FACE™

NFκB p65 Profiler Kit

(version A)

Catalog No. 48300 (Colorimetric)
Catalog No. 48400 (Chemiluminescent)

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Overview

The transcription factor NFκB (nuclear factor κB) is a key component for the inducible expression of a wide variety of cellular and viral genes. NFκB is composed of a heterodimer of p65 and p50 subunits in most cell types and is sequestered in the cytoplasm by its inhibitory proteins, the IκBs [1-3]. During the phosphorylation and degradation of IκBs, NFκB p65 is phosphorylated on multiple resides, each triggered by different stimuli but essential for maintaining NFκB transcriptional activation. Because of its role in regulating inflammatory and immune responses, high-throughput study methods for monitoring NFκB phosphorylation are in high demand.

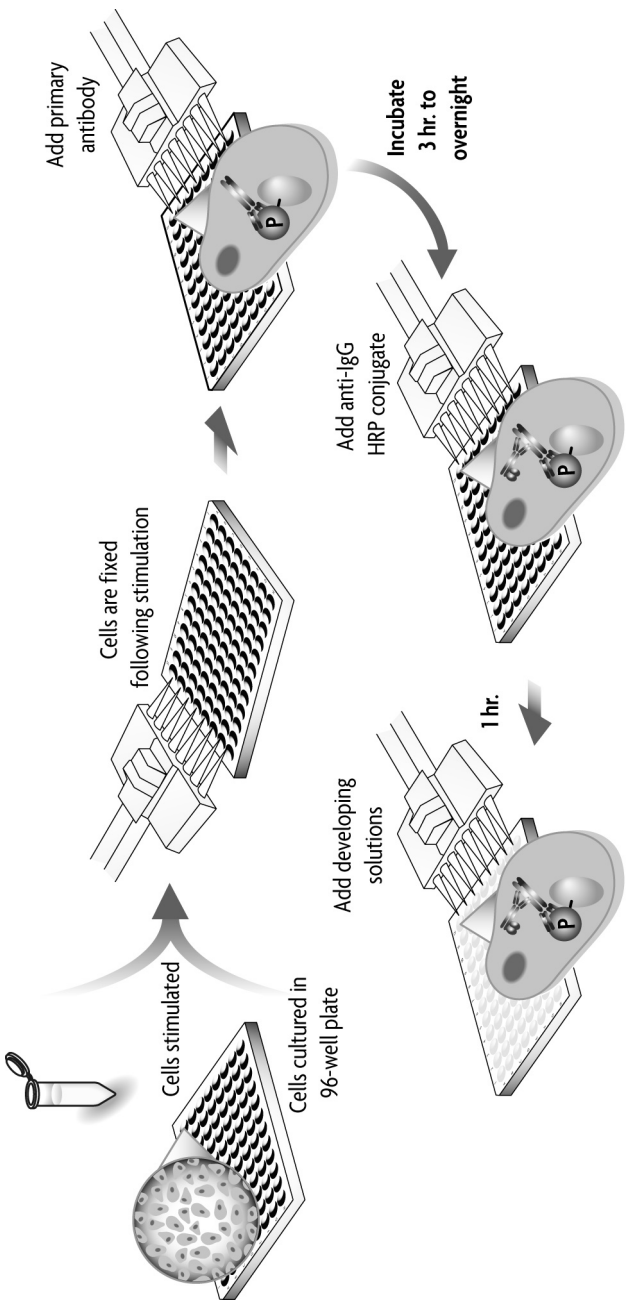
Fast Activated Cell-based ELISA (FACE™)* Kits provide a simple, efficient, cell-based method to monitor proteins activated by phosphorylation. FACE NFκB p65 Profiler Kits are designed specifically to quantify activated (phosphorylated) NFκB p65 and/or total NFκB p65[4]. In the FACE method, cells are 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 phosphorylated NFκB p65 at Serine 468, Serine 536 or total NFκB p65. Subsequent incubation with secondary HRP-conjugated antibody and developing solution provides an easily quantified colorimetric or 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:

product	format	catalog no.
FACE NFκB p65 Profiler	3 x 96 rxns	48300
FACE NFκB p65 Profiler Chemi	3 x 96 rxns	48400

See Active Motif products related to NFκB in Appendix, Section B.

* Developed in collaboration with Dr. M. Peppelenbosch and Dr. H. Versteeg.

Flow Chart of Process



Introduction

NFκB

The transcription factor NFκB is implicated in the regulation of many genes that code for mediators of the immune, acute phase and inflammatory responses[5]. The DNA-binding protein complex recognizes a discrete nucleotide sequence (5´-GGGACTTTCC-3´) in the upstream region of a variety of cellular and viral response genes[6]. NFκB is composed of homo- and heterodimeric complexes of members of the Rel (NFκB) family. There are five subunits of the NFκB family in mammals: p50, p65 (RelA), c-Rel, p52 and RelB[7]. These proteins share a conserved 300 amino acid sequence in the N-terminal region, known as the Rel homology domain, that mediates DNA binding, protein dimerization and nuclear localization. This domain is also a target of the IκB inhibitors, which include IκBα, IκBβ, IκBγ, Bcl-3, p105 and p100[8]. The p50/p65 (NFκB1/RelA) heterodimers and the p50 homodimers are the most common dimers found in the NFκB signaling pathway. In the majority of cells, NFκB exists in an inactive form in the cytoplasm, bound to the inhibitory IκB proteins[9].

