Echelon Biosciences Inc.

Autotaxin Inhibitor Screening Kit

K-4200 (96 tests)

Support: echelon@echelon-inc.com

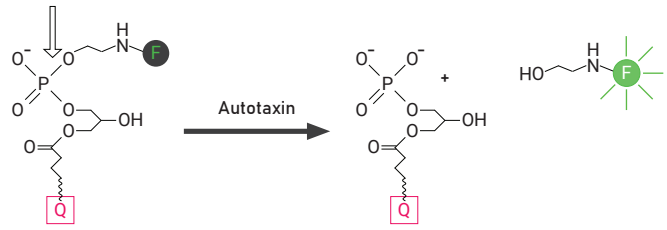
Description: Two-part fluorogenic assay for screening potential inhibitors of autotaxin.

Materials Provided

Catalog #	Description	Quantity
E-4000	Autotaxin enzyme	1 vial
K-4202	Fluorescent autotaxin substrate (FS-3)	1 vial
K-4102	Fluorescein dye	1 vial
K-4104	Buffer A (fluorescein dye suspension buffer)	1 bottle
K-4106	Buffer C (concentrated reaction buffer)	1 bottle
K-4107	Buffer D (protein stabilizer)	1 bottle
K-4204	Inhibitor positive control (BrP-LPA)	1 vial
---	96 well assay plates	2 plates
---	Plate sealer	2 seals

Additional Materials Provided by User:

- Fluorescence plate reader capable of exciting at 485 nm and reading at 528 nm.



Quick Protocol

Prepare buffers and reagents.

Pre-incubate ATX with inhibitor or compound.

Add FS-3 to start ATX reactions and collect kinetic data every minute for 30 minutes as primary screen.

Set up secondary screen with inhibitor, compounds, and fluorescein.

Read secondary screen plate once.

Analyze data.

Storage: Upon receipt, the kit should be stored at -20°C. With proper storage, the kit components should remain stable for at least six months from date of receipt. Do not freeze thaw kit components. Once thawed, use within five hours.

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Background

The phospholipid growth factor lysophosphatidic acid (LPA) activates cell surface receptors to elicit an array of responses that include platelet aggregation, chemotaxis, cytoskeletal remodeling, smooth muscle contraction and stimulation of cell proliferation[1-4]. Long associated with ovarian and breast cancer, LPA has been shown to regulate cancer cell proliferation, invasion, angiogenesis and biochemical resistance to chemotherapy and radiotherapy-induced apoptosis[5,6]. LPA is produced extracellularly largely through the lysophospholipase D (lysoPLD) activity of the enzyme autotaxin (ATX)[7,8]. ATX is a 100 kDa, secreted glycoprotein that is widely expressed with high levels in the serum. The lysoPLD activity of ATX extends beyond its ability to generate LPA from lysophosphatidylcholine (LPC) as it can also hydrolyze sphingosylphosphorylcholine (SPC) to sphinosine-1-phosphate (S1P). This activity, however, is of questionable physiologic significance since the Km of ATX for SPC is three orders of magnitude higher than normal serum levels of SPC[9,10].

ATX levels are elevated in the cerebrospinal fluid of multiple sclerosis (MS) patients[15], but ATX is more widely recognized for its involvement in cancer. ATX expression is increased in melanoma, breast cancer, renal cell cancer, non-small-cell lung cancer, neuroblastoma, hepatocellular carcinoma, glioblastoma multiform and thyroid cancer[16]. ATX expression is closely connected with breast cancer cell invasiveness, and a gene chip analysis found that ATX is among the forty most upregulated genes in highly metastatic cancers[15,16]. The notion that ATX is associated with tumor progression is supported by studies that show ATX-overexpressing Ras-transformed NIH3T3 cells injected into mouse models results in increased invasive and metastatic potential[17]. Further, ATX has been found to stimulate cell motility in several cultured cancer cell lines by increasing the production of LPA[17,18].

