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Western Immunoblotting Protocol (Primary Ab Incubation In BSA)

For Western blots, incubate membrane with diluted antibody in 5% w/v BSA, 1X TBS, 0.1% Tween-20 at 4°C with gentle shaking, overnight.

A. Solutions and Reagents

NOTE: Prepare solutions with Milli-Q or equivalently purified water.

- 1X Phosphate Buffered Saline (PBS)**
- 1X SDS Sample Buffer:** 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
- Transfer Buffer:** 25 mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5)
- 10X Tris Buffered Saline (TBS):** To prepare 1 liter of 10X TBS: 24.2 g Tris base, 80 g NaCl; adjust pH to 7.6 with HCl (use at 1X).
- Nonfat Dry Milk (weight to volume [w/v])
- Blocking Buffer:** 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk; for 150 ml, add 15 ml 10X TBS to 135 ml water, mix. Add 7.5 g nonfat dry milk and mix well. While stirring, add 0.15 ml Tween-20 (100%).
- Wash Buffer:** 1X TBS, 0.1% Tween-20 (TBS/T)
- Bovine Serum Albumin (BSA)
- Primary Antibody Dilution Buffer:** 1X TBS, 0.1% Tween-20 with 5% BSA; for 20 ml, add 2 ml 10X TBS to 18 ml water, mix. Add 1.0 g BSA and mix well. While stirring, add 20 µl Tween-20 (100%).
- Phototope®-HRP Western Blot Detection System #7071:** Includes biotinylated protein ladder, secondary anti-rabbit antibody #7074 conjugated to horseradish peroxidase (HRP), anti-biotin antibody conjugated to HRP, LumiGLO® chemiluminescent reagent and peroxide.
- Prestained Protein Marker, Broad Range (Premixed Format) #7720
- Biotinylated Protein Ladder Detection Pack #7727
- Blotting Membrane:** This protocol has been optimized for nitrocellulose membranes, which CST recommends. PVDF membranes may also be used.

B. Protein Blotting A general protocol for sample preparation is described below.

- Treat cells by adding fresh media containing regulator for desired time.
- Aspirate media from cultures; wash cells with 1X PBS; aspirate.
- 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.

- Sonicate for 10–15 seconds to shear DNA and reduce sample viscosity.
- Heat a 20 µl sample to 95–100°C for 5 minutes; cool on ice.
- Microcentrifuge for 5 minutes.
- Load 20 µl onto SDS-PAGE gel (10 cm x 10 cm).

NOTE: CST recommends loading prestained molecular weight markers (#7720, 10 µl/lane) to verify electrotransfer and biotinylated protein ladder (#7727, 10 µl/lane) to determine molecular weights.

- Electrotransfer to nitrocellulose or PVDF membrane.

C. Membrane Blocking and Antibody Incubations

NOTE: Volumes are for 10 cm x 10 cm (100 cm²) of membrane; for different sized membranes, adjust volumes accordingly.

- (Optional) After transfer, wash nitrocellulose membrane with 25 ml TBS for 5 minutes at room temperature.
- Incubate membrane in 25 ml of blocking buffer for 1 hour at room temperature.
- Wash three times for 5 minutes each with 15 ml of TBS/T.
- Incubate membrane and primary antibody (at the appropriate dilution) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.
- Wash three times for 5 minutes each with 15 ml of TBS/T.
- Incubate membrane with HRP-conjugated secondary antibody (1:2000) and HRP-conjugated anti-biotin antibody (1:1000) to detect biotinylated protein markers in 10 ml of blocking buffer with gentle agitation for 1 hour at room temperature.
- Wash three times for 5 minutes each with 15 ml of TBS/T.

D. Detection of Proteins

- Incubate membrane with 10 ml LumiGLO® (0.5 ml 20X LumiGLO®, 0.5 ml 20X Peroxide and 9.0 ml Milli-Q water) with gentle agitation for 1 minute at room temperature.

NOTE: LumiGLO® substrate can be further diluted if signal response is too fast.

- Drain membrane of excess developing solution (do not let dry), wrap in plastic wrap and expose to x-ray film. An initial 10-second 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 hours.

Western Blot Reprobing Protocol

A. Solutions and Reagents

NOTE: Prepare solutions with Milli-Q or equivalently purified water.

- Wash Buffer:** 1X Tris Buffered Saline, 0.1% Tween-20 (TBS/T)
- Stripping Buffer:** To prepare 100 ml, mix 0.76 g Tris base, 2 g SDS and 700 µl β-mercaptoethanol. Bring to 100 ml with deionized H₂O. Adjust pH to 6.8 with HCl.

