Echelon Biosciences Inc.

Lysosomal Phospholipase A2 (LPLA2) Inhibitor Screen

K-7000I (384 tests)

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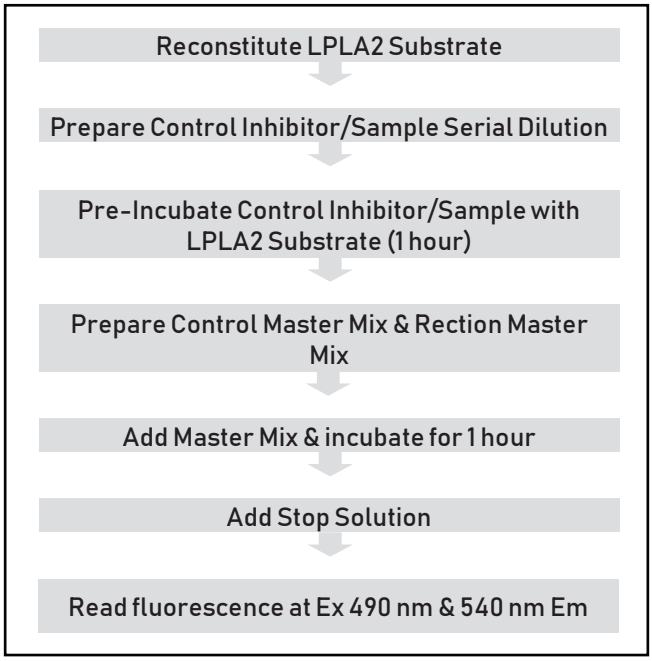
Description: LPLA2 Inhibitor Screen (K-7000I) is a homogeneous assay designed to screen molecules that potentially trigger phospholipidosis (PL) through inhibition of LPLA2.

Materials Provided

Catalog #	Description	Quantity
K-7001I	Human LPLA2 (Lyophilized)	1 vial
K-7002I	LPLA2 Substrate (Dried)	1 vial
K-7003I	4X Reaction Buffer	1 bottle
K-7004I	Control Inhibitor – Amiodarone (1 µmol)	1 vial
K-7005I	5X Stop Solution	1 bottle
K-DIL7	Diluent	1 bottle
---	384-well Low Volume Black Plate	1 plate
---	Microtiter Plate Seals	2 seals

Quick Protocol



Additional Materials Provided by User

- Pipettes (capable of delivering between 5 and 1,000 µL solution with appropriate tips)
- Reagent grade water
- Dimethyl sulfoxide (DMSO)
- (optional) plate shaker
- Microplate reader capable of reading with 490 nm excitation & 540 nm emission (see assay note #1)

Storage

Upon receipt, store assay kit at -20 °C. Opened and reconstituted reagents are less stable. Please see assay notes for additional information.

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Background

The human lysosomal phospholipase A2 (LPLA2) enzyme is responsible for normal lipid metabolism. It is unique from other known PLA2s in that LPLA2 is only active in an acidic environment such as the lysosome (~pH 4.5). Furthermore, it demonstrates negligible activity at pH 7.4 which is normally found for the cytosol. In addition, LPLA2 acts as a transacylase with C2-ceramide as a preferred lipophilic acceptor.[1] Research has shown LPLA2 to be involved in drug-induced phospholipidosis (DIPL).[2] Phospholipidosis (PL) is a condition resulting from the excessive accumulation of intracellular phospholipids, causing tissue inflammation and organ damage. PL commonly manifests in patients taking cationic amphiphilic drugs (CADs) such as fluoxetine (Prozac™, Sarafem) and Amiodarone. The FDA has determined DIPL a serious drug safety issue.[3] Although the complete cause of DIPL is unknown, evidence is accumulating that DIPL is the result of certain CADs directly inhibiting the actions of the lysosomal phospholipase A2 (LPLA2). Thus, LPLA2 inhibition is a potential predictor of drug-induced phospholipidosis.