Treatment of cells with various inducers results in the phosphorylation, ubiquitination and subsequent degradation of IκB proteins[10] (For studying the phosphorylation state of IκBα, see Active Motif's FunctionELISA™ IκBα Kit). During the phosphorylation and degradation of IκBs, NFκB p65 is phosphorylated on multiple resides, each triggered by different stimuli but essential for maintaining NFκB transcriptional activation. Phosphorylation at Ser536 occurs in the C-terminal transactivation domain[11] and is critical for p65 transcriptional activity. Phosphorylation at this site is triggered by several stimuli including phorbol ester, IL-1α and TNF-α as mediated by IκB kinase and p38 MAPK. Phosphorylation at Ser276 by PKA and/or MSK1 enables increased interaction with the transcriptional coactivator p300/CBP to further enhance the transcriptional activity of NFκB[12-14]. In contrast, PMA-induced NFκB transcriptional activity is dependent on the region between amino acids 442 and 470, suggesting a role for one or more of the potential phosphorylation sites (Ser457, Thr458, Thr464 or Ser468).

Traditional Kinase Assays

To date, two methods are widely used to perform kinase assays:

1. One method typically used is the in-gel kinase assay, which is an activity staining technique used to study protein kinases[15]. A given protein substrate is immobilized on a gel and phosphorylated by protein kinases, which are separated by SDS-PAGE. The bands of incorporated [^{32}P]phosphate are then visualized by autoradiography. While this method is sensitive, it is also cumbersome and is not suitable to high-throughput applications. In-gel kinase assays also require special precautions and equipment for handling radioactivity.
2. Another method used is Western blot analysis. Western blots are performed using antibodies that recognize only the phosphorylated version of the protein of interest. Although less tedious than in-gel kinase assays, Western blotting, like in-gel kinase, requires the preparation of nuclear or whole-cell extract and separation by SDS-PAGE. Furthermore, this process is expensive due to the large quantity of phospho-specific antibody required.

FACE NFκB p65 Profiler

The role of NFκB p65 in the regulation of inflammatory and immune responses has made it an interesting target for both basic and pharmaceutical research. However, these efforts have been hampered by the lack of convenient and high-throughput assays suitable for quantifying NFκB p65 activation (phosphorylation).

To overcome this, Active Motif has introduced its FACE™ (Fast Activated Cell-based ELISA) Kits. These are highly sensitive 96-well assays designed for detecting activated proteins within mammalian cells. Unlike Western blot, FACE assays do not require cell extracts, electrophoresis or membrane blotting. And, unlike typical kinase assays, FACE assays are non-radioactive and simple to perform. Each FACE NFκB p65 Profiler Kit contains three 96-well plates and three primary antibodies. The phospho-NFκB p65 antibodies are specific for phosphorylated NFκB p65 and do not cross-react with other family members. The phospho-NFκB p65 (S468) was raised against a synthetic phospho-peptide corresponding to residues surrounding Serine 468 of human NFκB p65 and does not cross-react with other sites. The phospho-NFκB p65 (S536) was raised against a synthetic phospho-peptide corresponding to residues surrounding Serine 536 of human NFκB p65 and does not cross-react with other sites. The total-NFκB p65 antibody recognizes NFκB p65 proteins regardless of the phosphorylation state.

FACE NFκB p65 Profiler Kits can be used to study the phosphorylation of NFκB p65 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 FACE protocol with only the phospho-NFκB p65 antibodies. The relative number of cells in each well is then determined through use of the Crystal Violet reagent. In this application, the other 96-well plates can be kept on reserve in case of culturing problems.

FACE NFκB p65 Kits can also be used to determine NFκB p65 phosphorylation relative to the total NFκB p65 protein found in the cells. In this application, the three 96-well plates are cultured as replicates, with the wells within each plate treated with reagents that may affect the phosphorylation state of NFκB p65. After the cells are fixed, two plates are studied with each of the phospho-NFκB p65 antibodies, while the third plate is studied with the total-NFκB p65 antibody. The relative number of cells in each well is then determined through use of the Crystal Violet reagent. Once the phospho-NFκB p65 and total-NFκB p65 signals have been normalized for cell number, a comparison of the ratio of phosphorylated NFκB p65 to total NFκB p65 for each of the cell growth conditions can be made.

In the FACE NFκB p65 assay, the provided total-NFκB p65 antibody can be used as a positive control to demonstrate that the cells contain NFκB p65, 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 FACE assay when desired. Fixed cells should be stored refrigerated in a zip-lock or heat-sealed bag with the formaldehyde solution in the wells.