B. Protocol

- After film exposure, wash membrane four times for 5 minutes each in TBS/T. Best results are obtained if the membrane is not allowed to dry.

- Incubate membrane for 30 minutes at 50°C in stripping buffer (with slight agitation).
- Wash membrane six times for 5 minutes each in TBS/T.
- (Optional) To assure that the original signal is removed, wash membrane twice for 5 minutes each with 10 ml of TBS/T. Incubate membrane with LumiGLO® with gentle agitation for 1 minute at room temperature. Drain membrane of excess developing solution. Do not let dry. Wrap in plastic wrap and expose to x-ray film.
- Wash membrane again four times for 5 minutes each in TBS/T.
- The membrane is now ready to reuse. Start detection at the "Membrane Blocking and Antibody Incubations" step in the Western Immunoblotting Protocol.

Immunoprecipitation Protocol (For Analysis By Western Immunoblotting)

A. Solutions and Reagents

NOTE: Prepare solutions with Milli-Q or equivalently purified water.

1. **1X Phosphate Buffered Saline (PBS)**
2. **1X Cell Lysis Buffer:** 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na_3VO_4 , 1 $\mu\text{g}/\text{ml}$ Leupeptin

NOTE: Add 1 mM PMSF immediately prior to use*.

3. **Transfer Buffer:** 25 mM Tris base, 0.2 mM glycine, 20% methanol (pH 8.5)
4. **Protein A or G Agarose Beads:** (Can be stored for 2 weeks at 4°C.) Please prepare according to manufacturer's instructions. Use Protein A for rabbit IgG pull down and Protein G for mouse IgG pull down.
5. **3X SDS Sample Buffer:** 187.5 mM Tris-HCl (pH 6.8 at 25°C), 6% w/v SDS, 30% glycerol, 150 mM DTT, 0.03% w/v bromophenol blue

B. Preparing Cell Lysates

1. Aspirate media. Treat cells by adding fresh media containing regulator 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 the plates on ice for 5 minutes.
4. Scrape cells off the plates and transfer to microcentrifuge tubes. Keep on ice.
5. Sonicate samples on ice four times for 5 seconds each.
6. Microcentrifuge for 10 minutes at 4°C, and transfer the supernatant to a new tube. If necessary, lysate can be stored at -80°C.

C. Immunoprecipitation

1. Add primary antibody to 200 μl cell lysate; incubate with gentle rocking overnight at 4°C.
2. Add either Protein A or G agarose beads (20 μl of 50% bead slurry). Incubate with gentle rocking for 1–3 hours at 4°C.
3. Microcentrifuge for 30 seconds at 4°C. Wash pellet five times with 500 μl of 1X cell lysis buffer. Keep on ice during washes.
4. Resuspend the pellet with 20 μl 3X SDS sample buffer. Vortex, then microcentrifuge for 30 seconds.
5. Heat the sample to 95–100°C for 2–5 minutes.
6. Load the sample (15–30 μl) on SDS-PAGE gel (12–15%).
7. Analyze sample by Western blotting (see Western Immunoblotting Protocol).

Immunoprecipitation of Denatured Proteins

A. Solutions and Reagents

NOTE: Prepare solutions with Milli-Q or equivalently purified water.

1. **Denaturing Cell Lysis Buffer:** 50 mM Tris (pH 7.5), 70 mM β -mercaptoethanol (β -ME) Add β -ME just prior to use. Pre-boil for 10 minutes.
2. **1X Cell Lysis Buffer:** 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na_3VO_4 , 1 $\mu\text{g}/\text{ml}$ Leupeptin

NOTE: Add 1 mM PMSF immediately prior to use.

3. **Protein A agarose Beads:** Add 5 ml of 1X PBS to 1.5 g of Protein A agarose beads. Incubate for 2 hours at 4°C with gentle agitation; Pellet beads by centrifugation. Wash pellet twice with PBS. Resuspend beads in 1 volume of PBS. (Can be stored for 2 weeks at 4°C.)
4. **3X SDS Sample Buffer:** 187.5 mM Tris-HCl (pH 6.8 at 25°C), 6% w/v SDS, 30% glycerol, 150 mM DTT, 0.03% w/v bromophenol blue

B. Preparing Cell Lysates

1. Aspirate media. Treat cells by adding fresh media containing regulator for desired time.
2. To harvest cells under denaturing conditions, remove media and rinse cells once with PBS.
3. Remove PBS and add 0.4 ml denaturing cell lysis buffer, (preboiled for 10 minutes immediately prior to use), to each plate (150 x 25 mm).
4. Immediately scrape cells off the plate and transfer to 2.5 ml tubes.
5. Boil for 10 minutes.
6. Add 4 volumes (1.6 mL) 1X ice-cold Cell Lysis Buffer. This is the cell lysate. If necessary, lysate can be stored at -80°C.

