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Technical Data Sheet

For research use only
Not intended or approved for diagnostic or therapeutic use.

Autotaxin Inhibitor Screening Kit

Product Number: **K-4200HTS**

Kit Components:

Autotaxin enzyme	E-4000
Fluorescent autotaxin substrate (FS-3)	K-4202
Fluorescein dye	K-4102
Buffer A (fluorescein dye suspension buffer)	K-4104
Buffer C (concentrated reaction buffer)	K-4106
Buffer D (protein stabilizer)	K-4107
Inhibitor positive control (BrP-LPA)	K-4204
2 X 384-Well assay plates	
2 X Plate sealer	

Each kit provides sufficient reagents for 384 assay points (including controls and standards).

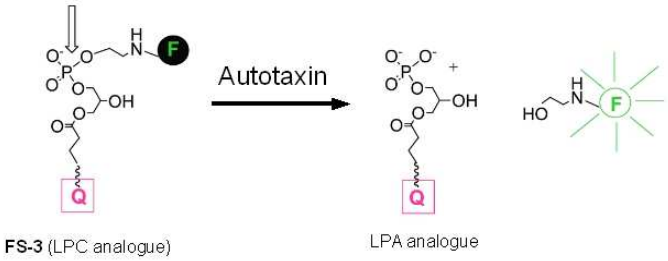
Researcher must provide:

- Fluorescence plate reader capable of exciting a 384 well plate at 485 nm and reading at 528 nm.

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.**

Assay reaction:



FS-3 (LPC analogue) LPA analogue

Background:

LPA and Autotaxin Biology

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); ATX can also hydrolyze sphingosylphosphorylcholine (SPC) to

sphinosine-1-phosphate (S1P). This activity, however, is of questionable physiologic significance since the K_m of ATX for SPC is three orders of magnitude higher than normal serum levels of SPC[9,10].

ATX and Disease

ATX was first isolated as the autocrine motility factor secreted from melanoma cells[11]. At that time the enzyme's lysoPLD activity had not been identified, so it was classified by homology to the ecto-nucleotide pyrophosphatase/phosphodiesterase (NPP) family of enzymes whose members hydrolyze phosphodiester bonds in various nucleotides and nucleotide derivatives. It was initially unclear how nucleotide hydrolysis could lead to the stimulation of cell motility. This mystery was solved when ATX was discovered to be identical to serum lysoPLD[9,12]. Since then, the cancer-related activities of ATX, at least in cultured cells, have been attributed to the enzyme's lysoPLD activity[13,14].

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].

Product Description:

The autotaxin inhibitor screening kit uses the fluorogenic ATX substrate FS-3. FS-3 is an LPC analogue that is conjugated with both a fluorophore (fluorescein) and a quencher. In its native state the quencher interferes with fluorescein's fluorescence. Once ATX cleaves FS-3, fluorescein becomes liberated from the quencher, resulting in increased fluorescence[19]. The Michaelis constant K_m of ATX for FS-3 is 6.3 µM[19].

In the initial screen, purified recombinant ATX is incubated with sample compounds in a 384-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, 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.

References:

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Assay Protocol:

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 ddH_2O to FS-3 (K-4202) for a 10 µM stock. Vortex. *Limit exposure to light.*

3. Add 1 mL ddH_2O to BrP-LPA (K-4204) for a 24.3 µM stock solution. Vortex. Label vial as tube C.

4. Prepare BrP-LPA standards. Label 5 tubes D – H. Add the appropriate volume of ddH_2O and BrP-LPA from step 3 or the previous dilution as indicated in the table below. Vortex after each addition.

Tube	Final BrP-LPA in Reaction (nM)	BrP-LPA (24.3 µM) or previous dilution (µL)	ddH_2O (µL)
C	2430	1 mL BrP-LPA from step 3	--
D	810	100 from tube C	200
E	270	100 from tube D	200
F	90	100 from tube E	200
G	30	100 from tube F	200
H	10	100 from tube G	200

5. Make 100 µL "control buffer" by adding the following components:
   - 75 µL ddH_2O
   - 12.5 µL Buffer C (K-4106)
   - 12.5 µL Buffer D (K-4107)

   Mix by gently inverting 5 times.

6. Pulse spin lyophilized ATX enzyme (E-4000). Add 200 µL ddH_2O 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 ddH_2O
   - 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 20 µL control buffer 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 20 µL reaction buffer from step 7 to remaining wells.

