Instructions for use

In Cytotox - PAC

1 - Parameter Cytotoxicity Kit

Acid Phosphatase PAC - Lysosomal Metabolism

Art. No. AKPA96.300
Art. No. AKPA96.310
Art. No. AKPA96.1200
Art. No. AKPA96.1210

For research use only

Version 2.4 04/2010

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Kit contents AKPA96.300/310

PAC I	Wash solution	1 x 65ml
PAC II	Substrate	1 x 66 ml (after reconstitution)
PAC III	Lysis solution	1 x 66 ml
PAC I V	Stop solution	1 x 16.5 ml

1 Instruction manual

8 sterile reagent reservoirs (AKPA96.310 only)
4 96-well microplates (AKPA96.310 only)

Kit contents AKPA96.1200/1210

PAC I	Wash solution	2 x 130 ml
PAC II	Substrate	2 x 132 ml (after reconstitution)
PAC III	Lysis solution	2 x 132 ml
PAC I V	Stop solution	1 x 66 ml

1 Instruction manual

32 sterile reagent reservoirs (AKPA96.1210 only)
16 96-well microplates (AKPA96.1210 only)

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Material required but not provided:

Test cells
Culture medium
Sterile water

96-well microplates (AKPA96.300/1200 only)
Sterile reagent reservoirs (AKPA96.300/1200 only)

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Storage conditions

All kit components must be stored at 4°C. The substrate PAC II is photosensitive and has to be stored protected from light. After reconstitution of PAC II with PAC III it can be stored frozen at -20°C for up to 12 months.
PAC IV may develop a dark precipitate which DOES NOT interfere with its function. You may remove the precipitate prior to use by centrifugation at 3000 rpm for 5 minutes.

Precautions

Some components of this kit are potentially carcinogenic or corrosive; it is advisable to work in a hood and to wear glasses, gloves and a mask. After skin contact with any reagents of this kit wash affected areas with water and soap.
PAC II, PAC III and PAC IV may be harmful if swallowed, inhaled or absorbed through skin. In case of contact of eyes with PAC II, PAC III or PAC IV solutions, immediately flush eyes with copious amounts of water and consult a physician.

Limitations of use and interfering factors

Mycoplasma, bacteria and other microbial contaminants may also convert the pNPP substrate and thus may produce erroneous results.

This kit is **for research use only**, and **not for human diagnostic purposes.**

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Technical information, questions

For any questions, supplementary information or suggestions, please contact the technical support department of Aniara:

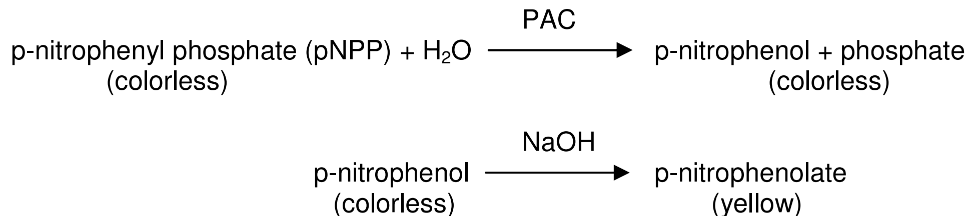
Phone : (866) 783-3797
Fax : (513) 573-9241
Email : info@aniara.com

Important note:
Please read the complete manual carefully before starting the assay!

PAC test principle

The acid phosphatase (PAC) is a hydrolase with a pH optimum below 7 and catalyzes the dephosphorylation of organic ortho-phosphoric esters. The enzyme has transferase activity leading to the fixation of a phosphate to the hydroxyl group of an alcohol. PAC is located in the golgi apparatus and therefore a marker for lysosomal activity.

The membrane associated PAC cleaves the colorless substrate p-nitrophenyl phosphate to the colorless p-nitrophenol which is measured spectrophotometrically at 405 nm after conversion to the yellow p-nitrophenolate form by addition of 1 M NaOH. An increase or decrease in cell numbers results in a concomitant increase or decrease in the amount of substrate converted by the test compound.