C. Immunoprecipitation

1. Add primary antibody to 200 μl cell lysate; incubate with gentle rocking overnight at 4°C.
2. Add Protein A agarose beads (20 μl of 50% bead slurry). Incubate with gentle rocking for 1–3 hours at 4°C.
3. Microcentrifuge for 30 seconds at 4°C. Wash pellet 5 times with 500 μl of 1X Cell Lysis Buffer. Keep on ice during washes.
4. Resuspend the pellet with 20 μl 3X SDS Sample Buffer. Vortex, then microcentrifuge for 30 seconds.
5. Heat the sample to 95–100°C for 2–5 minutes.
6. Load the sample (15–30 μl) on SDS-PAGE gel (12–15%).
7. Analyze sample by Western blotting (see Western Immunoblotting Protocol).

Immunoprecipitation Protocol (For Analysis By Kinase Assay)

A. Solutions and Reagents

NOTE: Prepare solutions with Milli-Q or equivalently purified water.

1. **1X Phosphate Buffered Saline (PBS)**
2. **1X Cell Lysis Buffer:** 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM Sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na_3VO_4 , 1 $\mu\text{g}/\text{ml}$ Leupeptin

NOTE: Add 1 mM PMSF immediately prior to use.

3. **Protein A Agarose Beads:** Add 5 ml of 1X PBS to 1.5 g of protein A agarose beads. Agitate for 2 hours at 4°C; pellet by centrifugation at 14,000 X g for 1 minute. Wash pellet twice with PBS. Resuspend beads in 1 volume of PBS. (Can be stored for 2 weeks at 4°C.)
4. **1X Kinase Buffer:** 25 mM Tris (pH 7.5), 5 mM β -glycerophosphate, 2 mM DTT, 0.1 mM Na_3VO_4 , 10 mM MgCl_2

B. Preparing Cell Lysates

1. Aspirate media. Treat cells by adding fresh media containing regulator 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 the plate on ice for 5 minutes.
4. Scrape cells off the plate and transfer to microcentrifuge tubes. Keep on ice.
5. Sonicate on ice three times for 5 seconds each.

6. Microcentrifuge for 10 minutes 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

NOTE: For immunoprecipitations with immobilized primary antibody: Add 20 μl of immobilized antibody bead slurry to 200 μl cell lysate. Incubate with gentle rocking overnight at 4°C. **Proceed to washing step 3.**

1. Add primary antibody to 200 μl cell lysate; incubate with gentle rocking overnight at 4°C.
2. Add Protein A agarose beads (20 μl of 50% bead slurry). Incubate with gentle rocking for 1–3 hours at 4°C.
3. Microcentrifuge for 30 seconds at 4°C. Wash pellet two times with 500 μl of 1X cell lysis buffer. Keep on ice during washes.
4. Wash pellet twice with 500 μl 1X kinase buffer. Keep on ice.

D. Kinase Assay

1. Suspend pellet in 40 μl 1X kinase buffer supplemented with 200 μM ATP and substrate.
2. Incubate 30 minutes at 30°C.
3. Terminate reaction with 20 μl 3X SDS sample buffer. Vortex, then microcentrifuge for 30 seconds.
4. Heat the sample to 95–100°C for 2–5 minutes.
5. Load the sample (15–30 μl) on SDS-PAGE gel.
6. Analyze sample by Western blotting (see Western Immunoblotting Protocol).

Immunofluorescence Protocol

A. Solutions and Reagents

NOTE: Prepare solutions with Milli-Q or equivalently 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.
 - Formaldehyde, 16%, methanol free (Polysciences, Inc., cat# 18814); use fresh, store opened vials at 4°C in dark, dilute in PBS for use.
 - Xylene
 - Ethanol, anhydrous denatured, histological grade, 100% and 95%
 - Distilled water (dH_2O)
 - 1X PBS/0.3% Triton X-100 (PBS/Triton):** To prepare 1 L, add 100 ml 10X PBS to 900 ml dH_2O . Add 3 ml Triton X-100 and mix.
 - 10 mM Sodium Citrate Buffer:** To prepare 1 L, add 2.94 g sodium citrate trisodium salt dihydrate ($\text{C}_6\text{H}_5\text{Na}_3\text{O}_7 \cdot 2\text{H}_2\text{O}$) to 1 L dH_2O . Adjust pH to 6.0.
 - 1X PBS, high salt (0.4M) (high salt PBS):** To prepare 1L, add 100 ml 10X PBS to 900 ml dH_2O . Add 23.38 g NaCl and mix.
 - 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.
- Vectashield Mounting Medium (Vector Labs, cat# H-1000) or Vectashield Mounting Medium with DAPI (cat# H-1200)

