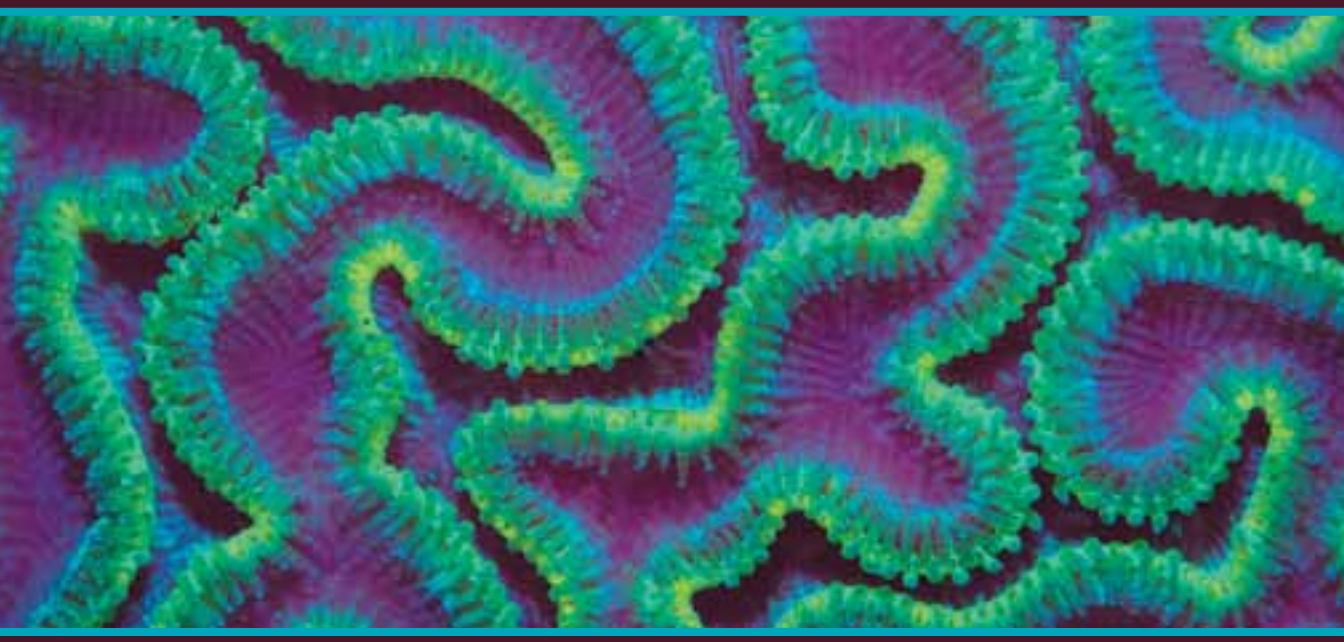
ANTIBODIES AND RELATED REAGENTS FOR SIGNAL TRANSDUCTION RESEARCH



2013/2014 Protocol Guide

Cell Signaling TECHNOLOGY®

XP® Monoclonal Antibodies

one antibody, multiple applications™

XP® monoclonal antibodies are a line of high quality rabbit monoclonal antibodies exclusively available from Cell Signaling Technology (CST). Any product labeled with XP has been carefully selected based on superior performance in all approved research applications.

XP monoclonal antibodies are generated using XMT® technology, a proprietary monoclonal method developed at CST. This technology provides access to a broad range of antibody-producing B cells unattainable with traditional monoclonal technologies, allowing more comprehensive screening and the identification of XP monoclonal antibodies with:

eXceptional specificity

As with all CST™ antibodies, the antibody is specific to your target of interest, saving you valuable time and resources.

+ eXceptional sensitivity

The antibody will provide a stronger signal for your target protein in cells and tissues, allowing you to monitor expression of low levels of endogenous proteins, saving you valuable materials.

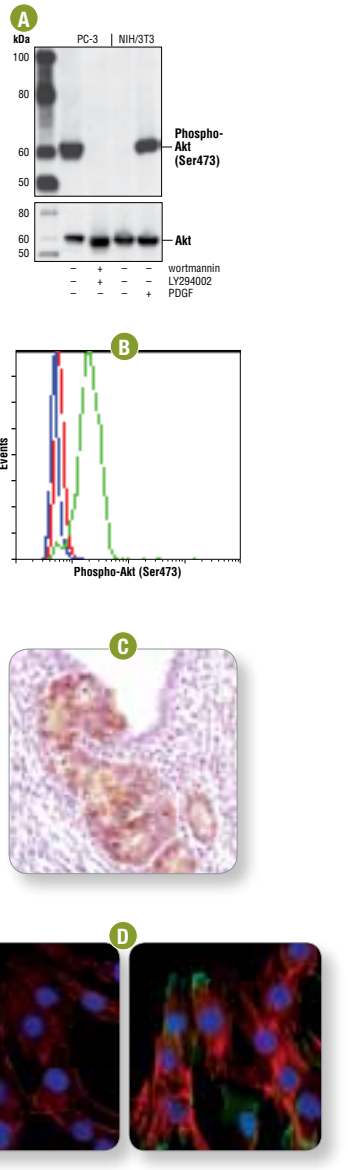
+ eXceptional stability and reproducibility

XMT technology combined with our stringent quality control ensures maximum lot-to-lot consistency and the most reproducible research results.

= eXceptional Performance™

XMT technology coupled with our extensive antibody validation and stringent quality control delivers XP monoclonal antibodies with eXceptional Performance in the widest range of research applications.

Visit our website for more experimental details, additional information, and a complete list of available XP® monoclonal antibodies.

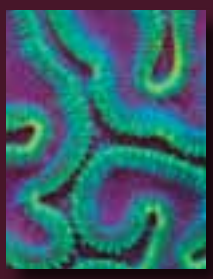


Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb #4060: Western blot analysis (**A**) of extracts from PC-3 cells, untreated or treated with LY294002 #9901 and Wortmannin #9951, and NIH/3T3 cells, serum-starved or PDGF-treated, using #4060 (upper) or an Akt total protein antibody (lower). Flow cytometric analysis (**B**) of Jurkat cells, untreated (green) or treated with LY294002 #9901, Wortmannin #9951, and U0126 #9903 (blue), using #4060 compared to a nonspecific negative control antibody (red). IHC analysis (**C**) of paraffin-embedded PTEN heterozygous mutant mouse endometrium using #4060. (*Tissue section courtesy of Dr. Sabina Signoretti, Brigham and Women's Hospital, Harvard Medical School, Boston, MA.*) Confocal IF analysis (**D**) of C2C12 cells treated with LY294002 #9901 (left) or insulin (right), using #4060 (green). Actin filaments were labeled with DY-554 phalloidin (red). Blue pseudocolor = DRAQ5® #4084 (fluorescent DNA dye).

Our Commitment to You...

As a company driven by science, our goal is to accelerate biomedical research by developing a "research tool box" enabling researchers to monitor and measure protein activity. We strive to meet contemporary and future research challenges by creating the highest quality, most specific, and thoroughly validated antibodies and related reagents.

As a committed member of the research community, we practice responsible and sustainable business methods and invest heavily in research and development. Moreover, we encourage thoughtful use of our limited resources by highlighting environmental issues in our catalog and promoting conservation and recycling.



Cover: Fluorescent photo taken to reveal the natural blue light fluorescence of the grooved brain coral (*Diploria labyrinthiformis)* at night with polyps extended to feed, Caribbean Sea. Coral reefs are biologically diverse and productive ecosystems, occupying just 0.1 percent of the oceans by volume while providing a home for about one third of all marine species.

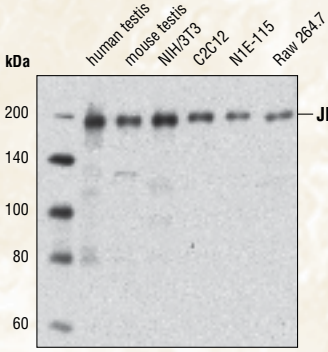
Antibody Validation for Western Blotting

Cell Signaling Technology (CST) provides the highest quality antibodies available for western blotting. CST™ antibodies are produced in-house and validated extensively according to a rigorous protocol.

Validation includes:

Analysis of Multiple Cell Lines:

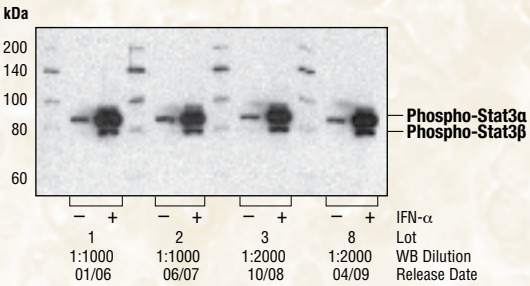
Examination of several cell lines and/or tissues of known expression levels allows accurate determination of species cross-reactivity and verifies specificity.



JIP4/SPAG9 (D72F4) XP® Rabbit mAb #5519: Western blot analysis of extracts from various tissues and cell lines using #5519.

Lot-to-lot Testing:

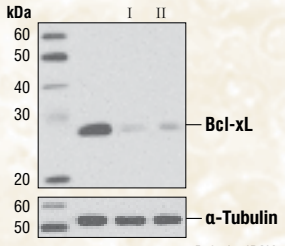
Side-by-side comparison of lots ensures lot-to-lot consistency.



Phospho-Stat3 (Tyr705) (D3A7) XP® Rabbit mAb #9145: Western blot analysis of HeLa cells, untreated or treated with IFN-α, comparing lots 1, 2, 3, and 8 of #9145. Note: Signal remains consistent from lot to lot. (Recommended dilution for western blot was changed to 1:2000 with release of lot 3.)

siRNA Knock-down:

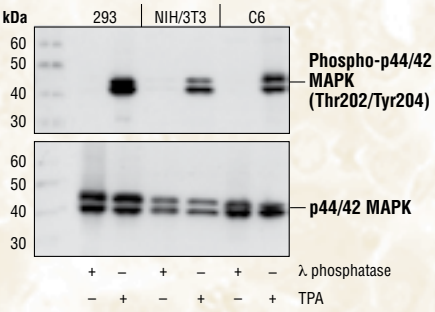
The use of siRNA transfection or knockout cell lines verifies target specificity.



SignalSilence® Control siRNA (Unconjugated) #6568, SignalSilence® Bcl-xL siRNA I #6362, SignalSilence® Bcl-xL siRNA II #6363, Bcl-xL (54H6) Rabbit mAb #2764, α-Tubulin (11H10) Rabbit mAb #2125: Western blot analysis of extracts from HeLa cells, transfected with 100 nM #6568 (-), #6362 (+), or #6363 (+), using #2764 (upper) or #2125 (lower). The Bcl-xL (54H6) Rabbit mAb confirms silencing of Bcl-xL expression, while the α-Tubulin (11H10) Rabbit mAb is used as a loading control.

Phosphatase and Activator Treatment:

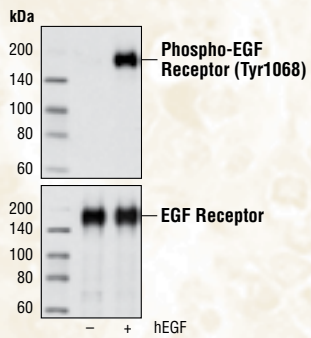
Treatment of cell lines with growth factors, chemical activators or inhibitors, which induce or inhibit target expression, verifies specificity. Phosphatase treatment confirms phospho-specificity.



Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb #4370, p44/42 MAPK (Erk1/2) (137F5) Rabbit mAb #4695: Western blot analysis of extracts from 293, NIH/3T3, and C6 cells, treated with λ phosphatase or TPA #4174 as indicated, using #4370 (upper) or #4695 (lower).

Optimization:

Optimal dilutions and buffers are predetermined, positive and negative cell extracts are specified, and detailed protocols are already optimized, saving valuable time and reagents.



EGF Receptor Control Cell Extracts #5634, EGF Receptor Control Cell Extracts #5634, Phospho-EGF Receptor (Tyr1068) (D7A5) XP® Rabbit mAb #3777, EGF Receptor (D38B1) XP® Rabbit mAb #4267: Western blot analysis of #5634 using #5634 using #3777 (upper) or #4267 (lower).

Western Immunoblotting (General)

For western blots, incubate membrane with diluted primary antibody in either 5% w/v BSA or nonfat dry milk, 1X TBS, 0.1% Tween-20 at 4°C with gentle shaking, overnight.
NOTE: Please refer to primary antibody datasheet for recommended primary antibody dilution buffer and recommended antibody dilution.

A. Solutions and Reagents

NOTE: Prepare solutions with purified water.

1. **20X Phosphate Buffered Saline (PBS):** (#9808) To prepare 1 L 1X PBS: add 50 ml 20X PBS to 950 ml dH_2O, mix.
2. **1X SDS Sample Buffer:** (#7722, #7723) 62.5 mM Tris-HCl (pH 6.8 at 25°C), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue or phenol red.
3. **Transfer Buffer:** 25 mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5).
4. **10X Tris Buffered Saline with Tween 20 (TBST-10X):** (#9997) To prepare 1 L 1X TBST: add 100 ml 10X TBST to 900 ml dH_2O, mix.
5. **Nonfat Dry Milk:** (#9999) (weight to volume [w/v])
6. **Blocking Buffer:** 1X TBST with 5% w/v nonfat dry milk; for 150 ml, add 7.5 g nonfat dry milk to 150 ml 1X TBST and mix well.
7. **Wash Buffer:** 1X TBST
8. **Bovine Serum Albumin (BSA):** (#9998)
9. **Primary Antibody Dilution Buffer:** 1X TBST with 5% BSA or 5% nonfat dry milk as indicated on primary antibody datasheet; for 20 ml, add 1.0 g BSA or nonfat dry milk to 20 ml 1X TBST and mix well.
10. **Phototope®-HRP Western Blot Detection System:** (anti-rabbit #7071) (anti-mouse #7072) Includes biotinylated protein ladder (#7727), secondary antibody conjugated to horseradish peroxidase (HRP) (anti-rabbit #7074) (anti-mouse #7076), anti-biotin HRP-linked antibody (#7075), LumiGLO® chemiluminescent reagent and peroxide (#7003).
11. **Prestained Protein Marker, Broad Range (Premixed Format):** (#7720)
12. **Blotting Membrane:** This protocol has been optimized for nitrocellulose membranes (recommended). PVDF membranes may also be used. Pore size 0.2 µm is generally recommended

B. Protein Blotting

A general protocol for sample preparation is described below.

1. Treat cells by adding fresh media containing regulator for desired time.
2. Aspirate media from cultures; wash cells with 1X PBS; aspirate.
3. Lyse cells by adding 1X SDS sample buffer (100 µl per well of 6-well plate or 500 µl per plate of 10 cm diameter plate). Immediately scrape the cells off the plate and transfer the extract to a microcentrifuge tube. Keep on ice.
4. Sonicate for 10–15 sec to complete cell lysis and shear DNA (to reduce sample viscosity).
5. Heat a 20 µl sample to 95–100°C for 5 min; cool on ice.
6. Microcentrifuge for 5 min.
7. Load 20 µl onto SDS-PAGE gel (10 cm x 10 cm). **NOTE:** Loading of prestained molecular weight markers (#7720, 10 µl/lane) to verify electrotransfer and biotinylated protein ladder (#7727, 10 µl/lane) to determine molecular weights are recommended.
8. Electrotransfer to nitrocellulose or PVDF membrane.

C. Membrane Blocking and Antibody Incubations

NOTE: Volumes are for 10 cm x 10 cm (100 cm^2) of membrane; for different sized membranes, adjust volumes accordingly.

I. Membrane Blocking

1. (Optional) After transfer, wash nitrocellulose membrane with 25 ml TBS for 5 min at room temperature.
2. Incubate membrane in 25 ml of blocking buffer for one hour at room temperature.
3. Wash once for 5 min each with 15 ml of TBST.

II. Primary Antibody Incubation

Proceed to one of the following specific set of steps depending on the primary antibody used.

For Unconjugated Primary Antibodies

1. Incubate membrane and primary antibody (at the appropriate dilution as recommended in the product datasheet) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.
2. Wash three times for 5 min each with 15 ml of TBST.
3. Incubate membrane with the species appropriate HRP-conjugated secondary antibody (#7074 or #7076) (1:2000) and Anti-biotin, HRP-linked Antibody (#7075) (1:1000) to detect biotinylated protein markers in 10 ml of blocking buffer with gentle agitation for one hr at room temperature.
4. Wash three times for 5 min each with 15 ml of TBST.
5. Proceed with detection (Section D).

For HRP Conjugated Primary Antibodies

1. Incubate membrane and primary antibody (at the appropriate dilution as recommended in the product datasheet) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.
2. Wash three times for 5 min each with 15 ml of TBST.
3. Incubate with Anti-biotin, HRP-linked Antibody (#7075) (1:1000) to detect biotinylated protein markers in 10 ml of blocking buffer with gentle agitation for one hour at room temperature.
4. Wash three times for 5 min each with 15 ml of TBST.
5. Proceed with detection (Section D).

For Biotinylated Primary Antibodies

1. Incubate membrane and primary antibody (at the appropriate dilution as recommended in the product datasheet) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.
2. Wash three times for 5 min each with 15 ml of TBST.
3. Incubate membrane with Streptavidin-HRP (at the appropriate dilution) in 10 ml of blocking buffer with gentle agitation for one hour at room temperature.
4. Wash three times for 5 min each with 15 ml of TBST.
5. Proceed to detection step in section D.

Do not add Anti-biotin, HRP-linked Antibody for detection of biotinylated protein markers. There is no need. The Streptavidin-HRP secondary antibody will also visualize the biotinylated markers.

D. Detection of Proteins

1. Incubate membrane with 10 ml LumiGLO® (0.5 ml 20X LumiGLO®, 0.5 ml 20X Peroxide, and 9.0 ml purified water) with gentle agitation for 1 min at room temperature. **NOTE:** LumiGLO® substrate can be further diluted if signal response is too fast.
2. Drain membrane of excess developing solution (do not let dry), wrap in plastic wrap and expose to x-ray film. An initial 10 sec exposure should indicate the proper exposure time. **NOTE:** Due to the kinetics of the detection reaction, signal is most intense immediately following LumiGLO® incubation and declines over the following 2 hr.

Western Immunoblotting (Fluorescent)

For western blots, incubate membrane with diluted primary antibody in either 5% w/v BSA or nonfat dry milk, 1X TBS, 0.1% Tween-20 at 4°C with gentle shaking, overnight. **NOTE:** Two-color western blots require primary antibodies from different species and appropriate secondary antibodies labeled with different dyes. If the primary antibodies require different primary antibody incubation buffers, test each primary individually in both buffers to determine the optimal one for the dual-labeling experiment.

A. Solutions and Reagents

NOTE: Prepare solutions with purified water.

1. **20X Phosphate Buffered Saline (PBS):** (#9808) To prepare 1 L 1X PBS: add 50 ml 20X PBS to 950 ml dH_2O, mix.
2. **1X SDS Sample Buffer:** (#7722, #7723) 62.5 mM Tris-HCl (pH 6.8 at 25°C), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue or phenol red.
3. **Transfer Buffer:** 25 mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5).
4. **10X Tris Buffered Saline with Tween 20 (TBST-10X):** (#9997) To prepare 1 L 1X TBST: add 100 ml 10X TBST to 900 ml dH_2O, mix.
5. **Nonfat Dry Milk:** (#9999) (weight to volume [w/v])
6. **Blocking Buffer:** 1X TBS with 5% w/v nonfat dry milk; for 150 ml, add 7.5 g nonfat dry milk to 150 ml 1X TBS and mix well.
7. **Wash Buffer:** 1X TBST
8. **Bovine Serum Albumin (BSA):** (#9998)
9. **Primary Antibody Dilution Buffer:** 1X TBST with 5% BSA or 5% nonfat dry milk as indicated on primary antibody datasheet; for 20 ml, add 1.0 g BSA or nonfat dry milk to 20 ml 1X TBST and mix well.
10. **Phototope®-HRP Western Blot Detection System:** (anti-rabbit #7071) (anti-mouse #7072) Includes biotinylated protein ladder (#7727), secondary antibody conjugated to horseradish peroxidase (HRP) (anti-rabbit #7074) (anti-mouse #7076), anti-biotin HRP-linked antibody (#7075), LumiGLO® chemiluminescent reagent and peroxide (#7003).
11. **Prestained Protein Marker, Broad Range (Premixed Format):** (#7720)
12. **Blotting Membrane:** This protocol has been optimized for nitrocellulose membranes (recommended). PVDF membranes may also be used. Pore size 0.2 µm is generally recommended.

B. Protein Blotting

A general protocol for sample preparation.

1. Treat cells by adding fresh media containing regulator for desired time.
2. Aspirate media from cultures; wash cells with cold 1X PBS; aspirate.
3. Lyse cells by adding 1X SDS sample buffer (100 µl per well of 6-well plate or 500 µl per plate of 10 cm diameter plate). Immediately scrape the cells off the plate and transfer the extract to a microcentrifuge tube. Keep on ice.
4. Sonicate for 10–15 sec to complete cell lysis and shear DNA (to reduce sample viscosity).
5. Heat a 20 µl sample to 95–100°C for 5 min; cool on ice.
6. Microcentrifuge for 5 min.
7. Load 20 µl onto SDS-PAGE gel (10 cm x 10 cm).

   NOTE: Loading of prestained molecular weight markers (#7720, 10 µl/lane) is recommended to verify electrotransfer and to determine molecular weights. Prestained markers are autofluorescent at near-infrared wavelengths.
8. Electrotransfer to nitrocellulose membrane.

C. Membrane Blocking and Antibody Incubations

NOTE: Volumes are for 10 cm x 10 cm (100 cm^2) of membrane; for different sized membranes, adjust volumes accordingly.

1. (Optional) After transfer, wash nitrocellulose membrane with 25 ml TBS for 5 min at room temperature.
2. Incubate membrane in 25 ml of blocking buffer for 1 hr at room temperature.
3. Wash three times for 5 min each with 15 ml of TBS/T.
4. Incubate membrane and primary antibody (at the appropriate dilution as recommended in the product datasheet) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.
5. Wash three times for 5 min each with 15 ml of TBS/T.
6. Incubate membrane with fluorophore-conjugated secondary antibody (1:5000-1:25,000 dilution of 1 mg/ml stock) in 10 ml of secondary antibody incubation buffer with gentle agitation for 1 hr at room temperature.
7. Wash three times for 5 min each with 15 ml of TBS/T.

D. Detection of Proteins

1. Drain membrane of excess TBS/T and allow to dry.
2. Scan membrane using an appropriate fluorescent scanner following the manufacturer's recommendations.

Western Immunoblotting Troubleshooting Guide

Problem: High Background

General background is high or nonspecific bands appear after a 1–30 second exposure of blot to film.