Assay Design

Echelon's LPLA2 Inhibitor Screen (K-7000l) is designed to screen a drug's ability to inhibit LPLA2 activity in vitro, a potential predictor of drug-induced phospholipidosis, in a high throughput format (HTS) using "smart probe" technology. A quenched fluorogenic substrate is liberated by LPLA2 activity, resulting in a bright fluorescent product (Figure 1). Therefore, the fluorescent signal is significantly reduced in the presence of an LPLA2 inhibitor. This direct biochemical approach provides a quantitative measurement in a robust and simple to use in vitro plate-based assay, providing a greatly improved throughput when compared to traditional microscopy methods of tissue cultured cells. The assay has been validated with a group of known PL-inducing and non-PL inducing drugs. A known PL-inducing cationic amphiphilic drug (CAD), amiodarone, is included in the assay as a control.

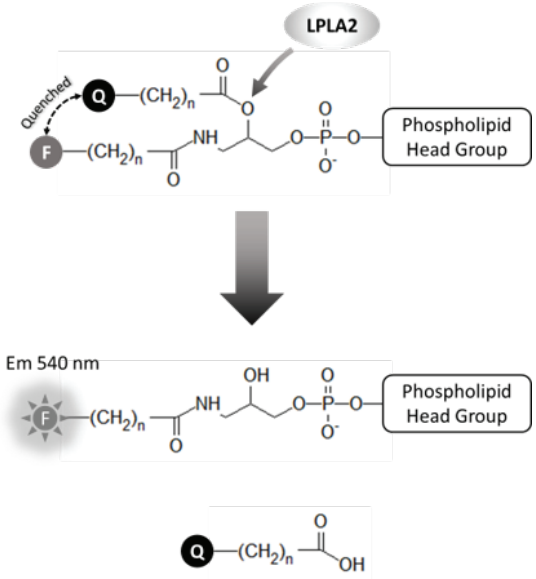


Figure 1. Assay Design
Q = qunecher, F = Fluorophore

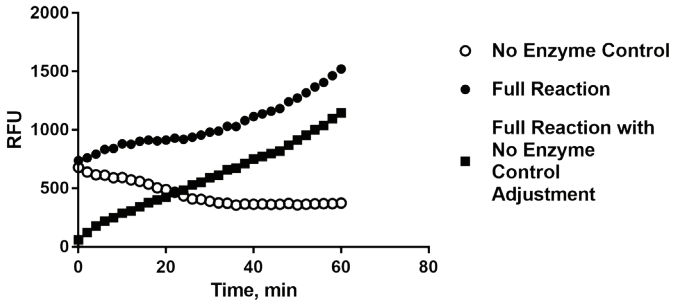
Assay Performance

For best results, please follow the protocols provided. Not following the instructions may result in suboptimal performance of the kit and failure to produce accurate data.

Assay Notes

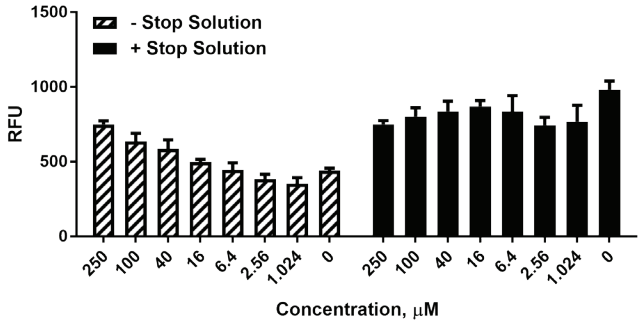
1. The LPLA2 Inhibitor Screen is designed to be run as a one-time use only assay. If the inhibitor screen is to be run on separate occasions, the remaining kit reagents can be stored at -20 °C. However, a reduction in the strength of the fluorescent signal has been observed after freeze-thaw. Always include the Control Inhibitor as a standard to ensure accurate results. DO NOT COMBINE different vials of reconstituted LPLA2 Substrate (K-7002l).
2. If an alternative solvent to DMSO is used in preparing the test compounds, include a "solvent only" control without the test compound in order to detect potential solvent effects. To ensure assay reproducibility, it is advised to use consistent solvent concentrations between experiments.
3. The LPLA2 Inhibitor Screen can also be read in kinetic mode, reading every 5 minutes for 1 hour, using the same excitation/emission settings as step 17. However, photobleaching might occur as demonstrated by the "No Enzyme Control" (Figure 2). Including this "No Enzyme Control" allows one to account for photobleaching and is recommended for data analysis.

