

FACE™
FKHR (FOXO1)
ELISA Kits

(version B)

Catalog Nos. 48160 & 48660 (FKHR)
Catalog Nos. 48260 & 48760 (FKHR Chemi)

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Overview

The Forkhead transcription factor FKHR (FOXO1) regulates expression of gluconeogenic factors, and plays a key role in insulin regulation of glucose levels. Besides its role in glucose homeostasis, FKHR has been shown to be involved in pathological processes such as type 2 diabetes, cancer and organismal aging. The transcriptional output of FKHR is controlled by a two-tiered mechanism of phosphorylation and acetylation, and alterations in this balance can result in profound effects.¹ Therefore, high-throughput study methods for monitoring FKHR phosphorylation are in high demand.

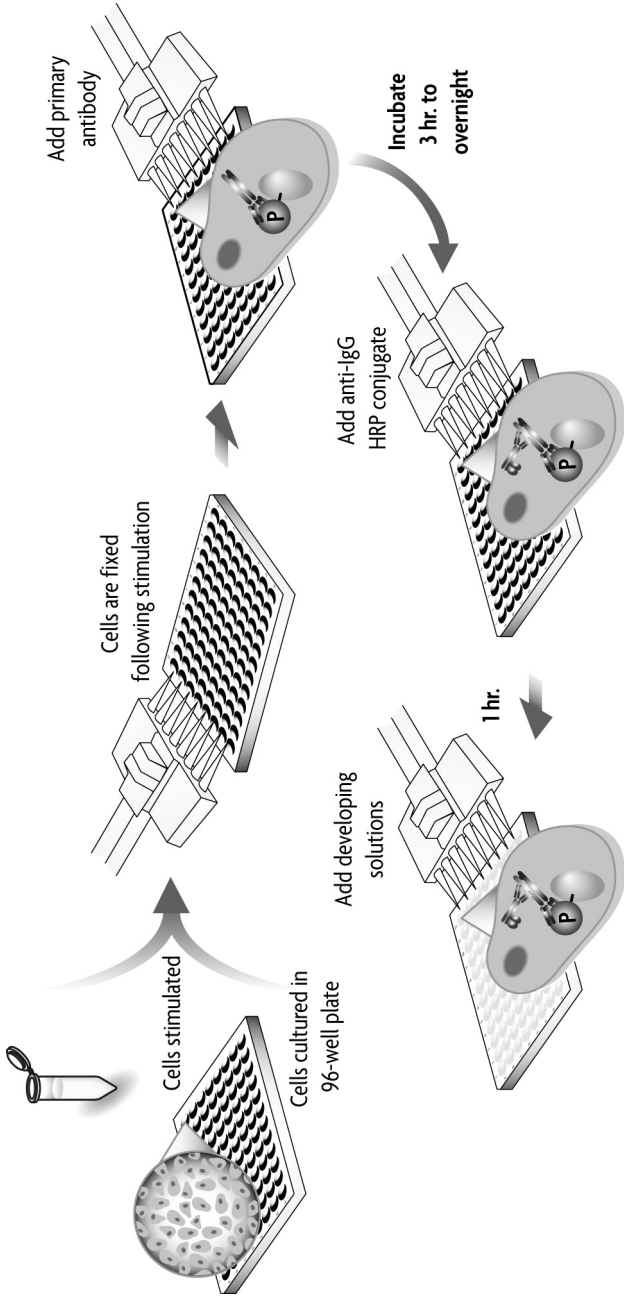
Fast Activated Cell-based ELISA (FACE™)* Kits provide a simple, efficient, cell-based method to monitor proteins activated by phosphorylation. FACE FKHR (FOXO1) Kits are designed specifically to quantify activated (phosphorylated) FKHR and/or total FKHR.² In the FACE method, cells are cultured in 96-well 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 either phosphorylated FKHR or total FKHR. 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. FACE FKHR (FOXO1) Kits are available in two sizes:

| product | format | catalog no. |
|-------------------------|-------------|-------------|
| FACE FKHR (FOXO1) | 1 x 96 rxns | 48160 |
| | 5 x 96 rxns | 48660 |
| FACE FKHR (FOXO1) Chemi | 1 x 96 rxns | 48260 |
| | 5 x 96 rxns | 48760 |