Cause:	Solution:
Tissue extract	In general, tissue extracts tend to contain more background bands and degradation products than cell line extracts. Using fresh, sonicated and clarified tissue extracts may lessen background. Lysing in RIPA Buffer (#9806) may provide a more thorough and consistent lysis of tissue.
Primary antibody was not incubated at the correct dilution or was in the incorrect buffer	Incubate primary antibody overnight at 4°C in TBS/T at the recommended dilution with the recommended blocking agent. In general, polyclonal and rabbit monoclonal antibodies give the highest signal:noise ratio in 5% BSA, and mouse monoclonal antibodies give the highest signal:noise ratio in 5% milk. For individual antibodies please consult the product datasheet for recommended dilution buffer.
Inadequate washing	Washing for less time than the recommended 3 x 5 min is common, and can result in high background. A mild detergent, such as Tween-20, should be included in the wash buffer. This applies to washing steps after both primary and secondary antibody incubations.
Membrane was inappropriate for chemiluminescent detection	Use only high quality nitrocellulose or PVDF membranes. Pore size 0.2 µm is generally recommended; membranes with a pore size of 0.45 µm are not recommended for proteins smaller than 30 kDa. Select a membrane found to exhibit high specific binding with low background. Nylon membranes are not recommended for western blotting.
Membrane blocking was not sufficient to prevent nonspecific binding of primary or secondary antibody	Block for 1 hr at room temperature in 5% milk in TBS/T. Do not block excessively.
Secondary antibody failed, was incubated at the incorrect dilution, or was in the incorrect buffer	Some secondary antibodies bind nonspecifically to proteins in cell extracts. To assess the quality of a secondary antibody, perform a blot (through to film exposure) without primary antibody. Serial dilutions of the secondary antibody can be performed on blots with the same cell extracts and primary antibody to optimize secondary antibody concentration. Always incubate the secondary antibody in 5% milk in TBS/T for 1 hr at room temperature.
LumiGLO® reagent was prepared using poor quality water	Chemiluminescent detection requires that high purity water be used to dilute LumiGLO® reagents A and B. Use only purified water with organic and inorganic impurities removed.
Long exposure times due to low expression or lack of treatment	Use cell lines or tissues with adequate protein expression levels. If necessary, utilize treatment to induce expression or modification.

Problem: Low Signal

The protein of interest cannot be detected after 1–30 second exposure of blot to film.

Cause:	Solution:
Lack of appropriate treatment	If basal levels of target protein, or protein modification are low, it may be necessary to induce expression or modification via chemical stimulant.
Insufficient incubation with primary antibody	Phospho-antibodies generated against a single or dual phosphorylation site are highly specific, but generally result in lower signal than total protein antibodies. It is critical that these antibodies be incubated with blots overnight at 4°C in the recommended buffer.
Incomplete transfer of proteins to membrane	Transfer longer or at higher voltage. Monitor transfer using prestained molecular weight markers (membrane staining can inhibit chemiluminescent detection). For high molecular weight proteins, reduce methanol in transfer buffer to 10% to improve transfer efficiency.
Overtransfer of proteins to membrane	For smaller proteins, use a 0.2 µm membrane with a 2 hr wet transfer. The use of a 0.45 µm membrane or transferring overnight may result in overtransfer and a lack of small protein signal.
Blocking was excessive	Blocking the membrane for too long can obscure antigenic epitopes and prevent the antibody from binding. Block for only 1 hr (never overnight).
Excessive washing	Washing for longer that the recommended 3 x 5 min is common, and can result in reduced signal. This applies to washing steps after both primary and secondary antibody incubations.
Protein of interest is below detectable levels	20 µg total protein per lane is usually sufficient for detection; more protein can be loaded if constitutive levels of the protein of interest are low. Load more sample or enrich through immunoprecipitation prior to SDS-PAGE. Investigate alternative cell lines or tissues in which the protein of interest is more abundant.
Secondary antibody was weak	Replace secondary antibody or increase concentration.
LumiGLO® reagent was improperly prepared or stock reagents A and B have become cross-contaminated	Remake LumiGLO® reagent. Biotinylated molecular weight standards detected with anti-biotin-HRP act as positive controls for chemiluminescent detection.

Immunoprecipitation (for Native Protein)

This protocol is intended for immunoprecipitation of native proteins for analysis by western immunoblot or kinase activity.

A. Solutions and Reagents

NOTE: Prepare solutions with purified water.

1. **20X Phosphate Buffered Saline (PBS):** (#9808) To prepare 1 L of 1X PBS, add 50 ml 20X PBS to 950 ml dH_2O, mix.
2. **10X Cell Lysis Buffer:** (#9803) To prepare 1 L of 1X Cell Lysis Buffer, add 100 ml Cell Lysis Buffer to 900 ml dH_2O, mix. **NOTE:** Add 1mM PMSF immediately prior to use.
3. **Blue Loading Buffer Pack (SDS loading buffer):** (#7722) Prepare fresh 3X reducing SDS loading buffer by adding 1/10 volume 30X reducing agent (1.25 M) to 1 volume of 3X SDS loading buffer.
4. **Protein A or G Agarose Beads (For unconjugated primary antibodies):** (can be stored for 2 weeks at 4°C). Please prepare according to manufacture's instructions. Use Protein A for rabbit IgG pull down and Protein G for mouse IgG pull down.
5. **Immobilized Streptavidin (Bead Conjugate) (For biotinylated antibodies):** (#3419) Gently vortex vial and use 10 µl per pull down.
6. **10X Kinase Buffer:** (#9802) To Prepare 1 ml of 1X Kinase Buffer, add 100 µl 10X Kinase Buffer to 900 µl dH_2O, mix.
7. **ATP (10 mM):** (#9804) To prepare 0.5 ml of ATP (200 µM), add 10 µl ATP (10 mM) to 490 µl 1X kinase buffer.

B. Preparing Cell Lysates

1. Aspirate media. Treat cells by adding fresh media containing modulator for desired time.
2. To harvest cells under nondenaturing conditions, remove media and rinse cells once with ice-cold PBS.
3. Remove PBS and add 0.5 ml 1X ice-cold cell lysis buffer to each plate (10 cm) and incubate on ice for 5 min.
4. Scrape cells off the plate and transfer to microcentrifuge tubes. Keep on ice.
5. Sonicate on ice three times for 5 sec each.
6. Microcentrifuge for 10 min at 4°C, 14,000 X g and transfer the supernatant to a new tube. The supernatant is the cell lysate. If necessary, lysate can be stored at -80°C.

C. Immunoprecipitation

Cell Lysate Pre-Clearing

(Optional step for unconjugated and biotinylated antibodies.)

1. Add 10–30 µl of 50% bead slurry, either Protein A or G agarose beads (for unconjugated primary antibodies) or 10 µl streptavidin beads (for biotinylated antibodies) to 200 µl cell lysate at 1 mg/ml.
2. Incubate at 4°C for 30–60 min.
3. Spin for 10 min at 4°C. Transfer the supernatant to a fresh tube.
4. Proceed to one of the following specific set of steps depending on the primary antibody used.

Using Unconjugated Primary Antibodies

1. Microcentrifuge lysate for 1 min at 14,000 X g. Add primary antibody (at the appropriate dilution as recommended in the product datasheet) to 200 µl cell lysate at 1 mg/ml. Incubate with gentle rocking overnight at 4°C.
2. Add either protein A or G agarose beads (10–30 µl of 50% bead slurry). Incubate with gentle rocking for 1–3 hr at 4°C.
3. Microcentrifuge for 30 sec at 4°C. Wash pellet five times with 500 µl of 1X cell lysis buffer. Keep on ice during washes.
4. Proceed to analyze pellet by western immunoblotting or kinase activity (Section D).

Using Biotinylated Primary Antibodies

1. Microcentrifuge lysate for 1 min at 14,000 X g. Add biotinylated antibody (at the appropriate dilution as recommended in the product datasheet) to 200 µl cell lysate at 1 mg/ml. Incubate with gentle rocking overnight at 4°C.
2. Vortex gently and add Immobilized Streptavidin (Bead Conjugate) (#3419) (10 µl). Incubate with gentle rocking for 2 hr at 4°C.
3. Microcentrifuge for 30 sec at 4°C. Wash pellet five times with 500 µl of 1X cell lysis buffer. Keep on ice during washes.
4. Proceed to analyze pellet by western immunoblotting or kinase activity (see below).

Using Immobilized Antibodies (Sepharose Bead Conjugate)

1. Microcentrifuge lysate for 1 min at 14,000 X g. Vortex gently and add immobilized bead conjugate (10 µl) to 200 µl cell lysate at 1 mg/ml. Incubate with gentle rocking overnight at 4°C.
2. Microcentrifuge for 30 sec at 4°C. Wash pellet five times with 500 µl of 1X cell lysis buffer. Keep on ice during washes.
3. Proceed to analyze pellet by western immunoblotting or kinase activity (Section D).

Using Immobilized Antibodies (Magnetic Bead Conjugate)

1. Microcentrifuge lysate for 1 min at 14,000 X g. Vortex gently and add immobilized bead conjugate (10 µl) to 200 µl cell lysate at 1 mg/ml. Incubate with gentle rocking overnight at 4°C.
2. Pellet magnetic beads by placing the tubes in a magnetic separation rack and wait 1 to 2 min for solution to clear. Wash pellet five times with 500 µl of 1X cell lysis buffer. Keep on ice during washes.
3. Proceed to analyze pellet by western immunoblotting or kinase activity (see below).

D. Sample Analysis

Proceed to one of the following specific set of steps. **NOTE:** For magnetic beads, do not centrifuge. Instead use a magnetic separation rack (#7017).

For Analysis by Western Immunoblotting

1. Resuspend the pellet with 20 µl 3X SDS sample buffer. Vortex, then microcentrifuge for 30 sec.
2. Heat the sample to 95–100°C for 2-5 min and microcentrifuge for 1 min at 14,000 X g.
3. Load the sample (15–30 µl) on SDS-PAGE (4–20%).
4. Analyze sample by western blot (see Western Immunoblotting Protocol).

NOTE: For proteins with molecular weights near 50 kDa, we recommend using Mouse Anti-rabbit IgG (Light-Chain Specific) (L57A3) mAb #3677 or Mouse Anti-rabbit IgG (Conformation Specific) (L27A9) mAb #3678 as a secondary antibody to minimize masking produced by denatured heavy chains. For proteins with molecular weights near 25 kDa, Mouse Anti-rabbit IgG (Conformation Specific) (L27A9) mAb #3678 or Mouse Anti-rabbit IgG (Conformation Specific) (L27A9) mAb (HRP Conjugate) #5127 is recommended.

For Analysis by Kinase Assay

1. Wash pellet twice with 500 µl 1X kinase buffer. Keep on ice.
2. Suspend pellet in 40 µl 1X kinase buffer supplemented with 200 µM ATP and appropriate substrate.
3. Incubate for 30 min at 30°C.
4. Terminate reaction with 20 µl 3X SDS sample buffer. Vortex, then microcentrifuge for 30 sec.
5. Transfer supernatant containing phosphorylated substrate to another tube.
6. Heat the sample to 95–100°C for 2–5 min and microcentrifuge for 1 min at 14,000 X g.
7. Load the sample (15–30 µl) on SDS-PAGE (4–20%).

Complementary Products for Western Immunoblotting and Immunoprecipitation

Epitope Tag Antibodies

#9962 Epitope Tag Antibody Sampler Kit
#2368 DYKDDDDK Tag Antibody (Binds to same epitope as Sigma's Anti-FLAG® M2 Antibody)
#5407 DYKDDDDK Tag Antibody (Binds to same epitope as Sigma's Anti-FLAG® M2 Antibody) (Alexa Fluor® 488 Conjugate)
#3768 DYKDDDDK Tag Antibody (Binds to same epitope as Sigma's Anti-FLAG® M2 Antibody) (Alexa Fluor® 555 Conjugate)
#9696 DYKDDDDK Tag Antibody (Binds to same epitope as Sigma's Anti-FLAG® M2 Antibody) (Alexa Fluor® 594 Conjugate)
#3916 DYKDDDDK Tag Antibody (Binds to same epitope as Sigma's Anti-FLAG® M2 Antibody) (Alexa Fluor® 647 Conjugate)
#2908 DYKDDDDK Tag Antibody (Binds to same epitope as Sigma's Anti-FLAG® M2 Antibody) (Biotinylated)
#2044 DYKDDDDK Tag Antibody (Binds to same epitope as Sigma's Anti-FLAG® M2 Antibody) (HRP Conjugate)
#2372 β-Gal (14B7) Mouse mAb
#2956 GFP (D5.1) XP® Rabbit mAb
#2912 GFP (D5.1) XP® Rabbit mAb (Biotinylated)
#2037 GFP (D5.1) XP® Rabbit mAb (HRP Conjugate)
#2555 GFP Antibody
#2955 GFP (4B10) Mouse mAb
#2448 Glu-Glu Tag Antibody
#9325 GSK-3β-Tag Antibody
#2625 GST (91G1) Rabbit mAb
#5475 GST (91G1) Rabbit mAb (HRP Conjugate)
#2622 GST Antibody
#2624 GST (26H1) Mouse mAb
#3368 GST (26H1) Mouse mAb (Alexa Fluor® 488 Conjugate)
#3720 GST (26H1) Mouse mAb (Alexa Fluor® 555 Conjugate)
#3445 GST (26H1) Mouse mAb (Alexa Fluor® 647 Conjugate)
#3513 GST (26H1) Mouse mAb (Sepharose Bead Conjugate)
#3724 HA-Tag (C29F4) Rabbit mAb
#5017 HA-Tag (C29F4) Rabbit mAb (Biotinylated)
#3956 HA-Tag (C29F4) Rabbit mAb (Sepharose Bead Conjugate)
#2367 HA-Tag (6E2) Mouse mAb
#2350 HA-Tag (6E2) Mouse mAb (Alexa Fluor® 488 Conjugate)
#3444 HA-Tag (6E2) Mouse mAb (Alexa Fluor® 647 Conjugate)
#2999 HA-Tag (6E2) Mouse mAb (HRP Conjugate)
#2365 His-Tag Antibody
#2366 His-Tag (27E8) Mouse mAb
#4079 His-Tag (27E8) Mouse mAb (Sepharose Bead Conjugate)
#2396 MBP (8G1) Mouse mAb
#2278 Myc-Tag (71D10) Rabbit mAb
#3946 Myc-Tag (71D10) Rabbit mAb (Biotinylated)
#2272 Myc-Tag Antibody
#2276 Myc-Tag (9B11) Mouse mAb
#2279 Myc-Tag (9B11) Mouse mAb (Alexa Fluor® 488 Conjugate)
#3756 Myc-Tag (9B11) Mouse mAb (Alexa Fluor® 555 Conjugate)
#9483 Myc-Tag (9B11) Mouse mAb (Alexa Fluor® 594 Conjugate)
#2233 Myc-Tag (9B11) Mouse mAb (Alexa Fluor® 647 Conjugate)
#2040 Myc-Tag (9B11) Mouse mAb (HRP Conjugate)
#3400 Myc-Tag (9B11) Mouse mAb (Sepharose Bead Conjugate)
#8476 S-Tag Antibody

Control Cell Extracts

#9273 Akt Control Cell Extracts
#9158 AMPK Control Cell Extracts
#9223 ATF-2 Control Cell Extracts
#9663 Caspase-3 Control Cell Extracts
#9193 CREB Control Cell Extracts
#5634 EGF Receptor Control Cell Extracts
#9263 c-Jun Control Cell Extracts
#2043 Jurkat Apoptosis Cell Lysates (etoposide)
#9160 MEK1/2 HeLa Control Cell Extracts
#9233 MKK3/MKK6 Control Cell Extracts
#9243 NF-κB Control Cell Extracts
#9213 p38 MAPK Control Cell Extracts
#9194 p44/42 MAPK (Erk1/2) Control Cell Extracts
#9203 p70 S6 Kinase Control Cell Extracts
#9253 SAPK/JNK Control Cell Extracts
#9173 Stat1 Control Cell Extracts
#9133 Stat3 Control Cell Extracts
#9353 Stat5 Control Cell Extracts

Control Proteins

#9293 Bad Control Proteins
#9113 cdc2 (Tyr15) Control Proteins
#9183 Elk-1 Control Proteins
#9103 p44/42 MAPK (Erk1/2) Control Proteins
#9303 Rb Control Proteins
#2904 VEGF Receptor 2 Control Proteins

Secondary Antibodies

#7055 Anti-biotin, AP-linked Antibody
#7056 Anti-mouse IgG, AP-linked Antibody
#7054 Anti-rabbit IgG, AP-linked Antibody
#7075 Anti-biotin, HRP-linked Antibody
#7076 Anti-mouse IgG, HRP-linked Antibody
#7074 Anti-rabbit IgG, HRP-linked Antibody
#7077 Anti-rat IgG, HRP-linked Antibody
#5946 Anti-mouse IgG (H+L), F(ab')$_2$ Fragment (Sepharose Bead Conjugate)
#6990 Anti-rabbit IgG F(ab')$_2$ Fragment (Sepharose Bead Conjugate)
#5597 Anti-biotin (D5A7) Rabbit mAb
#5571 Anti-biotin (D5A7) Rabbit mAb (HRP Conjugate)
#3678 Mouse Anti-rabbit IgG (Conformation Specific) (L27A9) mAb
#5127 Mouse Anti-rabbit IgG (Conformation Specific) (L27A9) mAb (HRP Conjugate)
#3677 Mouse Anti-rabbit IgG (Light-Chain Specific) (L57A3) mAb
#5470 Anti-mouse IgG (H+L) (DyLight® 680 Conjugate)
#5366 Anti-rabbit IgG (H+L) (DyLight® 680 Conjugate)
#5257 Anti-mouse IgG (H+L) (DyLight® 800 Conjugate)
#5151 Anti-rabbit IgG (H+L) (DyLight® 800 Conjugate)
#3999 Streptavidin (Sepharose Bead Conjugate)
#3419 Streptavidin-HRP

Detection Reagents

#7072 Phototope®-HRP Western Blot Detection System, Anti-mouse IgG, HRP-linked Antibody
#7071 Phototope®-HRP Western Blot Detection System, Anti-rabbit IgG, HRP-linked Antibody
#7003 20X LumiGLO® Reagent and 20X Peroxide
#6883 SignalFire™ ECL Reagent

Protein Markers & Loading Buffers

#7727 Biotinylated Protein Ladder Detection Pack
#7720 Prestained Protein Marker, Broad Range (Premixed Format)
#7722 Blue Loading Buffer Pack
#7723 Red Loading Buffer Pack

Solutions and Miscellaneous

#9998 BSA (BSA is not currently available in Japan)
#9803 Cell Lysis Buffer (10X)
#9852 Chaps Cell Extract Buffer (10X)
#9999 Nonfat Dry Milk
#9872 Phosphate Buffered Saline (PBS-1X) pH7.2 (Sterile)
#9808 Phosphate Buffered Saline (PBS-20X)
#9809 Phosphate Buffered Saline with Tween 20 (PBST-20X)
#5870 Phosphatase Inhibitor Cocktail (100X)
#5871 Protease Inhibitor Cocktail (100X)
#5872 Protease/Phosphatase Inhibitor Cocktail (100X)
#9806 RIPA Buffer (10X)
#9997 Tris Buffered Saline with Tween 20 (TBST-10X)
#4050 Tris-Glycine SDS Running Buffer (10X)

Agarose & Magnetic Beads & IP Controls

#9007 ChIP-Grade Protein G Agarose Beads
#9006 ChIP-Grade Protein G Magnetic Beads
#9863 Protein A Agarose Beads
#8687 Protein A Magnetic Beads
#8740 Protein G Magnetic Beads
#5946 Anti-mouse IgG (H+L), F(ab')$_2$ Fragment (Sepharose Bead Conjugate)
#6990 Anti-rabbit IgG F(ab')2 Fragment (Sepharose Bead Conjugate)
#3420 Mouse IgG (Sepharose Bead Conjugate)
#5873 Mouse IgG (Magnetic Bead Conjugate)
#3419 Streptavidin (Sepharose Bead Conjugate)
#7017 6-Tube Magnetic Separation Rack

Loading Control Antibodies

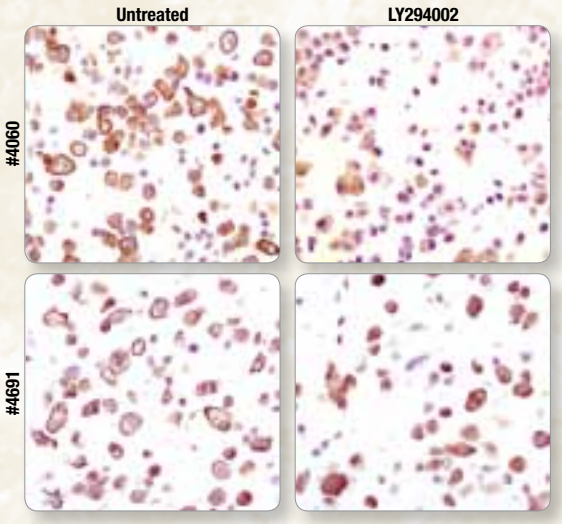
#5142 Loading Control Antibody Sampler Kit
#4670 Loading Control Antibody Sampler Kit (HRP Conjugate)
#9774 Loading Control Antibody Sampler Kit (Mouse)
#8457 β-Actin (D6A8) Rabbit mAb
#4970 β-Actin (13E5) Rabbit mAb
#5125 β-Actin (13E5) Rabbit mAb (HRP Conjugate)
#4967 β-Actin Antibody
#3700 β-Actin (8H10D10) Mouse mAb
#4850 COX IV (3E11) Rabbit mAb
#5247 COX IV (3E11) Rabbit mAb (HRP Conjugate)
#4844 COX IV Antibody
#5174 GAPDH (D16H11) XP® Rabbit mAb
#2118 GAPDH (14C10) Rabbit mAb
#5014 GAPDH (14C10) Rabbit mAb (Biotinylated)
#3683 GAPDH (14C10) Rabbit mAb (HRP Conjugate)
#4499 Histone H3 (D1H2) XP® Rabbit mAb
#9717 Histone H3 (3H1) Rabbit mAb
#5192 Histone H3 (3H1) Rabbit mAb (HRP Conjugate)
#9715 Histone H3 Antibody
#3638 Histone H3 (96C10) Mouse mAb
#2125 α-Tubulin (11H10) Rabbit mAb
#2144 α-Tubulin Antibody
#3873 α-Tubulin (DM1A) Mouse mAb
#2148 α/β-Tubulin Antibody
#2128 β-Tubulin (9F3) Rabbit mAb
#5346 β-Tubulin (9F3) Rabbit mAb (HRP Conjugate)
#2146 β-Tubulin Antibody

Antibody Validation for Immunohistochemistry

Cell Signaling Technology offers over 500 antibodies validated for immunohistochemistry (IHC). The determination of target specificity in research immunohistochemical analysis requires multiple validation steps. Scientists at Cell Signaling Technology use a variety of approaches for each antibody validated for IHC to demonstrate that staining achieved with the antibody is specific and believable.

Cell Pellets:

Paraffin-embedded cell pellets are subjected to activator or inhibitor treatments to verify target specificity.



Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb #4060, and Akt (pan) (C67E7) Rabbit mAb #4691: IHC analysis of SignalSlide® Phospho-Akt (Ser473) IHC Controls #8101 using #4060 (upper) or #4691 (lower).

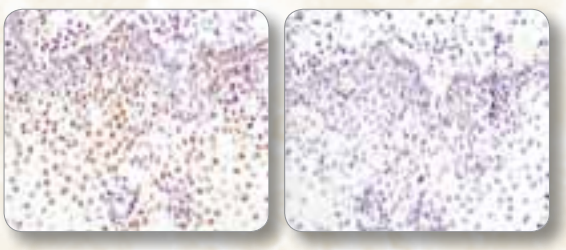
Mouse Models:

Antibody performance is assessed in relevant mouse models of cancer.

Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb #4060: IHC analysis of paraffin-embedded WT (left) and PTEN (-/-) (right) mouse prostate using #4060. Tissue courtesy of Dr. David Guertin, The Whitehead Institute for Biomedical Research, Cambridge, MA.

