ab207478

In-Cell ELISA Support Pack for Chemiluminescent Detection

In-Cell ELISA Support Pack for Chemiluminescent Detection) provide a cell-based method to monitor proteins activated by phosphorylation.

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

1. Overview 1
2. Protocol Summary 3
3. Precautions 4
4. Storage and Stability 4
5. Limitations 5
6. Materials Supplied 5
7. Materials Required, Not Supplied 6
8. Technical Hints 7
9. Reagent Preparation 8
10. Sample Preparation 11
11. Assay Procedure 13
12. Assay Specificity 15
13. Species Reactivity 15
14. Troubleshooting 16
15. Notes 19
1. Overview

In-Cell ELISA Support Pack for Chemiluminescent Detection (ab207478) provide a simple, efficient, cell-based method to monitor proteins activated by phosphorylation. The kit is designed specifically to quantify activated (phosphorylated) protein and/or total protein. 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 protein or total protein. Subsequent incubation with secondary HRP-conjugated antibody 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 kit can be used to study phosphorylated protein relative to cell number or to determine protein phosphorylation relative to the total protein found in the cells. Once the phospho-target and total-target signals have been normalized for cell number, a comparison of the ratio of phosphorylated protein to total protein for each of the cell growth conditions can be made. The provided total-protein antibody can be used as a positive control to demonstrate that the cells contain the protein of interest, 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.

Efforts to measure downstream effects of signal transduction events have been hampered by the lack of convenient and high-throughput assays suitable for quantifying target protein activation (phosphorylation).

In-Cell ELISA kits are highly sensitive 96-well assays designed for detecting activated proteins within mammalian cells. Unlike Western blot, In-Cell ELISA assays do not require cell extracts, electrophoresis or membrane blotting. And, unlike typical kinase assays, In-Cell ELISA assays are non-radioactive and simple to perform. Each In-Cell ELISA
Support Pack for Chemiluminescent Detection contains two 96-well plates and optimized assay reagents.

The In-Cell ELISA Support Pack for Chemiluminescent Detection (ab207478) can be used to study your choice of phosphorylated target protein relative to cell number. In this application, cells are cultured in the wells of one of the provided 96-well plates, treated as desired and then assayed using the In-Cell ELISA protocol with only the phospho-specific antibody. The relative number of cells in each well is then determined through use of the Crystal Violet reagent. In this application, the second 96-well plate can be kept on reserve in case of culturing problems or two 48-well assays can be performed.

The In-Cell ELISA Support Pack for Chemiluminescent Detection (ab207478) can also be used to determine target protein phosphorylation relative to the total target protein found in the cells. In this application, the two 96-well plates are cultured as replicates, with the wells within each plate treated with reagents that may affect the phosphorylation state of your desired target protein. After the cells are fixed, one plate is studied with the phospho-specific antibody, while the other plate is studied with the total-target protein antibody of your choice. The relative number of cells in each well is then determined through use of the Crystal Violet reagent. Once the phospho-target protein and total-target protein signals have been normalized for cell number, a comparison of the ratio of phosphorylated target protein to total target protein for each of the cell growth conditions can be made.

In the In-Cell ELISA Support Pack for Chemiluminescent Detection assay, intact cells are fixed with formaldehyde to preserve their characteristics at a chosen time point. Because fixed cells are stable for several weeks, you can prepare many plates simultaneously and then perform the In-Cell ELISA assay when desired. Fixed cells should be stored refrigerated in a zip-lock or heat-sealed bag with the formaldehyde solution in the wells.
2. Protocol Summary

Prepare all reagents, samples, and standards as instructed
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 Reagent

