#9300 Store at –20°C

PhosphoPlus®
Rb (Ser780, Ser795, Ser807/811) Antibody Kit

☑ (10 Western mini-blots for each primary antibody)

Cell Signaling TECHNOLOGY®

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rev. 03/30/06

This product is for *in vitro* research use only and is not intended for use in humans or animals.

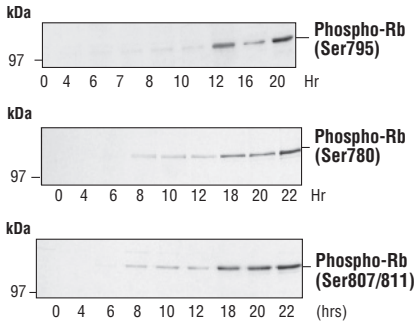
Products Included	Product #	Quantity	Applications	Species Cross-Reactivity	Mol. Wt.	Source
Phospho-Rb (Ser780) Antibody	9307	100 µl	W, IP	H, R, Mk, Z	110 kDa	rabbit
Phospho-Rb (Ser795) Antibody	9301	100 µl	W, IP	H, R, Mk, Z	110 kDa	rabbit
Phospho-Rb (Ser807/811) Antibody	9308	100 µl	W, IP, IHC-P, IF-P, IF-IC	H, R, Mk	110 kDa	rabbit
Rb (4H1) mAb	9309	100 µl	W, IP, IHC-P, IF-IC	H, R, Mk	110 kDa	mouse IgG2a
Rb Control Proteins	9303	(10 Western mini-blots)			76 kDa	recombinant fusion protein
Phototope®-HRP Western Detection System:						
Anti-rabbit IgG, HRP-linked Antibody	7074	50 µl				
Anti-biotin, HRP-linked Antibody	7075	100 µl				
20X LumiGLO® Reagent and Peroxide	7003	5 ml each				
Biotinylated Protein Ladder Detection Pack	7727	100 µl				

Background: The retinoblastoma tumor suppressor protein Rb regulates cell proliferation by controlling progression through the restriction point within the G1-phase of the cell cycle (1). Rb has three functionally distinct binding domains and interacts with critical regulatory proteins including the E2F family of transcription factors, c-Abl tyrosine kinase and proteins with a conserved LXCXE motif (2-4). Cell cycle-dependent phosphorylation by CDK's inhibits Rb target binding, thus allowing cell cycle progression (5). Rb inactivation and subsequent cell cycle progression likely requires first phosphorylation by cyclin D-CDK4/6 followed by cyclin E-CDK2 phosphorylation (6). Specificity of different CDK/cyclin complexes has been observed *in vitro* (6-8) and cyclin D1 is required for Ser780 phosphorylation *in vivo* (9).

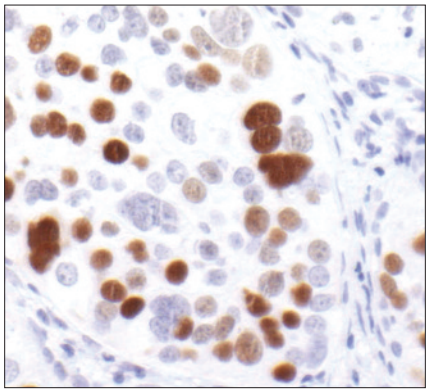
Description: The PhosphoPlus® Rb (Ser780, Ser795, Ser807/811) Antibody Kit provides reagents and protocols for the rapid analysis of Rb phosphorylation.

Specificity/Sensitivity: Phospho-Rb (Ser780, Ser795, Ser807/811) Antibodies detect endogenous levels of Rb only when phosphorylated at the target sites. The monoclonal Rb antibody does not recognize the Rb homologues p107 or p130, or other proteins.

Source/Purification: Polyclonal antibodies are produced by immunizing rabbits with synthetic peptides (KLH-coupled) corresponding to residues surrounding Ser780, Ser795 or Ser807/811 of human Rb. Polyclonal antibodies are purified by protein A and peptide affinity chromatography. Monoclonal antibody is produced by immunizing mice with a fusion protein (Rb-C Fusion Protein #6022) containing residues 701-928 of human Rb (KLH-coupled).



