Version 1 Last updated 23 June 2017

ab207483 Phospho-p38 MAPK (T180/Y182) + Total In-Cell ELISA Kit (Chemiluminescent)

For the quantitative measurement of total and T180/Y182 phosphorylated p38 MAPK in adherent and non-adherent cells.

This product is for research use only and is not intended for diagnostic use.

Table of Contents

1. Overview

Phospho-p38 MAPK (T180/Y182) + Total In-Cell ELISA Kit (Chemiluminescent) (ab207483) provides a simple, efficient, cell-based method to monitor proteins activated by phosphorylation. The kit is designed specifically to quantify activated (phosphorylated) p38 MAPK and/or total p38 MAPK. 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 p38 MAPK or total p38 MAPK. 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-p38 MAPK (T180/Y182) + Total In-Cell ELISA Kit (Chemiluminescent) contains two 96-well plates and two primary antibodies. The phospho-p38 antibody was raised in rabbit against a synthetic phospho-peptide corresponding to residues flanking the phosphorylation sites Thr180/Tyr182 of human p38 MAP kinase. This antibody recognizes only phosphorylated p38 MAPK. The total-p38 antibody was raised in rabbit and recognizes p38 MAPK regardless of its phosphorylation state. The kit can be used to study phosphorylated p38 MAPK relative to cell number or to determine p38 MAPK phosphorylation relative to the total p38 MAPK protein found in the cells. Once the phospho-p38 MAPK and total-p38 MAPK signals have been normalized for cell number, a comparison of the ratio of phosphorylated p38 MAPK to total p38 MAPK for each of the cell growth conditions can be made. The provided total-p38 antibody can be used as a positive control to demonstrate that the cells contain p38 MAPK, the kit reagents are functional and that the protocol is performed correctly. Also, because fixed cells are stable for several weeks, many plates can be prepared simultaneously and then the assay performed when desired.

p38 MAPK is a member of the mitogen-activated protein kinase (MAPKs) family of serine/threonine protein kinases. These proteins are widely conserved among eukaryotes and are involved in many cellular processes such as cell proliferation, cell movement and cell death.

MAPKs phosphorylate specific serines and threonines on target protein substrates and function in signaling cascades that convey external stimuli from the cell surface to cellular targets such as translational machinery, cytoskeletal proteins and transcription factors.

The p38 MAPK signaling cascade is initiated when external stimuli such as growth factors, cellular stress (e.g. osmotic shock, radiation) or inflammatory cytokines are recognized by cell-surface receptors. This results in the activation of the RHO family GTPases (Rac, Rho, Cdc42), which activate MAPKKKs (e.g. MLK, TAK, ASK1). These MAPKKKs in turn phosphorylate and activate p38 MAPK kinase, which then phosphorylates p38 MAPK. Targets of phosphorylated (activated) p38 MAPK include the transcription factors STAT1, Myc/Max, Elk-1, CHOP, MEF2, ATF-2 and (through Msk-1) CREB.

Aberrant regulation of p38 MAPK is thought to contribute to a variety of physiological pathologies, including human diseases such as asthma and autoimmunity.

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-p38 antibody	9 µL	5 x 9 µL	-20°C
Total-p38 antibody	9 µL	5 x 9 µL	+4°C
Anti-rabbit HRP-conjugated IgG	11 µL	5 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-p38 antibody and one 96-well assay with the total-p38 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-p38 antibody:

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

9.8 Diluted total-p38 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 1/2000 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	34.1 µL	1.64 mL	3.13 mL	6.25 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-p38 antibody	Antibody Dilution Buffer	45 µL	2080 µL	4160 µL	-
	Total-p38 antibody	0.09 µL	4.16 µL	8.32 µL	-
Diluted phospho-p38 antibody	Antibody Dilution Buffer	45 µL	2080 µL	4160 µL	-
	Phospho-p38 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	0.055 µL	2.64 µL	5.28 µL	10.56 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-p38 antibody, some with total-p38 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- p38 (*e.g.* macrophage-derived cell lines), 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-p38 and total-p38 antibodies can be used on equivalent cell cultures to determine the effects of various cell treatments on the ratio of phosphorylated p38 to total p38. However, if the signals with the phospho- p38 antibody and the total- p38 antibody are identical, one cannot conclude that the treatment resulted in phosphorylation of 100% of the p38.

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-p38 antibody is specific for phosphorylated p38 and was raised against a peptide phosphorylated on residues that correspond to the sequence surrounding Thr180 and Tyr182 of human p38. It recognizes p38 alpha, beta, gamma and delta isoforms. The total-p38 antibody recognizes p38 MAPK regardless of its phosphorylation state.

13. Species Reactivity

This kit recognizes phosphorylated and total p38 from human, mouse and rat origin.

14. Troubleshooting

Problem	Reason	Solution
No signal or weak signal in wells incubated with either phospho-p38 antibody or total-p38 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 p38 and/or total p38	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 1/2000 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-p38 antibody	Cell culture conditions did not induce phosphorylation of p38	Perform Western blot with phospho-p38 antibody to confirm that cells contain detectable levels of phosphorylated p38
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

Austria
wissenschaftlicherdienst@abcam.com | 019-288-259
France
supportscientifique@abcam.com | 01.46.94.62.96
Germany
wissenschaftlicherdienst@abcam.com | 030-896-779-154
Spain
soportecientifico@abcam.com | 91-114-65-60

Switzerland
technical@abcam.com
Deutsch: 043-501-64-24 | Français: 061-500-05-30
UK, EU and ROW
technical@abcam.com | +44(0)1223-696000

Canada
ca.technical@abcam.com | 877-749-8807
US and Latin America
us.technical@abcam.com | 888-772-2226

Asia Pacific
hk.technical@abcam.com | (852) 2603-6823
China
cn.technical@abcam.com | +86-21-5110-5938 | 400-628-6880
Japan
technical@abcam.co.jp | +81-(0)3-6231-0940
Singapore
sg.technical@abcam.com | 800 188-5244

Australia
au.technical@abcam.com | +61-(0)3-8652-1450
New Zealand
nz.technical@abcam.com | +64-(0)9-909-7829