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

<table>
<thead>
<tr>
<th>Item</th>
<th>1 x 96 tests</th>
<th>5 x 96 tests</th>
<th>Storage Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X Antibody Blocking Buffer</td>
<td>22 mL</td>
<td>5 x 22 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>1X Antibody Dilution Buffer</td>
<td>30 mL</td>
<td>5 x 30 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>10X PBS</td>
<td>120 mL</td>
<td>5 x 120 mL</td>
<td>RT</td>
</tr>
<tr>
<td>10% Triton X-100</td>
<td>10 mL</td>
<td>5 x 10 mL</td>
<td>RT</td>
</tr>
<tr>
<td>Crystal Violet Solution</td>
<td>22 mL</td>
<td>5 x 22 mL</td>
<td>+4°C</td>
</tr>
<tr>
<td>Chemiluminescent Reagent</td>
<td>2 x 2 mL</td>
<td>10 x 2 mL</td>
<td>+4°C</td>
</tr>
<tr>
<td>Reaction Buffer</td>
<td>2 x 4 mL</td>
<td>10 x 4 mL</td>
<td>+4°C</td>
</tr>
<tr>
<td>1% SDS Solution</td>
<td>22 mL</td>
<td>5 x 22 mL</td>
<td>RT</td>
</tr>
<tr>
<td>96-well tissue culture plate</td>
<td>2</td>
<td>10</td>
<td>RT</td>
</tr>
<tr>
<td>Plate sealing tape</td>
<td>2</td>
<td>10</td>
<td>RT</td>
</tr>
</tbody>
</table>
7. Materials Required, Not Supplied

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

- Primary antibodies specific to protein of interest.
- HRP-conjugated secondary antibodies.
- 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 (H₂O₂) in dH₂O (3 mL is required).
- 10 µg/mL poly-L-Lysine (if using non-adherent cells).
- 10% Sodium Azide (NaN₃) in dH₂O (250 µL are 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-target antibody and one 96-well assay with the total-target antibody.

9.1 11X PBS:
Prepare 1X PBS by adding 1 volume of 10X PBS (pH 7.4) to 9 volumes of dH$_2$O 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$_2$O$_2$ 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-specific antibody:**
We recommend using a dilution of 1/250 to 1/500 in Antibody Dilution Buffer. However, with antibodies that have not been tested in In-Cell ELISA, the optimal dilution may have to be determined empirically.

9.8 **Diluted total-target antibody:**
We recommend a dilution of 1/500 in Antibody Dilution Buffer. However, with antibodies that have not been tested in In-Cell ELISA, the optimal dilution may have to be determined empirically.

9.9 **Diluted HRP-conjugated secondary antibody:**
We recommend diluting the secondary 1/2000 in Antibody Dilution Buffer. However, the optimal dilution may have to be determined empirically.

