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ab207485 Phospho-PI 3 Kinase p85 + Total In-Cell ELISA Kit (Chemiluminescent)

For the quantitative measurement of total and phosphorylated PI 3 Kinase p85 in adherent and non-adherent cells.

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1. Overview

Phospho-PI 3 Kinase p85 + Total In-Cell ELISA Kit (Chemiluminescent) (ab207485) provides a simple, efficient, cell-based method to monitor proteins activated by phosphorylation. The kit is designed specifically to quantify activated (phosphorylated) phosphoinositide 3 Kinase (PI3K) p85 and/or total PI 3 Kinase p85. Cells are cultured in 96-well plates and stimulated to induce the pathway of interest. Following stimulation, the cells are rapidly fixed to preserve activation-specific protein modifications. Each well is then incubated with a primary antibody that recognizes either phosphorylated PI 3 Kinase p85 or total PI 3 Kinase p85. Subsequent incubation with secondary HRP-conjugated antibody and developing solution provides an easily quantified chemiluminescent readout. The relative number of cells in each well is then determined using the provided Crystal Violet solution. The 96-well plate format is suitable for high-throughput screening applications.

The Phospho-PI 3 Kinase p85 + Total In-Cell ELISA Kit (Chemiluminescent) contains two 96-well plates and two primary antibodies. The phospho-PI 3 Kinase p85 antibody recognizes peptides and proteins containing p85 PI3-Kinase phospho-tyrosine 467 (alpha) or 199 (gamma). No cross-reactivity is observed with corresponding non-phosphorylated sequences or with other phospho-tyrosine-containing motifs. The total-PI 3 Kinase p85 antibody recognizes the total level of PI 3 Kinase p85 proteins regardless of the phosphorylation state. The kit can be used to study phosphorylated PI 3 Kinase relative to cell number or to determine PI 3 Kinase phosphorylation relative to the total PI 3 Kinase protein found in the cells. Once the phospho-PI 3 Kinase and total-PI 3 Kinase signals have been normalized for cell number, a comparison of the ratio of phosphorylated PI 3 Kinase to total PI 3 Kinase for each of the cell growth conditions can be made. The provided total-PI 3 Kinase antibody can be used as a positive control to demonstrate that the cells contain PI 3 Kinase, the kit reagents are functional and that the protocol is performed correctly. Also, because fixed cells are stable for several weeks, you can prepare many plates simultaneously and then perform the assay when desired.

Phosphoinositide 3 Kinase (PI3 Kinase, PI3K) is a major signaling molecule that is located downstream of growth factor receptor tyrosine kinases (RTKs). PI 3 Kinase catalyzes the production of the lipid second messenger phosphatidylinositol-3,4,5-triphosphate (PIP3) at the

cell membrane. PIP3 then contributes to the recruitment and activation of a wide range of downstream targets, including the serine-threonine protein kinase AKT (PKB). The PI 3 Kinase-AKT signaling pathway regulates many normal cellular processes including cell proliferation, survival, growth and motility-processes that are critical for tumorigenesis.

PI 3 Kinase is a heterodimer with a p85 regulatory subunit and p110 catalytic subunit. The p85 subunit has two SH2 domains, which bind to phospho-tyrosine with the consensus motif (Y*XXM), where X denotes any amino acid and Y* is phosphorylated tyrosine. This motif exists in many receptor tyrosine kinases and their substrates, such as EGFR, FGFR, IRS-1, BCAP and GAB1. Activation of the PI 3 Kinase pathway occurs when the p85 subunit recognizes phosphorylated YXXM in signaling molecules. The small GTPase Ras can also recruit and activate PI 3 Kinase through direct binding to p110.

A large number of downstream effector targets have been described for PI 3 Kinase. AKT/PKB is a downstream effector of PI 3 Kinase as well as p85/p110. ERK1/2 MAPK is also a target of the PI 3 Kinase signaling cascade. Some Rho family GTPases (Rac, Cdc42) are regulated by PI 3 Kinase in several systems. A link between PI 3 Kinase, phospholipase D activation, actin rearrangement and vesicular trafficking may also exist.

2. Protocol Summary

Culture cells and treat as desired



Fix cells by replacing the growth medium with 4% (adherent cells) or 8% (non-adherent cells) formaldehyde in PBS for 20 minutes at RT. Wash cells



Quench endogenous peroxide by incubating in Quenching Buffer for 20 minutes at RT. Wash cells



Incubate with Antibody Blocking Buffer for 1 hour at RT. Wash cells



Incubate with primary antibody overnight at +4°C. Wash cells



Incubate with secondary antibody for 1 hour at RT. Wash cells



Add Chemiluminescent Working Solution and read chemiluminescence using a luminometer or CCD camera system



Crystal Violet cell staining (Optional)

3. Precautions

Please read these instructions carefully prior to beginning the assay.