*Western blot analysis of Rb phosphorylation in human fibroblasts synchronized by serum deprivation, using **Phospho-Rb (Ser795) Antibody #9301** (upper), **Phospho-Rb (Ser780) Antibody #9307** (middle) and **Phospho-Rb (Ser807/811) Antibody #9308** (lower). Cells were synchronized for 24 hours, then released by addition of serum and harvested at the times indicated. Cell cycle progression was verified by cyclin analysis and FACS. (Provided by John Boylan, Dupont/Merck, Delaware.)*



*Immunohistchemical analysis of paraffin-embedded human lung carcinoma, using **Phospho-Rb (Ser807/811) Antibody #9308.***

Storage: Supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100 µg/ml BSA, 50% glycerol and less than 0.02% sodium azide. Store at –20°C. *Do not aliquot the antibody.*

Recommended Antibody Dilutions:
See table on page 2.

Companion Products:

Phospho-CDK2 (Thr160) Antibody #2561

CDK4 (DCS156) mAb #2906

CDK7 (MO1) mAb #2916

CDK6 (DCS83) mAb #3136

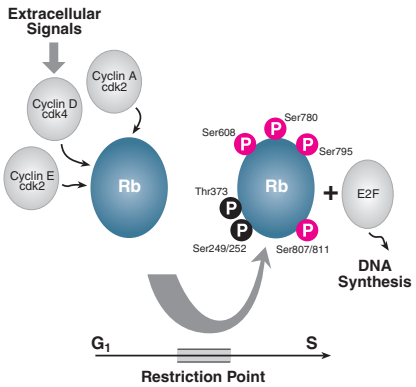
Rb-C Fusion Protein #6022

Rb Control Proteins #9303

Phototope-HRP Western Blot Detection System, Anti-rabbit IgG, HRP-linked Antibody #7071

Anti-rabbit IgG, HRP-linked Antibody #7074

Anti-mouse IgG, HRP-linked Antibody #7076



Rb Signaling Pathway

Kit Components:

Phospho Antibodies: Phospho-Rb (Ser780) Antibody; Phospho-Rb (Ser795) Antibody and Phospho-(Ser807/811) Antibody.

Control Antibody: Rb (4H1) mAb #9309 serves as the control antibody. Note that this monoclonal antibody requires an anti-mouse secondary antibody (not included in kit).

Control Proteins: Rb Control Proteins #9303 (kit contains controls for 10 Western mini-blots) with or without phosphorylation *in vitro* by cdc2/cyclin B, serve as positive and negative controls. Rb control protein is expressed as a recombinant fusion of maltose binding protein (MBP) and Rb residues 701–928. Load 10 µl per lane.

Secondary Antibodies: The Phototope®-HRP Western Detection System #7071 contains sufficient reagents for the chemiluminescent detection of rabbit antibodies on 10 (10 cm x 10 cm) Western blots. It includes a secondary anti-rabbit antibody conjugated to horseradish peroxidase (HRP), an anti-biotin antibody conjugated to HRP, a biotinylated protein ladder and LumiGLO® chemiluminescent reagent and peroxide. Avoid repeated LumiGLO exposure to skin (see MSDS enclosed with datasheet or refer to our website).

Selected Application References:

Wells, J. et al. (2003) Identification of novel pRb binding sites using CpG microarrays suggests that E2F recruits pRb to specific genomic sites during S phase. *Oncogene* 22, 1445–1460. Applications: W.

