

Application Note D100 | Echo Liquid Handler

# High throughput Miniaturization of Cytochrome P450 Inhibition Screening Using the Echo<sup>®</sup> Liquid Handler

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## Abstract

Early assessment of ADMET properties (absorption, distribution, metabolism, excretion and toxicity) of drug candidates has become an essential component of modern drug discovery screening to select the overall best scaffolds or leads to the pre-clinical candidate stage. One of the most important ADMET characterizations is the interaction between drug candidates and various human cytochrome P450 enzymes. Cytochrome P450 enzymes are the largest group of drug-metabolizing enzymes and their potential interactions with drug candidates could lead to idiosyncratic toxicity. CYP3A4 is the most abundant cytochrome P450 enzyme in the liver and plays a major role in metabolizing xenobiotics. The Promega P450-Glo<sup>™</sup> CYP3A4 Assay (Luciferin-IPA) offers a sensitive, specific and high-throughput luminescence assay for the examination of CYP3A4 inhibition. Here, we demonstrate the miniaturization and optimization of the P450-Glo<sup>™</sup> Assay with pooled Human Liver Microsomes (HLM) using the Echo 555 liquid handler.

## Experiment 1

### Introduction

The Echo 500 series of liquid handlers revolutionize liquid transfer by transferring fluids using focused acoustic energy. Non-contact transfer avoids the risk of cross-contamination and eliminates tip costs as part of a biochemical screen. Echo liquid handlers precisely and accurately transfer 2.5 nanoliter droplets which enables the miniaturization of many biochemical assays. This application note demonstrates transfer of enzymes, buffers, microsomes, inhibitors and substrates in small volumes, providing a sensitive and robust assay. Comparable  $IC_{50}$  values for four known CYP3A4 inhibitors are presented.

### Comparison between manual and acoustic transfer methods

The P450-Glo™ CYP450 Screening System from Promega Corporation was used as a test assay. The assay has a mix-and-read format that is amenable to a high-throughput screening methodology. In brief, pooled HLM, compound(s) of interest, a luciferin-labeled cytochrome P450 substrate, and a cofactor (NADPH) are transferred in a step-wise manner with brief incubation periods. The amount of light generated by cleavage of the substrate is directly proportional to cytochrome P450 activity. Results were compared between manual transfer of reagents and transfer with the Echo 555 liquid handler.

### Materials

- InVitro CYP H-Class 10-Donor Pooled Human Liver Microsomes, Mixed Gender (X008061, Celsis IVT)
- P450-Glo™ CYP3A4 Assay with Luciferin-IPA (V9001, Promega Corporation)
- GloMax®-Multi+ Detection System (Promega Corporation)
- 384-well, Echo® qualified polypropylene microplates (P-05525, Labcyte Inc.)
- 384-well, low volume microplates (3674, Corning Life Sciences)

### Methods

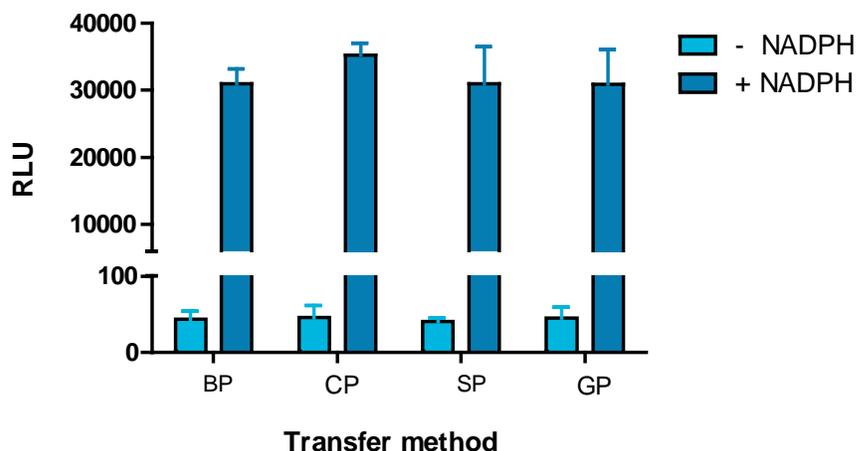
*Manual method:* HLM was diluted to 0.05 mg/mL in potassium phosphate buffer. NADPH regeneration system was made according to the P450-Glo™ assay protocol. 5  $\mu$ L HLM was pipetted into a 384-well assay plate. The assay was initiated with the addition of 5  $\mu$ L Luciferin-IPA in potassium phosphate buffer or NADPH regeneration system. CYP reaction was incubated at 37°C for 15 minutes. 10  $\mu$ L detection reagent was added after CYP reaction and incubated for 20 minutes. Light output was monitored on the GloMax®-Multi+ Detection System.

