A Miniaturized and Automated P450-Glo™ Screening System Using the Echo® Liquid Handler

Jing Wang[1], Joseph Barco[1], Tracy Worzella,[2] and Kevin Kershner[2]

[1] Labcyte Inc., Sunnyvale, CA, USA www.labcyte.com [2] Promega Corporation, Madison, WI, USA www.promega.com

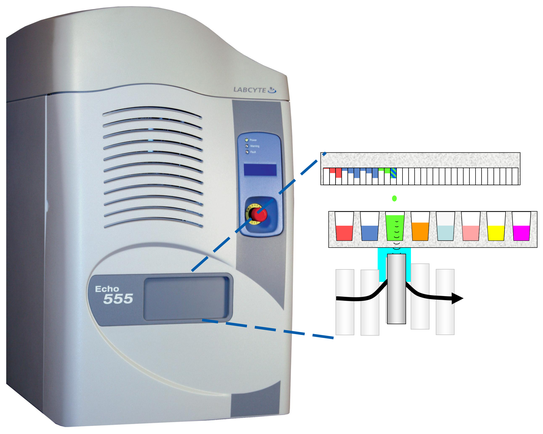
Abstract

Early assessment of cytochrome P450 (CYP) inhibition has become an essential component of drug discovery screening. The CYP3A4 enzyme is the most abundant enzyme in the liver and plays a major role in metabolizing many xenobiotics in humans. The Promega P450-Glo™ CYP3A4 Assay (Luciferin-IPA) offers an extremely sensitive, high throughput and specific luminescence assay for the examination of CYP3A4 inhibition with pooled Human Liver Microsomes. The Echo 555 liquid handler provides precise and accurate acoustic transfer free of cross-contamination risk. Here, we demonstrate optimization of the P450-Glo™ Assay with Human Liver Microsomes (HLM) in low volume format using the Echo 555 liquid handler. We show that the Echo 555 liquid handler can transfer microsomes, inhibitors and substrate in nanoliter volumes, providing a sensitive and robust small scale assay. IC_{50} results for four known CYP3A4 inhibitors using our miniaturized assay compared favorably to previously reported values in the literature.

Introduction

The Labcyte Echo 500 series revolutionizes liquid transfer by using acoustic energy to eject fluids. The Echo 500 series allows for assay miniaturization to previously unattainable volumes. Echo liquid handlers transfer 2.5 nL droplets repeatedly, so precision and accuracy are consistent over a larger volume range. Large volume transfer is achieved by transferring several hundred droplets per second. Transfer is non-contact and tipless, with increased cost savings from elimination of tip costs and washing fluids.

Miniaturization with the Echo liquid handler retains high assay performance, allowing quantitative results at higher assay well densities. The Echo liquid handler can be used to transfer from any source well position to any destination well position. These can be simple fluids (media for growing cells, buffers, DMSO) or viscous solutions (lysis buffers, antibodies with glycerol, or transfection reagents).



Absorption, Distribution, Metabolism, Excretion and Toxicity (ADMET) properties of drug candidates have been widely adopted as an integral part of a modern drug discovery for compound advancement and clinical candidate selection. They are critical factors in predicting pharmacokinetic behavior and drug-drug interaction potentials. One of the most important ADMET assays is inhibition of cytochrome P450 enzymes. It is inevitable that a large number of compounds will be screened against CYP enzymes. The ability of miniaturization of ADMET assays using the Echo liquid handler can be a valuable tool in drug discovery research.

P450-Glo™ Screening Assay

A fast, sensitive and easy to use assay to measure P450 activity

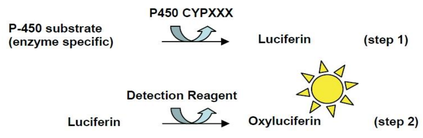


Figure 1. The P450-Glo™ assay was performed by incubating compounds with a luminogenic labeled P450 substrate, cytochrome P450 enzyme, and NADPH Regeneration System. CYP enzymes convert luciferin isopropyl acetal (Luciferin-IPA) to luciferin. Upon completion of the P450 reaction, detection reagent was added containing luciferase and ATP. These react with the luciferin generated from P450 reaction to produce light. The amount of light detected was directly proportional to the amount of cytochrome P450 activity.