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Protocol

Subculture of cells to 96-well plates

Note:
The cell seeding step can easily result in uneven cell densities in the wells of the microplates. Make sure your cell suspension is homogeneous and devoid of large cell aggregates!

Cell density:
Optimal sensitivity is obtained with near-confluent cells at the time of the assay. The actual cell number to be used depends on the size of the cells, the culture doubling time and the duration of the culture phase before the assay is performed. For average sized adherent cells such as mouse fibroblasts and a total culture phase of 48 hrs we recommend to seed 20'000 cells into each well. Fewer cells may be necessary for longer exposure times and larger cells.

- Trypsinize the cells according to the standard operating procedures of your laboratory. Optimally, cells in the log phase of growth should be used.
- Resuspend the trypsinized cells in complete culture medium such that the desired cell number per well is present in 200 µl of medium.
- Pour sterile water into a sterile reagent reservoir.

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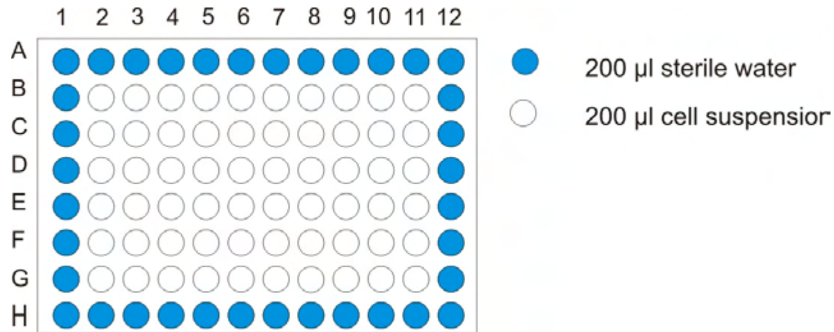
- Dispense 200 µl of the sterile water in each well of rows A and H, and in each well of columns 1 and 12 (see figure), to reduce culture medium evaporation during incubation. (Erroneous results may be obtained upon uneven evaporation of culture fluid.)
- Pour the cell suspension into the same sterile reagent reservoir.
- Dispense 200 µl of cell suspension into the remaining wells of the 96-well plates.



- Incubate the plate in a humidified incubator at 37°C, 5% of CO_2 overnight or for 24 hours.

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Preparation of test compound stock solutions

We recommend to use the plate layout described below. This allows to evaluate 2 compounds per plate in triplicate, 8 serial dilutions, with negative and positive controls.

- Prepare 8 sterile concentrations of each test compound that are 10X more concentrated than the concentrations to be used in the assay: Prepare the highest desired concentration and then the seven lower concentrations by serial dilutions with appropriate solvent. Use culture medium as solvent if possible.
- Dilute each 10X concentration 1:10 with culture medium

Note that it may be necessary to make more concentrated stock solutions in order to avoid final solvent concentrations that may interfere with cell growth. If DMSO, ethanol or methanol are used as solvents, the final solvent concentration should not exceed 2%. Equal solvent concentrations should be used for all compound solutions.

Preparation of the negative control

For 1 plate:

- Mix 500 µl of solvent with 4.5 ml of culture medium (if 10X concentration of test compounds is used) (SC).

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Exposure of the cells with test compounds

- Inspect the cells with a microscope. Cells should be distributed evenly between wells and look healthy.
- Remove the culture medium sterilly from each well either with a multi-channel pipette (recommended) or by using a sterile pipette attached to a vacuum source. Avoid scratching the bottom of the wells with the cells. The removed medium should be replaced rapidly with the test compounds in medium to avoid stress and cell death due to drying cell layers.

