FACE™ Maker

ELISA Kits

(version A)

Catalog Nos. 48000 & 48500 (FACE Maker)
Catalog Nos. 48050 & 48550 (FACE Maker Chemi)

Active Motif North America
1914 Palomar Oaks Way, Suite 150
Carlsbad, California 92008, USA
Toll free: 877 222 9543
Telephone: 760431 1263
Fax: 760431 1351

Active Motif Europe
104 Avenue Franklin Roosevelt
B-1330 Rixensart, Belgium
UK Free Phone: 0800 169 3147
France Free Phone: 0800 90 99 79
Germany Free Phone: 0800 181 99 10
Telephone: +32 (0)2 653 0001
Fax: +32 (0)2 653 0050

Active Motif Japan
Azuma Bldg, 7th Floor
2-21 Ageba-Cho, Shinjuku-Ku
Tokyo, 162-0824, Japan
Telephone: +81 3 5225 3638
Fax: +81 3 5261 8733

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Overview

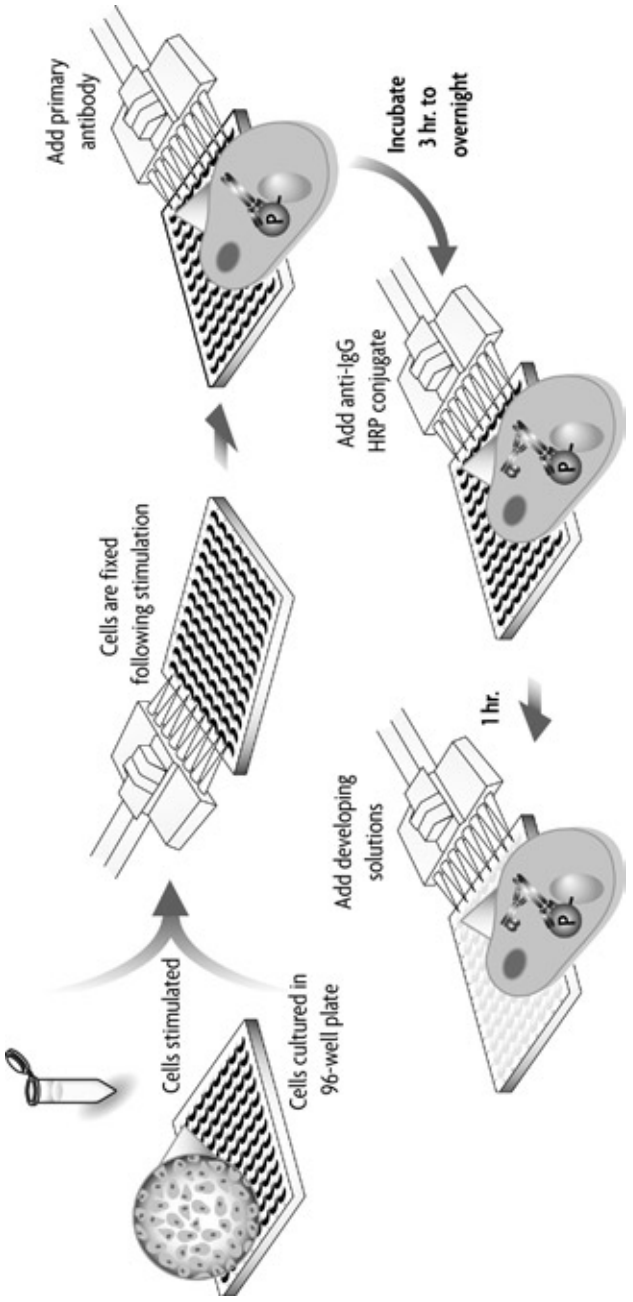
Fast Activated Cell-based ELISA (FACE™)* Kits provide a simple, efficient, cell-based method to monitor proteins activated by phosphorylation. FACE Maker Kits are designed specifically to quantify your choice of activated (phosphorylated) protein and/or total protein targets[1]. Using antibodies specific to your desired target protein, you can now investigate any activated target in any pathway. 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 or total protein. 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 Maker kits are available in two sizes:

product	format	catalog no.
FACE Maker	1 x 96 rxns	48000
	5 x 96 rxns	48500
FACE Maker Chemi	1 x 96 rxns	48050
	5 x 96 rxns	48550

See a listing of FACE Kits that have been optimized for specific targets in Appendix, Section B.