Figure 2. Photobleaching



4. Fluorescent interference, including self-quenching and inhibitor related auto-fluorescence, may result in false positives or false negatives when screening. If fluorescent interference is a concern, screening the inhibitor without the LPLA2 enzyme will serve as control. We have observed that the addition of Stop Solution (K-7005l) significantly improves this self-quenching or auto-fluorescence effect when screening drugs (Figure 3).

Figure 3. Clomipramine Background (No Enzyme Controls)



5. For optimal results, we suggest using a consistent pre-incubation time (step 8). Varying the pre-incubation time significantly affects the IC50 of the Control Inhibitor (K-7004l, Figure 4).

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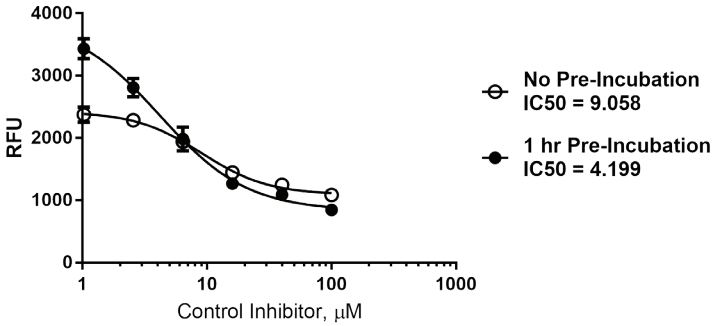
Figure 4. Drug-Substrate Pre-Incubation



Assay Protocol

Reagent volumes can be adjusted when not using the entire assay, please see assay note #1 for more details.

1. Place LPLA2 Enzyme (K-7001I) on ice. Bring the remaining reagents to room temperature (RT).
2. Reconstitute the Control Inhibitor (K-7004I) with 20 µL of DMSO for a 50 mM Stock. Vortex to mix. Keep at RT.
3. Add 2 mL of reagent grade water to the LPLA2 Substrate (K-7002I). VORTEX VIGOROUSLY FOR 5 MINUTES to form liposomes. Keep at RT.
4. Prepare the 7-point Control Inhibitor serial dilutions in micro-centrifuge tubes. See Table 1. Keep at RT and protect from light.

Table 1, Control Inhibitor Dilutions

Standard	1X Control Inhibitor	16X Control Inhibitor	Previous Dilution Needs	DMSO to add
A	100 µM	1600 µM	5 µL of K-7004I	151 µL
B	40 µM	640 µM	30 µL of 1600 µM	45 µL
C	16 µM	256 µM	30 µL of 640 µM	45 µL
D	6.4 µM	102.4 µM	30 µL of 256 µM	45 µL
E	2.56 µM	40.96 µM	30 µL of 102.4 µM	45 µL
F	1.024 µM	16.384 µM	30 µL of 40.96 µM	45 µL
G	0 µM	0 µM	None	45 µL