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

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

Flow Chart of Process



Introduction

FKHR

The Forkhead family of transcription factors are involved in regulation of the cell cycle, cell death, cell metabolism and oxidative stress (see 3 for review). The FOXO (Forkhead Box, class O) proteins form a subfamily of Forkhead transcription factors that are direct targets of phosphoinositide 3-kinase (PI3K) mediated signal transduction.⁴ In the presence of growth factor/survival signals, PI3K activation leads to PDK mediated downstream activation of PKB/c-AKT. Phosphorylation and activation of PKB causes nuclear translocation of PKB, which phosphorylates and inactivates nuclear FOXO. FOXO then binds 14-3-3 proteins, and the FOXO/14-3-3 complex is exported to the cytoplasm. Phosphorylated, inactive FOXO proteins remain bound to 14-3-3 proteins in the cytoplasm, thereby preventing nuclear import of FOXO. In the absence of survival signals, cytoplasmic FOXO is dephosphorylated, causing dissociation from 14-3-3 proteins and allowing nuclear import of FOXO to activate gene expression.⁴

There are three AKT phosphorylation sites in the FKHR protein: Thr24, Ser256 and Ser319.⁵ Specifically, phosphorylation at Ser256 is thought to play a role in masking a FKHR nuclear localization signal.⁶ In addition, phosphorylation at Ser256 may also mediate the effects of insulin on gene expression.⁷ Phosphorylation at Thr24 is critical for FKHR interaction with 14-3-3 proteins.⁶

The most well studied FOXO members include acute-lymphocytic-leukaemia-1 fused gene from chromosome X (AFX/FOXO4), Forkhead in rhabdomyosarcoma (FKHR/FOXO1) and FKHR-Like 1 (FKHRL1/FOXO3a).⁸ In different cell types, FOXO proteins modulate various cellular activities. In hepatocytes, FOXO proteins regulate the expression of factors involved in gluconeogenesis, such as peroxisome proliferators-activated receptor- γ coactivator-1, glucose-6-phosphate and phosphoenolpyruvate carboxykinase.⁹⁻¹² Using genetic gain and loss of function analysis in mice, FKHR has also been shown to control β cell compensation for insulin resistance and glucose production in type 2 diabetes.¹³ In addition, FOXO proteins such as FKHRL1 have been shown to regulate catalase and superoxide dismutase gene expression that protect cells from oxidative stress, suggesting that FOXO factors act to control the mammalian lifespan.^{14, 15}

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.¹⁶ A given protein substrate is immobilized on a gel and phosphorylated by protein kinases, which are separated by SDS-PAGE. The bands of incorporated [³²P]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 FKHR (FOXO1)

The role of FKHR in the regulation of cell survival and the cell cycle 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 FKHR 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 FKHR Kit contains two 96-well plates and two primary antibodies. The phospho-FKHR antibody is specific for phosphorylated FKHR and was raised against a synthetic phospho-peptide corresponding to residues surrounding Thr24 of human FKHR. This antibody recognizes FKHR only when phosphorylated at Thr24 and also recognizes FKHL1 when phosphorylated at Thr32 and AFX when phosphorylated at Thr28. The total-FKHR antibody recognizes FKHR proteins regardless of the phosphorylation state. Each antibody is provided in a quantity sufficient for one 96-well assay.

FACE FKHR Kits can be used to study phosphorylated FKHR 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-FKHR 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.

FACE FKHR Kits can also be used to determine FKHR phosphorylation relative to the total FKHR 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 FKHR. After the cells are fixed, one plate is studied with the phospho-FKHR antibody, while the other plate is studied with the total-FKHR antibody. The relative number of cells in each well is then determined through use of the Crystal Violet reagent. Once the phospho-FKHR and total-FKHR signals have been normalized for cell number, a comparison of the ratio of phosphorylated FKHR to total FKHR for each of the cell growth conditions can be made.

In the FACE FKHR assay, the provided total-FKHR antibody can be used as a positive control to demonstrate that the cells contain FKHR, 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 FKHR Kits are for research use only. Not for use in diagnostic procedures.