B. Specimen Preparation

I. Cultured Cell Lines (IF-IC)

IMPORTANT: Please check the **APPLICATIONS** section of the data sheet to verify that this product is validated and approved for **(IF-IC)**.

NOTE: This general fixation protocol will work with most antibodies and cell lines. However, we recommend you try different IF/IC fixation methods (methanol or acetone alone, aldehyde alone, or combinations of these) to identify the optimal fixation protocol for each antibody and/or cell line.

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

- Rinse cells briefly in PBS.
- Aspirate PBS, cover cells to a depth of 2-3 mm with 2-4% formaldehyde in PBS.

NOTE: Formaldehyde is toxic, use only in fume hood.

- Allow cells to fix for 15 minutes at room temperature.
- Aspirate fixative, rinse three times in PBS for 5 minutes each.

OPTION: After formaldehyde fixation, cover cells with ice-cold 100% methanol (use enough to cover cells completely to a depth of 3-5 mm, DO NOT LET CELLS DRY), incubate cells in methanol for 10 minutes in freezer, rinse in PBS for 5 minutes.

- Proceed with Immunostaining section C.

II. Paraffin Sections (IF-P)

IMPORTANT: Please check the **APPLICATIONS** section of the data sheet to verify that this product is validated and approved for **(IF-P)**.

Deparaffinization/Rehydration:

- Incubate sections in three washes of xylene for 5 minutes each.
- Incubate sections in two washes of 100% ethanol for 10 minutes each.
- Incubate sections in two washes of 95% ethanol for 10 minutes each.
- Rinse sections twice in dH_2O for 5 minutes each.

Antigen Unmasking:

- Place slides in room temperature 10 mM sodium citrate buffer pH 6.0.
- Bring slides to boiling in sodium citrate buffer using water bath or microwave, then maintain at 95-99°C for 10 minutes.
- Cool slides for 30 minutes on bench top.
- Rinse sections in dH_2O three times for 5 minutes each.
- Rinse sections in PBS for 5 minutes.
- Proceed with Immunostaining section C.

III. Frozen/Cryostat Sections (IF-F)

IMPORTANT: Please check the **APPLICATIONS** section of the data sheet to verify that this product is validated and approved for (IF-F).

NOTE: Fresh frozen/unfixed sections should be fixed immediately in 2-4% formaldehyde as follows to preserve signaling epitopes.

- Cover sections with 2-4% formaldehyde in PBS.

NOTE: Formaldehyde is toxic, use only in fume hood.

- Allow cells to fix for 15 minutes at room temperature.
- Rinse slides three times in PBS for 5 minutes each.

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.

- Block specimen in 5% normal serum from same species as secondary antibody (eg. normal goat serum, normal donkey serum) in PBS/Triton for 60 minutes.
- While blocking, prepare primary antibody by diluting as indicated on datasheet in PBS/Triton. You will need 50-100 μl per section, 25-50 μl per coverslip, chamber, or well (48 or 96 well plate).
- Aspirate blocking solution, apply diluted primary antibody.

NOTE: For double-labeling, prepare a cocktail of mouse and rabbit primary antibodies at their appropriate dilutions in PBS/Triton.

- Incubate overnight at 4°C.
- Rinse three times in PBS for 5 minutes each.

OPTION: To decrease background stain, rinse in high salt PBS for two minutes between second and third PBS rinses. Be aware, this may reduce specific staining of some antibodies.

NOTE: If using primary antibodies directly conjugated with AlexaFluor® fluorochromes, then skip to step C8.

- Incubate in fluorochrome-conjugated secondary antibody diluted in PBS/Triton for 1-2 hours at room temperature in dark.

NOTE: For double-labeling, prepare a cocktail of fluorochrome-conjugated anti-mouse and anti-rabbit primary antibodies at their appropriate dilutions in PBS/Triton.