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

Flow Chart of Process



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[2]. 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 Maker

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

To overcome this, Active Motif is introducing 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 Maker Kit contains two 96-well plates and optimized assay reagents.

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

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

In the FACE Maker assay, intact cells are fixed with formaldehyde to preserve their characteristics at a chosen time point. Because fixed cells are stable for several weeks, you can prepare many plates simultaneously and then perform the 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 Maker Kits are for research use only. Not for use in diagnostic procedures.

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

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

Note on data interpretation

For example, FACE ATF-2 phospho-ATF-2 and total-ATF-2 antibodies can be used on equivalent cell cultures to determine the effects of various cell treatments on the ratio of phosphorylated ATF-2 to total ATF-2. However, if the signals with the phospho-ATF-2 antibody and the total-ATF-2 antibody are identical, one cannot conclude that the treatment resulted in phosphorylation of 100% of the ATF-2.

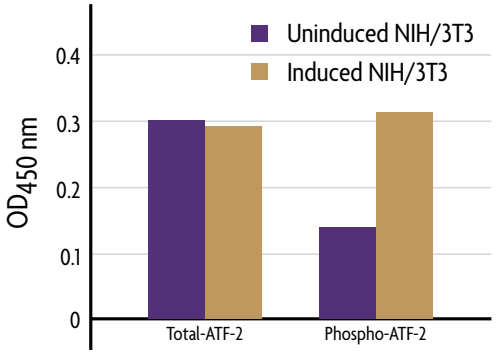


Figure 1: Measurement of phosphorylated and total ATF-2.
NIH/3T3 cells were cultured in 96-well plates and serum-starved for 16 hours. Cells were then treated with 25 µg/ml of anisomycin for 30 minutes and fixed. Total and phospho ATF-2 were each assayed in triplicate using the phospho and total ATF-2 antibodies included in the FACE ATF-2 Kit. Data was plotted after correction for cell number (performed through use of Crystal Violet).

FACE Maker Experimental Design

The FACE Maker assay is a high-throughput method for quantifying cellular levels of your desired target protein and its phosphorylated levels after treatment. The FACE method should be used with cell types that have been shown to contain readily detectable levels of your target protein and, under appropriate induction conditions, the phosphorylated state of the target protein.

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 protein levels to total? 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-protein 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
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

- Primary antibodies specific to protein of interest
- HRP-conjugated secondary antibodies
- Multi-channel pipettor
- Multi-channel pipettor reservoirs
- Rocking platform
- Parafilm
- Microplate spectrophotometer capable of reading at 595 nm and at 450 nm (655 as optional reference wavelength)
- Fresh 10% hydrogen peroxide (H_2O_2) in dH_2O (3 ml are required)
- 10 mg/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 1 plate / 5 plates	Storage / Stability
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

- Primary antibodies specific to protein of interest
- HRP-conjugated secondary antibodies
- Multi-channel pipettor
- Multi-channel pipettor reservoirs
- Rocking platform
- Parafilm
- Microplate spectrophotometer capable of reading at 595 nm for Crystal Violet staining
- Microplate luminometer or CCD camera-coupled imaging system for chemiluminescent detection
- Fresh 10% hydrogen peroxide (H_2O_2) in dH_2O (3 ml are required)
- 10 mg/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 the phospho-specific antibody and one 96-well FACE assay with the total-target protein 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-specific antibody

The phospho-specific antibody should recognize only the phosphorylated form of the protein of interest. We recommend using a dilution of 1/250 to 1/500 in Antibody Dilution Buffer (see the Quick Chart for Preparing Buffers in this section). However, with antibodies that have not been tested in FACE, the optimal dilution may have to be determined empirically.

Diluted total-target protein antibody

The total-target protein antibody should recognize both the non-phosphorylated and the phosphorylated forms of the protein. We recommend a dilution of 1/500 in Antibody Dilution Buffer (see the Quick Chart for Preparing Buffers in this section). However, with antibodies that have not been tested in FACE, the optimal dilution may have to be determined empirically.

Diluted HRP-conjugated secondary antibody

HRP-conjugated anti-species-appropriate IgG is used as the secondary antibody to detect bound primary antibodies. We recommend diluting the secondary 1/2000 in Antibody Dilution Buffer (see the Quick Chart for Preparing Buffers in this section). However, the optimal dilution may have to be determined empirically.