Antibody specificity: The phospho-FKHR antibody is specific for phosphorylated FKHR and was raised against a synthetic phospho-peptide corresponding to residues surrounding Thr24 of human FKHR. This antibody recognizes FKHR only when phosphorylated at Thr24 and also recognizes FKHL1 when phosphorylated at Thr32 and AFX when phosphorylated at Thr28. The total-FKHR antibody recognizes FKHR proteins regardless of the phosphorylation state.

Cross-reactivity: FACE FKHR Kits detect phosphorylated and total FKHR from human, mouse and rat origin.

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

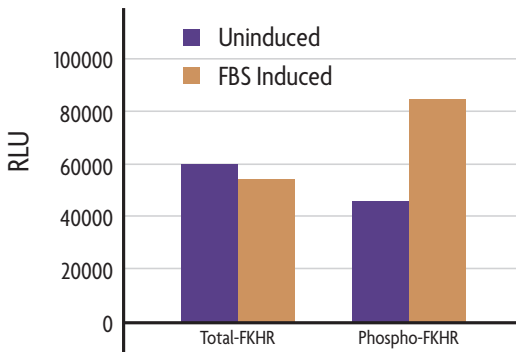


Figure 1: Measurement of phosphorylated and total FKHR.

CHO-K1 cells were cultured in 96-well plates and serum-starved for 16 hours. Cells were then treated with 20% FBS for 30 minutes and fixed. Total and phospho FKHR were each assayed in triplicate using the phospho and total FKHR antibodies included in the FACE FKHR Kit. Data was plotted after correction for cell number (performed through use of Crystal Violet).

Note on data interpretation

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

FACE FKHR Experimental Design

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

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 FKHR to total FKHR? If so, replicate wells must be cultured so that the two different antibodies can be used on equivalently grown cells.
3. Which wells will be used as positive controls (*e.g.* incubated with the total-FKHR 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 1 plate / 5 plates | Storage / Stability |
|--------------------------------|--------------------------------|-------------------------------|
| Phospho-FKHR antibody | 9 µl / 45 µl | -20°C for 6 months |
| Total-FKHR antibody | 9 µl / 45 µl | -20°C for 6 months |
| Anti-rabbit HRP-conjugated IgG | 22 µl / 110 µl | 4°C for 6 months |
| 1X Antibody Blocking Buffer | 22 ml / 110 ml | -20°C for 6 months |
| 1X Antibody Dilution Buffer | 30 ml / 150 ml | -20°C for 6 months |
| 10X PBS | 120 ml / 600 ml | Room temperature for 6 months |
| 10% Triton X-100 | 7 ml / 35 ml | Room temperature for 6 months |
| Crystal Violet Solution | 22 ml / 110 ml | 4°C for 6 months |
| Developing Solution | 22 ml / 110 ml | 4°C for 6 months |
| Stop Solution | 22 ml / 110 ml | 4°C for 6 months |
| 1% SDS Solution | 22 ml / 110 ml | Room temperature for 6 months |
| 96-well tissue culture plate* | 2 / 10 | |
| Plate sealing tape | 2 / 10 | |

* 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 (H₂O₂) in dH₂O (3 ml are 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 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 1 plate / 5 plates | Storage / Stability |
|--------------------------------|--------------------------------|-------------------------------|
| Phospho-FKHR antibody | 9 µl / 45 µl | -20°C for 6 months |
| Total-FKHR antibody | 9 µl / 45 µl | -20°C for 6 months |
| Anti-rabbit HRP-conjugated IgG | 22 µl / 110 µl | 4°C for 6 months |
| 1X Antibody Blocking Buffer | 22 ml / 110 ml | -20°C for 6 months |
| 1X Antibody Dilution Buffer | 30 ml / 150 ml | -20°C for 6 months |
| 10X PBS | 120 ml / 600 ml | Room temperature for 6 months |
| 10% Triton X-100 | 7 ml / 35 ml | Room temperature for 6 months |
| Crystal Violet Solution | 22 ml / 110 ml | 4°C for 6 months |
| Chemiluminescent Reagent | 4 ml / 20 ml | 4°C for 6 months |
| Reaction Buffer | 8 ml / 40 ml | 4°C for 6 months |
| 1% SDS Solution | 22 ml / 110 ml | Room temperature for 6 months |
| 96-well tissue culture plate* | 2 / 10 | |
| Plate sealing tape | 2 / 10 | |

* 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 (H₂O₂) in dH₂O (3 ml are 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 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.