- Rinse in PBS/high salt PBS as in step 5.
- Coverslip slides with Vectashield Mounting Medium or apply just enough to cover cells in multiwell plate.
- Seal slides by painting around edges of coverslips with nail polish.
- Examine specimens immediately using appropriate excitation wavelength, depending on fluorochrome for best results or store flat at 4°C in dark.

Immunohistochemistry Protocol (Paraffin)

A. Solutions and Reagents

NOTE: Prepare solutions with Milli-Q or equivalently purified water.

1. Xylene
2. Ethanol, anhydrous denatured, histological grade (100% and 95%)
3. Deionized water (dH₂O)
4. Hematoxylin (optional)
5. ***Wash Buffer:**
 - a. **Consult product datasheet for recommended wash buffers:**
 - 1X PBS/0.1% Tween-20 (wash buffer): To prepare 1 L add 100 ml 10X PBS to 900 ml dH₂O. 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 (Na₂HPO₄) and 2.4 g potassium phosphate, monobasic (KH₂PO₄) to 1 L dH₂O. Adjust pH to 7.4.
 - TBST:** 1X TBS/0.1% Tween-20 (wash buffer): To prepare 1 L add 100 ml 10X TBS to 900 ml dH₂O. Add 1 ml Tween-20 and mix.
 - 10X Tris Buffered Saline (TBS):** To prepare 1 L add 24.2 g Trizma® base (C₄H₁₁NO₃) and 80 g sodium chloride (NaCl) to 1 L dH₂O. Adjust pH to 7.6 with concentrated HCl.
6. **Antigen Unmasking Solution:**
 - a. **For Citrate/PBST OR Citrate/TBST:** 10 mM Sodium Citrate Buffer: To prepare 1 L, add 2.94 g sodium citrate trisodium salt dihydrate (C₆H₅Na₃O₇•2H₂O) to 1 L dH₂O. Adjust pH to 6.0.
 - b. **For EDTA/PBST OR EDTA/TBST:** 1 mM EDTA: To prepare 1 L add 0.372 g EDTA (C₁₀H₁₄N₂O₈Na₂•2H₂O) to 1 L dH₂O. Adjust pH to 8.0.
 - c. **Alternative Unmasking: 10 mM Tris:** To prepare 1 L add 1.21 g Trizma® Base (C₄H₁₁NO₃) to 1 L dH₂O. Adjust pH to 10.0.
7. **3% Hydrogen Peroxide:** To prepare, add 10 ml 30% H₂O₂ to 90 ml dH₂O.
8. **Blocking Solution:** 5% horse serum or goat serum diluted in recommended wash buffer.
9. Biotinylated secondary antibody.
10. **ABC Reagent:** (Vectastain ABC Kit, Vector Laboratories, Inc., Burlingame, CA) Prepare according to manufacturer's instructions 30 minutes before use.
11. **DAB Reagent or suitable substrate:** Prepare according to manufacturer's recommendations.

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 minutes each.
 - b. Incubate sections in two washes of 100% ethanol for 10 minutes each.
 - c. Incubate sections in two washes of 95% ethanol for 10 minutes each.
2. Wash sections twice in dH₂O for 5 minutes each.

C. Antigen Unmasking

NOTE: Consult product data sheet for specific recommendation for the unmasking solution.

1. **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 minutes. Cool slides on bench top for 30 minutes.
2. **For EDTA:** Bring slides to a boil in 1 mM EDTA pH 8.0 followed by 15 minutes at a sub-boiling temperature. No cooling is necessary.
3. **Alternate:** Bring slides to a boil in 10 mM Tris pH 10.0 followed by 10 minutes at a sub-boiling temperature. Cool slides on bench top for 30 minutes.

D. Staining

1. Wash sections in dH₂O three times for 5 minutes each.
2. Incubate sections in 3% hydrogen peroxide for 10 minutes.
3. Wash sections in dH₂O twice for 5 minutes each.