Blocking Peptide:

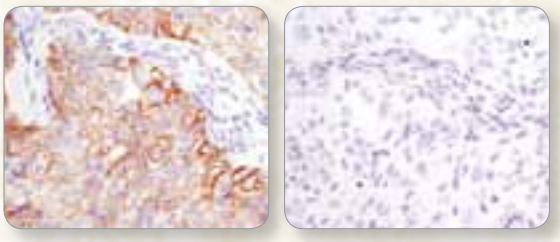
The use of blocking peptides verifies specificity and rules out Fc-mediated binding, biotin background, and other non-specific staining.



FoxP1 Antibody #2005: IHC analysis of paraffin-embedded human tonsil epithelium using #2005 in the presence of control peptide (left) or antigen-specific peptide (right).

Xenograft and Phosphatase Treatment:

Xenografts generated from cell lines with known target expression levels help verify target specificity. Phosphatase treatment verifies phospho-specificty.



Phospho-EGF Receptor (Tyr1068) (D7A5) XP® Rabbit mAb #3777: IHC analysis of paraffin-embedded HCC827 xenograft, control (left) or λ phosphatase-treated (right), using #3777.

Other Validation Steps Include:

- Western blot analysis is performed to demonstrate specific bands of the appropriate molecular weight(s), with minimal cross-reacting bands.
- Paraffin-embedded cell pellets of known target expression levels are used to verify target specificity.
- Tissue sections and cell pellets are subjected to phosphatase treatment to verify target phospho-specificity.
- The use of tissue arrays of human cancer tissues demonstrates antibody performance over a broad spectrum of tissue types.
- Thorough lot testing ensures the reproducibility necessary for accurate IHC research results.
- Dilutions and protocols are predetermined and specified; control reagents are also available.
- Staining on fresh frozen tissues is performed when appropriate.

Immunohistochemistry (Paraffin-embedded)

for SignalStain® Boost Detection Reagent

***IMPORTANT: See product datasheet for the appropriate antibody diluent, dilution, and antigen unmasking procedure.**

A. Solutions and Reagents

NOTE: Prepare solutions with purified water.

1. **Xylene**
2. **Ethanol**, anhydrous denatured, histological grade (100% and 95%)
3. **Deionized water** (dH_2O)
4. **Hematoxylin** (optional)
5. **Wash Buffer:**

   1X TBS/0.1% Tween-20 (1X TBST): To prepare 1 L, add 100 ml 10X TBS to 900 ml dH_2O. Add 1 ml Tween-20 and mix.

   10X Tris Buffered Saline (TBS): To prepare 1 L, add 24.2 g Trizma® base ($C_4H_{11}NO_3$) and 80 g sodium chloride (NaCl) to 1 L dH_2O. Adjust pH to 7.6 with concentrated HCl.
6. ***Antibody Diluent:**

   a. SignalStain® Antibody Diluent #8112

   b. TBST/5% normal goat serum: To 5 ml 1X TBST, add 250 µl normal goat serum (#5425).

   c. PBST/5% normal goat serum: To 5 ml 1X PBST, add 250 µl normal goat serum (#5425).

   1X PBS/0.1% Tween-20 (1X PBST): To prepare 1 L, add 100 ml 10X PBS to 900 ml dH_2O. Add 1 ml Tween-20 and mix.

   10X Phosphate Buffered Saline (PBS): To prepare 1 L, add 80 g sodium chloride (NaCl), 2 g potassium chloride (KCl), 14.4 g sodium phosphate, dibasic (Na_2HPO_4) and 2.4 g potassium phosphate, monobasic (KH_2PO_4) to 1 L dH_2O. Adjust pH to 7.4.
7. ***Antigen Unmasking:**

   a. Citrate: 10 mM Sodium Citrate Buffer: To prepare 1 L, add 2.94 g sodium citrate trisodium salt dihydrate ($C_6H_5Na_3O_7 \bullet 2H_2O$) to 1 L dH_2O. Adjust pH to 6.0.

   b. EDTA: 1 mM EDTA: To prepare 1 L add 0.372 g EDTA ($C_{10}H_{14}N_2O_8Na_2 \bullet 2H_2O$) to 1 L dH_2O. Adjust pH to 8.0.

   c. TE: 10 mM Tris/1 mM EDTA, pH 9.0: To prepare 1 L, add 1.21 g Trizma® base ($C_4H_{11}NO_3$) and 0.372 g EDTA ($C_{10}H_{14}N2O_8Na_2 \bullet 2H_2O$) to 950 ml dH_2O. Adjust pH to 9.0, then adjust final volume to 1 L with dH_2O.

   d. Pepsin: 1 mg/ml in Tris-HCl, pH 2.0.
8. **3% Hydrogen Peroxide:** To prepare, add 10 ml 30% H_2O_2 to 90 ml dH_2O.
9. **Blocking Solution:** TBST/5% normal goat serum: to 5 ml 1X TBST, add 250 µl normal goat serum (#5425).
10. **Detection System:** SignalStain® Boost IHC Detection Reagents (mouse #8125, rabbit #8114)
11. **Substrate:** SignalStain® DAB Substrate Kit (#8059).

B. Deparaffinization/Rehydration

NOTE: Do not allow slides to dry at any time during this procedure.

1. **Deparaffinize/hydrate sections:**

   a. Incubate sections in three washes of xylene for 5 min each.

   b. Incubate sections in two washes of 100% ethanol for 10 min each.

   c. Incubate sections in two washes of 95% ethanol for 10 min each.
2. Wash sections twice in dH_2O for 5 min each.

C. Antigen Unmasking*

NOTE: Consult product datasheet for specific recommendation for the unmasking solution.

1. **For Citrate:** Bring slides to a boil in 10 mM sodium citrate buffer, pH 6.0; maintain at a sub-boiling temperature for 10 min. Cool slides on bench top for 30 min.
2. **For EDTA:** Bring slides to a boil in 1 mM EDTA, pH 8.0: follow with 15 min at a sub-boiling temperature. No cooling is necessary.
3. **For TE:** Bring slides to a boil in 10 mM Tris/1 mM EDTA, pH 9.0: then maintain at a sub-boiling temperature for 18 min. Cool at room temperature for 30 min.
4. **For Pepsin:** Digest for 10 min at 37°C.

D. Staining

NOTE: Consult product datasheet for recommended antibody diluent.

1. Wash sections in dH_2O three times for 5 min each.
2. Incubate sections in 3% hydrogen peroxide for 10 min.
3. Wash sections in dH_2O twice for 5 min each.
4. Wash sections in wash buffer for 5 min.
5. Block each section with 100–400 µl blocking solution for 1 hr at room temperature.
6. Remove blocking solution and add 100–400 µl primary antibody diluted in recommended antibody diluent to each section*. Incubate overnight at 4°C.
7. Equilibrate SignalStain® Boost Detection Reagent to room temperature.
8. Remove antibody solution and wash sections in wash buffer three times for 5 min each.
9. Cover section with 1–3 drops SignalStain® Boost Detection Reagent as needed. Incubate in a humidified chamber for 30 min at room temperature.
10. Wash sections three times with wash buffer for 5 min each.
11. Add 1 drop (30 µl) SignalStain® DAB Chromogen Concentrate to 1 ml SignalStain® DAB Diluent and mix well before use.
12. Apply 100-400 µl SignalStain® DAB to each section and monitor closely. 1-10 minutes generally provides an acceptable staining intensity.
13. Immerse slides in dH_2O.
14. If desired, counterstain sections in hematoxylin per manufacturer's instructions.
15. Wash sections in dH_2O two times for 5 min each.
16. **Dehydrate sections:**

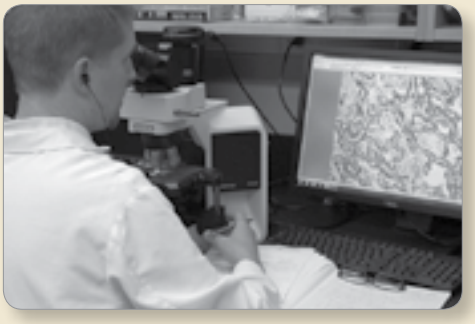
    a. Incubate sections in 95% ethanol two times for 10 sec each.

    b. Repeat in 100% ethanol, incubating sections two times for 10 sec each.

    c. Repeat in xylene, incubating sections two times for 10 sec each.
17. Mount coverslips

We are always improving our protocols and saving you precious time.

Some IHC-recommended antibodies have been validated using a shorter 2 hour incubation at 37°C, which yields the same results as the longer overnight incubation at 4°C. Please visit our website for optimal incubation conditions and recommendations specific for each product.



Immunohistochemistry (Frozen)

for SignalStain® Boost Detection Reagent

***IMPORTANT:** See product datasheet for the appropriate fixative, antibody diluent, and dilution.

A. Solutions and Reagents

NOTE: Prepare solutions with purified water.

1. **Xylene**
2. **Ethanol** (anhydrous denatured, histological grade 100% and 95%)
3. **Hematoxylin** (optional)
4. **Fixative:** For optimal fixative, please refer to the product datasheet.
   - **a. 10% neutral buffered formalin**
   - **b. Acetone**
   - **c. Methanol**
   - **d. 3% formaldehyde:** To prepare, add 18.75 ml 16% formaldehyde to 81.25 ml 1X PBS.
5. **10X Tris Buffered Saline (TBS):** To prepare 1 L, add 24.2 g Trizma® base ($C_4H_{11}NO_3$) and 80 g sodium chloride (NaCl) to 1 L dH_2O. Adjust pH to 7.6 with concentrated HCl.
6. **Wash buffer:** 1X Tris Buffered Saline (TBS): To prepare 1 L, add 100 ml 10X TBS to 900 ml dH_2O.
7. **Methanol/Peroxidase:** To prepare, add 10 mL 30% H_2O_2 to 90 ml methanol. Store at -20°C.
8. **Blocking Solution:** 1X TBS/0.3% Triton™-X 100/5% normal goat serum (#5425). To prepare, add 500 µl goat serum and 30 µl Triton™-X 100 to 9.5 ml 1X TBS.
9. **Detection System:** SignalStain® Boost IHC Detection Reagents (mouse #8125, rabbit #8114)
10. **Substrate:** SignalStain® DAB Substrate Kit (#8059).

B. Sectioning

1. **For tissue stored at -80°C:** Remove from freezer and equilibrate at -20°C for approximately 15 min before attempting to section. This may prevent cracking of the block when sectioning.
2. Section tissue at a range of 6–8 µm and place on positively charged slides.
3. Allow sections to air dry on bench for a few min before fixing (this helps sections adhere to slides).

C. Fixation

NOTE: Consult product datasheet to determine the optimal fixative.

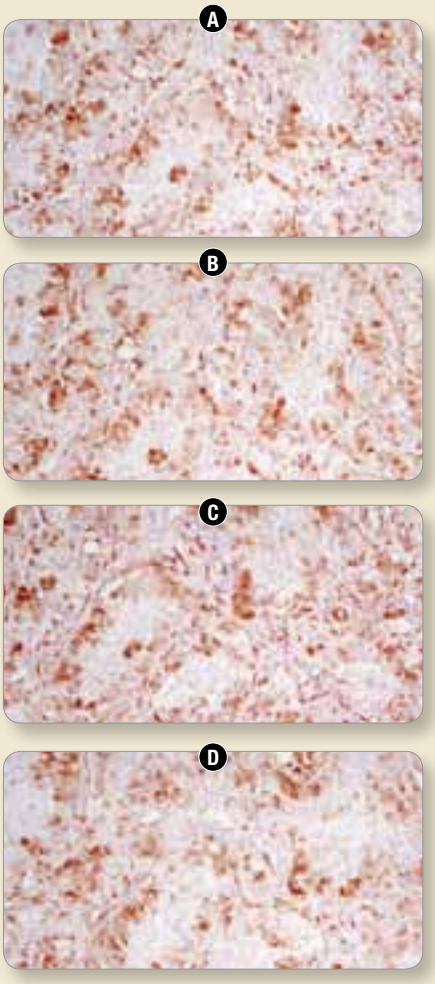
1. After sections have dried on the slide, fix in optimal fixative as directed below.
   - **a. 10% Neutral buffered formalin:** 10 min at room temperature. Proceed with staining procedure immediately.
   - **b. Cold acetone:** 10 min at -20°C. Air dry. Proceed with staining procedure immediately.
   - **c. Methanol:** 10 min at -20°C. Proceed with staining procedure immediately.
   - **d. 3% Formaldehyde:** 15 min at room temperature. Proceed with staining procedure immediately.
   - **e. 3% Formaldehyde/methanol:** 15 min at room temperature in 3% formaldehyde, followed by 5 min in methanol at -20°C (do not rinse in between). Proceed with staining procedure immediately.

D. Staining

1. Wash sections in wash buffer twice for 5 min.
2. Incubate for 10 min at room temperature in 3% H_2O_2 diluted in methanol.
3. Wash sections in wash buffer twice for 5 min.
4. Block each section with 100–400 µl blocking solution for 1 hr at room temperature.
5. Remove blocking solution and add 100–400 µl primary antibody diluted in blocking solution to each section.*
6. Incubate overnight at 4°C.
7. Equilibrate SignalStain® Boost Detection Reagent to room temperature.
8. Remove antibody solution and wash sections in wash buffer three times for 5 min each.
9. Cover section with 1–3 drops SignalStain® Boost Detection Reagent as needed. Incubate in a humidified chamber for 30 min at room temperature.
10. Wash sections three times with wash buffer for 5 min each.
11. Add 1 drop (30 µl) SignalStain® DAB Chromogen Concentrate to 1 ml SignalStain® DAB Diluent and mix well before use.
12. Apply 100-400 µl SignalStain® DAB to each section and monitor closely. 1-10 minutes generally provides an acceptable staining intensity.
13. Immerse slides in dH_2O.
14. If desired, counterstain sections in hematoxylin per manufacturer's instructions.
15. Wash sections in dH_2O two times for 5 min each.
16. **Dehydrate sections:**
    - **a.** Incubate sections in 95% ethanol two times for 10 sec each.
    - **b.** Repeat in 100% ethanol, incubating sections two times for 10 sec each.
    - **c.** Repeat in xylene, incubating sections two times for 10 sec each.
17. Mount coverslips.

Pre-optimization From Lot to Lot

Every antibody validated for IHC undergoes thorough testing prior to any new lot release. This ensures the stability and reproducibility necessary for consistently accurate results.



Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb #4370: IHC analysis of adjacent sections of paraffin-embedded human colon carcinoma using #4370 lot 7 at a 1:400 dilution (**A**), #4370 lot 9 at a 1:200 dilution (**B**), #4370 lot 9 at a 1:400 dilution (**C**) and #4370 lot 9 at a 1:800 dilution (**D**). The recommended dilution for lot 9 remained 1:400.

Complementary Products for Immunohistochemistry

SignalSlide® IHC Controls

#8101 SignalSlide® Phospho-Akt (Ser473) IHC Controls
For use with Antibodies: 2211, 2217, 2317, 2691, 2855, 2920, 2938, 2997, 3787, 4060, 4685, 4691, 4857, 4858, 5196, 5364, 5482, 9323, 9644

#8104 SignalSlide® Cleaved Caspase-3 (Asp175) IHC Controls
For use with Antibodies: 2035, 5625, 8109, 9541, 9661, 9662, 9664

#8102 SignalSlide® Phospho-EGF Receptor IHC Controls
For use with Antibodies: 2234, 2235, 2236, 2237, 3777, 4267, 4404, 4407, 9411, 9416, 9417

#8117 SignalSlide® Phospho-ErbB Family IHC Controls
For use with Antibodies: 2165, 2242, 2243, 3777, 4267, 4290, 4407, 4791, 8111

#8118 SignalSlide® Phospho-Met (Tyr1234/1235) IHC Controls
For use with Antibody: 3077, 8198

#8103 SignalSlide® Phospho-p44/42 MAPK (Thr202/Tyr204) IHC Controls
For use with Antibodies: 4370, 4376, 4695, 4696, 9102

#8106 SignalSlide® PTEN IHC Control Slides
For use with Antibodies: 9188, 9559

#8105 SignalSlide® Phospho-Stat1/3/5 IHC Controls
For use with Antibodies: 4113, 4904, 8113, 9132, 9139, 9314, 9359, 9145, 9167, 9175

Blocking Reagents & Buffers

#5425 Normal Goat Serum

#9808 Phosphate Buffered Saline (PBS-20X)

#9809 Phosphate Buffered Saline with Tween 20 (PBST-20X)

#9997 Tris Buffered Saline with Tween 20 (TBST-10X)

Isotype Control Antibodies

#3900 Rabbit (DA1E) mAb IgG XP® Isotype Control

#5415 Mouse (G3A1) mAb IgG1 Isotype Control

SignalStain® Boost IHC Detection Reagents

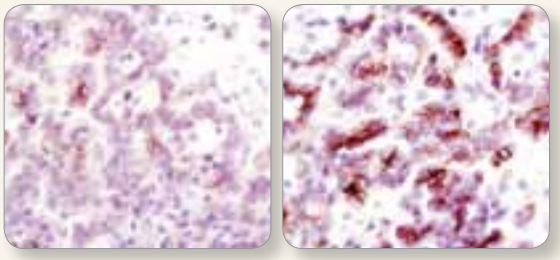
#8125 SignalStain® Boost IHC Detection Reagent (HRP, Mouse)

#8114 SignalStain® Boost IHC Detection Reagent (HRP, Rabbit)

These polymer-based detection reagents were developed to enhance sensitivity and eliminate complications arising from false positive staining due to endogenous biotin. Both products offer a one-step, highly sensitive alternative to traditional IHC detection methods. SignalStain® Boost IHC Detection Reagents are specific to either rabbit or mouse IgG, can be used to visualize targets in both paraffin-embedded and frozen tissue, and are compatible with all peroxidase-based substrates.

SignalStain® Boost IHC Detection Reagent offers several advantages over conventional ABC detection methods:

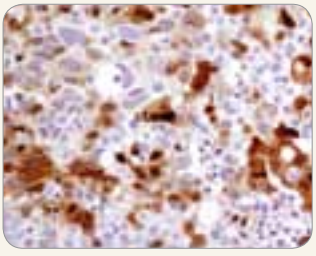
- Superior sensitivity, resulting in a stronger signal and greater confidence in your research results.
- A reduced number of steps in the detection procedure, saving valuable time.
- Less false positives resulting from endogenous biotin staining, providing a lower overall background.



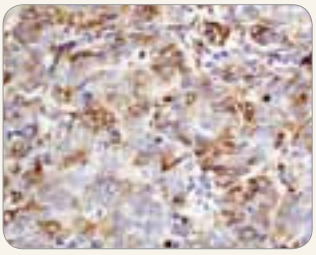
SignalStain® Boost IHC Detection Reagent (HRP, Rabbit) #8114: IHC analysis of paraffin-embedded human papillary renal cell carcinoma using Phospho-Met (Tyr1234/1235) (D26) XP® Rabbit mAb #3077 with biotin-based detection (left) or #8114 (right).

NEW! Substrate

#8059 SignalStain® DAB Substrate Kit



SignalStain® DAB Substrate Kit #8059: IHC analysis of paraffin-embedded human ovarian carcinoma using Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb #4370 and #8059.

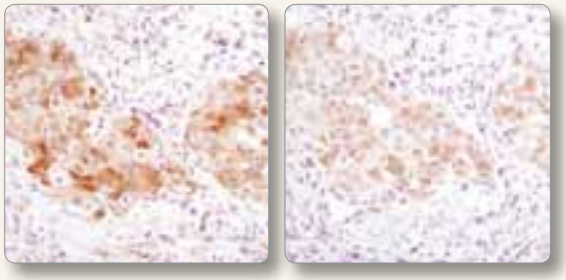


SignalStain® DAB Substrate Kit #8059: IHC analysis of paraffin-embedded human breast carcinoma using Phospho-S6 Ribosomal Protein (Ser235/236) (D57.2.2E) XP® Rabbit mAb #4858 and #8059.

SignalStain® Diluent

#8112 SignalStain® Antibody Diluent

SignalStain® Antibody Diluent #8112 yields superior staining with many of our IHC-validated antibodies. Please see product datasheets for diluent recommendations or visit the Antibody Diluent Table on our website.



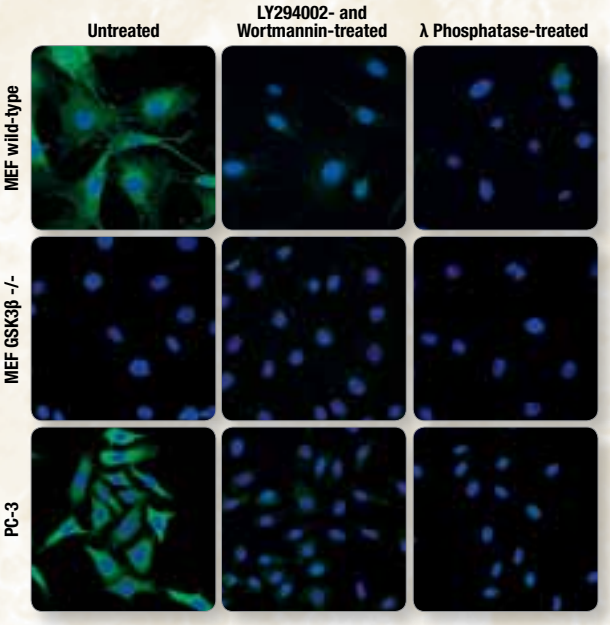
SignalStain® Antibody Diluent #8112: IHC analysis of paraffin-embedded human breast carcinoma, comparing #8112 (left) and TBST with 5% normal goat serum (right), using Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb #4060.

Antibody Validation for Immunofluorescence-based Assays

Scientists at Cell Signaling Technology (CST) have validated over 800 activation-state specific (e.g., phosphorylation-specific) and total protein antibodies for immunofluorescence (IF) applications, such as manual fluorescence microscopy or automated imaging and laser scanning high content platforms. All CST™ antibodies that are approved for use in immunofluorescent research assays have undergone a rigorous validation process.

Phosphatase Treatment and Knockout Cells:

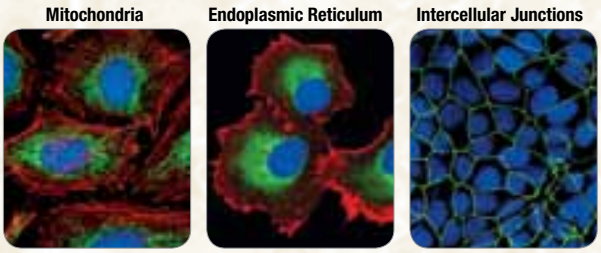
Cells are subjected to phosphatase treatment to verify phospho-specificity. Target specificity is also verified with the use of known knockout or null cell lines.



Phospho-GSK-3β (Ser9) (D85E12) XP® Rabbit mAb #5558: Confocal immunofluorescent analysis of wild type mouse embryonic fibroblasts (MEFs) (upper row), GSK-3β (-/-) MEFs (middle row), or PC-3 cells (lower row), untreated (left), LY294002 and Wortmannin-treated (#9901 and #9951 respectively; center), or λ phosphatase-treated (right), using #5558 (green). Blue pseudocolor = DRAQ5® #4084 (fluorescent DNA dye). (MEF wild type and GSK-3β (-/-) cells were kindly provided by Dr. Jim Woodgett, University of Toronto, Canada).

Subcellular Localization:

Appropriate cell lines and tissues are used to verify subcellular localization.