*Echo liquid handler method:* HLM was diluted to 5 mg/mL in potassium phosphate buffer. 40  $\mu$ L HLM at 5 mg/mL was added to a 384-well polypropylene Echo qualified microplate. 50 nL was transferred with an Echo 555 system to a 384-well low volume microplate containing 10  $\mu$ L potassium phosphate buffer. The reaction was initiated by the manual addition of 5  $\mu$ L Luciferin-IPA at 16  $\mu$ M in either potassium phosphate buffer or NADPH regeneration system. The CYP reaction was incubated at 37°C for 15 minutes. 10  $\mu$ L detection reagent was manually added after CYP reaction and incubated for 20 minutes. Light output was monitored on the GloMax®-Multi+ Detection System.

### Results

NADPH-dependent CYP3A4 activity was demonstrated with both manual and Echo transfer methods. Results from both experiments demonstrated essentially identical activity and signal/background ratios (Figure 1). The percent CV from both manual and Echo transfer are 10% and 8%, respectively. The results indicated that the Echo 555 liquid handler successfully transferred HLM.

**Figure 1 ▶**  
CYP3A4 activity comparison between the Echo 555 liquid handler and manual transfer.



## Experiment 2

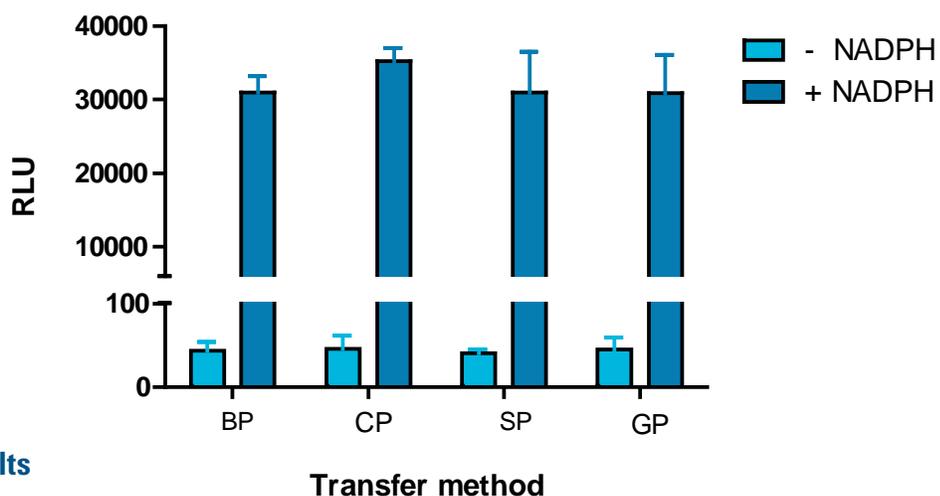
### Miniaturization of CYP inhibition assay in a low volume 384-well format

To miniaturize the CYP inhibition assay, the other reagent components were tested for transfer with the Echo liquid handler. 500 nL of HLM was transferred from a 384-well Echo qualified source plate into a 384-well low volume plate. Four Echo aqueous fluid types for Omics were tested to dispense HLM. The CYP reaction was initiated by addition of 500 nL of Luciferin-IPA which was also transferred by the Echo 555 liquid handler. CYP3A4 activity was compared among the various fluid types.