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
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_2O_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-antibody (1/250 example)	Antibody Dilution Buffer	45 µl	2080 µl	4160 µl	-
	Total-target antibody	0.09 µl	4.16 µl	8.32 µl	-
	TOTAL REQUIRED	**45.09 µl**	**2084.16 µl**	**4168.32 µl**	**-**
Diluted phospho-antibody (1/500 example)	Antibody Dilution Buffer	45 µl	2080 µl	4160 µl	-
	Phospho-target antibody	0.18 µl	8.32 µl	16.64 µl	-
	TOTAL REQUIRED	**45.18 µl**	**2088.32 µl**	**4176.64 µl**	**-**
Diluted HRP-conjugated secondary antibody (1/2000 example)	Antibody Dilution Buffer	110 µl	5280 µl	10.56 ml	21.12 ml
	HRP-conjugated secondary ab	0.055 µl	2.64 µl	5.28 µl	10.56 µl
	TOTAL REQUIRED	**110.05 µl**	**5282.64 µl**	**10.565 ml**	**21.13 ml**
1X PBS (for wash steps)	10X PBS	154 µl	7.39 ml	14.11 ml	28.22 ml
	dH_2O	1.39 ml	66.53 ml	127.01 ml	254.02 ml
	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 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 o f4% 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-target protein antibody, some with total-target protein 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 the phosphorylated form of the protein of interest, 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 at450 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-specific antibody and one 96-well FACE assay with the total-target protein 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-specific antibody

The phospho-specific antibody should recognize only the phosphorylated form of the protein of interest. We recommend using a dilution of 1/250 to 1/500 in Antibody Dilution Buffer (see the Quick Chart for Preparing Buffers in this section). However, with antibodies that have not been tested in FACE, the optimal dilution may have to be determined empirically.

Diluted total-target protein antibody

The total-target protein antibody should recognize both the non-phosphorylated and the phosphorylated forms of the protein. We recommend a dilution of 1/500 in Antibody Dilution Buffer (see the Quick Chart for Preparing Buffers in this section). However, with antibodies that have not been tested in FACE, the optimal dilution may have to be determined empirically.

Diluted HRP-conjugated secondary antibody

HRP-conjugated anti-species-appropriate IgG is used as the secondary antibody to detect bound primary antibodies. We recommend diluting the secondary 1/2000 in Antibody Dilution Buffer (see the Quick Chart for Preparing Buffers in this section). However, the optimal dilution may have to be determined empirically.

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
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_2O_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-antibody (1/250 example)	Antibody Dilution Buffer	45 µl	2080 µl	4160 µl	-
	Total-target antibody	0.09 µl	4.16 µl	8.32 µl	-
	TOTAL REQUIRED	**45.09 µl**	**2084.16 µl**	**4168.32 µl**	**-**
Diluted phospho-antibody (1/500 example)	Antibody Dilution Buffer	45 µl	2080 µl	4160 µl	-
	Phospho-target antibody	0.18 µl	8.32 µl	16.64 µl	-
	TOTAL REQUIRED	**45.18 µl**	**2088.32 µl**	**4176.64 µl**	**-**
Diluted HRP-conjugated secondary antibody (1/2000 example)	Antibody Dilution Buffer	110 µl	5280 µl	10.56 ml	21.12 ml
	HRP-conjugated secondary ab	0.055 µl	2.64 µl	5.28 µl	10.56 µl
	TOTAL REQUIRED	**110.05 µl**	**5282.64 µl**	**10.565 ml**	**21.13 ml**
1X PBS (for wash steps)	10X PBS	154 µl	7.39 ml	14.11 ml	28.22 ml
	dH_2O	1.39 ml	66.53 ml	127.01 ml	254.02 ml
	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 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-specific antibody, some with total-target protein 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 the phosphorylated form of the protein of interest, 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.

References

1. Versteeg H.H. *et al* (2000) *Biochem J.* 350 Pt 3: 717-22.*
2. Kameshita I. and Fujisawa H. (1989) *Analytical Biochem.* 183: 139-143.

* The FACE method was developed in the laboratory of Dr. Maikel P. Peppelenbosch, Laboratory for Experimental Internal Medicine, Academic Medical Centre, Amsterdam, The Netherlands. We thank Dr. Henri H. Versteeg and Dr. Peppelenbosch for their assistance in developing the FACE Kits.