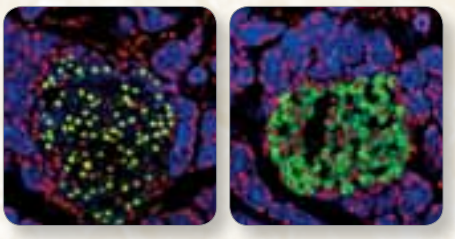
COX IV (3E11) Rabbit mAb #4850 (green)

ERp72 (D70D12) XP® Rabbit mAb #5033 (green)

ZO-3 (D57G7) XP® Rabbit mAb #3704 (green)

Tissue:

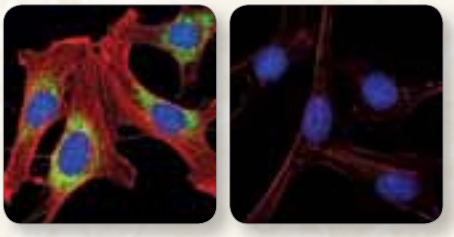
Antibody performance is assessed on appropriate tissues.



Pdx1 Antibody #2437: Confocal immunofluorescent analysis of normal rat pancreas using #2437 (green, left) or Insulin (C27C9) Rabbit mAb #3014 (green, right). Keratin filaments were labeled with Pan-Keratin (C11) Mouse mAb (Alexa Fluor® 647 Conjugate) #4528 (blue). Red = Propidium Iodide (PI)/RNase Staining Solution #4087 (fluorescent DNA dye).

Protocol Optimization:

Fixation and permeabilization conditions are optimized; alternative protocols are recommended if necessary.



PDI Antibody #2446 and β-Actin (8H10D10) Mouse mAb #3700: Confocal IF analysis of NIH/3T3 cells, permeabilized with methanol (left) or 0.3% Triton X-100 (right), using #2446 (green) and #3700 (red). Blue pseudocolor = DRAQ5® #4084 (fluorescent DNA dye).

Other Validation Steps Include:

- Requirement of threshold signal-to-noise ratio in antibody:isotype comparison and minimum fold-induction for phospho-specific antibodies ensures the greatest possible sensitivity.
- Activation state specification, target expression, or translocation are examined using ligands or inhibitors to modulate pathway activity.
- Stringent testing ensures lot-to-lot consistency.
- Cells are subjected to siRNA treatment or over-expression of the target protein to verify target specificity.
- Cell lines or tissues with known target expression levels are used to verify specificity.

Immunofluorescence (General)

IMPORTANT: Please refer to the APPLICATIONS section on the front page of product datasheet to determine if this product is validated and approved for use on cultured cell lines (IF-IC), paraffin-embedded samples (IF-P), or frozen tissue sections (IF-F). Please see product datasheet for appropriate antibody dilution and unmasking solution.

A. Solutions and Reagents

NOTE: Prepare solutions with purified water.

1. **10X Phosphate Buffered Saline (PBS):** To prepare 1 L add 80 g sodium chloride (NaCl), 2 g potassium chloride (KCl), 14.4 g sodium phosphate, dibasic (Na_2HPO_4) and 2.4 g potassium phosphate, monobasic (KH_2PO_4) to 1 L dH_2O. Adjust pH to 8.0.
2. **Formaldehyde:** 16%, methanol free, Polysciences, Inc. (cat# 18814), use fresh, store opened vials at 4°C in dark, dilute in PBS for use.
3. **Blocking Buffer:** (1X PBS / 5% normal goat serum (#5425) / 0.3% Triton™ X-100): To prepare 25 ml, add 2.5 ml 10X PBS, 1.25 ml normal serum from the same species as the secondary antibody (e.g., normal goat serum, normal donkey serum) and 21.25 ml dH_2O and mix well. While stirring, add 75 µl Triton™ X-100.
4. **Antibody Dilution Buffer:** (1X PBS / 1% BSA / 0.3% Triton™ X-100): To prepare 40 ml, add 4 ml 10X PBS and 120 µl Triton™ X-100 to 0.4 g BSA. Bring to final volume of 40 ml with dH_2O and mix well.
5. **Fluorochrome-conjugated secondary antibody**
   NOTE: When using any primary or fluorochrome-conjugated secondary antibody for the first time, titrate the antibody to determine which dilution allows for the strongest specific signal with the least background for your sample.
6. **Prolong® Gold Anti-Fade Reagent** (#9071), with DAPI (#8961)

Reagents specific to IF-P application:

1. **Xylene**
2. **Ethanol**, anhydrous denatured, histological grade, 100% and 95%.
3. **Antigen Unmasking:**

   For Citrate: 10 mM Sodium Citrate Buffer: To prepare 1 L add 2.94 g sodium citrate trisodium salt dihydrate ($C_6H_5Na_3O_7 \bullet 2H_2O$) to 1 L dH_2O. Adjust pH to 6.0.

   For EDTA: 1 mM EDTA: To prepare 1 L add 0.372 g EDTA ($C_{10}H_{14}N_2O_8Na_2 \bullet 2H_2O$) to 1 L dH_2O. Adjust pH to 8.0.

B. Specimen Preparation

I. Cultured Cell Lines (IF-IC)

NOTE: Cells should be grown, treated, fixed and stained directly in multi-well plates, chamber slides or on coverslips.

1. Aspirate liquid, then cover cells to a depth of 2–3 mm with 4% formaldehyde in PBS. **NOTE:** Formaldehyde is toxic, use only in fume hood.
2. Allow cells to fix for 15 min at room temperature.
3. Aspirate fixative, rinse three times in PBS for 5 min each.
4. Proceed with Immunostaining (Section C).

II. Paraffin Sections (IF-P)

NOTE: Do not allow slides to dry at any time during this process.

1. **Deparaffinization/Rehydration:**
   - **a.** Incubate sections in three washes of xylene for 5 min each.
   - **b.** Incubate sections in two washes of 100% ethanol for 10 min each.
   - **c.** Incubate sections in two washes of 95% ethanol for 10 min each.
   - **d.** Rinse sections twice in dH_2O for 5 min each.
2. **Antigen Unmasking:**
   NOTE: Consult product datasheet for specific recommendation for the unmasking solution.
   - **a. For Citrate:** Bring slides to a boil in 10 mM sodium citrate buffer pH 6.0, then maintain at a sub-boiling temperature for 10 min. Cool slides on bench top for 30 min.
   - **b. For EDTA:** Bring slides to a boil in 1 mM EDTA pH 8.0 followed by 15 min at a sub-boiling temperature. No cooling is necessary.
3. Proceed with Immunostaining (Section C).

III. Frozen/Cryostat Sections (IF-F)

1. For fixed frozen tissue proceed with Immunostaining (Section C).
2. For fresh, unfixed frozen tissue, please fix immediately, as follows:
   - **a.** Cover sections with 4% formaldehyde in PBS.
   - **b.** Allow sections to fix for 15 min at room temperature.
   - **c.** Rinse slides three times in PBS for 5 min each.
   - **d.** Proceed with Immunostaining (Section C).

C. Immunostaining

NOTE: All subsequent incubations should be carried out at room temperature unless otherwise noted in a humid light-tight box or covered dish/plate to prevent drying and fluorochrome fading.

1. Block specimen in Blocking Buffer for 60 min.
2. While blocking, prepare primary antibody by diluting as indicated on datasheet in Antibody Dilution Buffer.
3. Aspirate blocking solution, apply diluted primary antibody.
4. Incubate overnight at 4°C.
5. Rinse three times in PBS for 5 min each.
   NOTE: If using primary antibodies directly conjugated with Alexa Fluor® fluorochromes, then skip to (Section C, Step 8).
6. Incubate specimen in fluorochrome-conjugated secondary antibody diluted in Antibody Dilution Buffer for 1–2 hr at room temperature in dark.
7. Rinse in PBS (Section C, Step 5).
8. Coverslip slides with Prolong® Gold Anti-Fade Reagent (#9071), with DAPI (#8961).
9. For best results, allow mountant to cure overnight at room temperature. For long-term storage, store slides flat at 4°C protected from light.

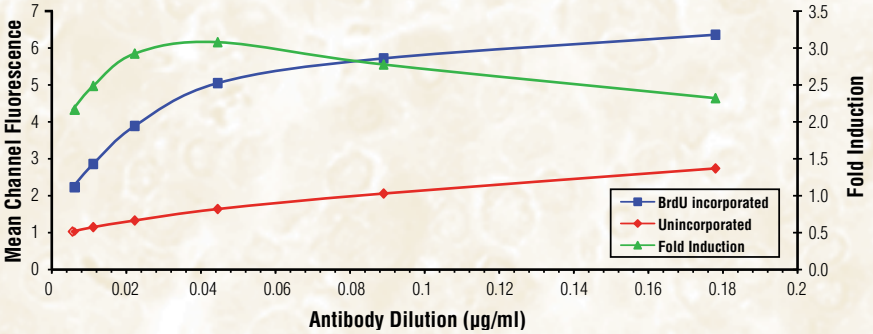
See page 18 for Complementary Products for Immunofluorescence-based applications.

Antibody Validation for Flow Cytometry

All of our over 550 antibodies validated for flow cytometry have been screened to determine optimal dilutions and to verify specificity.

Titration:

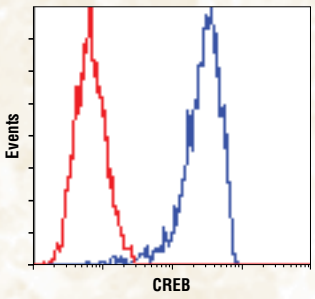
Serial dilution is used to determine optimal dilution.



BrdU (Bu20a) Mouse mAb #5292: Flow cytometric analysis of Jurkat cells, unincorporated (red) or after 30 minutes of BrdU incorporation (blue), using serial dilutions of #5292. The fold-induction ratio is shown in green. Optimal concentration of #5292 was determined to be 0.044 µg/ml.

Isotype Control:

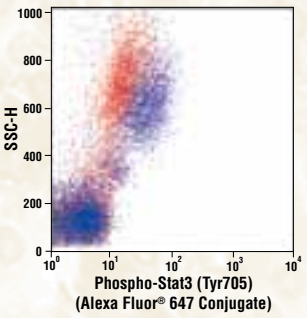
Comparison of signal to isotype control is used to estimate the nonspecific binding of primary antibodies.



Rabbit (DA1E) mAb IgG XP® Isotype Control #3900: Flow cytometric analysis of SH-SY5Y cells using CREB (D76D11) Rabbit mAb #4820 (blue) compared to concentration-matched #3900 (red).

Activation-state Specificity:

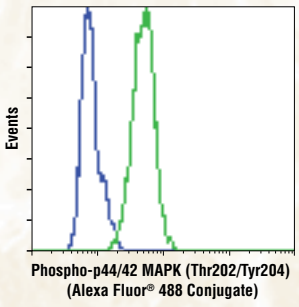
Comparison of the signal on treated and untreated cells helps to verify activation-state specificity.



Phospho-Stat3 (Tyr705) (D3A7) XP® Rabbit mAb (Alexa Fluor® 647 Conjugate) #4324: Flow cytometric analysis of human whole blood, untreated (red) or treated with Human Granulocyte Colony Stimulating Factor (hG-CSF) #8930 (blue), using #4324.

Inhibitor Treatment:

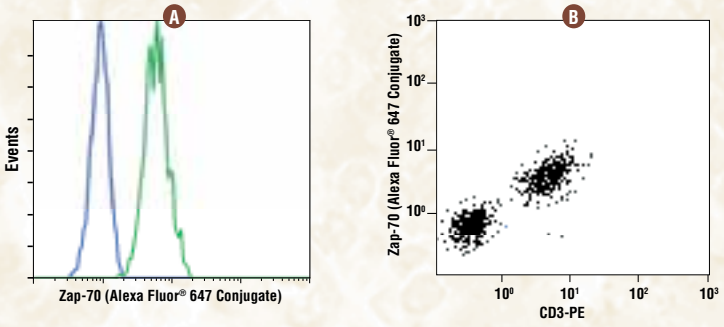
Treatment of cell lines with pathway-specific inhibitors helps to verify target specificity.



Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb (Alexa Fluor® 488 Conjugate) #4344: Flow cytometric analysis of Jurkat cells treated with U0126 #9903 (blue) or TPA #4174 (green), using #4344.

Positive and Negative Cell Lines:

Use of known positive and negative cell lines verifies target specificity.



Zap-70 (136F12) Rabbit mAb (Alexa Fluor® 647 Conjugate) #2707: Flow cytometric analysis **(A)** of Ramos B (blue) and Jurkat T (green) cells using #2707. Two-color flow cytometric analysis **(B)** of a mixed population of T and B cells (Jurkat and Ramos, respectively) using #2707 and a CD3 antibody. CD3-negative B cells have little or no Zap-70 staining, while CD3-positive T cells stain brightly for Zap-70 protein.

Other Validation Steps Include:

- The use of blocking peptides, siRNA, expression vectors, and positive and null cell lines verifies specificity of staining.
- Phosphatase treatment confirms phospho-specificity of the antibody.
- Extensive quality control testing guarantees stability over time and eliminates lot-to-lot variability.
- Optimized protocols are provided and dilutions are predetermined.

Flow Cytometry (General)

A. Solutions and Reagents

NOTE: Prepare solutions with purified water.

1. **1X Phosphate Buffered Saline (PBS):** Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4 in 800 ml dH_2O. Adjust the pH to 7.4 with HCl and the volume to 1 L. Store at room temperature.
2. **Formaldehyde** (methanol free).
3. **100% Methanol**
4. **Incubation Buffer:** Dissolve 0.5 g bovine serum albumin (BSA) in 100 ml 1X PBS. Store at 4°C.

B. Fixation

1. Collect cells by centrifugation and aspirate supernatant.
2. Resuspend cells briefly in 0.5–1 ml PBS. Add formaldehyde to a final concentration of 2-4% formaldehyde.
3. Fix for 10 min at 37°C.
4. Chill tubes on ice for 1 min.
5. For extracellular staining with antibodies that do not require permeabilization, proceed to step D1 or store cells in PBS with 0.1% sodium azide at 4°C; for intracellular staining, proceed to permeabilization (Section C, Step 1).

C. Permeabilization

1. Permeabilize cells by adding ice-cold 100% methanol slowly to pre-chilled cells, while gently vortexing, to a final concentration of 90% methanol. Alternatively, to remove fix prior to permeabilization, pellet cells by centrifugation and resuspend in 90% methanol.
2. Incubate 30 min on ice.
3. Proceed with immunostaining (Step D1.) or store cells at -20°C in 90% methanol.

D. Immunostaining

NOTE: Account for isotype matched controls for monoclonal antibodies or species matched IgG for polyclonal antibodies. Count cells using a hemocytometer or alternative method.

1. Aliquot 0.5–1x10^6 cells into each assay tube (by volume).
2. Add 2–3 ml Incubation Buffer to each tube and rinse by centrifugation. Repeat.
3. Resuspend cells in 100 µl Incubation Buffer per assay tube.
4. Block in Incubation Buffer for 10 min at room temperature.
5. Add the unconjugated, biotinylated, or fluorochrome-conjugated primary antibody at the appropriate dilution to the assay tubes (see individual antibody datasheet for the appropriate dilution).
6. Incubate for 1 hr at room temperature.
7. Rinse as before in Incubation Buffer by centrifugation.
8. If using a fluorochrome-conjugated primary antibody, resuspend cells in 0.5 ml PBS and analyze on flow cytometer; for unconjugated or biotinylated primary antibodies, proceed to immunostaining (Section D, Step 9).
9. Resuspend cells in fluorochrome-conjugated secondary antibody or fluorochrome-conjugated avidin, diluted in Incubation Buffer at the recommended dilution.
10. Incubate for 30 min at room temperature.
11. Rinse as before in Incubation Buffer by centrifugation.
12. Resuspend cells in 0.5 ml PBS and analyze on flow cytometer; alternatively, for DNA staining, proceed to optional DNA stain (Section E, Step 1).

E. Optional DNA Stain

1. Resuspend cells in 0.5 ml of DNA dye (e.g. Propidium Iodide (PI)/RNase Staining Solution #4087).
2. Incubate for at least 5 min at room temperature.
3. Analyze cells in DNA stain on flow cytometer.

Flow Cytometry (Alternate)

Protocol for Combined Staining of Intracellular Proteins and Cell Surface Markers in Blood

A. Solutions and Reagents

NOTE: Prepare solutions with purified water.

1. **10X Phosphate Buffered Saline (PBS):** To prepare 1 L add 80 g sodium chloride (NaCl), 2 g potassium chloride (KCl), 14.4 g sodium phosphate, dibasic (Na_2HPO_4) and 2.4 g potassium phosphate, monobasic (KH_2PO_4) to 1 L dH_2O. Adjust pH to 7.4. Store at room temperature.
2. **Formaldehyde** (methanol free)
3. **Triton™ X-100**
4. **50% Methanol**
5. **Incubation Buffer:** Dissolve 0.5 g bovine serum albumin (BSA) in 100 ml 1X PBS. Store at 4°C.

B. Preparation of Whole Blood (fixation, lysis, and permeabilization) for Immunostaining

1. Aliquot 100 µl fresh whole blood per assay tube.
2. **OPTIONAL:** Place tubes in rack in 37°C water bath for short-term treatments with ligands, inhibitors, drugs, etc.
3. Add 65 µl of 10% formaldehyde to each tube.
4. Vortex briefly and let stand for 15 min at room temperature.
5. Add 835 µl of Triton™ X-100 to each tube, for a final concentration of 0.1% Triton™ X-100.
6. Vortex and let stand for 30 min at room temperature.
7. Add 1 ml Incubation Buffer.
8. Pellet cells by centrifugation and aspirate supernatant.
9. Repeat steps 7 and 8.
10. Resuspend cells in 1 ml cold 50% methanol in PBS (store methanol solution at –20°C until just before use).
11. Incubate at least 10 min on ice.
12. Proceed with staining or store cells at –20°C in 50% methanol.

C. Staining Using Unlabeled Primary and Conjugated Secondary Antibodies

1. Add 1 ml Incubation Buffer to each tube and rinse by centrifugation. Repeat.
2. Add primary antibodies diluted as recommended on datasheet in Incubation Buffer.
3. Incubate for 30–60 min at room temperature.
4. Rinse as before in incubation buffer by centrifugation.
5. Resuspend cells in fluorochrome-conjugated secondary antibody diluted in Incubation Buffer according to the manufacturer's recommendations.
6. Incubate for 30 min at room temperature.
7. Rinse as before in Incubation Buffer by centrifugation.
8. Resuspend cells in 0.5 ml PBS and analyze on flow cytometer.

Reference: Chow S, Hedley D, Grom P, Magari R, Jacobberger JW, Shankey TV (2005) Whole blood fixation and permeabilization protocol with red blood cell lysis for flow cytometry of intracellular phosphorylated epitopes in leukocyte subpopulations. *Cytometry A* 67, 4–17.

Complementary Products for Immunofluorescence-based Assays and Flow Cytometry

Recommended Secondary Antibodies

#4408	Anti-mouse IgG (H+L), F(ab')$_2$ Fragment (Alexa Fluor® 488 Conjugate)
#4412	Anti-rabbit IgG (H+L), F(ab')$_2$ Fragment (Alexa Fluor® 488 Conjugate)
#4416	Anti-rat IgG (H+L) (Alexa Fluor® 488 Conjugate)
#4409	Anti-mouse IgG (H+L), F(ab')$_2$ Fragment (Alexa Fluor® 555 Conjugate)
#4413	Anti-rabbit IgG (H+L), F(ab')$_2$ Fragment (Alexa Fluor® 555 Conjugate)
#4417	Anti-rat IgG (H+L) (Alexa Fluor® 555 Conjugate)
#4410	Anti-mouse IgG (H+L), F(ab')$_2$ Fragment (Alexa Fluor® 647 Conjugate)
#4414	Anti-rabbit IgG (H+L), F(ab')$_2$ Fragment (Alexa Fluor® 647 Conjugate)
#4418	Anti-rat IgG (H+L) (Alexa Fluor® 647 Conjugate)
#5470	Anti-mouse IgG (H+L) (DyLight® 680 Conjugate)
#5366	Anti-rabbit IgG (H+L) (DyLight® 680 Conjugate)
#5257	Anti-mouse IgG (H+L) (DyLight® 800 Conjugate)
#5151	Anti-rabbit IgG (H+L) (DyLight® 800 Conjugate)

Fluorescent DNA Dyes

#4083	DAPI
#4084	DRAQ5®
#7406	DRAQ7™
#4082	Hoechst 33342
#4087	Propidium Iodide (PI)/RNase Staining Solution

Isotype Control Antibodies

#3900	Rabbit (DA1E) mAb IgG XP® Isotype Control
#2975	Rabbit (DA1E) mAb IgG XP® Isotype Control (Alexa Fluor® 488 Conjugate)
#3969	Rabbit (DA1E) mAb IgG XP® Isotype Control (Alexa Fluor® 555 Conjugate)
#2985	Rabbit (DA1E) mAb IgG XP® Isotype Control (Alexa Fluor® 647 Conjugate)
#4096	Rabbit (DA1E) mAb IgG XP® Isotype Control (Biotinylated)
#5742	Rabbit (DA1E) mAb IgG XP® Isotype Control (PE Conjugate)
#4340	Rabbit IgG Isotype Control (Alexa Fluor® 488 Conjugate)
#3452	Rabbit IgG Isotype Control (Alexa Fluor® 647 Conjugate)
#5415	Mouse (G3A1) mAb IgG1 Isotype Control
#9641	Mouse (G3A1) mAb IgG1 Isotype Control (Alexa Fluor® 555 Conjugate)
#6899	Mouse (G3A1) mAb IgG1 Isotype Control (PE Conjugate)
#4878	Mouse (MOPC-21) mAb IgG1 Isotype Control (Alexa Fluor® 488 Conjugate)
#4843	Mouse (MOPC-21) mAb IgG1 Isotype Control (Alexa Fluor® 647 Conjugate)
#4097	Mouse (MOPC-21) mAb IgG1 Isotype Control (Biotinylated)

Solutions and Miscellaneous

#9998	BSA
#5425	Normal Goat Serum
#9808	Phosphate Buffered Saline (PBS-20X)

PathScan® Sandwich ELISA (Colorimetric)

A. Solutions and Reagents

NOTE: Prepare solutions with purified water.

1. **Microwell strips:** Bring all to room temperature before use.
2. **1X Wash Buffer:** Prepare by diluting 20X Wash Buffer (included in each PathScan® Sandwich ELISA Kit) in purified water.
3. **1X Cell Lysis Buffer:** (10X Cell Lysis Buffer #9803): This buffer can be stored at 4°C for short-term use (1–2 weeks). **Recommended:** Add 1mM phenylmethylsulfonyl fluoride (PMSF) immediately before use.
4. **1X PBS:** (20X Phosphate Buffered Saline #9808).
5. **TMB Substrate** (#7004)
6. **STOP Solution** (#7002)

B. Preparing Cell Lysates

For adherent cells

1. Aspirate media when the cultures reaches 80-90% confluence. Treat cells by adding fresh media containing regulator for desired time.
2. Remove media and rinse cells once with ice-cold 1X PBS.
3. Remove PBS and add 0.5 ml ice-cold 1X Cell Lysis Buffer plus 1 mM PMSF to each plate (10 cm diameter) and incubate the plate on ice for 5 minutes.
4. Scrape cells off the plate and transfer to an appropriate tube. Keep on ice.
5. Sonicate lysates on ice.
6. Microcentrifuge for 10 min (x14,000 rpm) at 4°C and transfer the supernatant to a new tube. The supernatant is the cell lysate. Store at –80°C in single-use aliquots.