Protocols - Colorimetric Assay

Buffer Preparation and Recommendations

We provide an excess of buffer components in order to perform one 96-well FACE assay with the phospho-FKHR antibody and one 96-well FACE assay with the total-FKHR 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 dH₂O 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% H₂O₂ 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-FKHR antibody

The phospho-FKHR antibody recognizes FKHR when phosphorylated at Thr24. The supplied antibody will be diluted 1/500 in Antibody Dilution Buffer (see the Quick Chart for Preparing Buffers in this section).

Diluted total-FKHR antibody

The total-FKHR antibody recognizes both the non-phosphorylated and the phosphorylated forms of FKHR 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 1/1000 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 OD₅₉₅ 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 |
|---|-----------------------------------|-----------------|-------------------|-------------------|------------------|
| 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 |
| | TOTAL REQUIRED | 110 µl | 5.28 ml | 10.56 ml | 21.12 ml |
| Fixing Buffer for non-adherent cells | 1X PBS | 86.0 µl | 4.13 ml | 8.26 ml | 16.51 ml |
| | 37% Formaldehyde | 24.0 µl | 1.15 ml | 2.30 ml | 4.61 ml |
| | TOTAL REQUIRED | 110 µl | 5.28 ml | 10.56 ml | 21.12 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 | 6.26 ml |
| | TOTAL REQUIRED | 3.41 ml | 163.7 ml | 313 ml | 626 ml |
| Quenching Buffer | Wash Buffer | 97.9 µl | 4.7 ml | 9.40 ml | 18.8 ml |
| | 10% H ₂ O ₂ | 11 µl | 528 µl | 1.06 ml | 2.11 ml |
| | 10% Azide | 1.1 µl | 52.8 µl | 106 µl | 211 µl |
| | TOTAL REQUIRED | 110 µl | 5.28 ml | 10.56 ml | 21.12 ml |
| Blocking Buffer | TOTAL REQUIRED | 110 µl | 5.28 ml | 10.56 ml | 21.12 ml |
| Diluted total-FKHR antibody | Antibody Dilution Buffer | 45 µl | 2080 µl | 4160 µl | - |
| | Total-FKHR antibody | 0.09 µl | 4.16 µl | 8.32 µl | - |
| | TOTAL REQUIRED | 45.09 µl | 2084.16 µl | 4168.32 µl | - |
| Diluted phospho-FKHR antibody | Antibody Dilution Buffer | 45 µl | 2080 µl | 4160 µl | - |
| | Phospho-FKHR antibody | 0.09 µl | 4.16 µl | 8.32 µl | - |
| | TOTAL REQUIRED | 45.09 µl | 2084.16 µl | 4168.32 µl | - |
| Diluted HRP-conjugated secondary antibody | Antibody Dilution Buffer | 110 µl | 5280 µl | 10.56 ml | 21.12 ml |
| | HRP-conjugated secondary ab | 0.11 µl | 5.28 µl | 10.56 µl | 21.12 µl |
| | TOTAL REQUIRED | 110.1 µl | 5285.28 µl | 10.57 ml | 21.14 ml |
| 1X PBS (for wash steps) | 10X PBS | 154 µl | 7.39 ml | 14.11 ml | 28.22 ml |
| | dH ₂ O | 1.39 ml | 66.53 ml | 127.01 ml | 254.02 ml |
| | TOTAL REQUIRED | 1.54 ml | 73.92 ml | 141.12 ml | 282.24 ml |
| 1% SDS Solution | TOTAL REQUIRED | 110 µl | 5.28 ml | 10.56 ml | 21.12 ml |
| Developing Solution | TOTAL REQUIRED | 110 µl | 5.28 ml | 10.56 ml | 21.12 ml |
| Stop Solution | TOTAL REQUIRED | 110 µl | 5.28 ml | 10.56 ml | 21.12 ml |
| Crystal Violet Solution | TOTAL REQUIRED | 110 µl | 5.28 ml | 10.56 ml | 21.12 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 cm². 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-FKHR, 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 μ l 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 μ l Wash Buffer and then 2 times for 5 minutes with 200 μ l 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 μ l 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 μ l 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 OD₅₉₅ 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 μ l Wash Buffer and 2 times with 200 μ l 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 μ l 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 μ l 1X PBS for 5 minutes each.
4. Add 100 μ l 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 μ l) of the liquid from each well and replacing with an equivalent volume of dH₂O.
6. The measured OD₄₅₀ readings are corrected for cell number by dividing the OD₄₅₀ reading for a given well by the OD₅₉₅ 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.