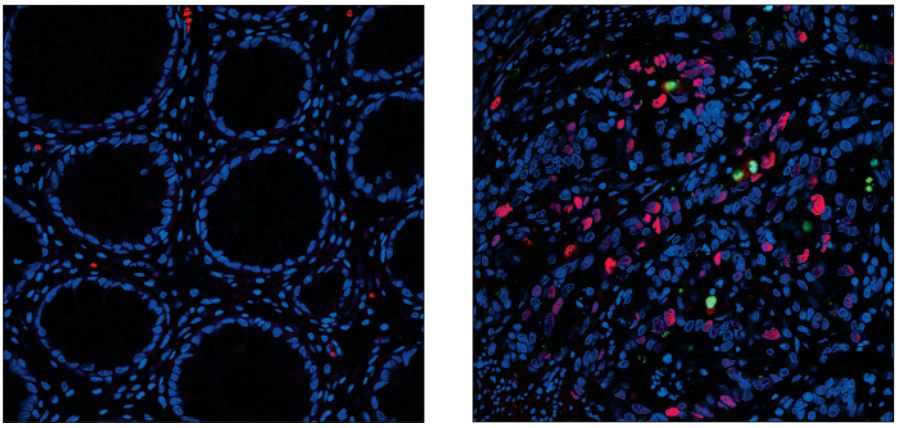
Background References:

(1) Sherr, C.J. (1996) Science 274, 1672–1677.
(2) Nevins, J.R. et al. (1992) Science 258, 424–429.
(3) Welch, P.J. and Wang, J.Y. (1993) *Cell* 75, 779–790.
(4) Hu, Q.J. et al. (1990) *EMBO J.* 9, 1147–1155.
(5) Knudsen, E.S. and Wang, J.Y. (1997) *Mol. Cell. Biol.* 17, 5771–5783.
(6) Lundberg, A.S. and Weinberg, R.A. (1998) *Mol. Cell. Biol.* 18, 753–761.
(7) Connell-Crowley, L. et al. (1997) *Mol. Cell. Biol.* 8, 287–301.
(8) Kitagawa, M. et al. (1996) *EMBO J.* 15, 7060–7069.
(9) Geng, Y. et al. (2001) *Proc. Natl. Acad. Sci. USA* 98, 194–199.

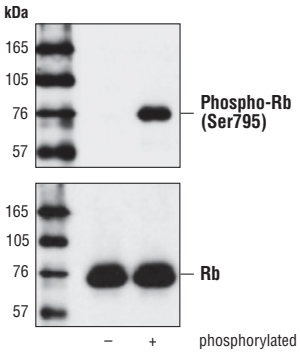
Recommended Antibody Dilutions

No.	Antibody	W	IP	IHC$^{F/P}$	IF	F
9307	Phospho-Rb (Ser780) Antibody	1:1000	1:100	N/A	N/A	N/T
9301	Phospho-Rb (Ser795) Antibody	1:1000	1:500	N/A	N/A	N/T
9308	Phospho-Rb (Ser807/811) Antibody	1:1000	1:100	1:1000^{F}/1:150^{P}	1:200	N/T
9309	Rb (4H1) mAb	1:2000	1:100	1:50^{P}	1:200	N/T

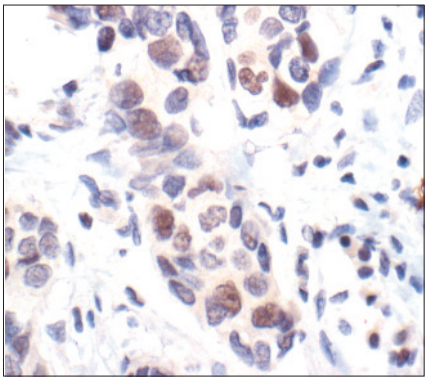
IHC$^{F/P}$ = Immunohistochemistry-Floating or -Paraffin IF = Immunofluorescence
N/A = Not Applicable NT = Not Tested



Confocal immunofluorescent images of normal human colon (left) and colon cancer (right) labeled with Phospho-Rb (Ser807/811) Antibody (red) and Phospho-Histone H3 (Ser10) (6G3) Mouse mAb #9706 (green). Blue pseudocolor = DRAQ5™ (fluorescent DNA dye).