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handle with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.
- Sodium Azide and Formaldehyde are highly toxic chemicals. Appropriate safety precautions (gloves and eye protection) should be used. In addition, formaldehyde is highly toxic by inhalation and should be used only in a ventilated hood.

4. Storage and Stability

Store kit at -20°C immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. Materials Supplied

Item	1 x 96 tests	5 x 96 tests	Storage Condition
Phospho-PI 3 Kinase p85 antibody	9 µL	5 x 9 µL	-20°C
Total-PI 3 Kinase p85 antibody	9 µL	5 x 9 µL	-20°C
Anti-rabbit HRP-conjugated IgG	2 x 11 µL	10 x 11 µL	+4°C
1X Antibody Blocking Buffer	22 mL	5 x 22 mL	-20°C
1X Antibody Dilution Buffer	30 mL	5 x 30 mL	-20°C
10X PBS	120 mL	5 x 120 mL	RT
10% Triton X-100	10 mL	5 x 10 mL	RT
Crystal Violet Solution	22 mL	5 x 22 mL	+4°C
Chemiluminescent Reagent	2 x 2 mL	10 x 2 mL	+4°C
Reaction Buffer	2 x 4 mL	10 x 4 mL	+4°C
1% SDS Solution	22 mL	5 x 22 mL	RT
96-well tissue culture plate	2	10	RT
Plate sealing tape	2	10	RT

7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Multi-channel pipettor.
- Multi-channel pipettor reservoirs.
- Rocking platform.
- Parafilm.
- Microplate spectrophotometer capable of reading at 595 nm for Crystal Violet staining.
- Microplate luminometer or CCD camera-coupled imaging system for chemiluminescent detection
- Fresh 10% hydrogen peroxide (H_2O_2) in dH_2O (3 mL is required)
- 10 µg/mL poly-L-Lysine (if using non-adherent cells).
- 10% Sodium Azide (NaN_3) in dH_2O (250 µL is required).
- 37% Formaldehyde (2.5 mL is required for adherent cells; 5.0 mL required for non-adherent cells).

8. Technical Hints

- This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Make sure all buffers and solutions are at room temperature before starting the experiment.
- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Make sure you have the right type of plate for your detection method of choice.
- Make sure the heat block/water bath and microplate reader are switched on before starting the experiment.

9. Reagent Preparation

- Equilibrate all reagents to room temperature (18-25°C) prior to use.
- Prepare only as much reagent as is needed on the day of the experiment.
- We provide an excess of buffer components in order to perform one 96-well assay with the phospho-PI 3 Kinase antibody and one 96-well assay with the total-PI 3 Kinase antibody.

9.1 1X PBS:

Prepare 1X PBS by adding 1 volume of 10X PBS (pH 7.4) to 9 volumes of dH_2O and mixing thoroughly.

9.2 Preparation of Fixing Buffer (4% or 8% Formaldehyde in PBS):

Fixing Buffer is used to fix cells after cell culturing. Prepare by adding formaldehyde to 1X PBS and mixing well.
4% formaldehyde is used with adherent cells; 8% formaldehyde is used with non-adherent cells. The recipe in the Quick Chart for Preparing Buffers is written for use with a stock solution of 37% formaldehyde.

9.3 Preparation of Wash Buffer (0.1% Triton X-100 in PBS):

Prepare Wash Buffer by adding the provided 10% Triton X-100 solution to 1X PBS and mixing thoroughly.

9.4 Quenching Buffer (Wash Buffer containing 1% H_2O_2 and 0.1% Sodium Azide):

Quenching Buffer is used to inactivate the cells' endogenous peroxidase activity. Prepare by adding fresh Sodium Azide and fresh hydrogen peroxide to the Wash Buffer.

9.5 Blocking Buffer:

Supplied ready-to-use. A small amount of white precipitate may form if thawed in a warm water bath. This does not interfere with buffer function.

9.6 Antibody Dilution Buffer:

Supplied ready-to-use. A small amount of white precipitate may form if thawed in a warm water bath. This does not interfere with buffer function.

9.7 Diluted phospho- PI 3 Kinase p85 antibody:

Prepare by diluting the supplied antibody 1/500 in Antibody Dilution Buffer (see the Quick Chart for Preparing Buffers).