Kit Performance and Benefits

FACE NFκB p65 Kits are for research use only. Not for use in diagnostic procedures.

Antibody specificity: The phospho-NFκB p65 (S468) was raised against a synthetic phospho-peptide corresponding to residues surrounding Serine 468 of human NFκB p65 and does not cross-react with other sites. The phospho-NFκB p65 (S536) was raised against a synthetic phospho-peptide corresponding to residues surrounding Serine 536 of human NFκB p65 and does not cross-react with other sites. The total-NFκB p65 antibody recognizes NFκB p65 proteins regardless of the phosphorylation state.

Cross-reactivity: FACE NFκB p65 Kits detect phosphorylated (S536) from human and mouse, (S468) from human, mouse and rat, and total NFκB p65 from human and monkey origin.

Assay time: < 3 hours of hands-on time.

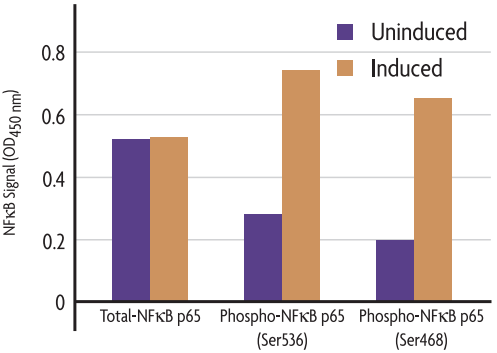


Figure 1: Measurement of phosphorylated and total NFκB p65.
HeLa cells were cultured in 96-well plates and serum-starved for 16 hours. Cells were then treated with 20 ng/ml TNF-α and 50 nM Calyculin A for 15 minutes and fixed. Total and phospho NFκB p65 were each assayed in triplicate using the phospho and total NFκB p65 antibodies included in the FACE NFκB p65 Profiler Kit. Data was plotted after correction for cell number (performed through use of Crystal Violet).

Note on data interpretation

The phospho-NFκB p65 and total-NFκB p65 antibodies can be used on equivalent cell cultures to determine the effects of various cell treatments on the ratio of phosphorylated NFκB p65 to total NFκB p65. However, if the signals obtained with the phospho-NFκB p65 antibody and the total-NFκB p65 antibody are identical, one cannot conclude that the treatment resulted in phosphorylation of 100% of the NFκB p65.

FACE NFκB p65 Experimental Design

The FACE NFκB p65 assay is a high-throughput method for quantifying cellular levels of NFκB p65 and phosphorylated NFκB p65. It should be used with cell types that have been shown to contain readily detectable levels of NFκB p65 and, under appropriate induction conditions, phosphorylated NFκB p65.

Before starting a FACE assay, it is necessary to determine the experimental conditions for each well of the 96-well plate to maximize the information obtained.

Points to consider:

1. Are you working with adherent or non-adherent cells? Protocol modifications for use of non-adherent cells are given after the protocol for adherent cells.
2. Do you want to compare phosphorylated NFκB p65 to total NFκB p65? If so, replicate wells must be cultured so that the different antibodies can be used on equivalently grown cells.
3 Which wells will be used as positive controls (*e.g.* incubated with the total-NFκB p65 antibody) and which will be used as negative controls (*e.g.* incubated with secondary antibody alone)?
4 Each experimental condition should be performed in duplicate or in triplicate to control for possible errors.
5 FACE assays are most easily performed when all 96 wells of the assay plate are used. This makes it possible to perform washing steps by "flicking" liquid from the plate into a sink. The inverted plate is then tapped gently onto several layers of paper towel to remove the remaining liquid. See "Kit Components" section if you need additional 96-well plates.
6. Fixed cells are stable for several weeks, so you can prepare many plates simultaneously and then perform the FACE assay when desired. Fixed cells should be stored with the formaldehyde solution in the wells and then sealed in a zip-lock bag or, preferably, a heat-sealed bag and refrigerated.

After planning the experiment, determine the amount of each buffer/reagent required and prepare according to the Quick Chart for Preparing Buffers. Multi-channel pipettors and pipettor reservoirs should be used when appropriate. The volumes given are appropriate for multi-channel pipetting if the assay is performed on 48 wells or more. Volumes may need to be adjusted if the assay is performed on less than 48 wells.

Kit Components and Storage - Colorimetric Assay

FACE Colorimetric Kit components can be stored at -20°C prior to first use. Then, we recommend storing each component at the temperature indicated in the table below.

Reagents	Quantity	Storage / Stability
Phospho-NFκB p65 (S468 & S536) antibodies	9 µl	-20°C for 6 months
Total-NFκB p65 antibody	9 µl	-20°C for 6 months
Anti-rabbit HRP-conjugated IgG	16 µl	4°C for 6 months
1X Antibody Blocking Buffer	32 ml	-20°C for 6 months
1X Antibody Dilution Buffer	45 ml	-20°C for 6 months
10X PBS	140 ml	Room temperature for 6 months
10% Triton X-100	10 ml	Room temperature for 6 months
Crystal Violet Solution	32 ml	4°C for 6 months
Developing Solution	32 ml	4°C for 6 months
Stop Solution	32 ml	4°C for 6 months
1% SDS Solution	32 ml	Room temperature for 6 months
96-well tissue culture plate*	3	
Plate sealing tape	3	

* Suitable tissue culture plates are Greiner part no. 655180 and Corning Costar part no. 3596.