*Western blot analysis of Rb Control Protein #9303, using **Phospho-Rb (Ser795) Antibody #9301** (upper) or Rb (4H1) mAb #9309 (lower).*



*Immunohistochemical staining of Rb in paraffin-embedded human breast carcinoma showing nuclear localization, using **Rb (4H1) mAb #9309.***

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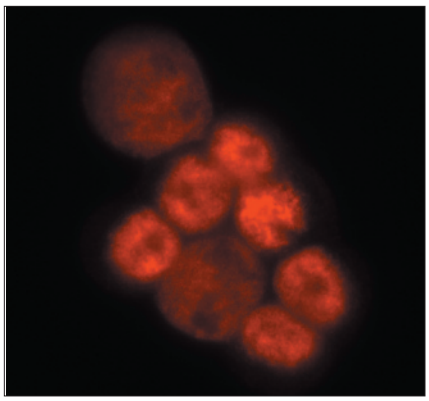
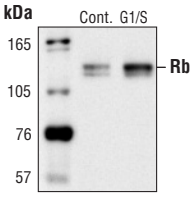
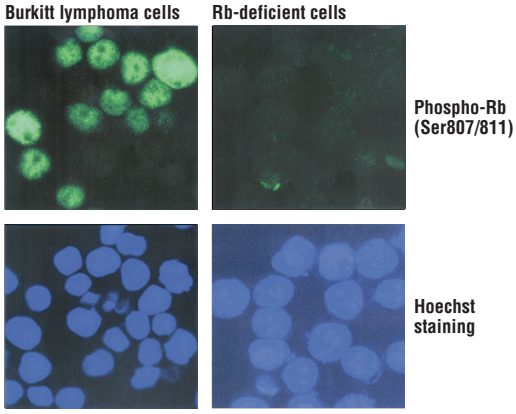


Immunofluorescent detection of Rb in the nuclei of HT29 cells, using ***Rb (4H1) mAb #9309.***



Western blot analysis of extracts from control (cont.) and 2 mM hydroxyurea treated (G1/S) COS-7 cell extracts, using ***Rb (4H1) mAb #9309.***



Immunocytochemical staining of Burkitt lymphoma cell line (Ramos) or Rb-deficient cell line (Weri-Rb-27), using ***Phospho-Rb (Ser807/811) Antibody #9308*** *(green). (Provided by Dr. Akihiko Maeda and Dr. George Klein, Karolinska Institute, Microbiology and Tumorbiology Center, Sweden.)*

Western Immunoblotting Protocol

For Western blots, incubate membrane with diluted antibody in 5% w/v nonfat dry milk (for monoclonal antibodies) or 5% BSA (for polyclonal antibodies, 1X TBS, 0.1% Tween-20 at 4°C with gentle shaking, overnight.

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)
- Primary Antibody Dilution Buffer:
  1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk (for a monoclonal antibody) or 5% BSA (for a polyclonal antibody); for 20 ml, add 2 ml 10X TBS to 18 ml water, mix. Add 1.0 g nonfat dry milk and mix well. While stirring, add 20 µl Tween-20 (100%).
- Phototope®-HRP Western Blot Detection System #7076:
  Includes biotinylated protein marker, secondary anti-mouse antibody 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.

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^2 plate). Immediately scrape the cells off the plate and transfer the extract to a microcentrifuge tube. Keep on ice.
4. Sonicate for 10–15 seconds to shear DNA and reduce sample viscosity.
5. Heat a 20 µl sample to 95–100°C for 5 minutes; cool on ice.
6. Microcentrifuge for 5 minutes.
7. 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 markers (#7726, 10 µl/lane) to determine molecular weights.

8. Electrotransfer to nitrocellulose membrane.

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 minutes at room temperature.
2. Incubate membrane in 25 ml of blocking buffer for 1 hour at room temperature.
3. Wash three times for 5 minutes each with 15 ml of TBS/T.
4. Incubate membrane and primary antibody (at the appropriate dilution) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.
5. Wash three times for 5 minutes each with 15 ml of TBS/T.
6. 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.
7. Wash three times for 5 minutes each with 15 ml of TBS/T.

Detection of Proteins

1. 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.

2. 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.

