RT-qPCR and HTG lysis buffer (HTG) for HTG Edge system, and the plates were frozen.

Compounds were selected based on their induction potency for the p450 enzymes being assessed. Table 1 summarizes the expected potency for the noted p450 enzyme. Treatment concentrations by compound are shown in Table 2.

Table 1. Compound induction potential for specific p450 enzymes

CYP Gene	Potent Inducer (+++)	Moderate Inducer (++)	Weak Inducer (+)	Negative Inducer (-)
CYP1A2	3-MC	BNF	OME	FLU PB RIF
CYP2B6	PB EFV	PHY RIF NVP	CMZ TGZ SIM	FLU
CYP3A4	RIF PB	TGZ PHY SIM	EFV CMZ NVP	FLU
CYP2C8	RIF PB	TGZ PHY SIM	EFV CMZ NVP	FLU
CYP2C9	RIF PB	TGZ PHY SIM	EFV CMZ NVP	FLU

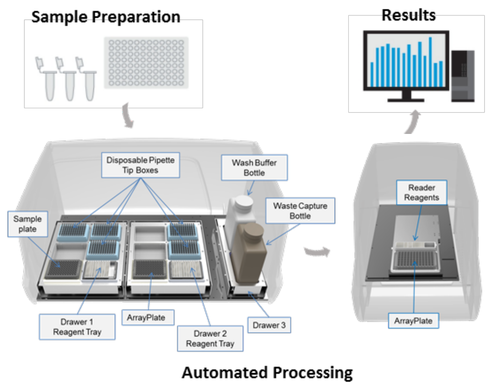
Table 2. Compound dosing range

Compound Name	# Points	Concentration Range
3-MC: 3-methylcholanthrene	8	0.05 - 6.4 µM
BNF: β-naphthoflavone	8	0.1 - 50 µM
CMZ: Carbamazepine	8	0.1 - 150 µM
EFV: Efavirenz	8	0.1 - 20 µM
NVP: Nevirapine	8	0.5 - 75 µM
OME: Omeprazole	11	0.1 - 75 µM
PHY: Phenytoin	8	0.1 - 50 µM
PB: Phenobarbital	11	1 - 1000 µM
RIF: Rifampin	11	0.01 - 50 µM
SIM: Simvastatin	8	0.1 - 20 µM
TGZ: Troglitazone	8	0.5 - 75 µM

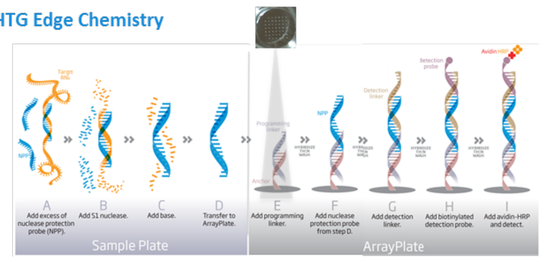
HTG Edge system & chemistry

The cell lysate plates were removed from -80°C storage and thawed at room temperature. The sample plate was loaded onto the HTG Edge processor along with the HTG Edge DMPK Comprehensive Assay reagents and other required consumables (HTG). Once the qNPA reaction was complete on the HTG Edge processor, the arrayplate (HTG) was subsequently loaded onto the HTG Edge reader with the required chemiluminescent substrates (HTG). Raw and normalized gene expression data for each p450 enzyme and sample were provided by the HTG Edge host software. Fold change was calculated by dividing the mean normalized expression value of a single compound concentration by the mean normalized expression value for the vehicle control (0.1% DMSO) for each p450 enzyme.