Additional materials required

- Multi-channel pipettor
- Multi-channel pipettor reservoirs
- Rocking platform
- Parafilm
- Microplate spectrophotometer capable of reading at 595 nm and at 450 nm (655 as optional reference wavelength)
- Fresh 10% hydrogen peroxide (H_2O_2) in dH_2O (3 ml are required)
- 10 µg/ml poly-L-Lysine (if using non-adherent cells)
- 10% Sodium Azide (NaN_3) in dH_2O (250 µl are required)
- 37% Formaldehyde (2.5 ml are required for adherent cells; 5.0 ml required for non-adherent cells)

WARNING: 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.

Kit Components and Storage - Chemiluminescent Assay

FACE Chemi Kit components can be stored at -20°C prior to first use. Then, we recommend storing each component at the temperature indicated in the table below.

Reagents	Quantity	Storage / Stability
Phospho-NFκB p65 (S468 & S536) antibodies	9 µl	-20°C for 6 months
Total-NFκB p65 antibody	9 µl	-20°C for 6 months
Anti-rabbit HRP-conjugated IgG	16 µl	4°C for 6 months
1X Antibody Blocking Buffer	32 ml	-20°C for 6 months
1X Antibody Dilution Buffer	45 ml	-20°C for 6 months
10X PBS	140 ml	Room temperature for 6 months
10% Triton X-100	10 ml	Room temperature for 6 months
Crystal Violet Solution	32 ml	4°C for 6 months
Chemiluminescent Reagent	5.5 ml	4°C for 6 months
Reaction Buffer	10.5 ml	4°C for 6 months
1% SDS Solution	32 ml	Room temperature for 6 months
96-well tissue culture plate*	3	
Plate sealing tape	3	

* Suitable tissue culture plates are Greiner part no. 655098.

Additional materials required

- 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 are required)
- 10 µg/ml poly-L-Lysine (if using non-adherent cells)
- 10% Sodium Azide (NaN_3) in dH_2O (250 µl are required)
- 37% Formaldehyde (2.5 ml are required for adherent cells; 5.0 ml required for non-adherent cells)

WARNING: 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.

Buffer Preparation and Recommendations

We provide an excess of buffer components in order to perform one 96-well FACE assay with each of the phospho-NFκB p65 antibodies and one 96-well FACE assay with the total-NFκB p65 antibody. Required reagents that are not supplied are listed on the previous page. Please review the Quick Chart for Preparing Buffers in this section prior to preparing the assay buffers.

Preparation of 1X PBS

1X PBS is the basis of several buffers used in the FACE protocol. 1X PBS is also used in several of the wash steps in the protocol (see the Quick Chart for Preparing Buffers). It is prepared by adding 1 volume of 10X PBS (pH 7.4) to 9 volumes of dH_2O and mixing thoroughly.

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

Fixing Buffer is used to fix cells after cell culturing. It is prepared 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.

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

Wash Buffer is used throughout the FACE protocol and is prepared by adding the provided 10% Triton X-100 solution to 1X PBS and mixing thoroughly.

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

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

Blocking Buffer

This is 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.

Antibody Dilution Buffer

This is 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.

Diluted phospho-NFκB p65 antibodies

The phospho-NFκB p65 antibodies recognize NFκB p65 when phosphorylated at Serine 468 or Serine 536. The supplied antibody will be diluted 1/500 in Antibody Dilution Buffer (see the Quick Chart for Preparing Buffers in this section).

Diluted total-NFκB p65 antibody

The total-NFκB p65 antibody recognizes both the non-phosphorylated and the phosphorylated forms of NFκB p65 proteins. The supplied antibody will be diluted 1/500 in Antibody Dilution Buffer (see the Quick Chart for Preparing Buffers in this section).

Diluted HRP-conjugated secondary antibody

HRP-conjugated anti-rabbit IgG is used as the secondary antibody to detect bound primary antibodies. The supplied antibody will be diluted 1/2000 in Antibody Dilution Buffer. (See the Quick Chart for Preparing Buffers in this section).

1% SDS Solution

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

Crystal Violet Solution

This is supplied ready-to-use. Crystal Violet is used to determine 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.

Developing Solution

The Developing Solution must be warmed to room temperature before use. This solution is light sensitive, therefore, we recommend avoiding direct exposure to intense light during storage. The Developing Solution may develop a yellow hue over time. This does not affect product performance. A blue color present in the solution indicates that it has been contaminated and must be discarded. Prior to use, transfer the amount of Developing Solution required for the assay into a secondary container (see the Quick Chart for Preparing Buffers in this section), avoid direct exposure to intense light and leave at room temperature for at least 1 hour. After use, discard any remaining solution that was transferred into the secondary container.