### Methods

HLM at 1 mg/mL diluted in phosphate buffer was transferred into a 384-well Echo qualified polypropylene source plate (Labcyte Inc.). Luciferin-IPA diluted to 16  $\mu$ M in both phosphate buffer and NADPH was also transferred into the same 384-well Echo qualified polypropylene source plate. The Echo 555 liquid handler was then used to transfer 500 nL of the HLM and 500 nL of Luciferin-IPA to a 384-well low volume plate (Corning Life Sciences). Four different fluid types (384PP\_AQ\_BP, 384PP\_AQ\_CP, 384PP\_AQ\_SP and 384PP\_AQ\_GP) were tested to choose the best and most efficient transfer scheme. The enzyme and substrate mixture was incubated for 10 minutes at 37°C. At the end of the incubation, 2  $\mu$ L detection reagent was added manually to the reaction and incubated for 20 minutes. Light output was monitored with the GloMax<sup>®</sup>-Multi+ Detection System.

**Figure 2 ▶**  
CYP3A4 activity comparing reagent transfer utilizing fluid types on the Echo 555 liquid handler. Blue is signal, light blue is background.



### Results

The P450-Glo™ assay was miniaturized to a 1 µL total assay volume in a 384-well plate format. The pooled HLM showed sensitive and NADPH-dependent CYP3A4 activity with Luciferin-IPA. HLM were transferred successfully with all four fluid types tested with similar precision and signal/background ratios (Figure 2). The 384PP\_AQ\_SP fluid type was used for further optimization and IC<sub>50</sub> determination as it has the fastest transfer rate.

## Experiment 3

### IC<sub>50</sub> determination of four reference compounds

To validate the miniaturized P450-Glo™ assay, IC<sub>50</sub> values of four control compounds were determined. The IC<sub>50</sub> value generated in the miniaturized format by the Echo liquid handler was compared to previously published IC<sub>50</sub> values.

### Materials

- In Vitro CYP H-Class 10-Donor Pooled Human Liver Microsomes, Mixed Gender (X008061, Celsis IVT)
- P450-Glo™ CYP3A4 Assay with Luciferin-IPA (V9001, Promega Corporation)
- Ketoconazole (Toronto Research Chemical A187500)
- Mibefradil (Sigma M5441)
- Mifepristone (Sigma M8046)
- Verapamil (Sigma V4629)
- GloMax®-Multi+ Detection System (Promega Corporation)
- 384-well, Echo qualified polypropylene microplates (P-05525, Labcyte Inc.)
- 384-well, low volume microplates (3674, Corning Life Sciences)

### Methods

Compounds were first dissolved in DMSO and transferred into a 384-well polypropylene Echo qualified microplate. Three intermediate concentrations were generated using Echo® Dose-Response software. Compounds from source and intermediate plates were transferred with the Echo 555 platform to create 10-point dose response curves. The assay plate was backfilled with DMSO to achieve the same percentage of organic solvent in all wells. The final DMSO concentration was 0.75%. Seven IC<sub>50</sub> replicates for each compound were generated in the same assay plate. Pooled HLM was diluted in potassium phosphate buffer. Luciferin-IPA was diluted in both potassium phosphate buffer and NADPH regeneration system. 40 µL HLM and substrate were first transferred to a 384-well Echo qualified source plate. 500 nL Luciferin-IPA was then transferred to the assay plate using the 384PP\_AQ\_SP fluid type, followed by transfer of 500 nL HLM using the same 384PP\_AQ\_SP with the Echo 555 platform. The reaction was incubated for 10 minutes at 37°C, and then 2 µL P450-Glo™ detection reagent was added manually to the mixture and incubated for 20 minutes. Light output was monitored with the GloMax®-Multi+ Detection System.