For suspension cells

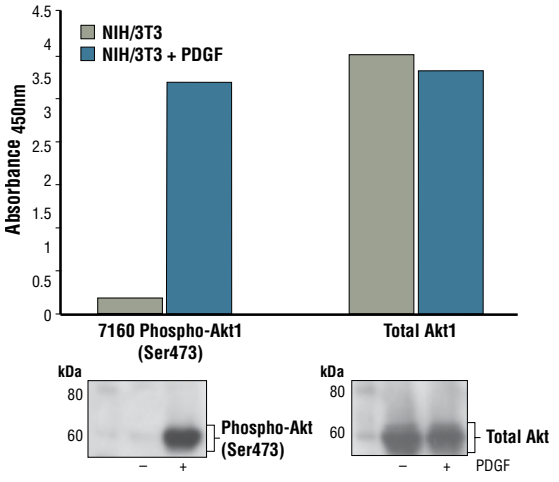
1. Remove media by low speed centrifugation (~1200 rpm) when the culture reaches 0.5–1.0 x 10^6 viable cells/ml. Treat cells by adding fresh media containing regulator for desired time.
2. Collect cells by low speed centrifugation (~1200 rpm) and wash once with 5-10 ml ice-cold 1X PBS.
3. Cells harvested from 50 ml of growth media can be lysed in 2.0 ml of 1X Cell Lysis Buffer plus 1 mM PMSF.
4. Sonicate lysates on ice.
5. Microcentrifuge for 10 min (x14,000 rpm) at 4°C and transfer the supernatant to a new tube. The supernatant is the cell lysate. Store at –80°C in single-use aliquots.

C. Test Procedure

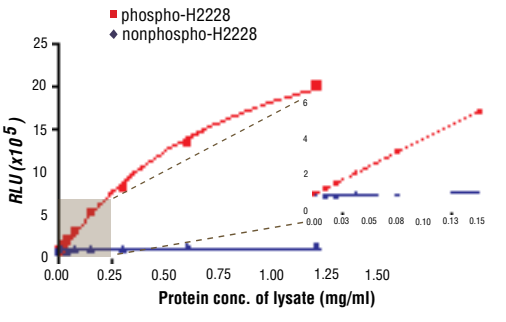
1. After the microwell strips have reached room temperature, break off the required number of microwells. Place the microwells in the strip holder. Unused microwells must be resealed and stored at 4°C immediately.
2. Cell lysates can be undiluted or diluted with Sample Diluent (supplied in each PathScan® Sandwich ELISA Kit, blue color). Individual datasheets for each kit provide information regarding an appropriate dilution factor for lysates and kit assay results.
3. Add 100 µl of each undiluted or diluted cell lysate to the appropriate well. Seal with tape and press firmly onto top of microwells. Incubate the plate for 2 hours at 37°C. Alternatively, the plate can be incubated overnight at 4°C.
4. **Gently remove the tape and wash wells:**
   - **a.** Discard plate contents into a receptacle.
   - **b.** Wash 4 times with 1X Wash Buffer, 200 µl each time for each well.
   - **c.** For each wash, strike plates on fresh towels hard enough to remove the residual solution in each well, but do not allow wells to completely dry at any time.
   - **d.** Clean the underside of all wells with a lint-free tissue.
5. Add 100 µl of Detection Antibody (green color) to each well. Seal with tape and incubate the plate at 37°C for 1 hr.
6. Repeat wash procedure (Section C, Step 4).
7. Add 100 µl of HRP-Linked secondary antibody (red color) to each well. Seal with tape and incubate the plate for 30 min at 37°C.
8. Repeat wash procedure (Section C, Step 4).
9. Add 100 µl of TMB Substrate to each well. Seal with tape and incubate the plate for 10 min at 37°C or 30 min at 25°C.
10. Add 100 µl of STOP Solution to each well. Shake gently for a few seconds.

NOTE: Initial color of positive reaction is blue, which changes to yellow upon addition of STOP Solution.

1. **Read results**
   - **a. Visual Determination:** Read within 30 min after adding STOP Solution.
   - **b. Spectrophotometric Determination:** Wipe underside of wells with a lint-free tissue. Read absorbance at 450 nm within 30 min after adding STOP Solution.



PathScan® Phospho-Akt1 (Ser473) Sandwich ELISA Kit #7160: Treatment of NIH/3T3 cells with PDGF stimulates phosphorylation of Akt at Ser473, detected by #7160 but does not affect the level of total Akt detected by PathScan® Total Akt1 Sandwich ELISA Kit #7170. Absorbance at 450 nm is shown in the top panel, while the corresponding western blots using Phospho-Akt (Ser473) (193H12) Rabbit mAb #4058 and Akt Antibody #9272 are shown in the bottom panel



PathScan® Phospho-ALK (Tyr1604) Chemiluminescent Sandwich ELISA Kit #7020: Relationship between protein concentrations of lysates prepared using NCI-H2228 cells lysed with (phospho) and without (nonphospho) the addition of phosphatase inhibitors to the lysis buffer. Graph inset corresponding to the shaded area shows high sensitivity and a linear response at the low protein concentration range.

PathScan® Sandwich ELISA (Chemiluminescent)

NOTE: Refer to product-specific datasheets for assay incubation temperature. This chemiluminescent ELISA is offered in low volume microplate. Samples and reagents only require 50 µl per microwell.

A. Solutions and Reagents

NOTE: Prepare solutions with purified water.

1. **Microwell strips:** Bring all to room temperature before use.
2. **1X Wash Buffer:** Prepare by diluting 20X Wash Buffer (included in each PathScan® Sandwich ELISA Kit) in purified water.
3. **1X Cell Lysis Buffer:** (10X Cell Lysis Buffer #9803) This buffer can be stored at 4°C for short-term use (1–2 weeks). **Recommended:** Add 1mM phenylmethylsulfonyl fluoride (PMSF) immediately before use.
4. **1X PBS:** (20X Phosphate Buffered Saline #9808)
5. **20X LumiGLO® Reagent and 20X Peroxide** (#7003)

B. Preparing Cell Lysates

For adherent cells

1. Aspirate media when the cultures reaches 80-90% confluence. Treat cells by adding fresh media containing regulator for desired time.
2. Remove media and rinse cells once with ice-cold 1X PBS.
3. Remove PBS and add 0.5 ml ice-cold 1X Cell Lysis Buffer plus 1 mM PMSF to each plate (10 cm diameter) and incubate the plate on ice for 5 min.
4. Scrape cells off the plate and transfer to an appropriate tube. Keep on ice.
5. Sonicate lysates on ice.
6. Microcentrifuge for 10 min (x14,000 rpm) at 4°C and transfer the supernatant to a new tube. The supernatant is the cell lysate. Store at –80°C in single-use aliquots.

For suspension cells

1. Remove media by low speed centrifugation (~1200 rpm) when the culture reaches 0.5–1.0 x 10^6 viable cells/ml. Treat cells by adding fresh media containing regulator for desired time.
2. Collect cells by low speed centrifugation (~1200 rpm) and wash once with 5-10 ml ice-cold 1X PBS.
3. Cells harvested from 50 ml of growth media can be lysed in 2.0 ml of 1X Cell Lysis Buffer plus 1 mM PMSF.
4. Sonicate lysates on ice.
5. Microcentrifuge for 10 min (x14,000 rpm) at 4°C and transfer the supernatant to a new tube. The supernatant is the cell lysate. Store at –80°C in single-use aliquots.

C. Test Procedure

1. After the microwell strips have reached room temperature, break off the required number of microwells. Place the microwells in the strip holder. Unused microwells must be resealed and stored at 4°C immediately.
2. Cell lysates can be undiluted or diluted with Sample Diluent (supplied in each PathScan® Sandwich ELISA Kit, blue color). Individual datasheets for each kit provide information regarding an appropriate dilution factor for lysates and kit assay results.
3. Add 50 µl of each undiluted or diluted cell lysate to the appropriate well. Seal with tape and press firmly onto top of microwells. Incubate the plate for 2 hours at room temperature. Alternatively, the plate can be incubated overnight at 4°C.
4. **Gently remove the tape and wash wells:**
   - **a.** Discard plate contents into a receptacle.
   - **b.** Wash 4 times with 1X Wash Buffer, 150 µl each time for each well.
   - **c.** For each wash, strike plates on fresh towels hard enough to remove the residual solution in each well, but do not allow wells to dry completely at any time.
   - **d.** Clean the underside of all wells with a lint-free tissue.
5. Add 50 µl of Detection Antibody (green color) to each well. Seal with tape and incubate the plate at room temperature for 1 hr.
6. Repeat wash procedure (Section C, Step 4).
7. Add 50 µl of HRP-linked secondary antibody (red color) to each well. Seal with tape and incubate the plate at room temperature for 30 min.
8. Repeat wash procedure (Section C, Step 4).
9. Prepare Detection Reagent Working Solution by mixing equal parts 2X LumiGLO® Reagent and 2X Peroxide.
10. Add 50 µl of the Detection Reagent Working Solution to each well.
11. Use a plate-based luminometer to measure Relative Light Units (RLU) within 1–10 min following addition of the substrate.
    - **a.** Optimal signal intensity is achieved when read within 10 min.

Complementary Products for PathScan® Sandwich ELISA

PathScan® Sandwich ELISA Control Extracts

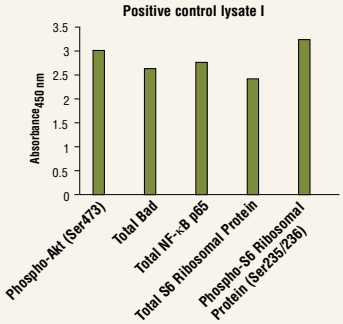
#7988	PathScan® Sandwich ELISA Control Phospho Cell Extract I
#7989	PathScan® Sandwich ELISA Control Phospho Cell Extract II

Detection Reagents

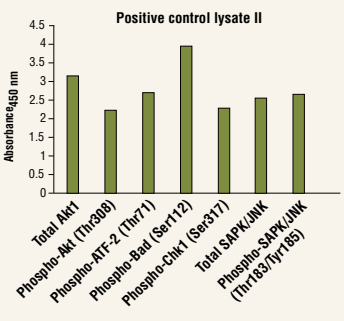
#7004	TMB Substrate
#7002	STOP Solution

Solutions and Miscellaneous

#7018	PathScan® Sandwich ELISA Lysis Buffer (1X)
#9998	BSA
#9808	Phosphate Buffered Saline (PBS-20X)
#9809	Phosphate Buffered Saline with Tween 20 (PBST-20X)
#9803	Cell Lysis Buffer (10X)



PathScan® Sandwich ELISA Control Phospho Cell Extract I #7988: Analysis of #7988 using multiple PathScan® Sandwich ELISA kits. Samples were prepared using the standard ELISA protocol to a final concentration of 0.25 mg/ml and assayed using the indicated ELISA kits.



PathScan® Sandwich ELISA Control Phospho Cell Extract II #7989: Analysis of #7989 using multiple PathScan® Sandwich ELISA kits. Samples were prepared using the standard ELISA protocol to a final concentration of 1.0 mg/ml and assayed using the indicated ELISA kits.

PathScan® Sandwich ELISA (Antibody Pair)

A. Solutions and Reagents

NOTE: Prepare solutions with purified water.

1. **1X PBS:** (20X Phosphate Buffered Saline #9808)
2. **Wash Buffer:** 1X PBS/0.05% Tween-20, (20X PBST #9809)
3. **Blocking Buffer:** 1X PBS/0.05% Tween-20, 1% BSA
4. **1X Cell Lysis Buffer:** (10X Cell Lysis Buffer #9803) This buffer can be stored at 4°C for short-term use (1–2 weeks). **Recommended:** Add 1mM phenylmethylsulfonyl fluoride (PMSF) immediately before use.
5. **TMB Substrate** (#7004)
6. **STOP Solution** (#7002)

NOTE: Reagents should be made fresh daily.

B. Preparing Cell Lysates

For adherent cells

1. Aspirate media when the cultures reaches 80-90% confluence. Treat cells by adding fresh media containing regulator for desired time.
2. Remove media and rinse cells once with ice-cold 1X PBS.
3. Remove PBS and add 0.5 ml ice-cold 1X Cell Lysis Buffer plus 1 mM PMSF to each plate (10 cm diameter) and incubate the plate on ice for 5 min.
4. Scrape cells off the plate and transfer to an appropriate tube. Keep on ice.
5. Sonicate lysates on ice.
6. Microcentrifuge for 10 min (x14,000 rpm) at 4°C and transfer the supernatant to a new tube. The supernatant is the cell lysate. Store at –80°C in single-use aliquots.

For suspension cells

1. Remove media by low speed centrifugation (~1200 rpm) when the culture reaches 0.5–1.0 x 10^6 viable cells/ml. Treat cells by adding fresh media containing regulator for desired time.
2. Collect cells by low speed centrifugation (~1200 rpm) and wash once with 5–10 ml ice-cold 1X PBS.
3. Cells harvested from 50 ml of growth media can be lysed in 2.0 ml of 1X Cell Lysis Buffer plus 1 mM PMSF.
4. Sonicate lysates on ice.
5. Microcentrifuge for 10 min (x14,000 rpm) at 4°C and transfer the supernatant to a new tube. The supernatant is the cell lysate. Store at –80°C in single-use aliquots.

C. Coating Procedure

1. Rinse microplate with 200 µl of dH_2O, discard liquid. Blot on paper towel to make sure wells are dry.
2. Dilute capture antibody 1:100 in 1X PBS. For a single 96 well plate, add 100 µl of Capture Antibody Stock to 9.9 ml 1X PBS. Mix well and add 100 µl/well. Cover plate and incubate overnight at 4°C (17–20 hr).
3. **After overnight coating, gently uncover plate and wash wells:**
   - **a.** Discard plate contents into a receptacle.
   - **b.** Wash 4 times with Wash Buffer, 200 µl each time for each well. For each wash, strike plates on fresh towels hard enough to remove the residual solution in each well, but do not allow wells to completely dry at any time.
   - **c.** Clean the underside of all wells with a lint-free tissue.
4. Block plates. Add 150 µl of Blocking Buffer/well, cover plate, and incubate at 37°C for 2 hr.
5. After blocking, wash plate as in Step 3. Plate is ready to use.

D. Test Procedure

1. Lysates can be used undiluted or diluted in Blocking Buffer. 100 µl of lysate is added per well. Cover plate and incubate at 37°C for 2 hr.
2. Wash plate (Section B, Step 3).
3. Dilute detection antibody 1:100 in Blocking Buffer. For a single 96 well plate, add 100 µl of Detector Antibody Stock to 9.9 ml of Blocking Buffer. Mix well and add 100 µl/well. Cover plate and incubate at 37°C for 1 hr.
4. Wash plate (Section B, Step 3).
5. Secondary antibody, either streptavidin anti-mouse or anti-rabbit-HRP, is diluted 1:1000 in Blocking Buffer. For a single 96 well plate, add 10 µl of secondary antibody stock to 9.99 ml of Blocking Buffer. Mix well and add 100 µl/well. Cover and incubate at 37°C for 30 min.
6. Wash plate as in Coating Procedure, Step 3.
7. Add 100 µl of TMB Substrate per well. Cover and incubate at 37°C for 10 min.
8. Add 100 µl of STOP Solution per well.
9. Read plate on a microplate reader at Absorbance 450 nm.

Enzymatic Chromatin Immunoprecipitation

for SimpleChIP® Chromatin IP Kits

Solutions and Reagents

Reagents included:

Reagents Included in
SimpleChIP® Enzymatic Chromatin IP Kit (Agarose Beads) #9002,
SimpleChIP® Enzymatic Chromatin IP Kit (Magnetic Beads) #9003,
SimpleChIP® Plus Enzymatic Chromatin IP Kit (Agarose Beads) #9004, or
SimpleChIP® Plus Enzymatic Chromatin IP Kit (Magnetic Beads) #9005:

1. **Glycine Solution** (10X)
2. **Buffer A** (4X)
3. **Buffer B** (4X)
4. **ChIP Buffer** (10X)
5. **ChIP Elution Buffer** (2X)
6. **5 M NaCl**
7. **0.5 M EDTA**
8. **1M DTT**
9. **DNA Binding Reagent A**
10. **DNA Wash Reagent B**
11. **DNA Elution Reagent C**
12. **DNA Spin Columns**
13. **Protease Inhibitor Cocktail** (200X)
14. **RNAse A** (10 mg/ml)
15. **Micrococcal Nuclease** (2000 gel units/µl)
16. **Proteinase K** (20 mg/ml)
17. **SimpleChIP® Human RPL30 Exon 3 Primers #7014**
18. **SimpleChIP® Mouse RPL30 Intron 2 Primers #7015**
19. **Histone H3 (D2B12) XP® Rabbit mAb (ChIP Formulated) #4620**
20. **Normal Rabbit IgG #2729**
21. **ChIP-Grade Protein G Magnetic Beads #9006 or ChIP-Grade Protein G Agarose Beads #9007**

Reagents not included:

1. **Formaldehyde** (37%)
2. **Ethanol** (96-100%)
3. **Isopropanol**
4. **1X PBS**
5. **Nuclease-free water**
6. **Taq DNA polymerase**
7. **dNTP Mix**
8. **For Kits #9002 and #9003 only:** PMSF (0.1 M stock)
9. **For Kits #9003 and #9005 only:** 6-Tube Magnetic Separation Rack #7017

I. Tissue Cross-linking and Sample Preparation

IMPORTANT: Section I exclusive to use of SimpleChIP® Plus Kits (#9004 & #9005)

When harvesting tissue, remove unwanted material such as fat and necrotic material from the sample. Tissue can then be processed and cross-linked immediately, or frozen on dry ice for processing later. For optimal chromatin yield and ChIP results, use 25 mg of tissue for each immunoprecipitation to be performed. The chromatin yield does vary between tissue types and some tissues may require more than 25 mg for each immunoprecipitation. Please see Troubleshooting Guide, Section A for more information regarding the expected chromatin yield for different types of tissue. One additional chromatin sample should be processed for Analysis of Chromatin Digestion and Concentration (Section IV).

Before starting:

- Remove and warm 200X Protease Inhibitor Cocktail (PIC) and 10X Glycine Solution. Make sure PIC is completely thawed.
- Prepare 3 ml of Phosphate Buffered Saline (PBS) + 15 µl 200X PIC per 25 mg of tissue to be processed and place on ice.
- Prepare 45 µl of 37% formaldehyde per 25 mg of tissue to be processed and keep at room temperature. Use fresh formaldehyde that is not past the manufacturer's expiration date.

A. Cross-linking

1. Weigh the fresh or frozen tissue sample. Use 25 mg of tissue for each IP to be performed.
2. Place tissue sample in a 60 mm or 100 mm dish and finely mince using a clean scalpel or razor blade. Keep dish on ice. It is important to keep the tissue cold to avoid protein degradation.
3. Transfer minced tissue to a 15 ml conical tube.
4. Add 1 ml of PBS + PIC per 25 mg tissue to the conical tube.
5. To crosslink proteins to DNA, add 45 µl of 37% formaldehyde per 1 ml of PBS + PIC and rock at room temp for 20 min. Final formaldehyde concentration is 1.5%.
6. Stop cross-linking by adding 100 µl of 10x glycine per 1 ml of PBS + PIC and mix for 5 min at room temperature.
7. Centrifuge tissue at 1,500 rpm in a bench top centrifuge for 5 min at 4°C.
8. Remove supernatant and wash one time with 1 ml PBS + PIC per 25 mg tissue.
9. Repeat centrifugation at 1,500 rpm in a bench top centrifuge for 5 min at 4°C.
10. Remove supernatant and resuspend tissue in 1 ml PBS + PIC per 25 mg tissue and store on ice. Disaggregate tissue into single-cell suspension using a Medimachine (Section B) or Dounce homogenizer (Section C).

B. Tissue Disaggregation Using Medimachine from BD Biosciences (part #340587)

1. Cut off the end of a 1000 µl pipette tip to enlarge the opening for transfer of tissue chunks.
2. Transfer 1 ml of tissue resuspended in PBS + PIC into the top chamber of a 50 mm medicone (Part #340592).
3. Grind tissue for 2 min according to manufacturer's instructions.
4. Collect cell suspension from the bottom chamber of the medicone using a 1 ml syringe and 18 gauge blunt needle. Transfer cell suspension to a 15 ml conical tube and place on ice.
5. Repeat steps 2 to 4 until all the tissue is processed into a homogenous suspension.
6. If more grinding is necessary, add more PBS + PIC to tissue. Repeat steps 2 to 5 until all tissue is ground into a homogeneous suspension.
7. Check for single-cell suspension by microscope (optional).
8. Centrifuge cells at 1,500 rpm in a bench top centrifuge for 5 min at 4°C.
9. Remove supernatant from cells and immediately continue with Nuclei Preparation and Chromatin Digestion (Section III).

C. Tissue Disaggregation Using a Dounce Homogenizer

1. Transfer tissue resuspended in PBS + PIC to a Dounce homogenizer.
2. Disaggregate tissue pieces with 20-25 strokes. Check for single-cell suspension by microscope (optional).
3. Transfer cell suspension to a 15 ml conical tube and centrifuge at 1,500 rpm in a bench top centrifuge for 5 min at 4°C.
4. Remove supernatant from cells and immediately continue with Nuclei Preparation and Chromatin Digestion (Section III).

II. Cell Culture Cross-linking and Sample Preparation

For optimal ChIP results, use approximately 4 X 10^6 cells for each immunoprecipitation to be performed. For HeLa cells, this is equivalent to half of a 15 cm culture dish containing cells that are 90% confluent in 20 ml of growth medium. One additional sample should be processed for Analysis of Chromatin Digestion and Concentration (Section IV). Include one extra dish of cells in experiment to be used for determination of cell number using a hemocytometer.

Before starting:

- Remove and warm 200X Protease Inhibitor Cocktail (PIC) and 10X Glycine Solution. Make sure PIC is completely thawed.
- Prepare 2 ml of Phosphate Buffered Saline (PBS) + 10 µl 200X PIC per 15 cm dish to be processed and place on ice.
- Prepare 40 ml of PBS per 15 cm dish to be processed and place on ice.
- Prepare 540 µl of 37% formaldehyde per 15 cm dish of cells to be processed and keep at room temperature. Use fresh formaldehyde that is not past the manufacturer's expiration date.

1. To crosslink proteins to DNA, add 540 µl of 37% formaldehyde to each 15 cm culture dish containing 20 ml medium. Swirl briefly to mix and incubate 10 min at room temperature. Final formaldehyde concentration is 1%. Addition of formaldehyde may result in a color change of the medium.
2. Add 2 ml of 10X glycine to each 15 cm dish containing 20 ml medium, swirl briefly to mix, and incubate 5 min at room temperature. Addition of glycine may result in a color change of the medium.
3. For suspension cells, transfer cells to a 50 ml conical tube, centrifuge at 1,500 rpm in a bench top centrifuge 5 min at 4°C and wash pellet two times with 20 ml ice-cold PBS. Remove supernatant and immediately continue with Nuclei Preparation and Chromatin Digestion (Section III).
4. For adherent cells, remove media and wash cells two times with 20 ml ice-cold 1X PBS, completely removing wash from culture dish each time.
5. Add 2 ml ice-cold PBS + PIC to each 15 cm dish. Scrape cells into cold buffer. Combine cells from all culture dishes into one 15 ml conical tube.
6. Centrifuge cells at 1,500 rpm in a bench top centrifuge for 5 min at 4°C. Remove supernatant and immediately continue with Nuclei Preparation and Chromatin Digestion (Section III).

III. Nuclei Preparation and Chromatin Digestion

One immunoprecipitation preparation (IP prep) is defined as 25 mg of disaggregated tissue or 4 x 10^6 tissue culture cells.