Stop Solution

Prior to use, transfer the amount of Stop Solution required for the assay into a secondary container (see the Quick Chart for Preparing Buffers in this section). After use, discard any remaining Stop Solution that was transferred into the secondary container.

WARNING: The Stop Solution is corrosive. Wear personal protective equipment when handling, *i.e.* labcoat, gloves and eye protection.

Quick Chart for Preparing Buffers - Colorimetric Assay

Reagents to prepare	Components	1 well	48 wells	96 wells	192 wells	288 wells
Fixing Buffer for adherent cells	1X PBS	98 µl	4.7 ml	9.41 ml	18.82 ml	28.22 ml
	37% Formaldehyde	12 µl	576 µl	1.15 ml	2.30 ml	3.46 ml
	TOTAL REQUIRED	**110 µl**	**5.28 ml**	**10.56 ml**	**21.12 ml**	**31.68 ml**
Fixing Buffer for non-adherent cells	1X PBS	86.0 µl	4.13 ml	8.26 ml	16.51 ml	24.77 ml
	37% Formaldehyde	24.0 µl	1.15 ml	2.30 ml	4.61 ml	6.91 ml
	TOTAL REQUIRED	**110 µl**	**5.28 ml**	**10.56 ml**	**21.12 ml**	**31.68 ml**
Wash Buffer	1X PBS	3.376 ml	162 ml	324 ml	648 ml	972 ml
	10% Triton X-100	34.1 µl	1.64 ml	3.27 ml	6.55 ml	9.82 ml
	TOTAL REQUIRED	**3.41 ml**	**163.7 ml**	**327.4 ml**	**654.7 ml**	**982.1 ml**
Quenching Buffer	Wash Buffer	97.9 µl	4.7 ml	9.40 ml	18.8 ml	28.2 ml
	10% H_2O_2	11 µl	528 µl	1.06 ml	2.11 ml	3.17 ml
	10% Azide	1.1 µl	52.8 µl	106 µl	211 µl	317 µl
	TOTAL REQUIRED	**110 µl**	**5.28 ml**	**10.56 ml**	**21.12 ml**	**31.68 ml**
Blocking Buffer	**TOTAL REQUIRED**	**110 µl**	**5.28 ml**	**10.56 ml**	**21.12 ml**	**31.68 ml**
Diluted total-NFκB p65 antibody	Ab Dilution Buffer	45 µl	2.16 ml	4.32 ml	-	-
	Total-NFκB p65 ab	0.09 µl	4.3 µl	8.6 µl	-	-
	TOTAL REQUIRED	**45.09 µl**	**2.16 ml**	**4.32 ml**	**-**	**-**
Diluted phospho-NFκB p65 antibody	Ab Dilution Buffer	45 µl	2.16 ml	4.32 ml	-	-
	Phospho-NFκB p65 ab	0.09 µl	4.3 µl	8.6 µl	-	-
	TOTAL REQUIRED	**45.09 µl**	**2.16 ml**	**4.32 ml**	**-**	**-**
Diluted HRP-conjugated secondary antibody	Ab Dilution Buffer	110 µl	5.28 ml	10.56 ml	21.12 ml	31.68 ml
	HRP-conj. secondary ab	0.055 µl	2.64 µl	5.28 µl	10.56 µl	15.84 µl
	TOTAL REQUIRED	**110 µl**	**5.28 ml**	**10.56 ml**	**21.13 ml**	**31.7 ml**
1X PBS (for wash steps)	10X PBS	154 µl	7.39 ml	14.78 ml	29.57 ml	44.35 ml
	dH_2O	1.39 ml	66.53 ml	133.0 ml	266.1 ml	399.2 ml
	TOTAL REQUIRED	**1.54 ml**	**73.92 ml**	**147.8 ml**	**295.7 ml**	**443.6 ml**
1% SDS Solution	**TOTAL REQUIRED**	**110 µl**	**5.28 ml**	**10.56 ml**	**21.12 ml**	**31.68 ml**
Developing Solution	**TOTAL REQUIRED**	**110 µl**	**5.28 ml**	**10.56 ml**	**21.12 ml**	**31.68 ml**
Stop Solution	**TOTAL REQUIRED**	**110 µl**	**5.28 ml**	**10.56 ml**	**21.12 ml**	**31.68 ml**
Crystal Violet Solution	**TOTAL REQUIRED**	**110 µl**	**5.28 ml**	**10.56 ml**	**21.12 ml**	**31.68 ml**

Adherent Cell Protocol - Colorimetric Assay

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING

Step 1: Culture, fix and block cells

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.
2. Grow and treat cells as desired.
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 ziplock bag. Incubate for 20 minutes at room temperature.

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

4. 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.
5. Remove Wash Buffer, add 100 µl Quenching Buffer and incubate for 20 minutes at room temperature.
6. Remove Quenching Buffer and wash cells 2 times for 5 minutes each with 200 µl Wash Buffer.
7. Remove Wash Buffer, add 100 µl Antibody Blocking Buffer and incubate 1 hour at room temperature.