NOTE: Consult product data sheet for recommended wash buffer.
4. Wash section in wash buffer for 5 minutes.
5. Block each section with 100-400 µl blocking solution for 1 hour at room temperature.
6. Remove blocking solution and add 100-400 µl diluted primary antibody to each section. (Dilute antibody in blocking solution.) Incubate overnight at 4°C.
7. Remove antibody solution and wash sections in wash buffer three times for 5 minutes each.
8. Add 100-400 µl secondary antibody, diluted in blocking solution per manufacturer's recommendation, to each section. Incubate 30 minutes at room temperature.
9. If using ABC avidin/biotin method, make ABC reagent according to the manufacturer's instructions and incubate solution for 30 minutes at room temperature.
10. Remove secondary antibody solution and wash sections three times with wash buffer for 5 minutes each.
11. Add 100-400 µl ABC reagent to each section and incubate for 30 minutes at room temperature.
12. Remove ABC reagent and wash sections three times in wash buffer for 5 minutes each.
13. Add 100-400 µl DAB or suitable substrate to each section and monitor staining closely.
14. As soon as the sections develop, immerse slides in dH₂O.
15. If desired, counterstain sections in hematoxylin per manufacturer's instructions.
16. Wash sections in dH₂O two times for 5 minutes each.
17. Dehydrate sections:
 - a. Incubate sections in 95% ethanol two times for 10 seconds each.
 - b. Repeat in 100% ethanol, incubating sections two times for 10 seconds each.
 - c. Repeat in xylene, incubating sections two times for 10 seconds each.
18. Mount coverslips.

Immunohistochemistry Frozen Section Protocol

A. Solutions and Reagents

NOTE: Prepare solutions with Milli-Q or equivalently 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 data sheet
 - a. 10% Neutral buffered formalin
 - b. Acetone
 - c. Methanol
 - d. 16% Formaldehyde
 - d1. **3% Formaldehyde:** To prepare, add 18.75 ml 16% formaldehyde to 81.25 ml 1X TBS.
5. **10X Tris Buffered Saline (TBS):** To Prepare 1 L add 24.2 g Trizma base ($C_2H_7NO_2$) 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 $-20^\circ C$.
8. **Blocking Solution:** 1X TBS/0.3% Triton-X 100/5% normal goat serum
To prepare: add 500 μl goat serum and 30 μl Triton-X 100 to 9.5 ml 1X TBS.
9. **Biotinylated Secondary Antibody.**
10. **ABC Reagent:** (Vectastain ABC Kit, Vector Laboratories, Inc., Burlingame, CA). Prepare according to manufacturer's instructions 30 minutes before use.
11. **DAB Reagent or suitable substrate:** Prepare according to manufacturer's recommendations.

B. Sectioning

1. For tissue stored at $-80^\circ C$: remove from freezer and equilibrate at $-20^\circ C$ for approximately 15 minutes 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 minutes before fixing (this helps sections adhere to slides).

C. Fixation

NOTE: Consult product data sheet to determine the optimal fixative.

1. After sections have dried on the slide, fix in optimal fixative as directed below.
 - 1a. **10% Neutral buffered formalin:** 10 minutes at room temperature.
Proceed with staining procedure immediately.

- 1b. **Cold acetone:** 10 minutes at $-20^\circ C$. Air dry.
Proceed with staining procedure immediately.
- 1c. **Methanol:** 10 minutes at $-20^\circ C$.
Proceed with staining procedure immediately.
- 1d. **3% Formaldehyde:** 15 minutes at room temperature.
Proceed with staining procedure immediately.
- 1e. **3% Formaldehyde/methanol:** 15 minutes at room temperature, followed by 5 minutes in methanol at $-20^\circ C$ (**do not rinse in between**).
Proceed with staining procedure immediately.

D. Staining

1. Wash sections in wash buffer twice for 5 minutes.
2. Incubate for 10 minutes in 3% H_2O_2 diluted in methanol at room temperature.
3. Wash sections in wash buffer twice for 5 minutes.
4. Block each section with blocking solution for one hour at room temperature.
5. Remove blocking solution and add 100-400 μl diluted primary antibody to each section. (Dilute antibody in blocking solution). Incubate overnight at $4^\circ C$.
**Refer to product datasheet to determine the recommended dilution.*
6. Remove antibody solution and wash sections three times with wash buffer for 5 minutes each.
7. Add 100-400 μl secondary antibody, diluted in blocking solution per manufacturer's recommendation, to each section. Incubate 30 minutes at room temperature.
8. If using ABC avidin/biotin method, make ABC reagent according to the manufacturer's instructions and incubate solution for 30 minutes at room temperature.
9. Remove secondary antibody solution and wash sections three times in wash buffer for 5 minutes each.
10. Add 100-400 μl ABC reagent to each section and incubate for 30 min. at room temperature.
11. Remove ABC reagent and wash sections three times in wash buffer for 5 minutes each.
12. Add 100-400 μl DAB or suitable substrate to each section and monitor staining closely.
13. As soon as the sections develop, 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 minutes each.
16. **Dehydrate sections:**
 - a. Incubate sections in 95% ethanol two times for 10 seconds each.
 - b. Repeat in 100% ethanol, incubating sections two times for 10 seconds each.
 - c. Repeat in xylene, incubating sections two times for 10 seconds each.
17. Mount coverslips.