HTG Edge System



HTG Edge Chemistry



RT-qPCR

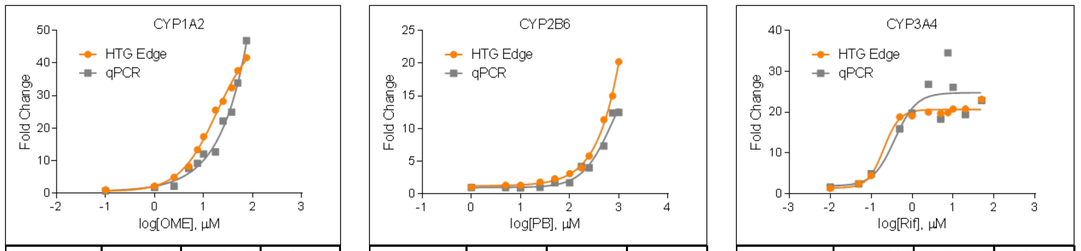
The cell lysate plates were removed from -80°C storage and thawed at room temperature. The RNA was isolated using an RNeasy Kit (Qiagen) according to manufacturer's instructions. Eluted RNA was quantified using a Nanodrop spectrophotometer (ThermoFisher). Reverse transcription (RT) was performed with the High Capacity cDNA Reverse Transcriptase Kit with RNase inhibitor (Life Technologies) using an ABI 9700 thermocycler. Subsequently, quantitative PCR analysis was performed on RT reactions using gene specific primer/probe sets for CYP1A2, CYP2B6, CYP2C8, CYP2C9, or CYP3A4 target cDNA and endogenous control (GAPDH). Samples were analyzed on an ABI 7900HT instrument. Relative-fold mRNA content was determined based on threshold cycle (CT) data of target gene relative to endogenous control for each reaction, and normalized to vehicle control.

Conclusions

- HTG Edge system & HTG Edge DMPK Comprehensive assay demonstrates comparable results to RT-qPCR in the prototypical compounds and appropriate p450 enzymes.
- Dose response data from HTG Edge system & chemistry generates smooth sigmoidal curves as demonstrated with tightness of data to curves.
- Complexity of RT-qPCR creates inherit variability in data as demonstrated by dispersal of data points relative to sigmoidal dose response curve.
- CYP2C8 & CYP2C9 data with HTG Edge system illustrates clear trends of induction, while RT-qPCR data is inconclusive.
- The multiplex capabilities of the HTG Edge chemistry support the use of the HTG Edge system as a more efficient alternative to RT-qPCR.

Results

Figure 1. Prototypical inducers consistent in HTG Edge system and RT-qPCR with primary target p450 enzymes



OME - CYP1A2	E_{MAX} (Fold)	EC_{50} (µM)	Slope
HTG Edge	49.4	18.8	1.135
RT–qPCR	NC	NC	0.773

NC = Not Calculated

PB - CYP2B6	E_{MAX} (Fold)	EC_{50} (µM)	Slope
HTG Edge	NC	NC	1.061
RT–qPCR	20.8	730.4	1.442

NC = Not Calculated

RIF - CYP3A4	E_{MAX} (Fold)	EC_{50} (µM)	Slope
HTG Edge	20.64	0.203	2.24
RT–qPCR	24.76	0.377	1.643

Figure 2. Concentration-dependent effects more discernible with HTG Edge system due to "fit" of data to sigmoidal curve