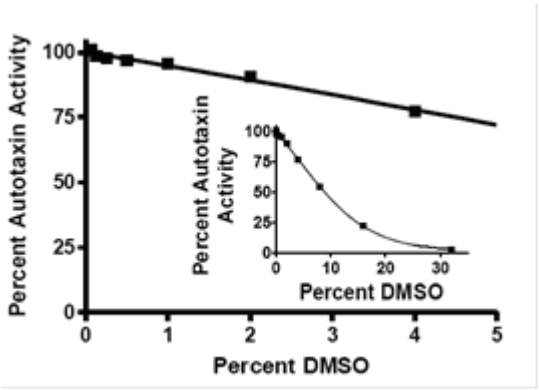
Assay Design

The autotaxin inhibitor screening kit uses the fluorogenic ATX substrate FS-3. Cleavage of FS-3 by ATX, liberates fluorescein from a quencher, resulting in increased fluorescence[19]. The Michaelis constant Km of ATX for FS-3 is 6.3 µM[19]. In the initial screen, recombinant ATX is incubated with sample compounds in a 96 well plate. FS-3 is added subsequently and fluorescein fluorescence is measured over time. This assay is continuous (not end point) and has the advantage of being less susceptible to error arising from mistiming the addition of reagents to different sample wells. A secondary screen is performed to evaluate whether hits revealed in the initial screen are false positives due to interference with the fluorescence of hydrolyzed FS-3. In this secondary screen the fluorescein fluorescence in the presence of sample compounds is measured. Those compounds that are positive in the initial screen and negative in the secondary screen are potential autotaxin inhibitors.

Assay Notes

1. Some organic solvents interfere with the assay (Figure 1). Limit to < 2% final concentration of organic solvents in the assay.
2. If your plate reader is not capable of reading time courses, incubate the plate at room temperature for 25 minutes. Read the plate at 5 min and 25 min respectively. Use these values to estimate the rates in step 18.
3. Do not freeze thaw kit components. Once thawed use within five hours.

Figure 1



Assay Protocol

Please read this entire section and the Assay Notes section before beginning the assay.

Initial Screen

1. Place ATX enzyme (E-4000) on ice and bring remaining kit components to room temperature before use. Once thawed, use within five hours.
2. Add 2 mL ddH2O to FS-3 (K-4202) for a 10 µM stock. Vortex. Limit exposure to light.
3. Add 0.5 mL ddH2O to BrP-LPA (K-4204). Vortex. This is 48.6 µM BrP-LPA solution. Label vial as tube C.
4. Prepare BrP-LPA dilutions. Label 5 tubes D – H. Add the appropriate volume of ddH2O and BrP-LPA from step 3 or the previous dilution as indicated in the table below (Table 1). Vortex after each addition.
5. Make 200 µL "control buffer 1" by adding the following components:
   - 150 µL ddH2O
   - 25 µL Buffer C (K-4106)
   - 25 µL Buffer D (K-4107)

   Mix by gently inverting 5 times.
6. Pulse spin lyophilized ATX enzyme (E-4000). Add 200 µL ddH2O to the vial of enzyme. Pipette up and down 5-10 times gently to mix. Do not vortex. Leave the vial on ice.
7. Make 8 mL "reaction buffer" by adding the following components:
   - 5.84 mL ddH2O

Table 1

Tube	Final BrP-LPA in Reaction (nM)	BrP-LPA (24.3 µM) or previous dilution (µL)	ddH_2O (µL)
C	4860	0.5 mL BrP-LPA from step 3	--
D	1620	200 from tube C	400
E	540	200 from tube D	400
F	180	200 from tube E	400
G	60	200 from tube F	400
H	20	200 from tube G	400

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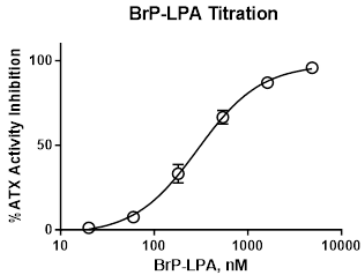
- 1 mL Buffer C (K-4106)
- 1 mL Buffer D (K-4107)
- 160 µL resuspended ATX enzyme (from step 6)

Mix by gently inverting 5 times.