Flow Cytometry Protocol for Intracellular Staining Using Conjugated Secondary Antibodies

A. Solutions and Reagents

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.
2. Formaldehyde (methanol free)
3. **Incubation Buffer:** Dissolve 0.5 g bovine serum albumin (BSA) in 100ml 1X PBS. Store at $4^\circ C$

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 minutes at $37^\circ C$.
4. Chill tubes on ice for 1 minute.

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 minutes on ice.
3. Proceed with staining or store cells at $-20^\circ C$ in 90% methanol.

D. Staining Using Unlabeled Primary and Conjugated Secondary Antibodies

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

1. Aliquot $0.5-1 \times 10^6$ 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 minutes at room temperature.
5. Add the primary antibody at the appropriate dilution to the assay tubes (see individual antibody data sheet for the appropriate dilution).
6. Incubate for 30-60 minutes at room temperature.
7. Rinse as before in incubation buffer by centrifugation.
8. Resuspend cells in fluorochrome-conjugated secondary antibody*, diluted in Incubation Buffer according to the manufacturer's recommendations.
9. Incubate for 30 minutes at room temperature.
10. Rinse as before in incubation buffer by centrifugation.
11. Resuspend cells in 0.5 ml PBS and analyze on flow cytometer.

***Recommended Secondary Antibodies from Invitrogen.**

A-11070 Alexa Fluor® 488 F(ab')₂ fragment of goat anti-rabbit IgG (H+L) (1:1000 dilution)
A-11017 Alexa Fluor® 488 F(ab')₂ fragment of goat anti-mouse IgG (H+L) (1:1000 dilution)

Flow Cytometry Alternate Protocol for Signaling Analysis in Peripheral Blood

A. Solutions and Reagents

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.
2. Formaldehyde (methanol free)
3. **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 minutes at room temperature.
5. Add 1 ml of Triton X-100 in PBS to make 0.1% Triton final concentration.
6. Vortex and let stand for 30 minutes at room temperature.
7. Add 1 ml incubation buffer.
8. Pellet cells by centrifugation and aspirate supernatant.
9. Resuspend cells in cold 50% methanol in PBS (store methanol solution at -20°C until just before use).
10. Incubate at least 10 minutes on ice.
11. 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 data sheet in incubation buffer.
3. Incubate for 30-60 minutes 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 minutes 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.

*Recommended Secondary Antibodies from Invitrogen:

A-11070 Alexa Fluor® 488 F(ab)2 fragment of goat anti-rabbit IgG (H+L) (1:1000 dilution) A-11017 Alexa Fluor® 488 F(ab)2 fragment of goat anti-mouse IgG (H+L) 1:1000 dilution).

Reference:

Chow, S. et al (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.

ELISA Protocol

A. Solutions and Reagents

1. **Carbonate Buffer:** 15 mM Na_2CO_3 , 35 mM NaHCO_3 , 0.2 g/L NaN_3 (pH 9.6). Use 1 μM synthetic peptide in carbonate buffer.
2. **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.
3. **Wash Buffer:** 1X PBS containing 0.05% Tween-20 (PBST)
4. **Blocking Buffer:** 10 mg/ml bovine serum albumin (BSA) in PBST
5. **Antibody Dilution Buffer:** 1.0 mg/ml BSA in PBST
6. **Enzyme Substrate:** 1 mg/ml p-Nitrophenyl Phosphate (Sigma #N-9389) in 0.1 M diethanolamine buffer
7. **0.1 M Diethanolamine Buffer:** 97 ml diethanolamine (Sigma #398179), 100 mg MgCl_2 and 0.2 g sodium azide; add 800 ml deionized water, adjust pH to 9.8 with 10 M HCl, then add deionized water to 1 liter.

B. Coating Plates

1. Coat the wells of a 96-well microtiter plate with 100 μl of 1 μM synthetic peptide in carbonate buffer by incubating overnight at 4°C or for 2 to 6 hours at 37°C. If the peptide does not bind or absorb, try other buffers in the pH 4–8 range.
2. Discard the uncoated synthetic peptide; wash the coated wells at least three times with PBST.
3. Block remaining active sites by incubating the plate with 200 μl /well of blocking buffer for 1 hour at 37°C.
4. Wash the wells at least three times with PBST.