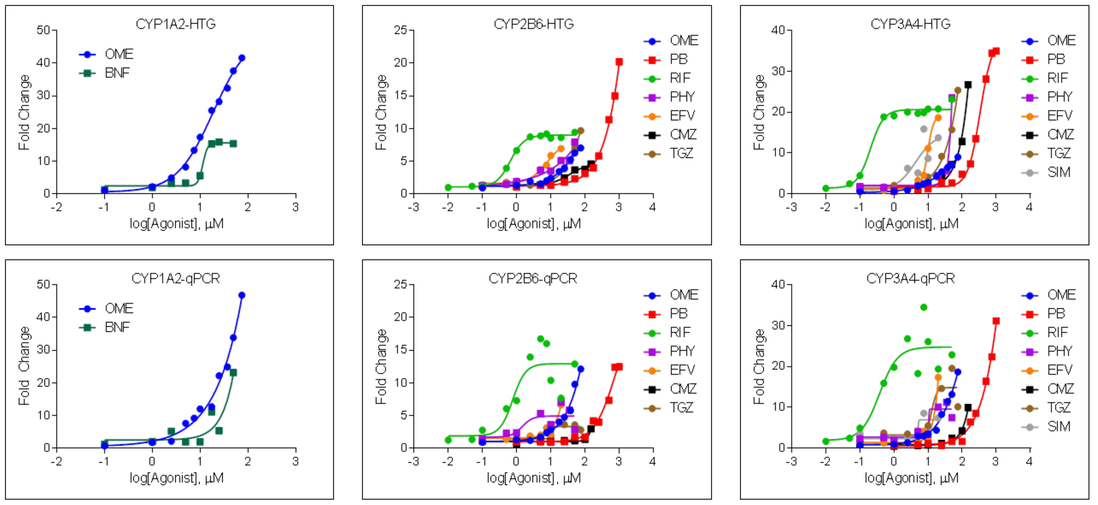


Figure 3. CYP2C data inconclusive with RT-qPCR

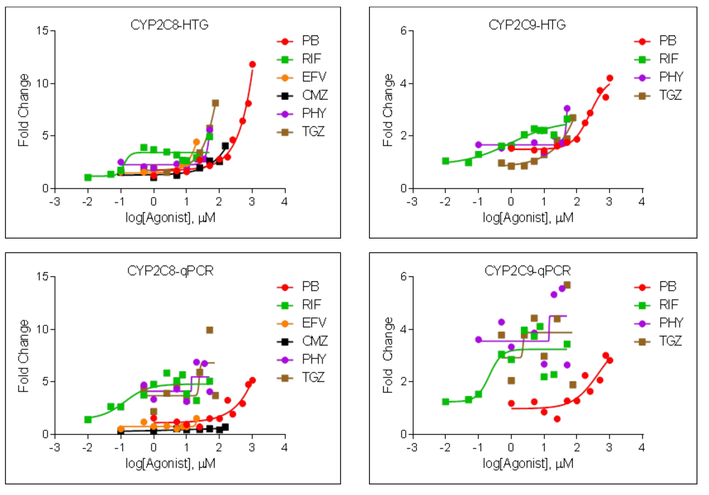
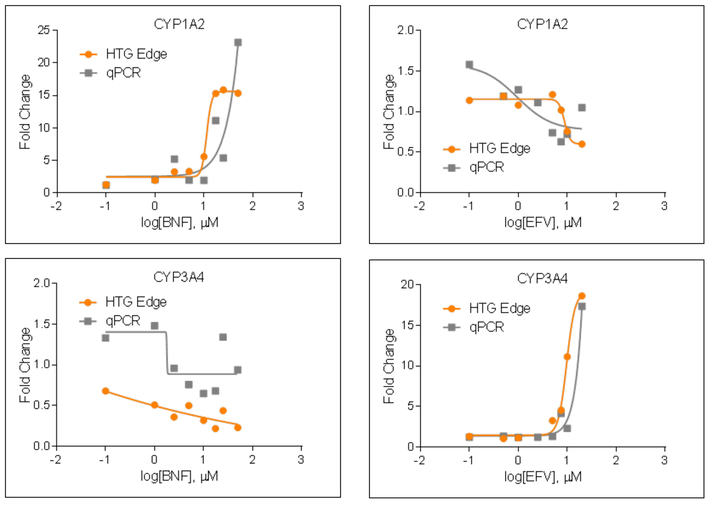


Figure 4. Expected p450 enzyme up/down regulation demonstrated



Data Analysis

Dose response curves were generated by plotting the fold change (y-axis) against the concentration of the compound tested (x-axis). EC_{50} and E_{max} for each parameter for CYP induction response were calculated using the Sigmoidal Hill 4-Parameter equation (also known as four-parameter logistic equation) using GraphPad Prism 6.05. The equation used was:

$$Y = a + \frac{(b - a)}{1 + 10^{(\log EC50 - X) - HillSlope}}$$

where: a = Y value at the bottom plateau
b = Y value at the top plateau (E_{max})
LogEC50 = X value 50% between a & b
HillSlope = steepness of the curve

Table 3. Flumazenil negative in all CYP's

Flumazenil 25µM - Fold Change				
p450 Enzyme	Plate Ctrl 1	Plate Ctrl 2	Plate Ctrl 3	Plate Ctrl 4
CYP1A2	0.58	0.64	0.81	0.78
CYP2B6	0.82	0.99	0.93	0.79
CYP3A4	0.48	0.52	0.33	0.43
CYP2C8	0.72	0.80	0.69	0.67
CYP2C9	0.73	0.48	1.03	0.74

References

1. US FDA Draft Guidance for Industry: Drug Interaction Studies (2012)
2. Fahmi OD, et al. Drug Metab Dispos 36:9 (2008)