Protocols - Chemiluminescent Assay

Buffer Preparation and Recommendations

We provide an excess of buffer components in order to perform one 96-well FACE assay with the phospho-FKHR antibody and one 96-well FACE assay with the total-FKHR 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 dH₂O 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% H₂O₂ 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-FKHR antibody

The phospho-FKHR antibody recognizes FKHR when phosphorylated at Thr24. The supplied antibody will be diluted 1/500 in Antibody Dilution Buffer (see the Quick Chart for Preparing Buffers in this section).

Diluted total-FKHR antibody

The total-FKHR antibody recognizes both the non-phosphorylated and the phosphorylated forms of FKHR 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 1/1000 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 OD₅₉₅ 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 |
|---|-----------------------------------|-----------------|-------------------|-------------------|------------------|
| 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 |
| | TOTAL REQUIRED | 110 µl | 5.28 ml | 10.56 ml | 21.12 ml |
| Fixing Buffer for non-adherent cells | 1X PBS | 86.0 µl | 4.13 ml | 8.26 ml | 16.51 ml |
| | 37% Formaldehyde | 24.0 µl | 1.15 ml | 2.30 ml | 4.61 ml |
| | TOTAL REQUIRED | 110 µl | 5.28 ml | 10.56 ml | 21.12 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 | 6.26 ml |
| | TOTAL REQUIRED | 3.41 ml | 163.7 ml | 313 ml | 626 ml |
| Quenching Buffer | Wash Buffer | 97.9 µl | 4.7 ml | 9.40 ml | 18.8 ml |
| | 10% H ₂ O ₂ | 11 µl | 528 µl | 1.06 ml | 2.11 ml |
| | 10% Azide | 1.1 µl | 52.8 µl | 106 µl | 211 µl |
| | TOTAL REQUIRED | 110 µl | 5.28 ml | 10.56 ml | 21.12 ml |
| Blocking Buffer | TOTAL REQUIRED | 110 µl | 5.28 ml | 10.56 ml | 21.12 ml |
| Diluted total-FKHR antibody | Antibody Dilution Buffer | 45 µl | 2080 µl | 4160 µl | - |
| | Total-FKHR antibody | 0.09 µl | 4.16 µl | 8.32 µl | - |
| | TOTAL REQUIRED | 45.09 µl | 2084.16 µl | 4168.32 µl | - |
| Diluted phospho-FKHR antibody | Antibody Dilution Buffer | 45 µl | 2080 µl | 4160 µl | - |
| | Phospho-FKHR antibody | 0.09 µl | 4.16 µl | 8.32 µl | - |
| | TOTAL REQUIRED | 45.09 µl | 2084.16 µl | 4168.32 µl | - |
| Diluted HRP-conjugated secondary antibody | Antibody Dilution Buffer | 110 µl | 5280 µl | 10.56 ml | 21.12 ml |
| | HRP-conjugated secondary ab | 0.11 µl | 5.28 µl | 10.56 µl | 21.12 µl |
| | TOTAL REQUIRED | 110.1 µl | 5285.28 µl | 10.57 ml | 21.14 ml |
| 1X PBS (for wash steps) | 10X PBS | 154 µl | 7.39 ml | 14.11 ml | 28.22 ml |
| | dH ₂ O | 1.39 ml | 66.53 ml | 127.01 ml | 254.02 ml |
| | TOTAL REQUIRED | 1.54 ml | 73.92 ml | 141.12 ml | 282.24 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 |
| | TOTAL REQUIRED | 54 µl | 2.592 ml | 5.184 ml | 10.37 ml |
| 1% SDS Solution | TOTAL REQUIRED | 110 µl | 5.28 ml | 10.56 ml | 21.12 ml |
| Crystal Violet Solution | TOTAL REQUIRED | 110 µl | 5.28 ml | 10.56 ml | 21.12 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 cm². 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-FKHR, 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 μ l 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 μ l Wash Buffer and then 2 times for 5 minutes with 200 μ l 1X PBS.
2. Remove PBS from plate wells and add 50 μ l 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 μ l Wash Buffer and 2 times with 200 μ l 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 μ l 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 μ l 1X PBS for 5 minutes each.
4. Add 100 μ l 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 μ l) 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-FKHR antibody or total-FKHR 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 FKHR and/or total FKHR | 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 1:1000 for the phospho antibody |
| | 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-antibody and 1:1000 for the secondary antibody. The sensitivity of the assay will be decreased |
| | Inadequate washing | Ensure all wells are filled with Wash Buffer and follow washing recommendations |
| | Inadequate quenching or blocking | Ensure that quenching and blocking steps were performed according to the protocol |