Before starting:

- Remove and warm 200X Protease Inhibitor Cocktail (PIC) and 1 M DTT. Make sure both are completely thawed and DTT crystals are completely in solution.
- Remove and warm 10X ChIP Buffer and ensure SDS is completely in solution.
- Prepare 1 ml 1X Buffer A (250 µl 4X Buffer A + 750 µl water) + 0.5 µl 1M DTT + 5 µl 200X PIC per IP prep and place on ice.
- Prepare 1.1 ml 1X Buffer B (275 µl 4X Buffer B + 825 µl water) + 0.55 µl 1M DTT per IP prep and place on ice.
- Prepare 100 µl 1X ChIP Buffer (10 µl 10X ChIP Buffer + 90 µl water) + 0.5 µl 200X PIC per IP prep and place on ice.

1. Resuspend cells in 1 ml ice-cold Buffer A + DTT + PIC per IP prep. Incubate on ice for 10 min. Mix by inverting tube every 3 min.
2. Pellet nuclei by centrifugation at 3,000 rpm in a bench top centrifuge for 5 min at 4°C. Remove supernatant and resuspend pellet in 1 ml ice-cold Buffer B + DTT per IP prep. Repeat centrifugation, remove supernatant, and resuspend pellet in 100 µl Buffer B + DTT per IP prep. Transfer sample to a 1.5 ml microcentrifuge tube, up to 1 ml total per tube.
3. Add 0.5 µl of Micrococcal Nuclease per IP prep, mix by inverting tube several times and incubate for 20 min at 37°C with frequent mixing to digest DNA to length of approximately 150-900 bp. Mix by inversion every 3 to 5 min. The amount of Micrococcal Nuclease required to digest DNA to the optimal length may need to be determined empirically for individual tissues and cell lines (see Troubleshooting Guide, Section B). HeLa nuclei digested with 0.5 µl Micrococcal Nuclease per 4 x 10^6 cells and mouse liver tissue digested with 0.5 µl Micrococcal Nuclease per 25 mg of tissue gave the appropriate length DNA fragments.
4. Stop digest by adding 10 µl of 0.5 M EDTA per IP prep and placing tube on ice.
5. Pellet nuclei by centrifugation at 13,000 rpm in a microcentrifuge for 1 min at 4°C and remove supernatant.
6. Resuspend nuclear pellet in 100 µl of 1X ChIP buffer + PIC per IP prep and incubate on ice for 10 min.
7. Sonicate up to 500 µl of lysate per 1.5 ml microcentrifuge tube with several pulses to break nuclear membrane. Incubate samples for 30 sec on wet ice between pulses. Optimal conditions required for complete lysis of nuclei can be determined by observing nuclei under light microscope before and after sonication. HeLa nuclei were completely lysed after 3 sets of 20-sec pulses using a VirTis Virsonic 100 Ultrasonic Homogenizer/Sonicator at setting 6 with a 1/8-inch probe. Alternatively, nuclei can be lysed by homogenizing the lysate 20 times in a Dounce homogenizer; however, lysis may not be as complete.
8. Clarify lysates by centrifugation at 10,000 rpm in a microcentrifuge for 10 min at 4°C.
9. Transfer supernatant to a new tube. This is the cross-linked chromatin preparation, which should be stored at -80°C until further use. Remove 50 µl of the chromatin preparation for Analysis of Chromatin Digestion and Concentration (Section IV).

IV. Analysis of Chromatin Digestion and Concentration (Recommended Step)

1. To the 50 µl chromatin sample (from Step 9 in Section III), add 100 µl nuclease-free water, 6 µl 5 M NaCl, and 2 µl RNAse A. Vortex to mix and incubate samples at 37°C for 30 min.
2. To each RNAse A-digested sample, add 2 µl Proteinase K. Vortex to mix and incubate samples at 65°C for 2 hr.
3. Purify DNA from samples using DNA purification spin columns as described in Section VII.
4. After purification of DNA, remove a 10 µl sample and determine DNA fragment size by electrophoresis on a 1% agarose gel with a 100 bp DNA marker. DNA should be digested to a length of approximately 150–900 bp (1 to 5 nucleosomes).
5. To determine DNA concentration, transfer 2 µl of purified DNA to 98 µl nuclease-free water to give a 50-fold dilution and read the OD_{260}. The concentration of DNA in µg/ml is OD_{260} x 2,500. DNA concentration should ideally be between 50 and 200 µg/ml.

NOTE: For optimal ChIP results, it is highly critical that the chromatin is of appropriate size and concentration. Over-digestion of chromatin may diminish signal in the PCR quantification. Under-digestion of chromatin may lead to increased background signal and lower resolution. Adding too little chromatin to the IP may result in diminished signal in the PCR quantification. A protocol for optimization of chromatin digestion can be found in Troubleshooting Guide, Section B.

V. Chromatin Immunoprecipitation

For optimal ChIP results, use approximately 5 to 10 µg of digested, cross-linked chromatin (as determined in Section IV) per immunoprecipitation. This should be roughly equivalent to a single 100 µl IP prep from 25 mg of disaggregated tissue or 4 x 10^6 tissue culture cells. Typically, 100 µl of digested chromatin is diluted into 400 µl 1X ChIP Buffer prior to the addition of antibodies. However, if more than 100 µl of chromatin is required per IP, the cross-linked chromatin preparation does not need to be diluted as described below. Antibodies can be added directly to the undiluted chromatin preparation for immunoprecipitation of chromatin complexes.

Before starting:

- Remove and warm 200X Protease Inhibitor Cocktail (PIC). Make sure PIC is completely thawed.
- Remove and warm 10X ChIP Buffer and ensure SDS is completely in solution.
- Thaw digested chromatin preparation (Section III, Step 9) and place on ice.
- Prepare low salt wash: 3 ml 1X ChIP Buffer (300 µl 10X ChIP Buffer + 2.7 ml water) per immunoprecipitation. Store at room temperature until use.
- Prepare high salt wash: 1 ml 1X ChIP Buffer (100 µl 10X ChIP Buffer + 900 µl water) + 70 µl 5M NaCl per immunoprecipitation. Store at room temperature until use.

1. In one tube, prepare enough 1X ChIP Buffer for the dilution of digested chromatin into the desired number of immunoprecipitations: 400 µl of 1X ChIP Buffer (40 µl of 10X ChIP Buffer + 360 µl water) + 2 µl 200X PIC per immunoprecipitation. When determining the number of immunoprecipitations, remember to include the positive control Histone H3 (D2B12) XP® Rabbit mAb and negative control Normal Rabbit IgG antibody samples. Place mix on ice.
2. To the prepared 1X ChIP buffer, add the equivalent of 100 µl (5–10 µg of chromatin) of the digested, cross-linked chromatin preparation (Section III, Step 9) per immunoprecipitation. For example, for 10 immunoprecipitations, prepare a tube containing 4 ml 1X ChIP Buffer (400 µl 10X ChIP Buffer + 3.6 ml water) + 20 µl 200X PIC + 1 ml digested chromatin preparation.
3. Remove a 10 µl sample of the diluted chromatin and transfer to a microfuge tube. This is your 2% Input Sample, which can be stored at -20°C until further use (Step 1 in Section VI).
4. For each immunoprecipitation, transfer 500 µl of the diluted chromatin to a 1.5 ml microcentrifuge tube and add the immunoprecipitating antibody. The amount of antibody required per IP varies and should be determined by the user. For the positive control Histone H3 (D2B12) XP® Rabbit mAb, add 10 µl to the IP sample. For the negative control Normal Rabbit IgG, add 1 µl (1 µg) to 2 µl (2 µg) to the IP sample. Incubate IP samples 4 h to overnight at 4°C with rotation.
5. **For Protein G Agarose Beads:** Resuspend ChIP-Grade Protein G Agarose Beads by gently vortexing. Immediately add 30 µl of Protein G Agarose Beads to each IP reaction and incubate for 2 hr at 4°C with rotation.

   For Protein G Magnetic Beads: Resuspend ChIP-Grade Protein G Magnetic Beads by gently vortexing. Immediately add 30 µl of Protein G Magnetic Beads to each IP reaction and incubate for 2 hr at 4°C with rotation.
6. **For Protein G Agarose Beads:** Pellet Protein G Agarose Beads in each immunoprecipitation by brief 1 min centrifugation at 6,000 rpm in a microcentrifuge and remove supernatant.

   For Protein G Magnetic Beads: Pellet Protein G Magnetic Beads in each immunoprecipitation by placing the tubes in a Magnetic Separation Rack. Wait 1 to 2 min for solution to clear and then carefully remove supernatant.
7. Wash Protein G Beads by adding 1 ml of low salt wash to the beads and incubate at 4°C for 5 min with rotation. Repeat steps 6 and 7 two additional times for a total of 3 low salt washes.
8. Add 1 ml of high salt wash to the beads and incubate at 4°C for 5 min with rotation.
9. **For Protein G Agarose Beads:** Pellet Protein G Agarose Beads in each immunoprecipitation by brief 1 min centrifugation at 6,000 rpm in a microcentrifuge. Remove supernatant and immediately proceed to Section VI.

   For Protein G Magnetic Beads: Pellet Protein G Magnetic Beads in each immunoprecipitation by placing the tubes in a Magnetic Separation Rack. Wait 1 to 2 min for solution to clear and then carefully remove supernatant and proceed to Section VI.

VI. Elution of Chromatin from Antibody/ Protein G Agarose Beads and Reversal of Cross-links

Before starting:

- Remove and warm 2X ChIP Elution Buffer in a 37°C water bath and ensure SDS is in solution.
- Set a water bath or thermomixer to 65°C.
- Prepare 150 µl 1X ChIP Elution Buffer (75 µl 2X ChIP Elution Buffer + 75 µl water) for each immunoprecipitation and the 2% input sample.

1. Add 150 µl of the 1X ChIP Elution Buffer to the 2% input sample tube and set aside at room temperature until Step 6.
2. Add 150 µl 1X ChIP Elution Buffer to each IP sample.
3. Elute chromatin from the antibody/Protein G Beads for 30 min at 65°C with gentle vortexing (1,200 rpm). A thermomixer works best for this step. Alternatively, elutions can be performed at room temperature with rotation, but may not be as complete.
4. **For Protein G Agarose Beads:** Pellet Protein G Agarose Beads by brief 1 min centrifugation at 6,000 rpm in a microcentrifuge.

   For Protein G Magnetic Beads: Pellet Protein G Magnetic Beads by placing the tubes in a Magnetic Separation Rack and wait 1 to 2 min for solution to clear.
5. Carefully transfer eluted chromatin supernatant to a new tube.
6. To all tubes, including the 2% input sample from Step 1, reverse cross-links by adding 6 µl 5M NaCl and 2 µl Proteinase K, and incubate 2 h at 65°C. This incubation can be extended overnight.
7. Immediately proceed to Section VII. Alternatively, samples can be stored at -20°C. However, to avoid formation of a precipitate, be sure to warm samples to room temperature before adding DNA Binding Reagent A (Section VII, Step 1).

VII. DNA Purification Using Spin Columns

Before starting:

- Add 12 ml of isopropanol to DNA Binding Reagent A and 24 ml of ethanol (96–100%) to DNA Wash Reagent B before use. These steps only have to be performed once prior to the first set of DNA purifications.
- Remove one DNA purification spin column and collection tube for each DNA sample (Section VI).

1. Add 600 µl DNA Binding Reagent A to each DNA sample and vortex briefly.
   - 4 volumes of DNA Binding Reagent A should be used for every 1 volume of sample.
2. Transfer 375 µl of each sample from Step 1 to a DNA purification spin column in collection tube.
3. Centrifuge at 14,000 rpm in a microcentrifuge for 30 sec.
4. Remove the spin column from the collection tube and discard the liquid. Replace spin column in the collection tube.
5. Transfer the remaining 375 µl of each sample from Step 1 to the spin column in collection tube. Repeat Steps 3 and 4.
6. Add 700 µl of DNA Wash Reagent B to the spin column in collection tube.
7. Centrifuge at 14,000 rpm in a microcentrifuge for 30 sec.
8. Remove the spin column from the collection tube and discard the liquid. Replace spin column in the collection tube.
9. Centrifuge at 14,000 rpm in a microcentrifuge for 30 sec.
10. Discard collection tube and liquid. Retain spin column.
11. Add 50 µl of DNA Elution Reagent C to each spin column and place into a clean 1.5 ml microcentrifuge tube.
12. Centrifuge at 14,000 rpm in a microcentrifuge for 30 sec to elute DNA.
13. Remove and discard DNA purification spin column. Eluate is now purified DNA. Samples can be stored at -20°C.

VIII. Quantification of DNA by PCR

Recommendations:

- Use Filter-tip pipette tips to minimize risk of contamination.
- The control primers included in the kit are specific for the human or mouse RPL30 gene and can be used for either standard PCR or quantitative real-time PCR. If the user is performing ChIPs from another species, it is recommended that the user design the appropriate specific primers to DNA and determine the optimal PCR conditions.
- A Hot-Start Taq polymerase is recommended to minimize the risk of non-specific PCR products.
- PCR primer selection is critical. Primers should be designed with close adherence to the following criteria:

  Primer length: 24 nucleotides

  Optimum Tm: 60°C

  Optimum GC: 50%

  Amplicon size: 150–200 bp (for standard PCR)
  80–160 bp (for real-time quantitative PCR)

Standard PCR Method:

1. Label the appropriate number of 0.2 ml PCR tubes for the number of samples to be analyzed. These should include the 2% input sample, the positive control histone H3 sample, the negative control normal rabbit IgG sample, and a tube with no DNA to control for DNA contamination.
2. Add 2 µl of the appropriate DNA sample to each tube.
3. Prepare a master reaction mix as described below, making sure to add enough reagent for two extra tubes to account for loss of volume. Add 18 µl of master mix to each reaction tube.

   Reagent : Volume for 1 PCR Reaction (18 µl)

   Nuclease-free H_2O : 12.5 µl

   10X PCR Buffer : 2.0 µl

   4 mM dNTP Mix : 1.0 µl

   5 µM RPL30 Primers : 2.0 µl

   Taq DNA Polymerase : 0.5 µl
4. Start the following PCR reaction program:

   a. Initial Denaturation 95°C 5 min

   b. Denature 95°C 30 sec

   c. Anneal 62°C 30 sec

   d. Extension 72°C 30 sec

   e. Repeat Steps b–d for a total of 34 cycles

   f. Final Extension 72°C 5 min
5. Remove 10 µl of each PCR product for analysis by 2% agarose gel or 10% poly-acrylamide gel electrophoresis with a 100 bp DNA marker. The expected size of the PCR product is 161 bp for human RPL30 and 159 bp for mouse RPL30.

Real-Time Quantitative PCR Method:

1. Label the appropriate number of PCR tubes or PCR plates compatible with the model of PCR machine to be used. PCR reactions should include the positive control histone H3 sample, the negative control normal rabbit IgG sample, a tube with no DNA to control for contamination, and a serial dilution of the 2% input chromatin DNA (undiluted, 1:5, 1:25, 1:125) to create a standard curve and determine the efficiency of amplification.
2. Add 2 µl of the appropriate DNA sample to each tube or well of the PCR plate.
3. Prepare a master reaction mix as described below. Add enough reagents for two extra reactions to account for loss of volume. Add 18 µl of reaction mix to each PCR reaction tube or well.

   Reagent : Volume for 1 PCR Reaction (18 µl)

   Nuclease-free H_2O : 6 µl

   5 µM RPL30 Primers : 2 µl

   2X SYBR®-Green Reaction Mix : 10 µl
4. Start the following PCR reaction program:

   a. Initial Denaturation 95°C, 3 min

   b. Denature 95°C, 15 sec

   c. Anneal and Extension: 60°C, 60 sec

   d. Repeat steps **b** and **c** for a total of 40 cycles.
5. Analyze quantitative PCR results using the software provided with the real-time PCR machine. Alternatively, one can calculate the IP efficiency manually using the Percent Input Method and the equation shown below. With this method, signals obtained from each immunoprecipitation are expressed as a percent of the total input chromatin.

Percent Input = 2% x $2^{(C[T]\ 2\%Input\ Sample\ -\ C[T]\ IP\ Sample)}$

C[T] = C_T = Threshold cycle of PCR reaction

Complementary Products for Chromatin Immunoprecipitation

SimpleChIP® Enzymatic Chromatin IP Kits

30 assays

#9002 SimpleChIP® Enzymatic Chromatin IP Kit (Agarose Beads)

#9003 SimpleChIP® Enzymatic Chromatin IP Kit (Magnetic Beads)

#9004 SimpleChIP® Plus Enzymatic Chromatin IP Kit (Agarose Beads)

#9005 SimpleChIP® Plus Enzymatic Chromatin IP Kit (Magnetic Beads)

Solutions and Miscellaneous

#7017 6-Tube Magnetic Separation Rack

#5415 Mouse (G3A1) mAb IgG1 Isotype Control

#3900 Rabbit (DA1E) mAb IgG XP® Isotype Control

#2729 Normal Rabbit IgG

#9006 ChIP-Grade Protein G Magnetic Beads

#9007 ChIP-Grade Protein G Agarose Beads

SimpleChIP® Control Primers are a mix of two control primers that can be used to amplify DNA that has been isolated using chromatin immunoprecipitation (ChIP). These primers will amplify positive control DNA sequences that contain known binding sites to the target protein detected by the antibody employed in the ChIP assay, and can also be used as negative controls to demonstrate antibody sensitivity. Please visit our website for a full primer listing.

- Primers are designed, tested, and optimized in-house in conjunction with our ChIP-validated antibodies and SimpleChIP® Kits, saving time and reagents.
- Primers are optimized for use in real-time PCR with SYBR® Green dye, which simplifies quantification of DNA enrichment.
- Technical Support is provided by the scientists who designed and use these products, and know them best.

Enzymatic Chromatin IP Troubleshooting Guide

A. Expected Chromatin Yield

When harvesting cross-linked chromatin from tissue samples, the yield of chromatin can vary significantly between tissue types. The table below provides a range for the expected yield of chromatin from 25 mg of tissue compared to 4 x 10^6 HeLa cells, and the expected DNA concentration, as determined in Section IV of the protocol. For each tissue type, disaggregation using a Medimachine (BD Biosciences) or a Dounce homogenizer yielded similar amounts of chromatin. However, chromatin processed from tissues disaggregated using the Medimachine typically gave higher IP efficiencies than chromatin processed from tissues disaggregated using a Dounce homogenizer. A Dounce homogenizer is strongly recommended for disaggregation of brain tissue, as the Medimachine does not adequately disaggregate brain tissue into a single-cell suspension. For optimal ChIP results, we recommend using 5 to 10 µg of digested, cross-linked chromatin per immunoprecipitation; therefore, some tissues may require harvesting more than 25 mg per each immunoprecipitation.

Tissue/Cell	Total Chromatin Yield	Expected DNA Concentration
Spleen	20–30 µg per 25 mg tissue	200–300 µg/ml
Liver	10–15 µg per 25 mg tissue	100–150 µg/ml
Kidney	8–10 µg per 25 mg tissue	80–100 µg/ml
Brain	2–5 µg per 25 mg tissue	20–50 µg/ml
Heart	2–5 µg per 25 mg tissue	20–50 µg/ml
HeLa	10–15 µg per 4 x 10^6 cells	100–150 µg/ml

B. Optimization of Chromatin Digestion

Optimal conditions for the digestion of cross-linked chromatin DNA to 150-900 base pairs in length is highly dependent on the ratio of Micrococcal Nuclease to the amount of tissue or number of cells used in the digest. Below is a protocol for determination of the optimal digestion conditions for a specific tissue or cell type.

1. Prepare cross-linked nuclei from 125 mg of tissue or 2 X 10^7 cells (equivalent of 5 IP preps), as described in Protocol Sections I, II, and III. Stop after Step 2 of Protocol Section III and proceed as described below.
2. Transfer 100 µl of the nuclei preparation into 5 individual 1.5 ml microcentrifuge tubes and place on ice.
3. Add 3 µl Micrococcal Nuclease stock to 27 µl of 1X Buffer B + DTT (1:10 dilution of enzyme).
4. To each of the 5 tubes in Step 2, add 0 µl, 2.5 µl, 5 µl, 7.5 µl, or 10 µl of the diluted Micrococcal Nuclease, mix by inverting tube several times and incubate for 20 min at 37°C with frequent mixing.
5. Stop each digest by adding 10 µl of 0.5 M EDTA and placing tubes on ice.
6. Pellet nuclei by centrifugation at 13,000 rpm in a microcentrifuge for 1 min at 4°C and remove supernatant.
7. Resuspend nuclear pellet in 200 µl of 1X ChIP buffer + PIC. Incubate on ice for 10 min.
8. Sonicate lysate with several pulses to break nuclear membrane. Incubate samples 30 sec on wet ice between pulses. Optimal conditions required for complete lysis of nuclei can be determined by observing nuclei under light microscope before and after sonication. HeLa nuclei were completely lysed after 3 sets of 20 sec pulses using a VirTis Virsonic 100 Ultrasonic Homogenizer/Sonicator set at setting 6 with a 1/8-inch probe. Alternatively, nuclei can be lysed by homogenizing the lysate 20 times in a Dounce homogenizer; however, lysis may not be as complete.
9. Clarify lysates by centrifugation at 10,000 rpm in a microcentrifuge for 10 min at 4°C.
10. Transfer 50 µl of each of the sonicated lysates to new microfuge tubes.
11. To each 50 µl sample, add 100 µl nuclease-free water, 6 µl 5 M NaCl and 2 µl RNAse A. Vortex to mix and incubate samples at 37°C for 30 min.
12. To each RNAse A-digested sample, add 2 µl Proteinase K. Vortex to mix and incubate sample at 65°C for 2 hr.
13. Remove 20 µl of each sample and determine DNA fragment size by electrophoresis on a 1% agarose gel with a 100 bp DNA marker.
14. Observe which of the digestion conditions produces DNA in the desired range of 150-900 base pairs (1 to 5 nucleosomes). The volume of diluted Micrococcal Nuclease that produces the desired size of DNA fragments using this optimization protocol is equivalent to 10 times the volume of Micrococcal Nuclease stock that should be added to one immunoprecipitation preparation (25 mg of disaggregated tissue cells or 4 X 10^6 tissue culture cells) to produce the desired size of DNA fragments. For example, if 5 µl of diluted Micrococcal Nuclease produces DNA fragments of 150-900 base pairs in this protocol, then 0.5 µl of stock Micrococcal Nuclease should be added to one immunoprecipitation preparation during the digestion of chromatin in Section III.
15. If results indicate that DNA is not in the desired size range, then repeat optimization protocol, adjusting the amount of Micrococcal Nuclease in each digest accordingly. Alternatively, the digestion time can be changed to increase or decrease the extent of DNA fragmentation.

