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Technical Data Sheet

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

PI 3-Kinase ELISA

Product No: K-1000

INTENDED USE: THIS PRODUCT IS FOR RESEARCH USE ONLY. NOT INTENDED FOR USE IN A DIAGNOSTIC OR CLINICAL SETTING.

ELISA Assay for Detection of PI 3-Kinase Activity

Kit includes:

5 vials PI(4,5)P_2 substrate (100 reactions, 200 pmol/reaction.)
2 vials PI(3,4,5)P_3 standard
1 vial PI(3,4,5)P_3 Detector
1 vial Secondary Detector
1 Incubation plate: 96-well polypropylene U-bottom plate
1 Detection plate: PI(3,4,5)P_3 coated flat-bottom clear 96-well plate (twelve 8-well strips), preblocked.
1 bottle TMB Solution

Researcher must provide:

Buffers for reactions and washes
Acetate plate sealers/parafilm
Absorbance plate reader
Source of PI 3-kinase
Microfuge tubes for kinase reaction
Stop Solution for TMB development

Background

The production of PI(3,4,5)P_3 from PI(4,5)P_2 by PI 3-kinases (PI3-K) is important in multiple cell signaling pathways. Typically, experiments to measure PI 3-K activity have involved phosphorylation of a phosphoinositide substrate using ^{32}P, then extraction of radioactive products, and separation using thin-layer chromatography. The assay plate method developed by Echelon Biosciences, Inc. allows the user to determine PI 3-K activity, using either recombinant or immunoprecipitated enzyme, by means of a standard ELISA format, eliminating the need for radioactivity, organic solvents, and thin layer chromatography.

The assay is a competitive ELISA in which the signal is inversely proportional to the amount of PI(3,4,5)P_3 produced. After the PI 3-K reactions are complete, reaction products are first mixed and incubated with a PI(3,4,5)P_3 detector protein, then added to the PI(3,4,5)P_3-coated microplate for competitive binding. A peroxidase-linked secondary detection reagent and colorimetric detection is used to detect PI(3,4,5)P_3 detector protein binding to the plate. The colorimetric signal is inversely proportional to the amount of PI(3,4,5)P_3 produced by PI 3-K activity.

Basic Protocol:

Kinase Reaction

1. Isolate or prepare PI 3-K according to usual protocols. See attached protocol suggestions on immunoprecipitation of PI 3-K from cells.

2. Each tube of PI(4,5)P_2 substrate (red capped tubes) contains enough for 20 reactions at 200 pmol (3.5 µg) of diC_8 PI(4,5)P_2 per reaction. There is enough substrate for 100 total reactions. Resuspend the PI(4,5)P_2 substrate to a concentration of 20 µM (200 pmol/10 µl) by adding 204 µl TBS. Vortex to resuspend the lipid. For each assay point, add 5 µl of 10X reaction buffer and 10 µl of substrate solution. Bring the total volume to 50µl per assay point. If you are using purified enzyme, add the volume of enzyme you plan to use and then add dH_2O to bring the final volume to 50 µl. If you are using immunoprecipitated enzyme bound onto beads, add the components listed above directly to the beads and then add dH_2O to bring the final volume to 50 µl. We suggest running duplicates or triplicates of each point.

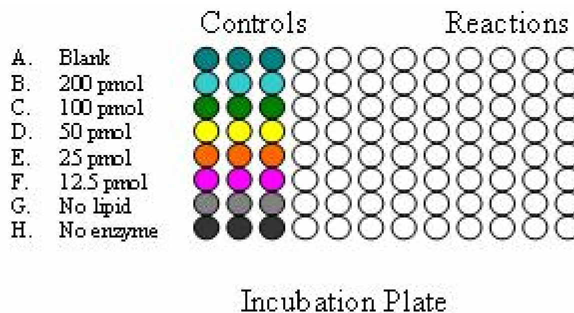
3. Let the kinase reaction proceed for appropriate amount of time, usually 1-3 hours, at room temperature.