| 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-FKHR antibody | Cell culture conditions did not induce phosphorylation of FKHR | Perform Western blot with phospho-FKHR antibody to confirm that cells contain detectable levels of phosphorylated FKHR |
| 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 μ l, 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™ FKHR | 1 x 96 rxns | 46396 |
| | 5 x 96 rxns | 46896 |
| TransAM™ MAPK Family | 2 x 96 rxns | 47296 |

| Cell-based ELISAs | Unit | Catalog No. |
|----------------------------|-------------|-------------|
| FACE™ AKT | 1 x 96 rxns | 48120 |
| | 5 x 96 rxns | 48620 |
| FACE™ AKT Chemi | 1 x 96 rxns | 48220 |
| | 5 x 96 rxns | 48720 |
| FACE™ ATF-2 | 1 x 96 rxns | 48115 |
| | 5 x 96 rxns | 48615 |
| FACE™ ATF-2 Chemi | 1 x 96 rxns | 48215 |
| | 5 x 96 rxns | 48715 |
| FACE™ EGFR (Y992) | 1 x 96 rxns | 48150 |
| | 5 x 96 rxns | 48650 |
| FACE™ EGFR (Y992) Chemi | 1 x 96 rxns | 48250 |
| | 5 x 96 rxns | 48750 |
| FACE™ EGFR (Y1173) | 1 x 96 rxns | 48190 |
| | 5 x 96 rxns | 48690 |
| FACE™ EGFR (Y1173) Chemi | 1 x 96 rxns | 48290 |
| | 5 x 96 rxns | 48790 |
| FACE™ ErbB-2 (Y877) | 1 x 96 rxns | 48130 |
| | 5 x 96 rxns | 48630 |
| FACE™ ErbB-2 (Y877) Chemi | 1 x 96 rxns | 48230 |
| | 5 x 96 rxns | 48730 |
| FACE™ ErbB-2 (Y1248) | 1 x 96 rxns | 48105 |
| | 5 x 96 rxns | 48605 |
| FACE™ ErbB-2 (Y1248) Chemi | 1 x 96 rxns | 48205 |
| | 5 x 96 rxns | 48705 |
| FACE™ ERK1/2 | 1 x 96 rxns | 48140 |
| | 5 x 96 rxns | 48640 |
| FACE™ ERK1/2 Chemi | 1 x 96 rxns | 48240 |
| | 5 x 96 rxns | 48740 |
| FACE™ GSK3β | 1 x 96 rxns | 48170 |
| | 5 x 96 rxns | 48670 |
| FACE™ GSK3β Chemi | 1 x 96 rxns | 48270 |
| | 5 x 96 rxns | 48770 |
| FACE™ JNK | 1 x 96 rxns | 48110 |
| | 5 x 96 rxns | 48610 |
| FACE™ JNK Chemi | 1 x 96 rxns | 48210 |
| | 5 x 96 rxns | 48710 |
| FACE™ MEK1/2 | 1 x 96 rxns | 48180 |
| | 5 x 96 rxns | 48680 |
| FACE™ MEK1/2 Chemi | 1 x 96 rxns | 48280 |
| | 5 x 96 rxns | 48780 |
| FACE™ p38 | 1 x 96 rxns | 48100 |
| | 5 x 96 rxns | 48600 |
| FACE™ p38 Chemi | 1 x 96 rxns | 48200 |
| | 5 x 96 rxns | 48700 |

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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