**Table 1 ▶**

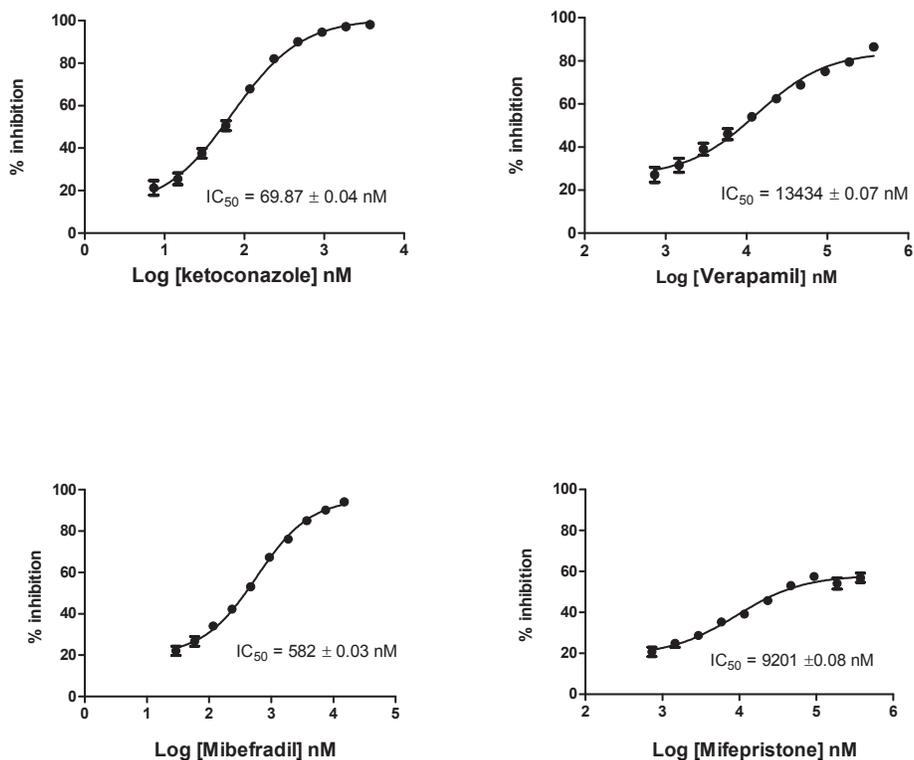
Comparison of IC<sub>50</sub> values determined in a miniaturized format (with the Echo 555 system) and previously published values

<i>Compound</i>	<i>Labcyte IC<sub>50</sub> (µM)</i>	<i>Literature IC<sub>50</sub> (µM) (Source)</i>
<b>Ketoconazole</b>	0.070 ± 0.04	0.0037 - 0.18 (FDA) 0.036 - 0.06 (Promega)
<b>Verapamil</b>	13.4 ± 0.07	10, 24 (FDA)
<b>Mibefradil</b>	0.58 ± 0.03	0.16 (Novartis) 0.8 (Vanderbilt University)
<b>Mifepristone</b>	9.2 ± 0.08	1.08 (Novartis) 4.7 (U. Michigan)

## Results

Results from the miniaturized P450-Glo™ CYP3A4 assay inhibition study are consistent with published literature values (Figure 3 and Table 1). IC<sub>50</sub> curve reproducibility was maintained despite the small scale of the volume delivery and the overall 1 µL assay volume.

**Figure 3 ▶**  
CYP3A4 IC<sub>50</sub> curves generated for four test compounds utilizing the Echo 555 liquid handler



## Summary

ADMET assays as demonstrated by the P450-Glo™ assay can be miniaturized to 1 µL utilizing the Echo liquid handler. Human Liver Microsomes were transferred with the same precision and accuracy as seen in higher volume assays. Generation of IC<sub>50</sub> values that are equivalent to published values can be obtained while saving on compounds and reagents. The Echo Dose-Response software can be incorporated into the assay design for ease of use of compound dispenses and generation of intermediate plates. A miniaturized P450-Glo™ assay has increased utility in screening ADMET profiles at earlier stage in the drug discovery process.

The authors wish to thank Tracy Worzella and Kevin Kershner from Promega Corporation for their technical support and providing the P450-Glo™ assays, and Ji Young Lee from Celsis In Vitro Technologies for the scientific assistance and providing Human Liver Microsomes.