C. Protocol

1. Prepare appropriate dilution of primary antibody with antibody dilution buffer. Add 100 μl to wells and incubate for 2 hours at 37°C or overnight at 4°C.
2. Wash the wells at least three times with PBST.
3. Dilute secondary antibody in PBST. Add 100 μl to wells and incubate for 1 hour at 37°C.
4. Wash the wells at least three times with PBST.
5. Add 100 μl enzyme substrate and incubate for 1–3 hours at 37°C.
6. Read the absorbance at 405 nm with a microtiter plate reader.

Sandwich ELISA Protocol

A. Reagent Preparation

1. Bring all microwell strips to room temperature before use.
2. Prepare 1X Wash Buffer by diluting 20X wash buffer (included in each Pathscan® Sandwich ELISA Kit) in Milli-Q or equivalently purified water.
3. **1X Cell Lysis Buffer from CST #9803:** 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM ethylene diamine tetraacetate (EDTA), 1 mM ethylene glycol-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA), 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na_3VO_4 , 1 $\mu\text{g}/\text{ml}$ leupeptin. This buffer can be stored at 4°C for short-term use (1–2 weeks).

B. Preparing Cell Lysates

1. Aspirate media. Treat cells by adding fresh media containing regulator 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 ice-cold 1X Cell Lysis Buffer plus 1 mM phenylmethylsulfonyl fluoride (PMSF) to each plate (10 cm^2) 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 minutes 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. Add 100 μl of Sample Diluent (supplied in each Pathscan® Sandwich ELISA Kit, blue color) to a microcentrifuge tube. Transfer 100 μl of cell lysate into the tube and vortex for a few seconds. (Sample applied to the well can be diluted 1:1 when the suggested

- cell lysis buffer is used for cell extraction.) Individual data sheets for each kit provide information regarding an appropriate dilution factor for lysates and kit assay results.
3. Add 100 μl of each 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, which gives the best detection of target protein.
 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 for 1 hour at 37°C.
 6. Repeat wash procedure as in Step C4.
 7. Add 100 μl of HRP-linked secondary antibody (red color) to each well. Seal with tape and incubate the plate for 30 minutes at 37°C.
 8. Repeat wash procedure as in Step C4.
 9. Add 100 μl of TMB Substrate to each well. Seal with tape and incubate the plate for 10 minutes at 37°C or 30 minutes 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.
 11. Read results.
 - a. Visual Determination — Read within 30 minutes after adding STOP Solution.
 - b. Spectrophotometric Determination — Wipe underside of wells with a lint-free tissue. Read absorbance at 450 nm within 30 minutes after adding STOP Solution.

DELFI[®] Assay Protocol

A. Solutions and Reagents

1. **Carbonate Buffer:** 15 mM Na_2CO_3 , 35 mM NaHCO_3 , 0.2 g/L NaN_3 (pH 9.6). Use 1 μM synthetic peptide in carbonate buffer.
2. **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.
3. **Wash Buffer:** 1X PBS containing 0.05% Tween-20 (PBST)
4. **Blocking Buffer:** 10 mg/ml bovine serum albumin (BSA) in PBST
5. **Antibody Dilution Buffer:** 3% BSA in PBST
6. DELFIA® Europium-labeled Anti-mouse IgG for mouse primary antibodies or Anti-rabbit IgG (PerkinElmer Life Sciences #AD0124) for rabbit primary antibodies.
7. DELFIA® Enhancement Solution (PerkinElmer Life Sciences #1244-105)

B. Protocol

1. Coat the wells of a 96-well microtiter plate with 100 μl of 1 μM synthetic peptide in carbonate buffer by incubating overnight at 4°C or for 2 to 6 hours at 37°C. If the peptide does not bind or absorb, try other buffers in the pH 4–8 range.
2. Wash plate three times 200 $\mu\text{l}/\text{well}$ with wash buffer.
3. Block plate with 200 $\mu\text{l}/\text{well}$ blocking buffer for 1 hour at 37°C. Wash plate three times with wash buffer. (May leave dry plate at 4°C for 1–2 months if desired.)
4. Prepare appropriate dilution of primary antibody with antibody dilution buffer. Add 100 μl to wells and incubate at 37°C for 1 hour.
5. Wash three times with wash buffer.
6. Add 67 ng/well DELFIA® Europium-labeled Anti-mouse IgG, diluted in 100 $\mu\text{l}/\text{well}$ antibody dilution buffer. Incubate at 37°C for 30 minutes.
7. Wash five times with wash buffer.
8. Add 100 μl enhancement solution and incubate at 37°C for 15 minutes. Read plate at 615 nm with an appropriate time-resolved plate reader.

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