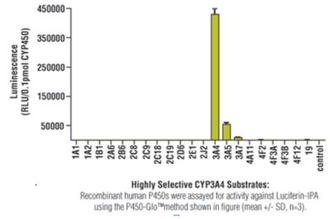


Figure 2. Luciferin-IPA is a CYP3A4 specific substrate.

CYP3A4 activity in Human Liver Microsomes using P450-Glo™ screening system

The purpose of this experiment was to evaluate the feasibility using HLM with Luciferin-IPA in a low volume 384-well format.

Methods

Pooled HLM (Celsis, IVT) at 20 mg/ml was diluted to 0.1 and 0.2 mg/mL in potassium phosphate buffer. Luciferin-IPA was also diluted in potassium phosphate buffer and NADPH regeneration system to 16 μM. 5 μL HLM at 0.1 and 0.2 mg/mL was first added manually into a 384-well low volume plate. The reaction was initiated by addition of 5 μL Luciferin-IPA. The reaction was incubated for 10 minutes at 37ºC. At the end of incubation, 10 μL P450-Glo detection reagent was added to the mixture. Light output was monitored with the GloMax®-Multi+ luminometer.

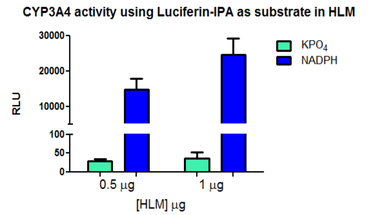


Figure 3. HLM showed NADPH dependent CYP3A4 activity with Luciferin-IPA as the substrate.

Results

Data demonstrated that pooled HLM showed sensitive and NADPH-dependent CYP3A4 activity with Luciferin-IPA as the substrate.

CYP3A4 activity comparison between Echo transfer and manual transfer

To test the ability of the Echo liquid handler to directly transfer HLM into a 384-well plate.

Methods

Pooled HLM (Celsis, IVT) at 20 mg/mL was diluted to 5 mg/mL in potassium phosphate buffer. Luciferin-IPA was also diluted in potassium phosphate buffer and NADPH regeneration system. 40 μL HLM at 5 mg/mL was first added to a 384-well, polypropylene Echo qualified source plate. 50 nL of HLM was then transferred from the source plate by the Echo 555 into a 384-well low volume assay plate containing 5 μL potassium phosphate buffer. Control wells of 5 μL HLM at 0.05 mg/mL were manually pipetted. The reaction was initiated by the addition of 5 μL diluted Luciferin-IPA for both experiments. The P450 reaction was incubated for 10 minutes at 37ºC, then 10 μL of P450-Glo detection reagent was added to the mixture. Light output was monitored on the GloMax®-Multi+ luminometer.

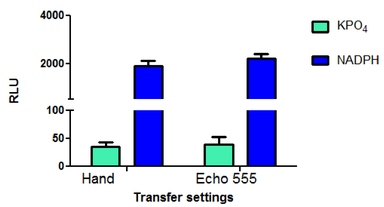


Figure 4. HLM transferred by the Echo 555 had similar CYP3A4 activity compared to manual transfer.

Results

CYP3A4 showed NADPH-dependent activity from both manual transfer and transfer by the Echo liquid handler. Enzymatic activity from Echo-transferred and hand-pipetted samples were similar, despite the more concentrated transfer of HLM by the Echo liquid handler.

Miniaturization of P450-Glo™ assay

Goal: to develop a miniaturized P450 assay with HLM in a 384-well plate.