C. Troubleshooting Table

Problem	Possible Causes	Recommendation
Concentration of the digested chromatin is too low (low chromatin yield).	Not enough tissue or cells were added to the chromatin digestion or cell nuclei were not completely lysed after digestion.	Add additional chromatin to each IP to give at least 5 µg/IP and continue with protocol.
		Weigh tissue or count a separate plate of cells prior to cross-linking to determine accurate cell number. Some tissues may require processing of more than 25 mg per IP. The amount of tissue can be increased to 50 mg per IP, while still maintaining efficient chromatin fragmentation and extraction
		Increase the number of sonications following chromatin digestion. Visualize cell nuclei under microscope before and after sonication to confirm complete lysis of nuclei.
Chromatin is under-digested and fragments are too large (greater than 900 bp). Large chromatin fragments can lead to increased background and lower resolution.	Too many cells or not enough Micrococcal Nuclease was added to the chromatin digestion.	Weigh tissue or count a separate plate of cells prior to cross-linking to determine accurate cell number. Add less tissue or cells, or more Micrococcal Nuclease to the chromatin digest. See Section B for optimization of chromatin digestion.
	Tissue or cells may have been over cross-linked. Cross-linking for longer than 10 min may inhibit digestion of chromatin.	Perform a time course at a fixed formaldehyde concentration. Shorten the time of cross-linking to 10 min or less.
Chromatin is over-digested and fragments are too small (exclusively 150 bp mono-nucleosome length). Complete digestion of chromatin to mono-nucleosome length DNA may diminish signal during PCR quantification, especially for amplicons greater than 150 bp in length.	Not enough cells or too much Micrococcal Nuclease added to the chromatin digestion.	Weigh tissue or count a separate plate of cells prior to cross-linking to determine accurate cell number. Add more tissue or cells, or less Micrococcal Nuclease to the chromatin digest. See Section B for optimization of chromatin digestion.
No product or very little product in the input PCR reactions.	Not enough DNA added to the PCR reaction or conditions are not optimal.	Add more DNA to the PCR reaction or increase the number of amplification cycles.
	PCR amplified region may span nucleosome-free region.	Optimize the PCR conditions for experimental primer set using purified DNA from cross-linked and digested chromatin. Design a different primer set and decrease length of amplicon to less than 150 bp (see primer design recommendations in Protocol Section VIII).
	Not enough chromatin added to the IP or chromatin is over-digested.	For optimal ChIP results, add 5 to 10 µg chromatin per IP.
No product in the positive control histone H3-IP RPL30 PCR reaction.	Not enough chromatin or antibody added to the IP reaction or IP incubation time is too short.	Be sure to add 5 to 10 µg of chromatin and 10 µl of antibody to each IP reaction and incubate with antibody overnight and an additional 2 hr after adding Protein G beads.
	Incomplete elution of chromatin from Protein G beads.	Elution of chromatin from Protein G beads is optimal at 65°C with frequent mixing to keep beads suspended in solution.
Quantity of product in the negative control Rabbit IgG-IP and positive control histone H3-IP PCR reactions is equivalent (high background signal).	Too much or not enough chromatin added to the IP reaction. Alternatively, too much antibody added to the IP reaction.	For optimal ChIP results, add 5 to 10 µg of chromatin and 10 µl of histone H3 antibody to each IP reaction. Reduce the amount of normal rabbit IgG to 1 µl per IP.
	Too much DNA added to the PCR reaction or too many cycles of amplification.	Add less DNA to the PCR reaction or decrease the number of PCR cycles. It is very important that the PCR products are analyzed within the linear amplification phase of PCR. Otherwise, the differences in quantities of starting DNA cannot be accurately measured. Alternatively, quantify immunoprecipitations using real-time quantitative PCR.
No product in the Experimental Antibody-IP PCR reaction.	Not enough DNA added to the PCR reaction.	Add more DNA to the PCR reaction or increase the number of amplification cycles.
	Not enough antibody added to the IP reaction.	Typically a range of 1 to 5 µg of antibody are added to the IP reaction; however, the exact amount depends greatly on the individual antibody. Increase the amount of antibody added to the IP.
	Antibody does not work for ChIP.	Find an alternate antibody source.

Controls Table

This table compiles treatments that can be used as a positive control for our activation-state specific antibodies and should be seen as a starting point for troubleshooting research assays. Please contact Technical Support for more detailed information or any further questions.

Note: To demonstrate phospho-specificity, extracts or nitrocellulose membranes should be subjected to phosphatase treatment. All UV treatments were carried out using a Stratagene Stratalinker UV Crosslinker.

Antibody	Cell Line	Treatment	Control Extract
Phospho-(Ser) 14-3-3 Binding Motif	Jurkat	Calyculin A (100 nM for 30 min)	#9273
Phospho-4E-BP1 (Thr37/Thr46/Ser65/Thr70)	HeLa	Untreated	
Phospho-53BP1 (Ser25/Ser29/Thr543/Ser1778)	HT-29	UV‡ (100 mJ/cm^2), 1 hr recovery	
Phospho-c-Abl (Tyr89/Tyr204/Tyr245/Tyr412/Thr735)	K-562	Untreated (K-562 cell line contains Bcr-Abl fusion)	
Acetylated-Lysine (Ac-K2-100)	COS-7	TSA (0.4 µM for 18 hrs)	
Phospho-Acetyl-CoA Carboxylase (Ser79)	C2C12	Serum-starve overnight, AICAR (0.5 mM for 30 min)	#9158
Acetyl-CoA Carboxylase	C2C12	Untreated	
Phospho-Afadrin (Ser1718)	A-431	Serum-starve overnight, hEGF (100 ng/ml for 5 min)	
Phospho-Akt Substrate (RXRXXS*/T*)	Jurkat	Calyculin A (100 nM for 30 min)	#9273
Phospho-Akt (Thr308/Thr450)	Jurkat	Calyculin A (100 nM for 30 min)	#9273
Phospho-Akt (Ser473)	Jurkat	Untreated or Calyculin A (100 nM for 30 min); alternative: serum-starve, insulin (100 nM for 10 min) works for most cell lines	#9273
Phospho-ALK (Tyr1078/Tyr1096/Tyr1278/Try1282/Tyr1283/ Tyr1586/Tyr1604)	KARPAS-299	Untreated (KARPAS-299 cell lines contains NPM-ALK fusion)	
Cleaved α-Fodrin (Asp1185)	HeLa	Serum-starve overnight, Staurosporine (1.0 µM for 4 hrs)	
Phospho-AML1 (Ser249)	Jurkat, HEL	Untreated	
Phospho-AMPKα (Thr172)	C2C12	Serum-starve overnight, AICAR (0.5 mM for 30 min)	#9158
Phospho-AMPKα1 (Ser485)	C2C12	Serum-starve overnight, AICAR (0.5 mM for 30 min)	#9158
Phospho-AMPKβ1 (Ser108/Ser182)	C2C12	Serum-starve overnight, AICAR (0.5 mM for 30 min)	#9158
Phospho-AP2M1 (Thr156)	HeLa	H_2O_2 (4 mM for 30 min)	
Phospho-APP (Thr668)	HeLa	Nocodazole (1 µg/ml for 18 hrs)	
Phospho-β-Arrestin 1 (Ser412)	293	Untreated	
Phospho-AS160 (Thr642)	HeLa	Serum-starve overnight, hIGF-I (100 ng/ml for 15 min)	
Phospho-ATF-2 (Thr69/Thr71)	NIH/3T3	Anisomycin (25 µg/ml for 30 min)	#9223
Phospho-ATM (Ser1981)	293	IR (10 Gy), 1 hr recovery	
Phospho-(Ser/Thr) ATM/ATR Substrate	293	UV‡ (50 mJ/cm^2), 30 min recovery	#9253
Phospho-ATR (Ser428)	HeLa	UV‡ (75 mJ/cm^2), 1 hr recovery	
Phospho-ATP-Citrate Lyase (Ser454)	NIH/3T3	Serum-starve overnight, PDGF (50 ng/ml for 10 min)	
Phospho-Na, K-ATPase a1 (Tyr10)	A-431	Serum-starve overnight, hEGF (100 ng/ml for 5 min)	
Phospho0Na, K-ATPase a1 (Ser16/Ser23)	PC-12	Serum-starve overnight, TPA (200 nM for 30 min)	
Phospho-ATRIP (Ser224)	HeLa	Untreated	
Phospho-Aurora A (Thr288)/Aurora B (Thr232)/Aurora C (Thr198)	HT-29	Nocodazole (100 ng/ml for 18 hrs)	
Phospho-Axl (Tyr702)	NCI-H1299	Gas6 (400 ng/mL for 5 min)	
Phospho-Bad (Ser112/Ser136/Ser155)	COS-7	Serum-starve overnight, TPA (200 nM for 30 min)	#9293
Phospho-BAP1 (Ser592)	HeLa	Serum-starve overnight, UV‡ (50 mJ/cm^2), 1 hr recovery	
Phospho-Bcl-2 (Thr56)	RL-7	Nocadazole (1 µg/ml for 20 hrs)	
Phospho-Bcl-2 (Ser70)	Jurkat	Paclitaxel (1 µM for 20 hrs)	
Phospho-Bcr (Tyr177)	K-562	Untreated (K-562 cell line contains Bcr-Abl fusion)	
Phospho-Bim (Ser55/Ser69)	A20	Serum-starve overnight, TPA (200 nM for 30 min)	
Phospho-BLNK (Tyr96)	Ramos	Anti-IgM (12 µg/ml for 2 min)	
Phospho-BRCA1 (Ser1524)	HeLa	UV‡ (75 mJ/cm^2), 1 hr recovery	
Phospho-Btk (Ser180)	THP-1	Serum-starve overnight, H_2O_2 (2 mM for 2 min)	
Phospho-Btk (Tyr223)	Ramos	Anti-IgM (12 µg/ml for 10 min)	
Phospho-C/EBPα (Ser21)	differentiated 3T3-L1	Serum-starve overnight, insulin (100 nM for 45 min)	
Phospho-C/EBPα (Thr222/226)	U-937	Untreated	
Phospho-C/EBPβ (Ser105)	PC-12	Untreated	
Phospho-C/EBPβ (Thr235)	differentiated 3T3-L1	Serum-starve overnight, insulin (100 nM for 10 min)	
Phospho-CaMKII (Thr286)	HeLa	TPA (200 nM for 15 min)	#9160
Cleaved Caspase-1 (Asp297)	THP-1	TPA (80 nM overnight) plus LPS (1 µg/ml for 8 hrs)	
Cleaved Caspase-3 (Asp175)	HeLa	Serum-starve overnight, Staurosporine (1 µM for 3 hrs)	#9663
Cleaved Caspase-6 (Asp162)	HeLa	Serum-starve overnight, Staurosporine (1 µM for 3 hrs)	#9663
Cleaved Caspase-7 (Asp198)	HeLa	Serum-starve overnight, Staurosporine (1 µM for 3 hrs)	#9663
Cleaved Caspase-8 (Asp391/Asp384/Asp387)	Jurkat	Etoposide (25 µM for 5 hrs)	#2043
Phospho-Caspase-9 (Thr125)	MCF7	hEGF (10 ng/ml for 30 min)	
Cleaved Caspase-9 (Asp315/Asp330)	Jurkat	Etoposide (25 µM for 5 hrs)	#2043
Phospho-β-Catenin (Ser33/Ser37/Thr41/Ser45)	SW480	Untreated	
Phospho-β-Catenin (Ser552/Ser675)	SK-N-MC	Forskolin (50 µM for 30 min)	

Antibody	Cell Line	Treatment	Control Extract
Phospho-Catenin δ-1 (Tyr228/Tyr904)	A-431	Serum-starve overnight, hEGF (100 ng/ml for 15 min)	
Phospho-Caveolin-1 (Tyr14)	HeLa	Serum-starve overnight, H_2O_2 (500 µM for 15 min)	
Phospho-c-Cbl (Tyr731/Tyr774)	K-562	Untreated	
Acetyl-CBP (Lys1535)/p300 (Lys1499)	NIH/3T3	TSA (400 nM overnight)	
Phospho-CD19 (Tyr531)	Ramos	Anti-IgM (12 µg/ml for 2 min)	
Phospho-cdc2 (Thr14/Tyr161)	HeLa	Nocodazole (50 ng/ml for 20 hrs)	
Phospho-cdc2 (Thr15)	HeLa	Untreated	
Phospho-cdc25C (Thr48/Ser216)	HT-29	Nocodazole (100 ng/ml for 18 hrs)	
Phospho-(Ser) CDKs Substrate	HeLa	Nocadazole (1 µg/ml for 12 hrs)	
Phospho-CDK2 (Thr160)	HeLa	Hydroxyurea (10 mM for 16 hrs)	
Phospho-CDK9 (Thr186)	HeLa	Untreated	
Phospho-Chk1 (Ser280/Ser296/Ser317/Ser345)	HeLa	UV‡ (75 mJ/cm^2), 1 hr recovery	
Phospho-Chk2 (Ser19/Ser33/Ser35/Thr68/Thr387/Thr432/Ser516)	HeLa	UV‡ (100 mJ/cm2), 1 hr recovery	
Phospho-Cofilin (Ser3)	C2C12, NIH/3T3, HeLa	Untreated	
Phospho-Connexin 43 (Ser368)	COS-7	Serum-starve overnight, TPA (200 nM for 30 min)	
Phospho-Cortactin (Tyr421)	HeLa	Serum-starve overnight, H_2O_2 (2 nM for 2 min)	
Phospho-CREB (Ser133)	SK-N-MC	Forskolin (30 µM) and IBMX (0.5 mM), for 30 min	
Phospho-CRMP-2 (Thr514)	rat brain	Untreated	
Phospho-CrkII (Tyr221)	K-562	Untreated	
Phospho-CrkL (Tyr207)	K-562	Untreated	
Phospho-M-CSF Receptor (Tyr699/Tyr723/Tyr809/Tyr923)	NKM-1	Untreated	
Phospho-CTDSPL2 (Ser104)	HeLa	Untreated	
Phospho-Cyclin B1 (Ser133/Ser147)	HeLa	Nocodazole (50 ng/ml for 20 hrs)	
Phospho-Cyclin D1 (Thr286)	HT-1080	MG132 (10 µM for 4 hrs)	
Phospho-Cyclin E (Thr62)	MCF7	Hydroxyurea (10 mM for 8 hrs)	
Phospho-DARPP-32 (Thr34/Thr75)	rat brain, mouse brain	PKA (12,500 U/100 µl for 1 hr)	
Phospho-DBC1 (Thr454)	293	UV‡ (100 mJ/cm^2), 4 hrs recovery	
Phospho-Doublecortin (Ser297)	fetal rat brain	Untreated	
Phospho-Doublecortin (Ser334)	rat brain, mouse brain	Untreated	
Cleaved DFF45 (Asp224)	Jurkat	Etoposide (25 µM for 5 hrs)	#2043
Phospho-DRP1 (Ser616)	HeLa	Nocadazole (100 ng/ml for 16 hrs)	
Phospho-DRP1 (Ser637)	PC-12	Serum-starve overnight, Forskolin (20 µM for 1 hr)	
Phospho-eEF2 (Thr56)	C6	Forskolin (10 µM for 1 hr)	
Phospho-eEF2k (Ser366)	HeLa, NIH/3T3, C6, COS-7	Untreated	
Phospho-EGF Receptor (Thr669/Tyr845/Tyr998/Tyr992/Tyr1045/Ser1046/Ser1047/Tyr1068/Tyr1086/Tyr1148/Tyr1173)	HT-29	Serum-starve overnight, hEGF (100 ng/ml for 5 min)	#5634
Phospho-eIF2α (Ser51)	HeLa	Thapsigargin (300 nM for 30 min)	
Phospho-eIF4B (Ser406)	NIH/3T3	Untreated	
Phospho-eIF4B (Ser422)	NIH/3T3	Serum-starve overnight, 20% calf serum for 20 min	
Phospho-eIF4E (Ser209)	MCF7	Serum-starve overnight, insulin (100 nM for 5–10 min)	#9203
Phospho-eIF4G (Ser1108)	HeLa	Serum-starve overnight, insulin (100 nM for 5–10 min)	
Phospho-Elk-1 (Ser383)	recombinant protein	GST-Elk1 fusion protein phosphorylated in vitro by Erk1/2	#9183
Phospho-eNOS (Ser113/Thr495)	BAEC	Untreated	
Phospho-eNOS (Ser1177)	BAEC	VEGF (50 ng/ml for 5 min)	
Phospho-Erk5 (Thr218/Tyr220)	NIH/3T3	Serum-starve overnight, PDGF (100 ng/ml for 5 min); IP with #3372 prior to western	
Phospho-Estrogen Receptor α (Ser104/Ser106/Ser118)	MCF7	Serum-starve overnight, hEGF (100 ng/ml) plus estradiol (100 nM) for 30 min	
Phospho-Etk (Tyr40)	Jurkat	Serum-starve overnight, anti-CD3 (1 µg/ml) plus anti-CD28 (0.5 µg/ml) for 10 min	
Phospho-Ezrin (Tyr353)	A-431	Serum-starve overnight, hEGF (100 ng/ml for 10 min)	
Phospho-Ezrin (Tyr567)/Radixin (Thr564)/Moesin (Thr558)	C2C12, NIH/3T3, HeLa	Untreated	
Phospho-FADD (Ser194)	Jurkat	Inhibit with hydroxyurea (4.0 mM for 24 hrs); activate with Nocodazole (1.0 µg/ml) for 24 hrs	
Phospho-FAK (Tyr397/Tyr576/Tyr577/Tyr925)	A549	Untreated	
Phospho-FANCD2 (Ser222)	HeLa	UV‡ (100 mJ/cm^2), 1 hr recovery	
Phospho-Filamin A (Ser2152)	293	Untreated	
Phospho-FLT3 (Tyr589/Tyr591/Tyr842/Tyr969)	SEM	Untreated	
Phospho-c-Fos (Ser32)	HeLa	Serum-starve overnight, TPA (200 nM for 4 hrs)	
Phospho-FRA1 (Ser265)	HeLa	Serum-starve overnight, TPA (200 nM for 4 hrs)	
Phospho-FoxO1 (Thr24/Ser256/Ser319)	HeLa	Serum-starve overnight, 20% serum for 30 min	
Phospho-FoxO3a (Thr32/Ser318/Ser321/Ser253)	HeLa	Serum-starve overnight, 20% serum for 30 min	
Phospho-FoxO4 (Ser193)	HeLa	Serum-starve overnight, 20% serum for 30 min	
Phospho-FRS2-α (Tyr196/Tyr436)	NIH/3T3	Serum-starve overnight, hFGF basic/FGF2 (100 ng/ml for 10 min)	
Phospho-Gab1 (Tyr307/Tyr627)	Hep G2	Serum-starve overnight, HGF (40 ng/ml for 10 min)	
Phospho-Gab2 (Ser159/Tyr452)	H-4-II-E	Serum-starve overnight, HGF (40 ng/ml for 10 min)	

Antibody	Cell Line	Treatment	Control Extract
Phospho-Glucocorticoid Receptor (Ser211)	A549	Serum-starve overnight, dexamethasone (100 nM for 1 hr)	
Phospho-AMPA Receptor (GluR 2) (Tyr869/873/876)	Rat brain	ischemia and reperfusion	
Phospho-Glycogen Synthase (Ser641)	293	Serum-starve overnight, insulin (100 nM for 5 min)	
Phospho-GRB10 (Tyr67)	H-4-II-E	Serum-starve overnight, HGF (40 ng/ml for 10 min)	
Phospho-GSK-3α (Ser21)	NIH/3T3	Serum-starve overnight, PDGF (100 ng/ml for 30 min)	
Phospho-GSK-3β (Ser9)	NIH/3T3	Serum-starve overnight, PDGF (100 ng/ml for 30 min)	
Phospho-GSK-3β (Thr390)	HeLa	Paclitaxel (100 nM/ml for 20 hrs)	
Phospho-HDAC3 (Ser424)	NIH/3T3	Untreated	
Phospho-HDAC4 (Ser246/Ser632)/HDAC5 (Ser259/Ser498)/ HDAC7 (Ser155/Ser486)	HeLa	Serum-starve overnight, Pervanadate	
Phospho-HER2/ErbB2 (Tyr877/Tyr1221/Tyr1222/Tyr1248)	MCF7	Serum-starve overnight, neuregulin (100 ng/ml for 5 min)	
Phospho-HER3/ErbB3 (Tyr1197/Tyr1222/Tyr1289)	MCF7	Serum-starve overnight, neuregulin (100 ng/ml for 5 min)	
Phospho-HER4/ErbB4 (Tyr984/Tyr1284)	MCF7	Serum-starve overnight, neuregulin (100 ng/ml for 5 min)	
Acetyl-Histone H2A (Lys5)	NIH/3T3	TSA (400 nM overnight)	
Phospho-Histone H2A.X (Ser139/Tyr142)	293	UV‡ (40 mJ/cm^2), 2 hrs recovery	
Acetyl-Histone H2B (Lys5/Lys12/Lys20)	NIH/3T3	TSA (400 nM overnight)	
Phospho-Histone H3 (Thr3/Ser10/Thr11/Ser28)	NIH/3T3	Serum-starve overnight, add 20% serum for 15 min, then Calyculin A (100 nM) 15 min	
Methyl-Histone H3 (Arg2)	MCF7	Untreated	
Acetyl- and Phospho-Histone H3 (Lys9/Ser10)	NIH/3T3	Serum-starve plus TSA (400nM overnight), add 20% serum for 15 min, then add Calyculin A (100 nM for 15 min)	
Acetyl-Histone H3 (Lys9/Lys14/Lys18/Lys23)	NIH/3T3	TSA (400 nM overnight)	
Mono/Di/Tri-Methyl-Histone H3 (Lys4/Lys27/Lys9/Lys36/ Lys79)	NIH/3T3	Untreated	
Acetyl-Histone H4 (Lys5/Lys8/Lys12)	NIH/3T3	TSA (400 nM overnight)	
Mono/Di/Tri-Methyl-Histone H4 (Lys20)	NIH/3T3	Untreated	
Phospho-HP1γ (Ser83)	HeLa	IBMX (30 µM) plus Forskolin (500 µM) for 30 min	
Phospho-HS1 (Tyr397)	Ramos	Serum-starve overnight, IgM (12 µg/ml for 10 min)	
Phospho-HSL (Ser563/Ser565/Ser660)	differentiated 3T3-L1	Isoproterenol (10 µM for 20 min)	
Phospho-HSP27 (Ser15/Ser78/Ser82)	HeLa	Serum-starve overnight, UV‡ (40 mJ/cm^2), 20 min recovery	
Phospho-HSP90α (Thr5/Thr7)	HeLa	Serum-starve overnight, UV‡ (40 mJ/cm^2), 20 min recovery	
Phospho-IGF-I Receptor β (Tyr1316/Tyr980/Tyr1131/ Tyr1135)	293	Serum-starve overnight, hIGF-I (50 ng/mL for 5 min)	
Phospho-IκBα (Ser32/36)	HeLa	hTNF-α (20 ng/ml for 5 min)	#9243
Phospho-IκBβ (Ser19/Ser23)	HeLa	hTNF-α (20 ng/ml) plus Calyculin A (50 nM for 5 min)	#9243
Phospho-IκBε (Ser18/Ser22)	HeLa	hTNF-α (20 ng/ml) plus Calyculin A (50 nM for 5 min)	#9243
Phospho-IKKα/β (Ser176/Ser180)	HeLa	hTNF-α (20 ng/ml for 10 min)	#9243
Phospho-IKKγ (Ser376)	HeLa	hTNF-α (20 ng/ml for 10 min)	#9243
Cleaved IL-1β (Asp116)	THP-1	Differentiate with TPA (80 nM for 24 hrs), rest for 24 hrs, then treat with LPS (1 µg/ml for 24 hrs). Protein is secreted into media.	
Phospho-IP3 Receptor (Ser1756)	mouse brain, rat brain		
Phospho-IRAK1 (Thr209/Ser376/Thr387)	HeLa, 293, MCF7	Untreated	
Phospho-IRF-3 (Ser396)	Raw 264.7	LPS (1 µg/ml for 2 hrs)	
Phospho-IRS-1 (Ser302/Ser307/Ser318/Ser332/Ser336/ Ser612/Ser636/Ser639/Ser1101)	MCF7	Serum-starve overnight, insulin (100 nM for 5 min)	
Phospho-Jak1 (Tyr1022/1023)	HT-29	Serum-starve overnight, IL-4 (100 ng/ml for 5–10 min)	
Phospho-Jak2 (Tyr221/Tyr1007/Try1008)	CTLL-2	Untreated	
Phospho-Jak2 (Tyr1007/Tyr1008)	TF-1	Serum-starve overnight, GM-CSF (25 ng/ml for 15 min); GM-CSF (2 ng/ml) should be included during growth and serum-starvation	
Phospho-c-Jun (Ser63/Ser73/Thr91/Ser243)	NIH/3T3	UV‡ (40 mJ/cm^2), 30 min recovery	#9263
Phospho-c-Jun (Thr93)	NIH/3T3	Untreated	
Phospho-c-Kit (Tyr703/Tyr719)	NCI-H526	Serum-starve overnight, hSCF (100 ng/ml for 5 min)	
Phospho-KSR1 (Ser392)	HeLa	Untreated	
Cleaved Lamin A (Small Subunit)	HeLa	Serum-starve overnight, Staurosporine (1.0 µM for 4 hrs)	
Cleaved Lamin A (Asp230)	HeLa	Serum-starve overnight, Staurosporine (1.0 µM for 4 hrs)	
Phospho-Lamin A/C (Ser22)	THP-1	Nocadazole (1 µg/ml for 20 hrs)	
Phospho-LAT (Tyr171/Tyr191)	Jurkat	Anti-CD3 (10 µg/ml for 2 min)	
Phospho-LATS1 (Thr1079)	HeLa	Okadaic Acid (1 µM for 1 hr)	
Phospho-LATS1 (Ser909)	HeLa	TPA (200 nM for 30 min)	
LC3B	HeLa, NIH/3T3, KNRK	Chloroquine (50 µM for 20 hrs)	
Phospho-Lck (Tyr505)	Jurkat	Serum-starve overnight, H_2O_2 (2 mM for 2 min)	