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

<table>
<thead>
<tr>
<th>Reagents to prepare</th>
<th>Components</th>
<th>1 well</th>
<th>48 wells</th>
<th>96 wells</th>
<th>192 wells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fixing Buffer for adherent cells</strong></td>
<td>1X PBS</td>
<td>98 µL</td>
<td>4.7 mL</td>
<td>9.41 mL</td>
<td>18.82 mL</td>
</tr>
<tr>
<td></td>
<td>37% Formaldehyde</td>
<td>12 µL</td>
<td>576 µL</td>
<td>1.15 mL</td>
<td>2.30 mL</td>
</tr>
<tr>
<td><strong>Fixing Buffer for non-adherent cells</strong></td>
<td>1X PBS</td>
<td>86 µL</td>
<td>4.13 mL</td>
<td>8.26 mL</td>
<td>16.51 mL</td>
</tr>
<tr>
<td></td>
<td>37% Formaldehyde</td>
<td>24 µL</td>
<td>1.15 mL</td>
<td>2.30 mL</td>
<td>4.61 mL</td>
</tr>
<tr>
<td><strong>Wash Buffer</strong></td>
<td>1X PBS</td>
<td>3.376 mL</td>
<td>162 mL</td>
<td>310 mL</td>
<td>620 mL</td>
</tr>
<tr>
<td></td>
<td>10% Triton X-100</td>
<td>34.1 µL</td>
<td>1.64 mL</td>
<td>3.13 mL</td>
<td>6.26 mL</td>
</tr>
<tr>
<td><strong>Quenching Buffer</strong></td>
<td>Wash Buffer</td>
<td>97.9 µL</td>
<td>4.7 mL</td>
<td>9.40 mL</td>
<td>18.8 mL</td>
</tr>
<tr>
<td></td>
<td>10% H₂O₂</td>
<td>11 µL</td>
<td>528 µL</td>
<td>1.06 mL</td>
<td>2.11 mL</td>
</tr>
<tr>
<td></td>
<td>10% Sodium Azide</td>
<td>1.1 µL</td>
<td>52.8 µL</td>
<td>106 µL</td>
<td>211 µL</td>
</tr>
<tr>
<td><strong>Diluted total-antibody (1/500 example)</strong></td>
<td>Antibody Dilution Buffer</td>
<td>45 µL</td>
<td>2080 µL</td>
<td>4160 µL</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Total-target antibody</td>
<td>0.09 µL</td>
<td>4.16 µL</td>
<td>8.32 µL</td>
<td>-</td>
</tr>
<tr>
<td><strong>Diluted phospho-antibody (1/250 example)</strong></td>
<td>Antibody Dilution Buffer</td>
<td>45 µL</td>
<td>2080 µL</td>
<td>4160 µL</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Phospho-target antibody</td>
<td>0.18 µL</td>
<td>8.32 µL</td>
<td>16.64 µL</td>
<td>-</td>
</tr>
<tr>
<td><strong>Diluted HRP-conjugated secondary antibody (1/2000 example)</strong></td>
<td>Antibody Dilution Buffer</td>
<td>110 µL</td>
<td>5.28 mL</td>
<td>10.56 mL</td>
<td>21.12 mL</td>
</tr>
<tr>
<td></td>
<td>HRP-conjugated secondary antibody</td>
<td>0.055 µL</td>
<td>2.64 µL</td>
<td>5.28 µL</td>
<td>10.56 µL</td>
</tr>
<tr>
<td><strong>1X PBS (for wash steps)</strong></td>
<td>10X PBS</td>
<td>154 µL</td>
<td>7.39 mL</td>
<td>14.11 mL</td>
<td>28.22 mL</td>
</tr>
<tr>
<td></td>
<td>dH₂O</td>
<td>1.39 mL</td>
<td>66.53 mL</td>
<td>127.01 mL</td>
<td>254.02 mL</td>
</tr>
<tr>
<td>Reagents to prepare</td>
<td>Components</td>
<td>1 well</td>
<td>48 wells</td>
<td>96 wells</td>
<td>192 wells</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>---------------------------------</td>
<td>--------</td>
<td>----------</td>
<td>------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Chemiluminescent Working</td>
<td>Chemiluminescent Reagent</td>
<td>18 µL</td>
<td>864 µL</td>
<td>1.728 mL</td>
<td>3.46 mL</td>
</tr>
<tr>
<td>Solution</td>
<td>Reaction Buffer</td>
<td>36 µL</td>
<td>1.728 mL</td>
<td>3.456 mL</td>
<td>6.91 mL</td>
</tr>
</tbody>
</table>

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 cm². 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.

△ 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.

10.3 Note: Fixed cells should be stored refrigerated in a zip-lock or heat-sealed bag with the formaldehyde solution in the wells.
11. Assay Procedure

1. Equilibrate all materials and prepared reagents to room temperature prior to use.
2. We recommend that you assay all standards, controls and samples in duplicate.
3. 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

△ Note: Depending on experiment design, some wells may be incubated with diluted phospho-specific antibody, some with total-target 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.

△ Note: In cells known to generate high amounts of the phosphorylated form of the protein of interest, 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. Read chemiluminescence using a luminometer or CCD camera system. Readings should be taken within 10 minutes to minimize changes in signal intensity.

\[\textbf{Note:}\] The phospho-specific and total-target antibodies can be used on equivalent cell cultures to determine the effects of various cell treatments on the ratio of phosphorylated protein of interest to total protein of interest. However, if the signals with the phospho-specific antibody and the total-target antibody are identical, one cannot conclude that the treatment resulted in phosphorylation of 100% of the protein of interest.

11.4 OPTIONAL - Crystal Violet cell staining

Crystal Violet is an intense stain that binds to the cell nuclei and gives an \(\text{OD}_{595}\) 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.
△ 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 dH₂O.
11.4.6 The measured OD₅₉₅ 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

Choose an antibody specific for your desired phosphorylated protein that recognizes the targeted protein only when phosphorylated. Also, choose an antibody that recognizes the target protein regardless of its phosphorylation state if you want to compare phosphorylation levels versus normal levels of protein.