4. Stop the kinase reaction by adding 2.5 µl 100mM EDTA per 50 µl reaction volume (final concentration approximately 5 mM). If enzyme is bound to beads or otherwise immobilized, the enzyme reaction can be stopped by simply pelleting the beads by centrifugation and transferring the reaction mixture to the incubation plate.

> **NOTE:** Two different recipes for 10X kinase reaction buffer are provided. One is suitable for determining the activity of PI 3-Kα isoforms and the other is suitable for determining activity of both PI 3-Kα and PI 3-Kγ isoforms. If you have a preferred reaction buffer composition for running PI 3-K assays, you may substitute this for the suggested reaction buffers.
>
> **NOTE:** The amount of enzyme to use per 100 pmol of substrate will vary according to your individual experiment. Whether you are using purified PI 3-K or enzyme immunoprecipitated from cell extracts, you will need to try reactions using different amounts of enzyme to determine the optimum amount. In our testing, we found 500 ng of purified recombinant PI 3-Kα per point, or enzyme immunoprecipitated from cell lysates containing 50 µg cellular protein (approximately 500,000 cells), to be sufficient.

Incubation

Stock solution of PI(3,4,5)P_3	pmol/50µl standard solution
4 µM	200
2 µM	100
1 µM	50
0.5 µM	25
0.25 µM	12.5
No lipid control	0
No enzyme control	0



1. Set up the incubation plate (colored polystyrene U-bottom microtiter plate). First, prepare the standard curve using one of the two blue-capped tubes containing PI(3,4,5)P_3 provided for this purpose. Add 450 µl of TBS to the tube for a total volume of 500 µl. Vortex to resuspend the lipid. This will make a 4µM stock solution.

2. We suggest that controls, standards, and samples be run in duplicate or triplicate. From the 4 µM stock of PI(3,4,5)P_3, make four 2-fold serial dilutions in TBS, as outlined in the above table. Reserve some wells for blank controls, in which secondary detection reagent alone will be added. Pipet 50 µl of each standard dilution (in duplicate or triplicate) into the wells of the incubation plate. One suggested setup for the incubation plate is shown above. For the blank controls in row A, add 100µl TBS per well. Add 50 µl/well of TBS to the no lipid controls in row G. Add 40 µl/well of TBS to row H plus 10 µl/well of PI(4,5)P_2 substrate as the no enzyme control. Use the other wells of the plate to run your samples. Add 50 µl of reaction mixture per well.

3. Dilute the PI(3,4,5)P_3 detector 1:200 in TBS-0.05%Tween. (Make only enough working stock of detector for use that day.) Add 50 µl of diluted PI(3,4,5)P_3 detector to all wells except the blank controls in row A. Seal the incubation plate and incubate at room temperature in a dark location. For best results, incubate for one hour at room temperature. The plate may also be incubated at 4 °C overnight if desired. PI(3,4,5)P_3 detector may be stored at 4° C for up to one month. For longer storage, aliquot into single use tubes and store at -20 °C, or below. Avoid repeated freeze-thaw cycles.

Detection

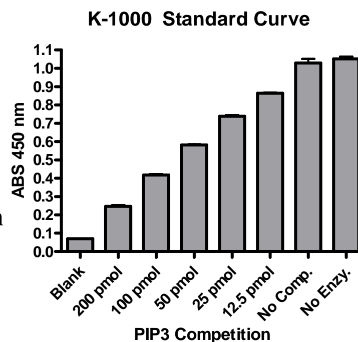
1. Following the incubation, transfer the reacted mixtures to the detection plate (clear flat-bottom strip plate). Transfer 100µl from each well to the corresponding well in the detection plate. (This can easily be accomplished with a multi-channel pipettor.) Seal the plate and incubate 30-60 minutes at room temperature in a dark location. Discard the solution and wash the wells 3 times with 200-300µl TBS-0.05%Tween. Increase time and number of washes if background is observed.

2. Dilute the secondary detection reagent solution 1:40 with TBS-T. Dilute ONLY the amount you will use for the current assay and store the remainder of the secondary reagent at 4°C for future use. Add 100µl of secondary detection reagent solution to all wells of the detection plate. Seal the plate and incubate for another 30-60 minutes in a dark location. Discard the solution and wash the wells 3 times with 200-300 µl TBS-Tween.