III Immunoprecipitation / Western Immunoblotting Protocol

A. Solutions and Reagents

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

A1. ■ 1X Phosphate Buffered Saline (PBS)

A2. ■ 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-Glycerolphosphate
1 mM Na_3VO_4
1 µg/ml Leupeptin

Note: CST recommends adding 1 mM PMSF before use.*

A3. ■ Transfer Buffer:
25 mM Tris base, 0.2 mM glycine, 20% methanol (pH 8.5)

A4. ■ 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.

A5. ■ 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

B1. Aspirate media. Treat cells by adding fresh media containing regulator for desired time.

B2. To harvest cells under nondenaturing conditions, remove media and rinse cells once with ice-cold PBS.

B3. Remove PBS and add 0.5 ml 1X ice-cold cell lysis buffer plus 1 mM PMSF* to each plate (10 cm) and incubate the plates on ice for 5 minutes.

B4. Scrape cells off the plates and transfer to microcentrifuge tubes. Keep on ice.

B5. Sonicate samples on ice four times for 5 seconds each.

B6. Microcentrifuge for 10 minutes at 4°C, 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

C1. Take 200 µl cell lysate and add primary antibody; incubate with gentle rocking overnight at 4°C.

C2. 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.

C3. Microcentrifuge for 30 seconds at 4°C. Wash pellet five times with 500 µl of 1X cell lysis buffer. Keep on ice during washes.

C4. Resuspend the pellet with 20 µl 3X SDS sample buffer. Vortex, then microcentrifuge for 30 seconds.

C5. Heat the sample to 95–100°C for 2–5 minutes.

C6. Load the sample (15–30 µl) on SDS-PAGE gel (12–15%).

C7. Analyze sample by Western blotting (see Western Immunoblotting Protocol).

Immunohistochemistry Protocol (Paraffin)

***IMPORTANT:** See product data sheet for the appropriate wash buffer and antigen unmasking procedure.

- For Citrate/PBST, use steps A5a, A6a and C1.
- For Citrate/TBST, use steps A5b, A6a and C1.
- For EDTA/PBST, use steps A5a, A6b and C2.
- For EDTA/TBST, use steps A5b, A6b and C2.

A Solutions and Reagents

A1 Xylene

A2 Ethanol, anhydrous denatured, histological grade (100% and 95%)

A3 Deionized water (dH_2O)

A4 Hematoxylin (optional)

A5 *Wash Buffer:

A5a For Citrate/PBST OR EDTA/PBST: 1X PBS/0.1% Tween-20 (wash buffer): To prepare 1 L add 100 ml 10X PBS to 900 ml dH_2O. Add 1ml 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 phophate, dibasic (Na_2HPO_4) and 2.4 g potassium phosphate, monobasic (KH_2PO_4) to 1 L dH_2O. Adjust pH to 7.4.

A5b For Citrate/TBST OR EDTA/TBST: 1X TBS/0.1% Tween-20 (wash buffer): 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.

A6 *Antigen Unmasking Solution:

A6a For Citrate/PBST OR Citrate/TBST: 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.

A6b For EDTA/PBST OR EDTA/TBST: 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.

A6c Alternative Unmasking: 10 mM Tris: To prepare 1 L add 1.21 g Trizma® Base ($C_4H_{11}NO_3$) to 1 L dH_2O. Adjust pH to 10.0.

A7 3% Hydrogen Peroxide: To prepare, add 10 ml 30% H_2O_2 to 90 ml dH_2O.

A8 Blocking Solution: 5% horse serum or goat serum diluted in recommended wash buffer.

A9 Biotinylated secondary antibody.

A10 ABC Reagent: (Vectastain ABC Kit, Vector Laboratories, Inc., Burlingame, CA) Prepare according to manufacturer's instructions 30 minutes before use.

A11 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.

Note: Consult product data sheet for recommended wash buffer.

B1 Deparaffinize/hydrate sections:

B1a Incubate sections in three washes of xylene for 5 minutes each.

B1b Incubate sections in two washes of 100% ethanol for 10 minutes each.

B1c Incubate sections in two washes of 95% ethanol for 10 minutes each.

B2 Wash sections twice in dH_2O for 5 minutes each.

C *Antigen Unmasking

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

C1 For Citrate/PBST OR Citrate/TBST: 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.