9.8 Diluted total- PI 3 Kinase p85 antibody:

Prepare by diluting the supplied antibody 1/500 in Antibody Dilution Buffer (see the Quick Chart for Preparing Buffers).

9.9 Diluted HRP-conjugated secondary antibody:

Prepare by diluting the supplied antibody 1/1000 in Antibody Dilution Buffer (see the Quick Chart for Preparing Buffers).

9.10 Preparation of Chemiluminescent Working Solution:

The Chemiluminescent Reagent and Reaction Buffer should be warmed to room temperature before use. These components are light sensitive. Therefore, we recommend avoiding direct exposure to intense light during storage. Prior to use, place the Chemiluminescent Reagent and Reaction Buffer at room temperature for at least 1 hour. In a separate container, mix 1 volume of Chemiluminescent Reagent with 2 volumes of Reaction Buffer to prepare the Chemiluminescent Working Solution (see the Quick Chart for Preparing Buffers). The Chemiluminescent Working Solution is stable for several hours. After the Chemiluminescent Working Solution is aliquoted into the wells, discard the remaining solution.

9.11 1% SDS Solution:

Supplied ready-to-use. 1% SDS Solution is used in the Crystal Violet counting procedure to solubilize cells and release the dye for subsequent quantification at 595 nm.

9.12 Crystal Violet Solution:

Supplied ready-to-use. Crystal Violet is used to estimate the relative number of cells in each well. This stain binds to cell nuclei and gives an OD_{595} reading that is proportional to cell number.

Quick Chart for Preparing Buffers

Reagents to prepare	Components	1 well	48 wells	96 wells	192 wells
Fixing Buffer for adherent cells	1X PBS	98 µL	4.7 mL	9.41 mL	18.82 mL
	37% Formaldehyde	12 µL	576 µL	1.15 mL	2.30 mL
Fixing Buffer for non-adherent cells	1X PBS	86 µL	4.13 mL	8.26 mL	16.51 mL
	37% Formaldehyde	24 µL	1.15 mL	2.30 mL	4.61 mL
Wash Buffer	1X PBS	3.376 mL	162 mL	310 mL	620 mL
	10% Triton X-100	31.4 µL	1.64 mL	3.13 mL	6.26 mL
Quenching Buffer	Wash Buffer	97.9 µL	4.7mL	9.40 mL	18.8 mL
	10% H_2O_2	11 µL	528 µL	1.06 mL	2.11 mL
	10% Sodium Azide	1.1 µL	52.8 µL	106 µL	211 µL
Diluted total-PI 3 Kinase p85 antibody	Antibody Dilution Buffer	45 µL	2080 µL	4160 µL	-
	Total- PI 3 Kinase p85 antibody	0.09 µL	4.16 µL	8.32 µL	-
Diluted phospho- PI 3 Kinase p85 antibody	Antibody Dilution Buffer	45 µL	2080 µL	4160 µL	-
	Phospho- PI 3 Kinase p85 antibody	0.09 µL	4.16 µL	8.32 µL	-
Diluted HRP-conjugated secondary antibody	Antibody Dilution Buffer	110 µL	5280 µL	10.56 mL	21.12 mL
	HRP-conjugated secondary antibody	0.11 µL	5.28 µL	10.56 µL	21.12 µL
1X PBS (for wash steps)	10X PBS	154 µL	7.39 mL	14.11 mL	28.22 mL
	dH_2O	1.39 mL	66.53 mL	127.01 mL	254.02 mL
Chemiluminescent Working Solution	Chemiluminescent Reagent	18 µL	864 µL	1.728 mL	3.46 mL
	Reaction Buffer	36 µL	1.728 mL	3.456 mL	6.91 mL

10. Sample Preparation

10.1 Adherent cell protocol:

10.1.1 Seed cells in the 96-well plate so that they will be approximately 80% confluent at the time of fixing, after they have been treated as desired. The growth area in each well of the 96-well plate is 0.32 cm^2. The provided plates are sterile and treated for tissue culture.

10.1.2 Grow and treat cells as desired.

10.1.3 Fix cells by replacing the growth medium with 100 µL of 4% formaldehyde in PBS. To minimize the escape of formaldehyde vapors, place a 10 cm x 17 cm piece of parafilm over the plate and then cover the plate with the lid. The covered plate can also be placed in a zip-lock bag. Incubate for 20 minutes at room temperature.

Δ Note: Formaldehyde is highly toxic. Confine vapors to a chemical hood and wear appropriate gloves and eye protection when using this chemical.