Antibody	Cell Line	Treatment	Control Extract
Phospho-5-Lipoxygenase (Ser663)	mouse brain, rat brain	Untreated	
Phospho-LRP6 (Ser1490)	HeLa	Wnt3a-conditioned media for 5 hrs (Conditioned media from L cells transfected with Wnt3a ligand)	
Phospho-Lyn (Tyr507)	Ramos	Serum-starve overnight, anti-IgM (12 µg/ml for 2 min)	
Acetylated-Lysine	COS-7	TSA (0.4 µM for 18 hrs)	
LC3A	HeLa, NIH/3T3, KNRK	Chloroquine (50 µM for 20 hrs)	
Phospho-MAP2 (Ser136/Thr1620/Thr1623)	PC-12	Positive control: Nocodazole (1 µg/ml for 16 hrs)	
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	Jurkat	TPA (200 nM for 10 min)	#9194
Phospho-MAPKAPK-2 (Thr222/Thr334)	HeLa	Serum-starve overnight, UV‡ (40 mJ/cm^2), 20 min recovery	
Phospho-p38 MAPK (Thr180/Tyr182)	C6	Anisomycin (25 µg/ml for 20 min)	#9213
Phospho-MAPK/CDK Substrates (PXSP or SPXR/K)	HeLa	Nocadazole (1 µg/ml for 12 hrs)	
Phospho-(Thr) MAPK/CDK Substrate	Jurkat	Nocadazole (1 µg/ml for 12 hrs)	
Phospho-MARCKS (Ser152/Ser156)	HeLa, NIH/3T3	Serum-starve overnight, TPA (200 nM for 15 min)	#9160
Phospho-MARK Family (Activation Loop)	Raji, BaF3	Untreated	
Phospho-Mcl-1 (Ser159/Thr163)	H929	MG132 (10 µM for 2 hrs)	
Phospho-MDM2 (Ser166)	MCF7	hIGF-I (100 ng/ml for 15 min)	
Phospho-MEK1/2 (Ser217/Ser221)	HeLa	Serum-starve overnight, TPA (200 nM for 15 min)	#9160
Phospho-MEK1 (Thr286/Ser298)	HeLa	Serum-starve overnight, Nocodazole (0.2 µg/ml for 16 hrs)	#9160
Phospho-Merlin (Ser518)	rat brain, mouse brain, human brain	Untreated	
Phospho-Met (Tyr1003/Tyr1234/Tyr1235/Tyr1349)	H-4-II-E	Serum-starve overnight, HGF (40 ng/ml for 10 min)	
Phospho-MKK3/MKK6 (Ser189/Ser207)	NIH/3T3	UV‡ (40 mJ/cm^2), 30 min recovery	#9233
Phospho-MKK7 (Ser271/Thr275)	U-937	Sorbitol (400 mM for 15 min)	
Phospho-Mnk1 (Thr197/Thr202)	HeLa	Serum-starve overnight, 20% serum for 20 min or Anisomycin (25 µg/ml for 30 min)	#9213
Phospho-MKP1 (Ser359)	HeLa	ALLN (50 µM for 5–6 hrs)	
Phospho-Mre11 (Ser676)	HeLa	UV‡ (100 mJ/cm^2), 1 hr recovery	
Phospho-MSK1 (Ser376/Thr581)	293	UV‡ (40 mJ/cm^2), 30 min recovery	
Phospho-Mst1 (Thr183)/Mst2 (Thr180)	WEHI	Staurosporine (1 µM for 30 min – 3 hrs)	
Phospho-mTOR (Ser2448/Ser2481)	MCF7	Serum-starve overnight, insulin (100 nM for 5–10 min)	#9203
Phospho-c-Myc (Thr58/Ser62)	A-431	Serum-starve overnight, TPA (200 nM for 30 min)	
Phospho-Myosin IIa (Ser1943)	HeLa, A-431, 293T	Untreated	
Phospho-Myosin Light Chain 2 (Thr18/Ser19)	C2C12	Untreated	
Phospho-MYPT1 (Ser507/Ser668/Thr853)	HeLa	Serum-starve overnight, Calyculin A (100 nM for 30 min)	
Phospho-Myt1 (Ser83)	HeLa	Nocodazole (50 ng/ml for 20 hrs)	
Phospho-NDRG1 (Ser330)	Jurkat	Calyculin A (100 nM for 20 min)	
Phospho-NDRG1 (Thr346)	C2C12	Insulin (30 min)	
Phospho-NF-κB p105 (Ser933)	HeLa	hTNF-α (20 ng/ml for 5 min)	#9243
Phospho-NF-κB p65 (Ser276)	Jurkat	Calyculin A (100 nM for 30 min)	#9273
Phospho-NF-κB p65 (Ser468/Ser536)	HeLa	hTNF-α (20 ng/ml for 5 min)	
Phospho-NMDAR2A (Tyr1246)	rat brain	Untreated	
Phospho-NMDAR2B (Tyr1070/Tyr1472)	rat brain	Untreated	
Cleaved Notch1 (Val1744)	MOLT-4, Jurkat	Untreated	
Phospho-NPM (Ser4/Thr95/Thr199)	HeLa	Nocodazole (50 ng/ml for 20 hrs)	
Phospho-NuMA (Ser395)	M059K	UV‡ (100 mJ/cm^2), 1 hr recovery	
Phospho-Numb (Ser276)	Ramos	TPA (200 nM for 30 min)	
Phospho-Nur77 (Ser351)	Jurkat	TPA (40 nM), A23187 (2 µM), both for 4 hours	
Phospho-p130 Cas (Tyr165/Tyr249/Tyr410)	NIH/3T3	Untreated	
Phospho-p40phox (Thr154)	THP-1	Serum-starve overnight, TPA (200 nM for 15 min)	
Phospho-p53 (Ser6/Ser9/Ser15/Thr18/Ser20/Ser37/Ser46/Ser315)	293	UV‡ (75 mJ/cm^2), 2 hrs recovery	#9253
Phospho-p53 (Ser33/Thr81/Ser315)	HeLa	Nocodazole (50 ng/ml for 24-48 hrs)	
Phospho-p53 (Ser392)	293	Hydroxyurea (20 mM for 30 hrs)	
Acetyl-p53 (Lys379/Lys382)	MCF7	Doxorubicin (0.5 µM for 24 hrs); 400 nM TSA can be used in conjunction to stabilize activated protein	
Phospho-p57 Kip2 (Thr310)	HeLa	Dexamethasone (50 nM for 16 hrs)	
Phospho-p63 (Ser160/Ser162)	ME-180	Nocodazole (100 ng/ml for 18 hrs)	
Phospho-p70 S6 Kinase (Thr389/Ser371/Thr421/Ser424)	MCF7	Serum-starve overnight, insulin (100 nM for 5–10 min)	#9203
Phospho-Drosophila p70 S6 Kinase (Thr398)	S2	hEGF (100 ng/ml for 30 min)	
Phospho-p73 (Tyr99)	HT-1376	Pervanadate (1 mM for 20 min)	
Phospho-p95/NBS1 (Ser343)	HeLa	UV‡ (40 mJ/cm^2), 90 min recovery	
Methyl-PABP1 (Arg455/Arg460)	HeLa, C6, COS-7	Untreated	
Phospho-PAK1 (Ser144/Ser199/Ser204/Thr423)/PAK2 (Ser20/Ser141/Ser192/Ser197/Thr402)	guinea pig neutrophils	fMLP (1 µM for 30 sec)	
Phospho-PAK4 (Ser474)/PAK5 (Ser602)/PAK6 (Ser560)	guinea pig neutrophils	fMLP (1 µM for 30 sec)	
Phospho-PAR-4 (Thr163)	HeLa	Calyculin A (100 nM for 5 min)	
Cleaved PARP (Asp214)	HeLa	Serum-starve overnight, Staurosporine (1 µM for 3 hrs)	#2043

Antibody	Cell Line	Treatment	Control Extract
Phospho-Paxillin (Tyr118)	C2C12, NIH/3T3, A-431, C6	Untreated	
Phospho-PBK/TOPK (Thr9)	HT-29	Nocodazole (50 ng/ml for 20 hrs)	
Phospho-PDGF Receptor α (Tyr754/Tyr849/Tyr1018)	NIH/3T3	Serum-starve overnight, PDGF-β (100 ng/ml for 5 min)	
Phospho-PDGF Receptor β (Tyr740/Tyr751/Tyr771/Tyr857/Tyr1009/Tyr1021)	NIH/3T3	Serum-starve overnight, PDGF-β (100 ng/ml for 5 min)	
Phospho-PDK1 (Ser241)	Jurkat, HeLa	Untreated	
Phospho-(Ser/Thr) PDK1 Docking Motif	Jurkat	Calyculin A (100 nM for 30 min)	#9273
Phospho-PEA-15 (Ser104)	C6	TPA (200 nM for 1 hr)	
Phospho-PERK (Thr980)	AR42J	Thapsigargin (1 µM for 20 min)	
Phospho-(Ser/Thr) Phe	Jurkat	Calyculin A (100 nM for 30 min)	#9273
Phospho-PI3K p85 (Tyr458)/p55 (Tyr199)	NIH/3T3/Src	Untreated	
Phospho-Pin1 (Ser16)	OVCAR8	Forskolin (30 µM for 30 min)	
Phospho-PKA C (Thr197)	HeLa	Untreated	
Phospho-PKA Substrate (RRXS/T)	Jurkat	Calyculin A (100 nM for 30 min)	#9273
Phospho-PKC (pan)	HeLa, NIH/3T3	Serum-starve overnight, TPA (200 nM for 15 min)	#9160
Phospho-(Ser) PKC Substrate	HeLa	TPA (200 nM for 30 min)	#9160
Phospho-(Ser/Thr) PKD Substrate	HeLa	TPA (200 nM for 30 min)	#9160
Phospho-PKD/PKCμ (Ser744/Ser748/Ser916)	HeLa, NIH/3T3	Serum-starve overnight, TPA (200 nM for 15 min)	#9160
Phospho-PKM2 (Tyr105)	A549	Untreated	
Phospho-cPLA2 (Ser505)	HeLa	Serum-starve overnight, TPA (200 nM for 15 min)	#9160
Phospho-PLCβ3 (Ser537/Ser1105)	HeLa	Serum-starve overnight, TPA (200 nM for 15 min)	#9160
Phospho-PLCγ1 (Tyr783/Ser1248)	NIH/3T3	Serum-starve overnight, PDGF (50 ng/ml for 30 min)	
Phospho-PLCγ2 (Tyr759/Tyr1217)	Ramos, Raji	IgM (12 µg/ml for 2 min)	
Phospho-PLD1 (Thr147/Ser561)	HeLa	Serum-starve overnight, TPA (200 nM for 15 min)	#9160
Phospho-PNK1 (Ser114/Thr118)	HeLa	UV‡ (100 mJ/cm^2), 1 hr recovery	
Phospho-PP1α (Thr320)	HeLa, COS-7, NIH/3T3, C6	Untreated	
Phospho-PPIG (Ser374)	Jurkat	Untreated	
Phospho-PRAS40 (Thr246)	NIH/3T3	Serum-starve overnight, insulin (100 nM for 5–10 min)	
Phospho-PRK1 (Thr774)/PRK2 (Thr816)	HeLa	Untreated	
Phospho-Progesterone Receptor (Ser190)	T-47D	Serum-starve overnight, promegestone (100 nM for 1 hr)	
Phospho-PSD93 (Tyr340)	rat brain	Untreated	
Phospho-PSD95 (Tyr236/Tyr240)	rat brain	Untreated	
Phospho-PTEN (Ser380/Thr382/Thr383)	HeLa	Untreated	
Phospho-PTPα (Tyr789)	293, COS-7, C6, C2C12	Untreated	
Phospho-Pyk2 (Tyr402)	Jurkat	Serum-starve overnight, anti-CD3 plus anti-CD28 (both 1 µg/ml for 10 min)	
Phospho-Rac1/cdc42 (Ser71)	A-431	Serum-starve overnight, hEGF (100 ng/ml for 10 min)	
Phospho-Rad17 (Ser645)	COS-7	UV‡ (100 mJ/cm^2), 1 hr recovery	
Phospho-A-Raf (Ser299)	HeLa	Serum-starve overnight, TPA (200 nM for 15 min)	#9160
Phospho-B-Raf (Ser445)	HeLa	Serum-starve overnight, TPA (200 nM for 15 min)	#9160
Phospho-c-Raf (Ser259/Ser289/Ser296/Ser301/Ser338)	HeLa	Serum-starve overnight, TPA (200 nM for 15 min)	#9160
Phospho-RanBP3 (Ser58)	HeLa	Serum-starve overnight, hEGF (100 ng/ml for 10 min)	
Phospho-Raptor (Ser792)	293	Oligomycin (0.5 µM for 30 min)	
Phospho-Rb (Ser608/Ser780/Ser795/Ser807/Ser811)	HT-29	Nocodazole (100 ng/ml for 18 hrs); treatment induces slight phospho-shift	
Phospho-RCC1 (Ser11)	HT-29	Thymidine (2 mM for 16 hrs) followed by Nocodazole (100 ng/ml for 24 hrs)	
Phospho-RelB (Ser552)	Jurkat	TPA (200 nM for 30 min)	
Phospho-Ret (Tyr905)	TT	Untreated	
Phospho-Rictor (Thr1135)	HeLa	Serum-starve overnight, hIGF-I (50 ng/ml for 30 min)	
Phospho-RIP2 (Ser176)	THP-1	Treat overnight with TPA (50 ng/ml), then culture in fresh medium 48 hrs to form differentiated macrophages. Induce with LPS (1 µg/ml for 10 min).	
Phospho-Rpb1 CTD (Ser2/5)	MCF7	Doxorubicin (0.5 µM for 30 hrs)	
Phospho-Ros (Tyr2274)	HCC78	HCC78 cells express a SLC3482-Ros fusion protein	
Phospho-p90RSK (Thr359/Ser363/Ser380/Thr573)	HeLa	TPA (200 nM for 30 min)	#9160
Phospho-RSK2 (Ser227)	HeLa	TPA (200 nM for 30 min)	#9160
Phospho-RSK2 (Tyr529)	SK-N-MC	Serum-starve overnight, FGF (100 ng/ml for 10 min)	
Phospho-RSK3 (Thr356/Ser360)	HeLa	Serum-starve overnight, TPA (200 nM for 15 min)	#9160
Phospho-S6 Ribosomal Protein (Ser235/Ser236/Ser240/Ser244)	MCF7	Serum-starve overnight, insulin (100 nM for 5–10 min)	#9203
Phospho-SAPK/JNK (Thr183/Tyr185)	293	UV‡ (40 mJ/cm^2), 30 min recovery time	#9253
Phospho-SATB1 (Ser47)	Jurkat	Untreated	
Phospho-SEK1/MKK4 (Ser80)	NIH/3T3	PDGF (100 ng/ml for 5 min)	
Phospho-SEK1/MKK4 (Ser257/Thr261)	HeLa	UV‡ (50 mJ/cm^2), 30 min recovery time	
Phospho-Semaphorin 4B (Ser825)	MKN-45	Untreated	
Phospho-(Ser) Arg-X-Tyr/Phe-X-pSer Motif	Jurkat	Calyculin A (100 nM for 30 min)	#9273
Phospho-SGK1 (Ser78)	SK-MEL-28	H_2O_2 (4 mM for 15 min)	
Phospho-SGK3 (Thr320)	MCF7	H_2O_2 (4 mM for 15 min)	

Antibody	Cell Line	Treatment	Control Extract
Phospho-Shc (Tyr239/Tyr240/Tyr317)	Hep G2	Serum-starve overnight, hEGF (100 ng/ml for 5 min)	
Phospho-SHIP1 (Tyr1020)	Ramos	Anti-human IgM (12 µg/ml for 2 min)	
Phospho-SHIP2 (Tyr986/Tyr987/Tyr1135)	K-562	Untreated	
Phospho-SHP-2 (Tyr542/Tyr580)	NIH/3T3	Serum-starve overnight, PDGF (100 ng/ml for 5 min)	
Phospho-SirT1 (Ser27/Ser47)	293	Untreated	
Phospho-Smad1(Ser206/Ser463/Ser465)	HeLa	Serum-starve overnight, BMP (50 ng/ml for 30 min)	
Phospho-Smad2 (Ser245/Ser250/Ser255/Ser465/Ser467)	HeLa	Serum-starve overnight, TPA (200 nM for 30 min)	
Phospho-Smad3 (Ser423/Ser425)	HT-1080	Serum-starve overnight, TGF-β (10 ng/ml for 30 min)	
Phospho-Smad5 (Ser463/Ser465)	HeLa	Serum-starve overnight, BMP (50 ng/ml for 30 min)	
Phospho-Smad8 (Ser426/Ser428)	HeLa	Serum-starve overnight, BMP (50 ng/ml for 30 min)	
Phospho-SMC1 (Ser360/Ser957)	293	UV‡ (100 mJ/cm^2), 4 hrs recovery	
Phospho-Src Family (Tyr416)	COLO201	Serum-starve overnight, treat 5 min 20% FBS	
Phospho-Src (Tyr527)	COLO201	Untreated	
Phospho-SRC-3 (Thr24)	MCF7	Untreated	
Phospho-SRF (Ser103)	HeLa	Serum-starve overnight, TPA (200 nM for 15 min)	#9160
Phospho-Stat1 (Tyr701/Ser727)	HeLa	Serum-starve overnight, IFN-α (100 ng/ml for 5 min)	#9173
Phospho-Stat2 (Tyr690)	HeLa	Serum-starve overnight, IFN-α (100 ng/ml for 15 min)	
Phospho-Stat3 (Tyr705/Ser727)	HeLa	Serum-starve overnight, IFN-α (100 ng/ml for 5 min)	#9133
Phospho-Stat4 (Tyr693)	NK-92	Cytokine-starve overnight, IL-2 (10 ng/ml for 15 min); IL-2 (5 ng/ml) should be included during growth	
Phospho-Stat5 (Tyr694)	HeLa	Serum-starve overnight, IFN-α (100 ng/ml for 5 min)	#9353
Phospho-Stat6 (Tyr641)	HeLa	Serum-starve overnight, IL-4 (100 ng/ml for 15 min)	
Phospho-Stathmin (Ser16/Ser38)	HeLa	Nocadazole (100 ng/ml for 16 hrs)	
Phospho-Synapsin (Ser9)	PC-12	NGF (100 ng/ml for 2 hrs)	
Phospho-TACC3 (Ser558)	HT-29	Thymidine (2 mM for 16 hrs), add fresh media	
Phospho-TAK1 (Thr184/Thr187/Ser412)	HeLa	hTNF-α or IL-1β (20 ng/ml), both with Calyculin A (100 nM) for 10 min	
Phospho-Tau (Ser396)	mouse brain, rat brain	Untreated	
Phospho-TCTP (Ser46)	HT-29	2 mM Thymidine for 16 hrs, then wash plate, add fresh media and Nocodazole (100 ng/ml overnight)	
Phospho-Threonine	NIH/3T3	PDGF-BB (100 ng/ml for 5 min)	
Phospho-Threonine-X-Arginine	Jurkat	Calyculin A (100 nM for 30 min)	#9273
Phospho-TIF1β (Ser824)	HeLa	IR (10 Gy), 1 hr recovery	
Phospho-TLK1 (Ser695)	SK-N-MC	Serum-starve overnight	
Phospho-TNK1 (Tyr277)	KARPAS-299	Untreated (KARPAS-299 cell lines contains NPM-ALK fusion)	
Phospho-TrkA (Tyr490/Tyr674/Tyr785)	PC-12	NGF (100 ng/ml for 2–5 min)	
Phospho-TrkB (Tyr516/Tyr706/Tyr707/Tyr816)	PC-12	NGF (100 ng/ml for 2–5 min)	
Phospho-Troponin I (Cardiac) (Ser23/24)	Primary cardiac myocytes	Isoproterenol (1 µM for 5 min)	
Phospho-Tuberin/TSC2 (Ser939/Ser1254/Thr1462/Tyr1571)	NIH/3T3	PDGF (50 ng/ml for 30 min)	
Acetyl-α-Tubulin (Lys40)	HeLa	TSA (400 nM for 16 hrs)	
Phospho-Tyk2 (Tyr1054/Tyr1055)	HeLa	Serum-starve overnight, IFN-α (100 ng/ml for 15 min)	
Phospho-Tyrosine	Jurkat	Pervanadate (1 mM for 30 min)	
Phospho-Tyrosine Hydroxylase (Ser31/Ser40)	rat brain, mouse brain, human brain, SH-SY5Y, Neuro-2a, PC-12	Untreated	
Phospho-ULK1 (Ser555)	MCF7	Oligomycin (0.5 µM for 30 min)	
Phospho-VASP (Ser157/Ser239)	A-431	Serum-starve overnight, Forskolin (10 µM for 30 min)	
Phospho-VEGF Receptor 2 (Tyr1175/Tyr1212)	HUVEC	Serum-starve overnight, VEGF (100 ng/ml for 5 min)	#2904
Phospho-Vimentin (Ser56)	HeLa	Paclitaxel (100 ng/ml for 20 hrs)	
Phospho-Vimentin (Ser82)	C2C12, C6	Untreated	
Phospho-Wee1 (Ser642)	A-431	Serum-starve overnight, hEGF (100 nM for 5 min)	
Phospho-WNK1 (Thr60)	HT-29	Serum-starve overnight, hIGF-I (100 ng/ml for 30 min)	
Phospho-YAP (Ser127)	HeLa	Untreated	
Phospho-YB1 (Ser102)	MCF7	Serum-starve overnight, hIGF-I (100 ng/ml for 30 min)	
Phospho-Zap-70 (Tyr319/Tyr493)/Syk (Tyr352)	Jurkat	Serum-starve overnight, H_2O_2 (2 mM for 2 min)	

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