8. Add 80 µL control buffer 1 from step 5 to wells A1 and A2. Use only one assay plate for steps 8 through 17. The other plate supplied in the kit will be used in the secondary screen.
9. Add 80 µL reaction buffer from step 7 to remaining wells.
10. Add 10 µL of ddH2O to wells A1, A2, B1 and B2.
11. Add BrP-LPA dilutions to columns 1 and 2. Add 10 µL of tube C to wells C1 and C2. Add 10 µL of tube D to D1 and D2. Continue through row H. Do not discard remainder of BrP-LPA dilutions; contents will be used in the secondary screen.
12. Add 10 µL of your sample compounds to the remaining wells. It is suggested that you run your samples in duplicate. A suggested plate layout is shown below in Table 2. Please see assay note #1 if organic solvent is used to dissolve the test compound(s). Do not discard remainder of sample; contents will be used in the secondary screen.
13. Mix plate on orbital shaker for 1 minute. If you do not have an orbital shaker, gently tap the plate to mix, being careful to keep all liquid in the wells.
14. Incubate plate at room temperature for 10 minutes.
    Note: You may wish to increase pre-incubation time if you suspect your compounds are slow-binding competitive inhibitors.
15. Add 10 µL of resuspended FS-3 from step 2 to each well to start reaction. Each 100 µL in a reaction well contains 1 µM FS-3 substrate and 2 nM ATX enzyme.
16. Mix plate on orbital shaker for 1-3 minutes. If you do not have an orbital shaker, gently tap the plate to mix, being careful to keep all liquid in the wells.
17. Set a fluorescence plate reader for excitation at 485 nm and reading at 528 nm at room temperature. Read the plate every minute for thirty minutes. See note #2 if your plate reader cannot read a time course.
18. Plot each data point on a fluorescence versus time graph. Fit a linear regression to the linear portion of the graph. Often the reaction will not have a steady state in first five minutes. Consequently, nonlinear fluorescence increases may be observed in this time window. Nonlinearity at later time points may also occur. Alternatively, you can use the fluorescence readings at 5 minutes and 25 minutes as a substitute for the kinetic fitting. However, this method will yield less reliable results.
19. For each control or sample, average the slopes obtained from the linear fittings in step 18. For example, average the slopes from wells B1 and B2 to obtain the slope for ATX in the absence of inhibitor.
20. For each BrP-LPA dilution or sample, subtract spontaneous FS-3 hydrolysis by subtracting the average slope of wells A1 and A2 from each averaged value obtained in step 19.
21. Determine the percent inhibition for each sample or BrP-LPA dilution by dividing the results obtained in step 20 by the average slope minus background for ATX in the absence of inhibitor.
22. Below is an example of the calculations performed in steps 19-21 to determine the percent inhibition of BrP-LPA Tube C. Coordinates indicate the slopes obtained in step 18. e.g. C2 is the slope of the linear portion of the graph for well C2.

Percent inhibition Tube C =
[1-((C1 + C2)/2 – (A1+ A2)/2) / ((B1 + B2)/2 – (A1 + A2)/2)] x 100



Secondary Screen

1. Add 1 mL Buffer A (K-4104) to Fluorescein Dye (K-4102). Vortex. Limit exposure to light.
2. Make 250 µL "control buffer 2" by adding the following components:
   - 200 µL ddH2O
   - 25 µL Buffer C (K-4106)
   - 25 µL Buffer D (K-4107)

   Mix by gently inverting 5 times.
3. Make 9 mL "fluorescein buffer" by adding the following components:
   - 6.75 mL ddH2O
   - 250 µL resuspended fluorescein from step 1.