5. Prepare the compounds to be tested in DMSO at 16X the desired final concentration. See assay note #2 if using a solvent other than DMSO.
6. Add 1 µL/well of the 16X Control Inhibitor serial dilutions (step 4.) and 1 µL/well of the 16X compounds to be tested. For the "No LPLA2" control, add 1 µL/well of DMSO or the solvent of your choice. It is suggested that samples be run in triplicate. A suggested plate layout is shown in Figure 5. See assay note #4 for information on drug auto-fluorescence.
7. Add 4 µL of the LPLA2 Substrate (K-7002I), prepared in step 3, to all wells.
8. Cover the plate with a plate seal and pre-incubate the control inhibitors or test compounds with the LPLA2 substrate for 1 hour at RT with shaking and protected from light. See assay note #5 for more information on pre-incubation.
9. Reconstitute the Human LPLA2 (K-7001I) with reagent grade water with the volume labeled on the vial. GENTLY PIPETTE UP & DOWN AND INCUBATE ON ICE FOR 10 MINUTES to ensure full reconstitution. Keep on ice.
10. Control Master Mix: Prepare 137.5 µL of "Control Master Mix" solution by combining the following in a Microcentrifuge tube:
    a. 50 µL 4X Reaction Buffer (K-7003I)
    b. 50 µL Diluent (K-DIL7)
    c. 37.5 µL Reagent Grade Water
    d. Vortex to mix. Keep at RT.
11. Prepare 1,700 µL of the human LPLA2 by further diluting the reconstituted human LPLA2 (step 9) with Diluent (K-DIL7) according to the dilution factor labeled on the vial. Vortex for 3 seconds to mix. Keep at RT.
12. Reaction Master Mix: Prepare 4,675 µL of "Reaction Master Mix" by combining the following in an appropriate container.
    a. 1,700 µL 4X Reaction Buffer (K-7003I)
    b. 1,700 µL LPLA2 Enzyme diluted in Diluent (step 11.)
    c. 1,275 µL Reagent Grade Water
    d. Vortex to mix. Keep at RT.
13. Following the 1-hour LPLA2 Substrate / Inhibitor pre-incubation step, add 11 µL of the Control Master Mix into the "No LPLA2" control wells. Add 11 µL of the Reaction Master Mix into the remaining wells to start the reactions.
14. Cover the plate with a plate seal and incubate at RT for 1 hour with shaking. Protect from light. If the plate shaker is not available, gently tap plate to mix before incubation. See assay note #3 for

Table 2, Suggested Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	*No LPLA2*			Compound 9			Compound 25			Compound 41			Compound 57			Compound 73			Compound 89			Compound 105		
B	**Standard A**			Compound 10			Compound 26			Compound 42			Compound 58			Compound 74			Compound 90			Compound 106		
C	**Standard B**			Compound 11			Compound 27			Compound 43			Compound 59			Compound 75			Compound 91			Compound 107		
D	**Standard C**			Compound 12			Compound 28			Compound 44			Compound 60			Compound 76			Compound 92			Compound 108		
E	**Standard D**			Compound 13			Compound 29			Compound 45			Compound 61			Compound 77			Compound 93			Compound 109		
F	**Standard E**			Compound 14			Compound 30			Compound 46			Compound 62			Compound 78			Compound 94			Compound 110		
G	**Standard F**			Compound 15			Compound 31			Compound 47			Compound 63			Compound 79			Compound 95			Compound 111		
H	**Standard G**			Compound 16			Compound 32			Compound 48			Compound 64			Compound 80			Compound 96			Compound 112		
I	Compound 1			Compound 17			Compound 33			Compound 49			Compound 65			Compound 81			Compound 97			Compound 113		
J	Compound 2			Compound 18			Compound 34			Compound 50			Compound 66			Compound 82			Compound 98			Compound 114		
K	Compound 3			Compound 19			Compound 35			Compound 51			Compound 67			Compound 83			Compound 99			Compound 115		
L	Compound 4			Compound 20			Compound 36			Compound 52			Compound 68			Compound 84			Compound 100			Compound 116		
M	Compound 5			Compound 21			Compound 37			Compound 53			Compound 69			Compound 85			Compound 101			Compound 117		
N	Compound 6			Compound 22			Compound 38			Compound 54			Compound 70			Compound 86			Compound 102			Compound 118		
O	Compound 7			Compound 23			Compound 39			Compound 55			Compound 71			Compound 87			Compound 103			Compound 119		
P	Compound 8			Compound 24			Compound 40			Compound 56			Compound 72			Compound 88			Compound 104			Compound 120		

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for additional information regarding kinetic analysis.