Recommended dosing configuration (see figure below):

- Add 200 µl of culture medium to wells B2, C2 , D2, E11, F11, and G11 (cell growth control = GC).
- Add 200 µl of culture medium - solvent mixture (page 10) to wells E2, F2, G2, B11, C11 and D11 (solvent control = SC) and to the wells of columns 1 and 12 (blank = B).
- Add 200 µl of the lowest test compound concentrations (X1) to wells B, C, D of column 3, 200 µl of test compound concentration X2 to wells B, C, D of column 4 and proceed through column 10 by adding 200 µl of test compound concentrations X3-X8.

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- Add 200 µl of the lowest test compound concentrations (Y1) to wells E, F, G of column 3, 200 µl of test compound concentration Y2 to wells E, F, G of column 4 and proceed through column 10 by adding 200 µl of test compound concentrations Y3-Y8.
- Incubate the plate for the desired length of time.

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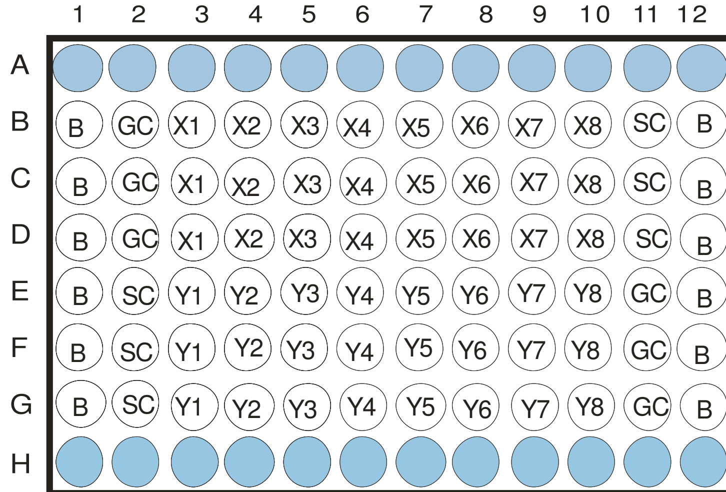
Recommended 96-well plate configuration



Rows A + H: sterile water
B: culture medium + solvent without cells (negative control)
GC: culture medium + cells (cell growth control)
SC: culture medium + cells + solvent (solvent control)
X1, X2,…, X8 and Y1, Y2,…, Y8: culture medium + cells + different concentrations of two compounds X, Y.

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PAC Test Procedure

- Dissolve the contents of 1 flask of PAC II by adding all of the lysis solution of 1 flask of PAC III. Part of the contents of the PAC II flask may stick to the inside of the cap and the walls of the flask. Invert several times to ensure complete dissolution. Don't shake vigorously to avoid foam formation.
- Remove the culture medium, with the cells remaining attached to the bottom of the wells
- Wash the cells with 200 µl/well wash solution PAC I
- Discard the wash solution PAC I
- Add 200 µl/well of the reconstituted substrate/lysis solution PAC II. Store unused PAC II solution at -20°C for later use.
- Incubate the plate for 2 hours at 37°C. The incubation period may be lengthen up to 4 hours for low cell densities or for cells with low metabolic activity.
- Add 50 µl/well of stop solution PAC IV. (PAC IV may develop a dark precipitate which DOES NOT interfere with its function. You may remove the precipitate prior to use by centrifugation at 3000 rpm for 5 minutes.)
- Mix by pipetting up and down or with a microplate shaker and read the OD at 405 nm. Measure the background absorbance with a reference filter at 690 nm.