Table 2. Suggested Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	No inhibitor, no enzyme	No inhibitor, no enzyme	Sample 1	Sample 1	Sample 9	Sample 9	Sample 17	Sample 17	Sample 25	Sample 25	Sample 33	Sample 33
B	No Inhibitor	No Inhibitor	Sample 2	Sample 2	Sample 10	Sample 10	Sample 18	Sample 18	Sample 26	Sample 26	Sample 34	Sample 34
C	BrP-LPA Tube C	BrP-LPA Tube C	Sample 3	Sample 3	Sample 11	Sample 11	Sample 19	Sample 19	Sample 27	Sample 27	Sample 35	Sample 35
D	Tube D	Tube D	Sample 4	Sample 4	Sample 12	Sample 12	Sample 20	Sample 20	Sample 28	Sample 28	Sample 36	Sample 36
E	Tube E	Tube E	Sample 5	Sample 5	Sample 13	Sample 13	Sample 21	Sample 21	Sample 29	Sample 29	Sample 37	Sample 37
F	Tube F	Tube F	Sample 6	Sample 6	Sample 14	Sample 14	Sample 22	Sample 22	Sample 30	Sample 30	Sample 38	Sample 38
G	Tube G	Tube G	Sample 7	Sample 7	Sample 15	Sample 15	Sample 23	Sample 23	Sample 31	Sample 31	Sample 39	Sample 39
H	Tube H	Tube H	Sample 8	Sample 8	Sample 16	Sample 16	Sample 24	Sample 24	Sample 32	Sample 32	Sample 40	Sample 40

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- 1 mL Buffer C (K-4106)
- 1 mL Buffer D (K-4107)

Mix by gently inverting 5 times. Limit exposure to light.

4. Add 100 µL control buffer (from step 2) to wells A1 and A2 of a clean 96 well assay plate.
5. Add 90 µL fluorescein buffer 2 (from step 3) to remaining wells. Limit exposure to light.
6. Add 10 µL of ddH2O to wells B1 and B2.
7. Add BrP-LPA dilutions to columns 1 and 2. Add 10 µL of tube C to wells C1 and C2. Add 10 µL of tube D to D1 and D2. Continue through row H.
8. Add 10 µL of sample compounds to remaining wells. It is suggested that you use the same layout as in the primary screen.
9. Mix plate on orbital shaker for 1 minute. If you do not have an orbital shaker, gently tap the plate to mix, being careful to keep all liquid in the wells. Limit exposure to light.
10. Incubate at room temperature for 10 minutes. Limit exposure to light.
11. Read plate once in a fluorescence plate reader set for excitation at 485 nm and reading at 528 nm.
12. Average the fluorescence of wells A1 and A2 to give you background fluorescence.
13. Average the fluorescence of wells B1 and B2 then subtract the background fluorescence value obtained in step 12 to determine fluorescence in the absence of inhibitor.
14. Average control and sample wells then subtract the background fluorescence value obtained in step 12 to determine the fluorescence for each data point. For example average C1 and C2 then subtract the value obtained in step 12 to determine fluorescence of fluorescein in the presence of BrP-LPA Dilution C.
15. Determine percent fluorescein fluorescence inhibition by dividing values obtained in step 14 by the value obtained in step 13 Below is an example of the calculations performed in steps 12-15 to determine the percent inhibition of Dilution C. Coordinates indicate the fluorescence obtained in step 11.

Percent inhibition Dilution C =
$[1-((C1 + C2)/2 - (A1 + A2)/2)/ ((B1 + B2)/2 - (A1 + A2)/2)] \times 100\%$

References (Background)

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Related Products

Products	Catalog Number
Assays	
Autotaxin Activity Assay Kit	K-4100
Autotaxin Inhibitor Screening Kit	K-4200
Autotaxin Sandwich ELISA Kit	K-5600
Lysophosphatidic Acid ELISA Kit	K-2800S
Antibodies & Proteins	
Goat anti-human ATX antibody	Z-P400
Lysophosphatidic Acid Antibody	Z-P200
ATX Enzyme, active	E-4000

Products	Catalog Number
Services	
Autotaxin Activity Assay	T-4100
Autotaxin Inhibitor Screening	T-4200
Autotaxin Sandwich ELISA	T-5600
Lysophosphatidic Acid ELISA	T-2800S
ATX Inhibitors & Substrates	
HA-130	B-0701
PF-8380	B-0702
FS-3	L-2000
ATX-Red AR-2	L-2010

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