Step 2: Binding of primary and secondary antibodies

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.

1. Remove Antibody Blocking Buffer and wash cells 2 times with 200 µl Wash Buffer.
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 phosphorylated-NFκB, a three hour primary antibody incubation is sufficient. For maximum sensitivity an overnight incubation is recommended.

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

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.
5. During this incubation, transfer the amount of Developing Solution required for the assay into a secondary container and leave at room temperature for at least an hour (avoid light).

Step 3: Colorimetric reaction

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.
2. Transfer the amount of Developing Solution required for the assay into a secondary container. Remove PBS from plate wells and add 100 µl Developing Solution to each well.
3. Incubate 2-20 minutes at room temperature protected from direct light. Monitor the blue color development until the darkest-staining wells are medium- to dark-blue. Do not over-develop.
4. Add 100 µl Stop Solution. This acidic solution turns the blue color to yellow. Take care with pipetting to ensure that each well is developed for the same amount of time.

   WARNING: The Stop Solution is corrosive. Wear personal protective equipment when handling, *i.e.* labcoat, gloves and eye protection.

5. Read absorbance on a spectrophotometer within 5 minutes at 450 nm with an optional reference wavelength of 655 nm.

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.

1. After reading at 450 nm is complete, 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.
2. Add 100 µl Crystal Violet solution to each well and incubate 30 minutes at room temperature.

   WARNING: Crystal Violet is an intense stain. Avoid contact with skin and clothing.

3. Wash wells 3 times with 200 µl 1X PBS for 5 minutes each.
4. Add 100 µl of 1% SDS Solution to each well and incubate on shaker for 1 hour at room temperature.
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.
6. The measured OD_{450} readings are corrected for cell number by dividing the OD_{450} reading for a given well by the OD_{595} reading for that well.

Non-Adherent Cell Protocol - Colorimetric Assay

The protocol given above can be modified for use with non-adherent cells by culturing and fixing the cells as follows:

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.
2. Seed 17,000 cells/well, or whatever amount is appropriate for your particular cell line.
3. Grow and treat cells as desired.
4. Fix cells by replacing the growth medium with 100 µl of 8% formaldehyde in PBS. Incubate 20 minutes at room temperature.
5. Continue with Step 1, No. 4 of the Adherent Cell Protocol above.

Buffer Preparation and Recommendations

We provide an excess of buffer components in order to perform one 96-well FACE assay with the phospho-NFκB p65 antibody and one 96-well FACE assay with the total-NFκB p65 antibody. Required reagents that are not supplied are listed on the previous page. Please review the Quick Chart for Preparing Buffers in this section prior to preparing the assay buffers.

Preparation of 1X PBS

1X PBS is the basis of several buffers used in the FACE protocol. 1X PBS is also used in several of the wash steps in the protocol (see the Quick Chart for Preparing Buffers). It is prepared by adding 1 volume of 10X PBS (pH 7.4) to 9 volumes of dH_2O and mixing thoroughly.

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

Fixing Buffer is used to fix cells after cell culturing. It is prepared 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.

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

Wash Buffer is used throughout the FACE protocol and is prepared by adding the provided 10% Triton X-100 solution to 1X PBS and mixing thoroughly.

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

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

Blocking Buffer

This is 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.

Antibody Dilution Buffer

This is 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.

Diluted phospho-NFκB p65 antibodies

The phospho-NFκB p65 antibodies recognize NFκB p65 when phosphorylated at Serine 468 or Serine 536. The supplied antibody will be diluted 1/500 in Antibody Dilution Buffer (see the Quick Chart for Preparing Buffers in this section).

Diluted total-NFκB p65 antibody

The total-NFκB p65 antibody recognizes both the non-phosphorylated and the phosphorylated forms of NFκB p65 proteins. The supplied antibody will be diluted 1/500 in Antibody Dilution Buffer (see the Quick Chart for Preparing Buffers in this section).

Diluted HRP-conjugated secondary antibody

HRP-conjugated anti-rabbit IgG is used as the secondary antibody to detect bound primary antibodies. The supplied antibody will be diluted 1/2000 in Antibody Dilution Buffer. (See the Quick Chart for Preparing Buffers in this section).

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 in this section). The Chemiluminescent Working Solution is stable for several hours. After the Chemiluminescent Working Solution is aliquoted into the wells, discard the remaining solution.