C2 For EDTA/PBST OR EDTA/TBST: 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.

C3 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

D1 Wash sections in dH_2O three times for 5 minutes each.

D2 Incubate sections in 3% hydrogen peroxide for 10 minutes.

D3 Wash sections in dH_2O twice for 5 minutes each.

Note: Consult product data sheet for recommended wash buffer.

D4 Wash section in wash buffer for 5 minutes.

D5 Block each section with 100-400 µl blocking solution for 1 hour at room temperature.

D6 Remove blocking solution and add 100-400 µl diluted primary antibody to each section. (Dilute antibody in blocking solution.) Incubate overnight at 4°C.

D7 Remove antibody solution and wash sections in wash buffer three times for 5 minutes each.

D8 Add 100-400 µl secondary antibody, diluted in blocking solution per manufacturer's recommendation, to each section. Incubate 30 minutes at room temperature.

D9 If using ABC avidin/biotin method, make ABC reagent according to the manufacturer's instructions and incubate solution for 30 minutes at room temperature.

D10 Remove secondary antibody solution and wash sections three times with wash buffer for 5 minutes each.

D11 Add 100-400 µl ABC reagent to each section and incubate for 30 minutes at room temperature.

D12 Remove ABC reagent and wash sections three times in wash buffer for 5 minutes each.

D13 Add 100-400 µl DAB or suitable substrate to each section and monitor staining closely.

D14 As soon as the sections develop, immerse slides in dH_2O.

D15 If desired, counterstain sections in hematoxylin per manufacturer's instructions.

D16 Wash sections in dH_2O two times for 5 minutes each.

D17 Dehydrate sections:

D17a Incubate sections in 95% ethanol two times for 10 seconds each.

D17b Repeat in 100% ethanol, incubating sections two times for 10 seconds each.

D17c Repeat in xylene, incubating sections two times for 10 seconds each.

D18 Mount coverslips.

Immunofluorescence Protocol

***IMPORTANT:** Please refer to the **APPLICATIONS** section on the front page of the data sheet to determine **IF THIS PRODUCT** is validated and approved for the specific protocol you will be using

A Solutions and Reagents

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

A1 **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.

A2 Formaldehyde, 16%, methanol free, Polysciences, Inc. (cat# 18814), use fresh, store opened vials at 4°C in dark, dilute in PBS for use.

A3 Xylene

A4 Ethanol, anhydrous denatured, histological grade, 100% and 95%

A5 Distilled water (dH_2O)

A6 **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.

A7 **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.

A8 **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.

A9 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.

A10 Vectashield Mounting Medium (Vector Labs, Burlingame, CA, 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.

1. Rinse cells briefly in PBS.

2. 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.

3. Allow cells to fix for 15 minutes at room temperature.

4. 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.

5. 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:

1. Incubate sections in three washes of xylene for 5 minutes each.

2. Incubate sections in two washes of 100% ethanol for 10 minutes each.

3. Incubate sections in two washes of 95% ethanol for 10 minutes each.

4. Rinse sections twice in dH_2O for 5 minutes each.

Antigen Unmasking:

1. Place slides in room temperature 10 mM sodium citrate buffer pH 6.0.

2. Bring slides to boiling in sodium citrate buffer using water bath or microwave, then maintain at 95-99°C for 10 minutes.

3. Cool slides for 30 minutes on bench top.

4. Rinse sections in dH_2O three times for 5 minutes each.

5. Rinse sections in PBS for 5 minutes.

6. 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.

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

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

2. Allow cells to fix for 15 minutes at room temperature.

3. 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.

C1 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.

C2 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).

C3 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.

C4 Incubate overnight at 4°C.

C5 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.

C6 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.

C7 Rinse in PBS/high salt PBS as in step 5.

C8 Coverslip slides with Vectashield Mounting Medium or apply just enough to cover cells in multiwell plate.

C9 Seal slides by painting around edges of coverslips with nail polish.

C10 Examine specimens immediately using appropriate excitation wavelength, depending on fluorochrome for best results or store flat at 4°C in dark.