10.2 Non-Adherent cell protocol:

The protocol is suitable for use with non-adherent cells if the cells are cultured and fixed as follows:

10.2.1 Treat the 96-well culture plate with 10 µg/mL poly-L-Lysine for 30 minutes at 37°C. Wash twice for 5 minutes with PBS.

10.2.2 Seed 17,000 cells/well, or whatever amount is appropriate for your particular cell line.

10.2.3 Grow and treat cells as desired.

10.2.4 Fix cells by replacing the growth medium with 100 µL of 8% formaldehyde in PBS. Incubate for 20 minutes at room temperature.

Δ Note: Fixed cells are stable for several weeks. Several plates can be prepared simultaneously and then the assay performed when desired. Fixed cells should be stored refrigerated in a zip-lock or heat-sealed bag with the formaldehyde solution in the wells.

11. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all standards, controls and samples in duplicate.
- Prepare all reagents, working standards, and samples as directed in the previous sections.

11.1 Block cells

11.1.1 Remove formaldehyde solution and wash cells 3 times with 200 µL Wash Buffer. Each wash step should be performed for 5 minutes with gentle shaking.

11.1.2 Remove Wash Buffer, add 100 µL Quenching Buffer and incubate for 20 minutes at room temperature.

11.1.3 Remove Quenching Buffer and wash cells 2 times for 5 minutes each with 200 µL Wash Buffer.

11.1.4 Remove Wash Buffer, add 100 µL Antibody Blocking Buffer and incubate 1 hour at room temperature.

11.2 Binding of primary and secondary antibodies

Δ Note: Depending on experiment design, some wells may be incubated with diluted phospho antibody, some with total antibody and some with secondary antibody alone (negative controls). For negative control wells, incubate with 40 µL Antibody Dilution Buffer during primary antibody incubation step.

11.2.1 Remove Antibody Blocking Buffer and wash cells 2 times with 200 µL Wash Buffer.

11.2.2 Remove Wash Buffer, add 40 µL of diluted primary antibody (or Antibody Dilution Buffer for negative control wells) and seal plate with sealing tape. Place a 10 cm x 17 cm piece of parafilm over the plate, cover with lid and incubate overnight at 4°C. Be sure that the plate is level and that each well is tightly sealed with the sealing tape to prevent evaporation.
Δ Note: In cells known to generate high amounts of phosphorylated-PI 3 Kinase, a 3-hour primary antibody incubation is sufficient. For maximum sensitivity, an overnight incubation is recommended.

11.2.3 Remove primary antibody, wash cells 3 times for 5 minutes each with 200 µL Wash Buffer.

11.2.4 Remove Wash Buffer, add 100 µL diluted secondary antibody, cover plate with tissue culture plate lid or sealing tape, and incubate 1 hour at room temperature.

11.2.5 During this incubation, place the Chemiluminescent Reagent and Reaction Buffer at room temperature.

11.3 Chemiluminescent detection

11.3.1 Remove secondary antibody, wash cells 3 times for 5 minutes with 200 µL Wash Buffer and then 2 times for 5 minutes with 200 µL 1X PBS.

11.3.2 Remove PBS from plate wells and add 50 µL room temperature Chemiluminescent Working Solution to each well.

11.3.3 Read chemiluminescence using a luminometer or CCD camera system. Readings should be taken within 10 minutes to minimize changes in signal intensity.

Δ Note: The phospho-PI 3 Kinase p85 and total-PI 3 Kinase p85 antibodies can be used on equivalent cell cultures to determine the effects of various cell treatments on the ratio of phosphorylated PI 3 Kinase to total PI 3 Kinase. However, if the signals obtained with the phospho-PI 3 Kinase p85 antibody and the total-PI 3 Kinase p85 antibody are identical, one cannot conclude that the treatment resulted in phosphorylation of 100% of the PI 3 Kinase.

11.4 OPTIONAL - Crystal Violet cell staining

Crystal Violet is an intense stain that binds to the cell nuclei and gives an OD_{595} reading that is proportional to cell number. If you wish to normalize your readings from above, simply follow the steps below.

11.4.1 After reading chemiluminescence, wash wells twice with 200 µL Wash Buffer and 2 times with 200 µL 1X PBS. Tap plates onto paper towels to remove excess liquid from wells and air-dry at room temperature for 5 minutes.

11.4.2 Add 100 µL Crystal Violet solution to each well and incubate 30 minutes at room temperature.
Δ Note: Crystal Violet is an intense stain. Avoid contact with skin and clothing.