1% SDS Solution

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

Crystal Violet Solution

This is 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 - Chemiluminescent Assay

Reagents to prepare	Components	1 well	48 wells	96 wells	192 wells	288 wells
Fixing Buffer for adherent cells	1X PBS	98 µl	4.7 ml	9.41 ml	18.82 ml	28.22 ml
	37% Formaldehyde	12 µl	576 µl	1.15 ml	2.30 ml	3.46 ml
	TOTAL REQUIRED	**110 µl**	**5.28 ml**	**10.56 ml**	**21.12 ml**	**31.68 ml**
Fixing Buffer for non-adherent cells	1X PBS	86.0 µl	4.13 ml	8.26 ml	16.51 ml	24.77 ml
	37% Formaldehyde	24.0 µl	1.15 ml	2.30 ml	4.61 ml	6.91 ml
	TOTAL REQUIRED	**110 µl**	**5.28 ml**	**10.56 ml**	**21.12 ml**	**31.68 ml**
Wash Buffer	1X PBS	3.376 ml	162 ml	324 ml	648 ml	972 ml
	10% Triton X-100	34.1 µl	1.64 ml	3.27 ml	6.55 ml	9.82 ml
	TOTAL REQUIRED	**3.41 ml**	**163.7 ml**	**327.4 ml**	**654.7 ml**	**982.1 ml**
Quenching Buffer	Wash Buffer	97.9 µl	4.7 ml	9.40 ml	18.8 ml	28.2 ml
	10% H_2O_2	11 µl	528 µl	1.06 ml	2.11 ml	3.17 ml
	10% Azide	1.1 µl	52.8 µl	106 µl	211 µl	317 µl
	TOTAL REQUIRED	**110 µl**	**5.28 ml**	**10.56 ml**	**21.12 ml**	**31.68 ml**
Blocking Buffer	**TOTAL REQUIRED**	**110 µl**	**5.28 ml**	**10.56 ml**	**21.12 ml**	**31.68 ml**
Diluted total-NFκB p65 antibody	Ab Dilution Buffer	45 µl	2.16 ml	4.32 ml	-	-
	Total-NFκB p65 ab	0.09 µl	4.3 µl	8.6 µl	-	-
	TOTAL REQUIRED	**45.09 µl**	**2.16 ml**	**4.32 ml**	**-**	**-**
Diluted phospho-NFκB p65 antibody	Ab Dilution Buffer	45 µl	2.16 ml	4.32 ml	-	-
	Phospho-NFκB p65 ab	0.09 µl	4.3 µl	8.6 µl	-	-
	TOTAL REQUIRED	**45.09 µl**	**2.16 ml**	**4.32 ml**	**-**	**-**
Diluted HRP-conjugated secondary antibody	Ab Dilution Buffer	110 µl	5.28 ml	10.56 ml	21.12 ml	31.68 ml
	HRP-conj. secondary ab	0.055 µl	2.64 µl	5.28 µl	10.56 µl	15.84 µl
	TOTAL REQUIRED	**110 µl**	**5.28 ml**	**10.56 ml**	**21.13 ml**	**31.7 ml**
1X PBS (for wash steps)	10X PBS	154 µl	7.39 ml	14.78 ml	29.57 ml	44.35 ml
	dH_2O	1.39 ml	66.53 ml	133.0 ml	266.1 ml	399.2 ml
	TOTAL REQUIRED	**1.54 ml**	**73.92 ml**	**147.8 ml**	**295.7 ml**	**443.6 ml**
Chemiluminescent Working Solution	Chemiluminescent Reagent	18 µl	864 µl	1.728 ml	3.46 ml	5.18 ml
	Reaction Buffer	36 µl	1.728 ml	3.456 ml	6.91 ml	10.37 ml
	TOTAL REQUIRED	**54 µl**	**2.592 ml**	**5.184 ml**	**10.37 ml**	**15.55 ml**
1% SDS Solution	**TOTAL REQUIRED**	**110 µl**	**5.28 ml**	**10.56 ml**	**21.12 ml**	**31.68 ml**
Crystal Violet Solution	**TOTAL REQUIRED**	**110 µl**	**5.28 ml**	**10.56 ml**	**21.12 ml**	**31.68 ml**

Adherent Cell Protocol - Chemiluminescent Assay

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING

Step 1: Culture, fix and block cells

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.
2. Grow and treat cells as desired.
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.

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

4. 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.
5. Remove Wash Buffer, add 100 µl Quenching Buffer and incubate for 20 minutes at room temperature.
6. Remove Quenching Buffer and wash cells 2 times for 5 minutes each with 200 µl Wash Buffer.
7. Remove Wash Buffer, add 100 µl Antibody Blocking Buffer and incubate 1 hour at room temperature.

Step 2: Binding of primary and secondary antibodies

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.

1. Remove Antibody Blocking Buffer and wash cells 2 times with 200 µl Wash Buffer.
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 phosphorylated-NFκB p65, a three hour primary antibody incubation is sufficient. For maximum sensitivity an overnight incubation is recommended.

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

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

Step 3: Chemiluminescent detection

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.
2. Remove PBS from plate wells and add 50 µl room temperature Chemiluminescent Working Solution to each well.
3. Read chemiluminescence using a luminometer or CCD camera system. Readings should be taken within 10 minutes to minimize changes in signal intensity.

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.

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.
2. Add 100 µl Crystal Violet solution to each well and incubate 30 minutes at room temperature.

   WARNING: Crystal Violet is an intense stain. Avoid contact with skin and clothing.

3. Wash wells 3 times with 200 µl 1X PBS for 5 minutes each.
4. Add 100 µl of 1% SDS Solution to each well and incubate on shaker for 1 hour at room temperature.
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.
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 3.