13. Species Reactivity

The species reactivity depends on the species reactivity of the phospho-specific and total-target antibody used in the In-Cell ELISA assay and varies.
## Troubleshooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Reason</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>No signal or weak signal in wells incubated with either phospho-specific antibody or total-target antibody.</td>
<td>Omission of key reagent</td>
<td>Check that all reagents have been added in the correct order</td>
</tr>
<tr>
<td></td>
<td>Substrate or conjugate is no longer active</td>
<td>Test conjugate and substrate for activity</td>
</tr>
<tr>
<td></td>
<td>Enzyme inhibitor present</td>
<td>Sodium azide will inhibit the peroxidase reaction, follow our recommendations to prepare buffers</td>
</tr>
<tr>
<td></td>
<td>Plate reader or CCD camera settings not optimal</td>
<td>Verify the wavelength (measurement mode) and filter settings in the plate reader</td>
</tr>
<tr>
<td></td>
<td>Developing Solution was cold</td>
<td>Bring Developing Solution to room temperature</td>
</tr>
<tr>
<td></td>
<td>Inadequate volume of Developing Solution</td>
<td>Check to make sure that correct volume is delivered by pipette</td>
</tr>
<tr>
<td></td>
<td>Cells do not contain detectable levels of phospho and/or total target protein</td>
<td>Use Western blotting to confirm that cells contain detectable levels of protein(s) of interest</td>
</tr>
<tr>
<td></td>
<td>Insufficient number of cells were plated</td>
<td>Plate cells so that they are 80% confluent at time of fixing</td>
</tr>
<tr>
<td></td>
<td>Cells did not adhere correctly to plate</td>
<td>Follow protocol for use of non-adherent cells</td>
</tr>
<tr>
<td></td>
<td>Cells are not from correct origin</td>
<td>Refer to species reactivity information</td>
</tr>
<tr>
<td></td>
<td>Excessive washing</td>
<td>Wash steps should be 5 minutes each</td>
</tr>
<tr>
<td></td>
<td>Incubation of secondary antibody was too long</td>
<td>Incubate secondary antibody for 1 hour</td>
</tr>
<tr>
<td>Issue</td>
<td>Description</td>
<td>Solution</td>
</tr>
<tr>
<td>-------</td>
<td>-------------</td>
<td>----------</td>
</tr>
<tr>
<td>High background in all wells</td>
<td>Measurement time too long</td>
<td>Reduce integration time or exposure time on luminometer or CCD camera</td>
</tr>
<tr>
<td></td>
<td>Concentration of antibodies too high</td>
<td>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</td>
</tr>
<tr>
<td></td>
<td>Inadequate washing</td>
<td>Ensure all wells are filled with Wash Buffer and follow washing recommendations</td>
</tr>
<tr>
<td></td>
<td>Inadequate quenching or blocking</td>
<td>Ensure that quenching and blocking steps were performed according to the protocol</td>
</tr>
<tr>
<td>Uneven signal development</td>
<td>Incomplete washing of wells</td>
<td>Ensure all wells are filled with Wash Buffer and follow washing recommendations</td>
</tr>
<tr>
<td></td>
<td>Well cross-contamination</td>
<td>Follow washing recommendations</td>
</tr>
<tr>
<td>No signal or weak signal in wells incubated with phospho-specific antibody</td>
<td>Cell culture conditions did not induce phosphorylation of target protein</td>
<td>Perform Western blot with phospho-specific antibody to confirm that cells contain detectable levels of phosphorylated target protein</td>
</tr>
<tr>
<td>Antibody solution evaporates from well during overnight incubation with primary antibody</td>
<td>Sealing tape was incorrectly applied</td>
<td>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</td>
</tr>
<tr>
<td>Insufficient sensitivity</td>
<td>Antibody concentration incorrect</td>
<td>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</td>
</tr>
<tr>
<td>Poor precision</td>
<td>Cross-well read through</td>
<td>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</td>
</tr>
</tbody>
</table>
15. Notes
Technical Support

Copyright © 2017 Abcam, All Rights Reserved. The Abcam logo is a registered trademark. All information / detail is correct at time of going to print.

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

UK, EU and ROW
technical@abcam.com | +44(0)1223-696000

Canada
c.a.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