11.4.3 Wash wells 3 times with 200 µL 1X PBS for 5 minutes each.

11.4.4 Add 100 µL of 1% SDS Solution to each well and incubate on shaker for 1 hour at room temperature.

11.4.5 Read absorbance on a spectrophotometer at 595 nm. If the signals obtained are greater than the range of your

spectrophotometer, the signal can be reduced by removing some (*e.g.* 50 µL) of the liquid from each well and replacing with an equivalent volume of dH_2O.

11.4.6 The measured OD_{595} readings indicate the relative number of cells in each well. This relative cell number is then used to normalize each reading from Step 11.3.

12. Assay Specificity

The phospho-PI 3 Kinase p85 antibody preferentially recognizes peptides and proteins containing p85 PI 3-Kinase phospho-tyrosine 467 (alpha) or 199 (gamma). No cross-reactivity is observed with corresponding non-phosphorylated sequences or with other phospho-tyrosine-containing motifs. The total-PI 3 Kinase p85 antibody recognizes the total level of PI 3 Kinase p85 proteins regardless of the phosphorylation state.

13. Species Reactivity

This kit recognizes phosphorylated PI 3 Kinase p85 from all species and total PI 3 Kinase p85 from human, mouse and rat origin.

14. Troubleshooting

Problem	Reason	Solution
No signal or weak signal in wells incubated with either phospho-PI 3 Kinase p85 antibody or total-PI 3 Kinase p85 antibody.	Omission of key reagent	Check that all reagents have been added in the correct order
	Substrate or conjugate is no longer active	Test conjugate and substrate for activity
	Enzyme inhibitor present	Sodium azide will inhibit the peroxidase reaction, follow our recommendations to prepare buffers
	Plate reader or CCD camera settings not optimal	Verify the wavelength (measurement mode) and filter settings in the plate reader
	Cells do not contain detectable levels of phospho PI 3 Kinase p85 and/or total PI 3 Kinase p85	Use Western blotting to confirm that cells contain detectable levels of protein(s) of interest.
	Insufficient number of cells were plated	Plate cells so that they are 80% confluent at time of fixing
	Cells did not adhere correctly to plate	Follow protocol for use of non-adherent cells
	Cells are not from correct origin	Follow protocol for use of non-adherent cells
	Excessive washing	Wash steps should be 5 minutes each
	Incubation of secondary antibody was too long	Incubate secondary antibody for 1 hour

High background in all wells	Measurement time too long	Reduce integration time or exposure time on luminometer or CCD camera
	Concentration of antibodies too high	Perform antibody titration to determine optimal working concentration. Start using 1/500 for the phospho- and the total-antibody and 1/1000 for the secondary antibody. The sensitivity of the assay will be decreased
	Inadequate washing	Ensure all wells are filled with Wash Buffer and follow washing recommendations
	Inadequate quenching or blocking	Ensure that quenching and blocking steps were performed according to the protocol
Uneven signal development	Incomplete washing of wells	Ensure all wells are filled with Wash Buffer and follow washing recommendations
	Well cross-contamination	Follow washing recommendations
No signal or weak signal in wells incubated with phospho-PI 3 Kinase p85 antibody	Cell culture conditions did not induce phosphorylation of PI 3 Kinase	Perform Western blot with phospho-PI 3 Kinase p85 antibody to confirm that cells contain detectable levels of phosphorylated PI 3 Kinase p85
Antibody solution evaporates from well during overnight incubation with primary antibody	Sealing tape was incorrectly applied	Ensure that each well is sealed when sealing tape is applied and ensure that the parafilm sheet covers the plate completely before the lid is placed on the plate. The plate can also be placed in a zip-lock or heat-sealed bag

Insufficient sensitivity	Antibody concentration incorrect	If the cells studied have very low levels of the protein of interest, the sensitivity of detection may be improved by increasing the concentration of primary antibody used and by minimizing the incubation volume. It is possible to perform the overnight incubation in as little as 25 µL, however, this will make multichannel pipetting difficult and requires the plate be carefully sealed and incubated on a level surface. Alternatively, if the cells have easily detectable levels of the phosphorylated protein and the detection of small changes in phosphorylation is desired, sensitivity of the assay may be improved by decreasing the concentration of the phospho antibody used
Poor precision	Cross-well read through	The 96-well plates provided are designed to minimize signal cross-well contamination. If possible, do not use the phospho and total antibodies in adjoining wells. If this is not possible, use the total antibody at a higher dilution

15. Notes

Technical Support

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