10. Add 2.5 µL of ddH_2O to wells A1, A2, B1 and B2.

11. Add BrP-LPA standards to columns 1 and 2. Add 2.5 µL of tube C to wells C1 and C2. Add 2.5 µL of tube D to D1 and D2. Continue through row H. *Do not discard remainder of standard solutions; contents will be used in the secondary screen.*

12. Add 2.5 µL of your sample compounds to the remaining wells. It is suggested that you run your samples in duplicate or triplicate. *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 2.5 µL of resuspended FS-3 (K-4202) from step 2 to each well to start reaction. Each 25 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 reach a steady state within 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 standard 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.

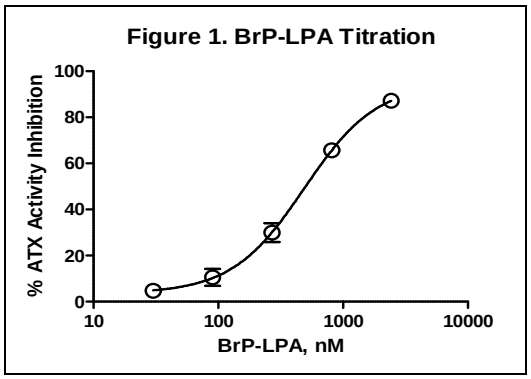
20. For each standard or sample, subtract spontaneous FS-3 hydrolysis by subtracting the average slope of wells A1 and A2 from each averaged value obtained in step 19.

21. Determine the percent inhibition for each sample or standard by dividing the results obtained in step 20 by the average slope minus background for ATX in the absence of inhibitor. A*n example of the calculations performed in steps 19-21 to determine the percent inhibition of Standard C is provided below.*

    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$_{\text{Standard C}}$ = [1-((C1 + C2)/2 – (A1 + A2)/2)/ ((B1 + B2)/2 – (A1 + A2)/2)] x 100

22. Plot percent inhibition versus BrP-LPA concentration in log scale (Figure 1). See the table in step 4 for BrP-LPA concentrations. 2.43 µM of BrP-LPA should inhibit over 80% of ATX activity. The IC_{50} (concentration of BrP-LPA sufficient to inhibit ATX activity by 50%) is typically about 0.5 µM BrP-LPA.



Secondary Screen

23. Add 1 mL Buffer A (K-4104) to Fluorescein Dye (K-4102). Vortex. *Limit exposure to light.*

24. Make 250 µL "control buffer" by adding the following components:
    - 200 µL ddH_2O
    - 25 µL Buffer C (K-4106)
    - 25 µL Buffer D (K-4107)

    Mix by gently inverting 5 times.

25. Make 9 mL "fluorescein buffer" by adding the following components:
    - 6.75 mL ddH_2O
    - 250 µL resuspended fluorescein from step 23.
    - 1 mL Buffer C (K-4106)
    - 1 mL Buffer D (K-4107)

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

26. Add 25 µL control buffer from step 24 to wells A1 and A2 of a clean 384 well assay plate.

27. Add 22.5 µL fluorescein buffer from step 25 to remaining wells. *Limit exposure to light.*

28. Add 2.5 µL of ddH_2O to wells B1 and B2.

29. Add BrP-LPA standards to columns 1 and 2. Add 2.5 µL of tube C to wells C1 and C2. Add 2.5 µL of tube D to D1 and D2. Continue through row H.

30. Add 2.5 µL of sample compounds to remaining wells excluding A1 and A2. It is suggested that you use the same layout as in the primary screen.

31. 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.*

32. Incubate at room temperature for 10 minutes. *Limit exposure to light.*

33. Read plate once in a fluorescence plate reader set for excitation at 485 nm and reading at 528 nm.

34. Average the fluorescence of wells A1 and A2 to give you background fluorescence.

35. Average the fluorescence of wells B1 and B2 then subtract the background fluorescence value obtained in step 34 to determine fluorescence in the absence of inhibitor.

36. Average control and sample wells then subtract the background fluorescence value obtained in step 34 to determine the fluorescence for each data point. *For example average C1 and C2 then subtract the value obtained in step 34 to determine fluorescence of fluorescein in the presence of Standard C.*

37. Determine percent fluorescein fluorescence inhibition by dividing values obtained in step 36 by the value obtained in step 35. *Below is an example of the calculations performed in steps 34-37 to determine the percent inhibition of Standard C. Coordinates indicate the fluorescence obtained in step 32.*

$$\text{Percent inhibition}_{\text{Standard C}} = [1-((C1 + C2)/2 – (A1+ A2)/2)/ ((B1 + B2)/2 – (A1 + A2)/2)] \text{ X } 100\%$$

Assay Notes:

1. Some organic solvents interfere with the assay (Figure 2). 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 2: DMSO Titration