Non-Adherent Cell Protocol - Chemiluminescent Assay

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

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.
2. Seed 17,000 cells/well, or whatever amount is appropriate for your particular cell line.
3. Grow and treat cells as desired.
4. Fix cells by replacing the growth medium with 100 µl of 8% formaldehyde in PBS. Incubate 20 minutes at room temperature.
5. Continue with Step 1, No. 4 of the Adherent Cell Protocol above.

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Appendix

Section A. Troubleshooting Guide

PROBLEM	POSSIBLE CAUSE	RECOMMENDATION
No signal or weak signal in wells incubated with either phospho-NFκB p65 antibody or total-NFκB p65 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
	Developing Solution was cold	Bring Developing Solution to room temperature
	Inadequate volume of Developing Solution	Check to make sure that correct volume is delivered by pipette
	Cells do not contain detectable levels of phospho NFκB p65 and/or total NFκB p65	Use Western blotting to confirm that cells contain detectable levels of protein(s) of interest. If you do not require all of the included antibodies for FACE assays, they can be used in Colorimetric Western blotting at a 1:400 dilution for the total and a 1:500 for the phospho antibodies
	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	Refer to cross reactivity information on page 5
	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	Developing time too long (Colorimetric Assay)	Stop enzymatic reaction as soon as the positive wells turn medium-dark blue
	Measurement time too long (Chemiluminescent Assay)	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-antibodies 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

PROBLEM	POSSIBLE CAUSE	RECOMMENDATION
Uneven color 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-NFκB p65 antibody	Cell culture conditions did not induce phosphorylation of NFκB p65	Perform Western blot with phospho-NFκB p65 antibodies to confirm that cells contain detectable levels of phosphorylated NFκB p65
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

Section B. Related Products

TransAM™ Kits	Unit	Catalog No.
TransAM™ NFκB Family	2 x 96 rxns	43296
TransAM™ NFκB p50	1 x 96 rxns	41096
	5 x 96 rxns	41596
TransAM™ NFκB p50 Chemi	1 x 96 rxns	41097
	5 x 96 rxns	41597
TransAM™ NFκB p65	1 x 96 rxns	40096
	5 x 96 rxns	40596
TransAM™ NFκB p65 Chemi	1 x 96 rxns	40097
	5 x 96 rxns	40597

Sandwich ELISAs	Unit	Catalog No.
FunctionELISA™ IκBα	1 x 96 rxns	48005
	5 x 96 rxns	48505

Cell-based ELISAs	Unit	Colorimetric Kit Catalog No.	Chemi Kit Catalog No.
FACE™ AKT	1 x 96 rxns	48120	48220
	5 x 96 rxns	48620	48720
FACE™ ATF-2	1 x 96 rxns	48115	48215
	5 x 96 rxns	48615	48715
FACE™ Bad	1 x 96 rxns	48165	48265
	5 x 96 rxns	48665	48765
FACE™ c-Jun (S63)	1 x 96 rxns	48125	48225
	5 x 96 rxns	48625	48725
FACE™ c-Jun (S73)	1 x 96 rxns	48135	48235
	5 x 96 rxns	48635	48735
FACE™ c-Src	1 x 96 rxns	48155	48255
	5 x 96 rxns	48655	48755
FACE™ EGFR (Y992)	1 x 96 rxns	48150	48250
	5 x 96 rxns	48650	48750
FACE™ EGFR (Y1173)	1 x 96 rxns	48190	48290
	5 x 96 rxns	48690	48790
FACE™ ErbB-2 (Y877)	1 x 96 rxns	48130	48230
	5 x 96 rxns	48630	48730
FACE™ ErbB-2 (Y1248)	1 x 96 rxns	48105	48205
	5 x 96 rxns	48605	48705
FACE™ ERK1/2	1 x 96 rxns	48140	48240
	5 x 96 rxns	48640	48740
FACE™ FAK	1 x 96 rxns	48145	48245
	5 x 96 rxns	48645	48745
FACE™ FKHR (FOXO1)	1 x 96 rxns	48160	48260
	5 x 96 rxns	48660	48760
FACE™ GSK3β	1 x 96 rxns	48170	48270
	5 x 96 rxns	48670	48770
FACE™ JNK	1 x 96 rxns	48110	48210
	5 x 96 rxns	48610	48710
FACE™ JAK1	1 x 96 rxns	48185	48285
	5 x 96 rxns	48685	48785
FACE™ JNK	1 x 96 rxns	48110	48210
	5 x 96 rxns	48610	48710
FACE™ MEK1/2	1 x 96 rxns	48180	48280
	5 x 96 rxns	48680	48780
FACE™ p38	1 x 96 rxns	48100	48200
	5 x 96 rxns	48600	48700
FACE™ PI3 Kinase p85	1 x 96 rxns	48175	48275
	5 x 96 rxns	48675	48775

Technical Services

If you need assistance at any time, please call Active Motif Technical Service at one of the numbers listed below.

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