Appendix

Section A. Troubleshooting Guide

PROBLEM	POSSIBLE CAUSE	RECOMMENDATION
No signal or weak signal in wells incubated with either phospho-specific antibody or total-target protein 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- and/or total target protein	Use Western blotting to confirm that cells contain detectable levels of protein(s) of interest
	Insufficient number of cells were plated	Plate cells so that they are 80% confluent at time of fixing
	Cells did not adhere correctly to plate	Follow protocol for use of non-adherent cells
	Cells are not from correct origin	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 1:2000 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-specific antibody	Cell culture conditions did not induce phosphorylation of target protein	Perform Western blot with phospho-specific antibody to confirm that cells contain detectable levels of phosphorylated target protein
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

Cell-based ELISAs	Unit	Catalog No. Colorimetric Kit	Catalog No. Chemiuminescent Kit
FACE™ AKT	1 x 96 rxns	48120	48220
FACE™ ATF-2	1 x 96 rxns	48115	48215
FACE™ Bad	1 x 96 rxns	48165	48265
FACE™ c-Jun (S63)	1 x 96 rxns	48125	48225
FACE™ c-Jun (S73)	1 x 96 rxns	48135	48235
FACE™ c-Src	1 x 96 rxns	48155	48255
FACE™ EGFR (Y1173)	1 x 96 rxns	48190	48290
FACE™ EGFR (Y845)	1 x 96 rxns	48340	48440
FACE™ EGFR (Y992)	1 x 96 rxns	48150	48250
FACE™ ErbB-2 (Y1248)	1 x 96 rxns	48105	48205
FACE™ ErbB-2 (Y877)	1 x 96 rxns	48130	48230
FACE™ ERK1/2	1 x 96 rxns	48140	48240
FACE™ FAK	1 x 96 rxns	48145	48245
FACE™ FKHR (FOXO1)	1 x 96 rxns	48160	48260
FACE™ GSK3b	1 x 96 rxns	48170	48270
FACE™ JAK1	1 x 96 rxns	48185	48285
FACE™ JNK	1 x 96 rxns	48110	48210
FACE™ HSP27	1 x 96 rxns	48350	48450
FACE™ MEK1/2	1 x 96 rxns	48180	48280
FACE™ NFkB p65 Profiler	3 x 96 rxns	48300	48400
FACE™ p38	1 x 96 rxns	48100	48200
FACE™ PI3 Kinase p85	1 x 96 rxns	48175	48275
FACE™ STAT2	1 x 96 rxns	48310	48410
FACE™ STAT4	1 x 96 rxns	48320	48420
FACE™ STAT6	1 x 96 rxns	48330	48430

TransAM™ Kits	Unit	Catalog No.
TransAM™ pCREB	1 x 96 rxns	43096
TransAM™ MAPK Family	2 x 96 rxns	47296
TransAM™ STAT Family	2 x 96 rxns	42296

Methylation Detection	Unit	Catalog No.
MethylDetector	50 rxns	55001

SUMOylation Detection	Unit	Catalog No.
SUMOlink™ SUMO-1 Kit	20 rxns	401201
SUMOlink™ SUMO-2/3 Kit	20 rxns	40220

Protein Labeling	Unit	Catalog No.
LigandLink™ pLL-1 Kit	1 kit	34001
LigandLink™ pLL-1-AKT1 Kit	1 kit	34002
LigandLink™ pLL-1-p53 Kit	1 kit	34005
LigankLink™ Fluorescein Label	1 kit	34101
LigankLink™ Hexachlorofluorescein Label	1 kit	34104

Technical Services

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

Active Motif North America
1914 Palomar Oaks Way, Suite 150
Carlsbad, CA 92008
USA
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Telephone: 760 431 1263
Fax: 760 431 1351
E-mail: tech_service@activemotif.com

Active Motif Europe
1104 Avenue Franklin Roosevelt
B-1330 Rixensart, Belgium
UK Free Phone: 0800 169 3147
France Free Phone: 0800 90 99 79
Germany Free Phone: 0800 181 99 10
Telephone: +32 (0)2 653 0001
Fax: +32 (0)2 653 0050
E-mail: eurotech@activemotif.com

Active Motif Japan
Azuma Bldg, 7th Floor
2-21 Ageba-Cho, Shinjuku-Ku
Tokyo, 162-0824, Japan
Telephone: +81 3 5225 3638
Fax: +81 3 5261 8733
E-mail: japantech@activemotif.com

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