3. Add 100 µl of TMB solution to each well. Allow color to develop for approximately 1-5 minutes. Its progression can be monitored by reading the blue end product at 370 nm or 655 nm. (watch for color development and DO NOT overdevelop). Stop the reaction by adding 50 µl of stop solution (0.5 M H_2SO_4) to each well. Read on an absorbance plate reader at 450 nm. (NOTE: You may not be able to accurately tell the difference between the intensities of color by eye.)

Results

Enzyme activity can be estimated by comparing the values from the wells containing enzymatic reaction products to the values in the standard curve. Plot the absorbance values obtained vs. amount of PI(3,4,5)P_3 per standard to generate a standard curve. Determine where the values obtained from the enzymatic reactions lie on the curve to obtain a measure of PI 3-K activity in your samples.



Solutions

10X Reaction Buffer for PI 3-Kα

25 mM $MgCl_2$
50 mM Hepes pH 7
250 µM ATP

10X Reaction Buffer (for PI 3-Kα and γ)

40mM MgCl2
200 mM Tris pH 7.4
100 mM NaCl
250 µM ATP

TBS

150 mM NaCl
10 mM Tris pH 7.5

TBS-Tween

TBS + 0.05% Tween-20

Stop Solution for ELISA Color Development

0.5 M H_2SO_4

Storage:

Store kit at 4° C in a dark location. The PI(3,4,5)P_3 detector can be stored at 4° C and used up to one month, although signal may decrease with storage. For longer storage periods, store the detector at –20° C.

SUPPORT PROTOCOL- IMMUNOPRECIPITATION OF PI 3-KINASE FROM CELLS

1. Grow cells to 80% confluence in 10 cm dishes.

2. Induce quiescence by incubating overnight in serum-free medium containing 0.5% insulin-free BSA.

3. Remove medium, and stimulate cells with 100 nM insulin for 10 minutes at 37° C.

4. Remove solution and place cells on ice. Add 10 ml per dish of ice-cold Buffer A (137mM NaCl, 20 mM Tris-HCl, pH 7.4, 1 mM CaCl2, 1 mM MgCl2, and 0.1 mM sodium orthovanadate). Rinse three times with this solution.

5. Remove Buffer A and add 1 ml of ice cold Lysis Buffer (Buffer A plus 1% NP-40 and I mM PMSF). Keep plates on ice for 20 minutes.

6. Scrape cells from dish, transfer to 1.5 ml microfuge tubes. Centrifuge for 10 minutes to sediment insoluble material.

7. Transfer supernatant to new tubes, add 5 µl of anti-PI 3 kinase antibody (Upstate Biotechnology, catalog # 06-195) to each tube. Incubate for one hour at 4° C.

8. Add 60 µl of a 50% slurry of Protein A-agarose beads in PBS to each tube. Incubate with mixing for one hour at 4°C.

9. Collect immunoprecipitated enzyme by centrifuging 5 seconds, and wash with freshly prepared buffers as follows:

   - Three times with Buffer A plus 1% NP-40
   - Three times with 0.1 M Tris-HCl, pH 7.4; 5 mM LiCl, and 0.1 mM sodium orthovanadate.
   - Twice with TNE (10mM Tris-HCl, pH 7.4, 150 mM NaCl, 5mM EDTA) containing 0.1 mM sodium orthovanadate.

10. Aspirate last wash as completely as possible, and proceed with kinase reactions as described in the Basic Protocol.

Notice to Purchaser
Echelon Biosciences products are sold for research and development purposes only and are not to be incorporated into products for resale without written permission from Echelon Biosciences. **This kit and all non-radioactive, competitive assays for determining phosphoinositide-3-kinase (PI3-K) activity are protected by Echelon Biosciences Inc. U.S. Patent 7,067,269**. The purchase of this product includes a limited, non-transferable immunity from suit under the foregoing patent claims for using only this amount of product for the purchaser's own internal research. For inquiries email busdev@echelon-inc.com.