Methods

Pooled HLM was diluted in potassium phosphate buffer to 1 mg/mL. Luciferin-IPA was diluted in both potassium phosphate buffer and NADPH regeneration system to 16 μM. 40 μL HLM and 40 μL Luciferin-IPA in either potassium phosphate buffer and NADPH were added to a 384-well, polypropylene Echo qualified source plate. 500 nL of HLM was then transferred by the Echo 555 into a 384-well low volume plate. The reaction was initiated by the addition of 500 nL IPA in either NADPH regeneration system or potassium phosphate buffer with the Echo 555. The reaction was incubated for 10 minutes at 37ºC, then 2 μL luciferin detection reagent was added to the reaction. Light output was monitored with the GloMax®-Multi+ luminometer.

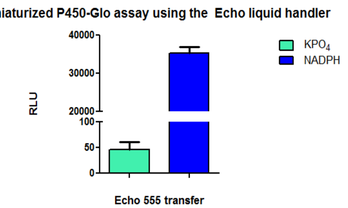


Figure 5. HLM showed NADPH-dependent CYP3A4 activity in a miniaturized P450-Glo™ format.

Results

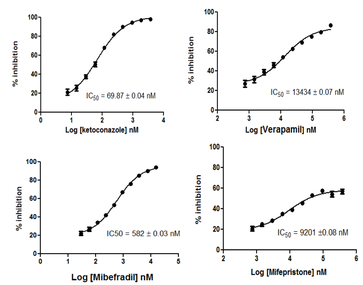
The P450-Glo assay works properly in a small scale format in 384-well plates. The HLM showed sensitive and NADPH dependent CYP3A4 activity, making it a suitable target in the miniaturized format assay.

CYP compounds profiling in the miniaturizing assay format

To validate the miniaturized P450-Glo™ assay, IC_{50} curves were generated for four control compounds. The IC_{50} concentrations generated by the Echo liquid handler were compared to previously published IC_{50} concentrations.

Methods

Control compounds were first dissolved in 100% DMSO and transferred into a 384-well, polypropylene Echo qualified plate. Three intermediate concentrations were generated using Echo® Dose-Response software. 7.5 nL of compound from the source plate and the three concentrations from the intermediate plate were transferred to create 10-point dose response curves with the Echo 555. DMSO was backfilled to achieve the same percentage of organic solvent in all wells. Seven replicates for each compound were performed 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. 500 nL Luciferin-IPA was first transferred to the assay plate containing compounds, then 500 nL HLM with Echo 555. The reaction was incubated for 10 minutes at 37ºC, then 2 μL P450-Glo detection reagent was added to the mixture. Light output was monitored with the GloMax®-Multi+ luminometer.



Compound	Labcyte IC_{50} (μM)	Literature IC_{50} (μM) / (Source)
Ketoconazole	0.069 ± 0.04	0.0037 - 0.18 (FDA) 0.036 - 0.06 (Promega)
Verapamil	13.4 ± 0.07	10, 24 (FDA)
Mibefradil	0.58 ± 0.03	0.16 (Novartis) 0.8 (Vanderbilt)
Mifepristone	9.2 ± 0.08	1.08 (Novartis) 4.7 (U. Michigan)

Figure 6. Compound inhibition curves were generated in the miniaturized assay format. IC_{50} values generated in miniaturized assay were comparable with published literature values.

Results

The data showed the miniaturized P450-Glo™ assay provided inhibition data that were consistent with published literature values. IC_{50} curve reproducibility was maintained despite the small scale of the volume delivery and the overall assay volume.

Summary

- Successfully miniaturized the P450-Glo™ assay to a 1 μL total reaction volume in a 384-well format.
- Transferred HLM with the Echo liquid handler.
- A miniaturized P450-Glo™ assay has increased utility in screening ADMET profiles in an earlier stage in the drug discovery process.
- Compounds and other reagents can be transferred efficiently using Echo® applications software.