15. After 1 hour, add 4 µL of the 5X Stop Buffer (K-7005I) to each well to stop the reactions. Incubate at RT in the dark for 15 minutes. Stop Solution must be added prior to fluorescence recording for end-point assay. See assay note #4 for additional information.
16. Record the fluorescence using 490 nm excitation and 540 nm emission settings. See assay note #3 for additional information. additional information regarding kinetic analysis.

References

1. Abe, A.; Gregory, S.; Lee, L.; Shayman, J. A., Use of sulfobutyl ether beta-cyclodextrin as a vehicle for D-threo-1-phenyl-2-decanoylamino-3-morpholinopropanol-relat ed glucosylceramide synthase inhibitors. Anal Biochem 2000, 287 (2), 344-7.
2. Halliwell, W. H., Cationic amphiphilic drug-induced phospholipidosis. Toxicol Pathol 1997, 25 (1), 53-60.
3. Shayman, J. A.; Kelly, R.; Kollmeyer, J.; He, Y.; Abe, A., Group XV phospholipase A(2), a lysosomal phospholipase A(2). Prog Lipid Res 2011, 50 (1), 1-13.

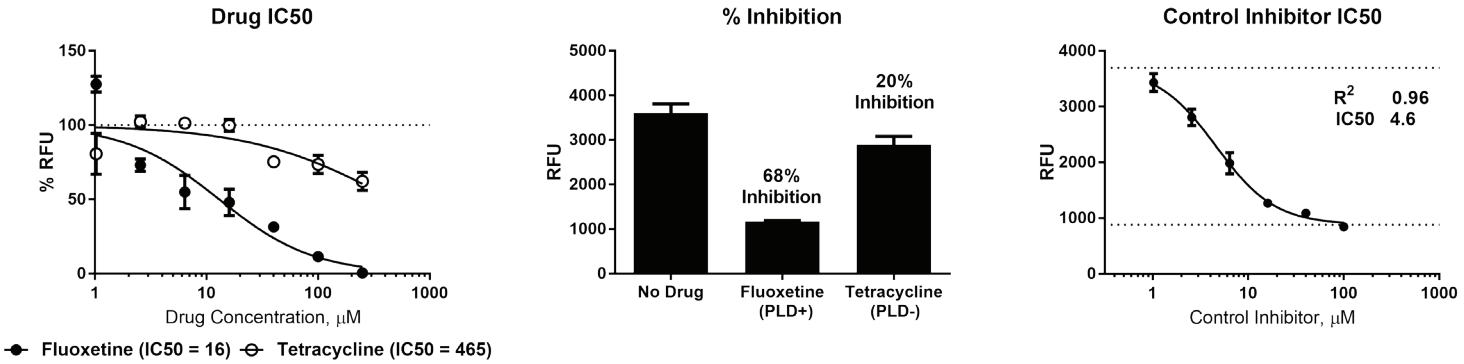

Figure 5. LPLA2 Inhibitor Screen

Examples of the Control Inhibitor's IC50 curve (Left), an end-point inhibitor screen (Middle), and the analysis of the drugs, fluoxetine and tetracycline, and their respective IC50 curves (Right). IC50 is analyzed using the log(agonist) vs. response – variable slope curve fit from GraphPad Software with top & bottom constrained by "No Enzyme" and "No Inhibitor" controls respectively.

Related Products

Products	Catalog Number
Enzyme	
Human LPLA2	E-7000
Antibody	
Purified Anti-LPLA2	Z-PLPLA2
Assay	
LPLA2 Activity Assay	K-7000A
LPLA2 ELISA	Coming Soon

Please visit our website at www.echelon-inc.com for more enzyme and lipid products.

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