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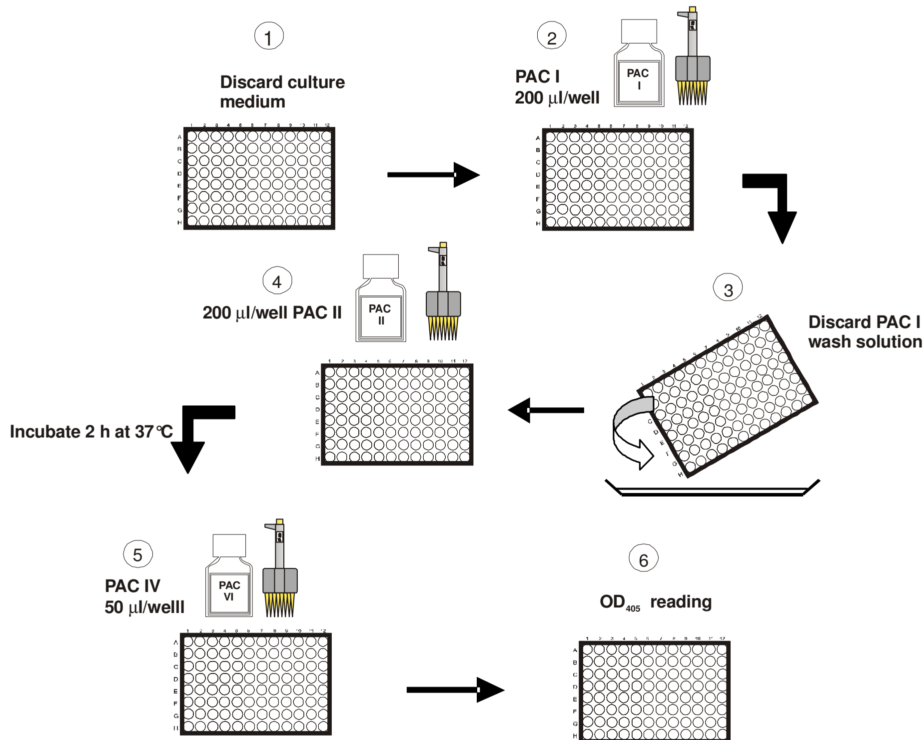
PAC assay



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Quality control of the assay

The mean OD values in the wells without test sample (solvent control, SC) correspond to a viability of 100% (wells E2, F2, G2, B11, C11 and D11).

The solvent controls (SC) are placed at the left and right side of the 96-well microplate to detect systematic errors. The assay is acceptable, if the left and right sided mean values do not differ more than 15% from the mean of all solvent controls (+/- 15%)

The cell growth controls (GC) allow to detect solvent effects. If the solvent control values differ significantly from the growth control values, inhibition values of test compounds are to be interpreted with caution. If possible chose a different solvent.

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Data Analysis

For each well subtract the OD_{690} values from the OD_{405} values.

Calculate the mean OD values for every test sample concentration.

Calculate the mean OD values of the blanks (columns 1 and 12). Correct the sample and solvent control OD's:

Mean OD of samples/controls – mean OD of blanks

Relative inhibition activity is then expressed as percent of solvent control:

% inhibition = 100 - (corrected mean OD sample x 100 / corrected mean OD solvent controls)

Plot the % inhibition of activity against the test compound concentration. For serial dilutions plot the concentrations on a log scale.

The 50% inhibiting concentration IC_{50} can be determined graphically.

The example below shows the toxicity of a toxic compound on L929 cells measured with the PAC test. IC_{50} is around 20 µM.

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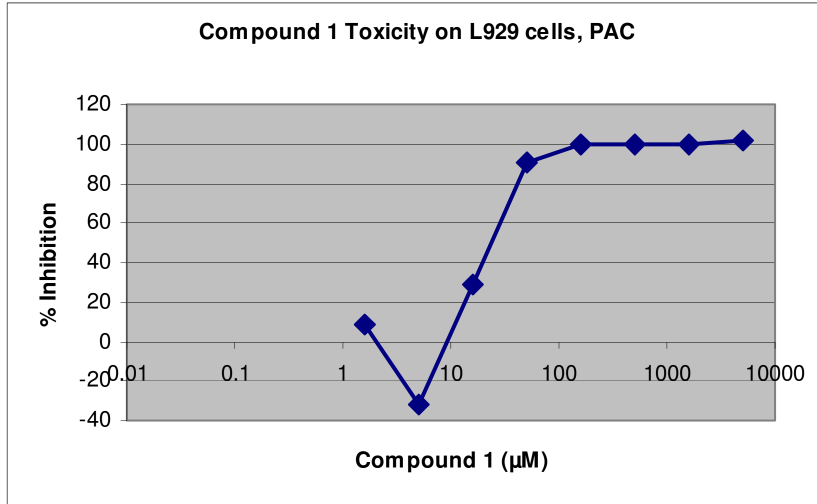
Example:

	Blank	GC	SC	1.58	5	15.8	50	158	500	1580	5000
	0.025	1.576	1.561	1.428	2.044	1.112	0.176	0.0346	0.0321	0.03	0.0028
Corrected	0.000	1.551	1.536	1.403	2.019	1.087	0.151	0.010	0.007	0.005	-0.022
% Inhibition		-0.976	0.000	8.658	-31.441	29.228	90.158	99.362	99.525	99.662	101.432
%inh/(100%-%inh)			0.000	0.095	-0.239	0.413	9.160	155.755	209.438	294.423	-70.827
log (%inh/(100%-%inh)			#ZAHL!	-1.023	#ZAHL!	-0.384	0.962	2.192	2.321	2.469	#ZAHL!
log conc.				0.19865709	0.69897	1.19865709	1.69897	2.19865709	2.69897	3.19865709	3.69897
log (%inh/(100%-%inh)				-1.023	#ZAHL!	-0.384	0.962	2.192	2.321	2.469	#ZAHL!



Note the stimulation (= negative inhibition) by this compound at 5 µM!

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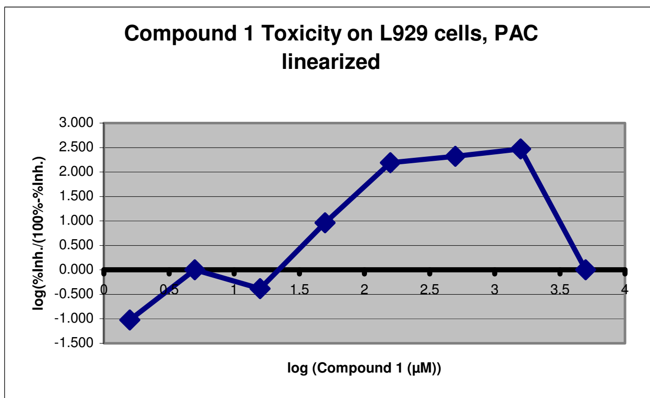
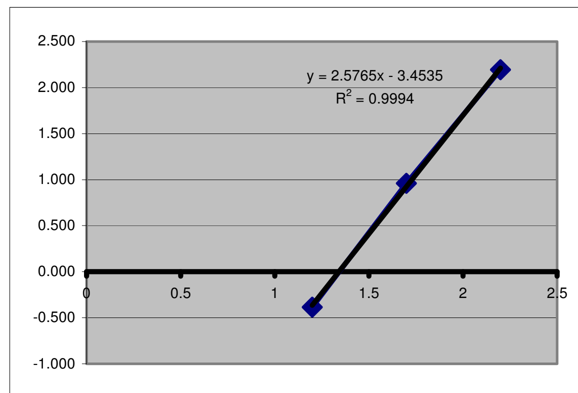
Alternatively you can plot:

Log (% inhibition / (100 -% inhibition)) vs. log (concentration)

The IC_{50} is obtained from the intercept on the x-axis. Note that values ≥ 100% and ≤ 0% can not be used with this analysis! A regression line using the linear part of the curve crossing the y-axis at 0, can be used to obtain a calculated estimation of the IC_{50}. In this example one can not use the values from 5 µM (neg. logarithm. So for (y=0): (log IC_{50}) = (3.4535 / 2.5765) = 1.34, giving an IC_{50} of 22 µM.





IC_{50} values can also be calculated using the CelTox software available from Aniara.

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