#4058 Store at –20°C

Phospho-Akt (Ser473) (193H12) Rabbit mAb

☐ Small 100 µl
(10 Western mini-blots)

☐ Large 300 µl
(30 Western mini-blots)

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rev. 11/14/07

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

Applications	Species Cross-Reactivity*	Molecular Wt.	Source	Isotype
W, IP, IF-IC, F	H, M, R	60 kDa	Rabbit**	IgG

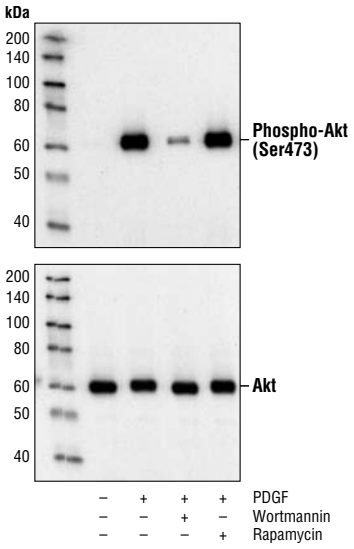
Background: Akt, also referred to as PKB or Rac, plays a critical role in controlling survival and apoptosis (1-3). This protein kinase is activated by insulin and various growth and survival factors to function in a wortmannin-sensitive pathway involving PI3 kinase (2,3). Akt is activated by phospholipid binding and activation loop phosphorylation at Thr308 by PDK1 (4) and by phosphorylation within the carboxy terminus at Ser473. The previously elusive PDK2 responsible for phosphorylation of Akt at Ser473 has been identified as mammalian target of rapamycin (mTor) in a rapamycin-insensitive complex with rictor and Sin1 (5,6). Akt promotes cell survival by inhibiting apoptosis by phosphorylating and inactivating several targets, including Bad (7), forkhead transcription factors (8), c-Raf (9) and caspase-9. PTEN phosphatase is a major negative regulator of the PI3 kinase/Akt signaling pathway (10). LY294002 is a specific PI3 kinase inhibitor (11).

Another essential Akt function is the regulation of glycogen synthesis through phosphorylation and inactivation of GSK-3α and β (12,13). Akt may also play a role in insulin stimulation of glucose transport (12).

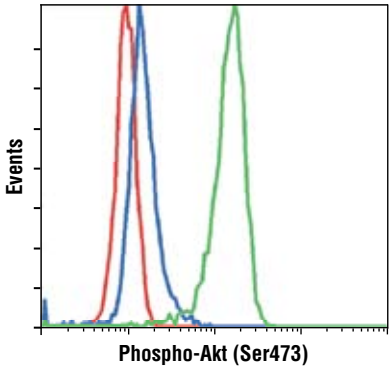
In addition to its role in survival and glycogen synthesis, Akt is involved in cell cycle regulation by preventing GSK-3β mediated phosphorylation and degradation of cyclin D1 (14) and by negatively regulating the cyclin dependent kinase inhibitors p27 Kip (15) and p21 Waf1 (16). Akt also plays a critical role in cell growth by directly phosphorylating mTOR in a rapamycin-sensitive complex containing raptor (17). More importantly, Akt phosphorylates and inactivates tuberin (TSC2), an inhibitor of mTOR within the mTOR-raptor complex (18). Inhibition of mTOR stops the protein synthesis machinery due to inactivation of its effector, p70 S6 kinase and activation of the eukaryotic initiation factor 4E binding protein 1 (4E-EP1), an inhibitor of translation (18,19).

Specificity/Sensitivity: Phospho-Akt (Ser473) (193H12) Rabbit mAb detects endogenous levels of Akt only when phosphorylated at Ser473.

Source/Purification: Monoclonal antibodies are produced by immunizing rabbits with a synthetic phospho-peptide (KLH-coupled) corresponding to residues around Ser473 of mouse Akt.



Western blot analysis of extracts from untreated or PDGF-treated NIH/3T3 cells, pretreated with wortmannin and/or rapamycin as indicated, using Phospho-Akt (Ser473) (193H12) Rabbit Monoclonal Antibody (upper) or Akt Antibody #9272 (lower).



Flow cytometric analysis of Jurkat cells, untreated (green) or LY294002 and Wortmannin treated (blue), using Phospho-Akt (Ser473) (193H12) Rabbit mAb compared to a nonspecific negative control antibody (red).

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.*

***Species cross-reactivity is determined by Western blot.**

****Anti-rabbit secondary antibodies must be used to detect this antibody.**

Recommended Antibody Dilutions:

Western blotting	1:1000
Immunoprecipitation	1:200
Immunofluorescence (IF-IC)	1:200
Flow Cytometry	1:100

Companion Products:

Phospho-Akt (Ser473) (D9E) Rabbit mAb #4060

Phospho-Akt (Thr308) (244F9) Rabbit mAb #4056

Akt2 (5B5) Rabbit mAb #2964

Akt2 (54G8) Rabbit mAb (IHC Specific) #4057

Phospho-Akt (Ser473) (736E11) Rabbit mAb (IHC Specific) #3787

SignalSilence® Akt siRNA Kit #6210

SignalSilence® Akt2 siRNA Kit #6395

PathScan® Phospho-Akt1 (Ser473) Sandwich ELISA Kit #7160

PathScan® Akt1 Sandwich ELISA Kit #7170

Akt Antibody #9272

Phospho-Akt Pathway Sampler Kit #9916

LY294002 (PI3 Kinase Inhibitor) #9901

Phospho-Akt (Ser473) Antibody #9271

Immobilized Akt (1G1) Mouse mAb #9279

Phospho-Akt (Ser473) (587F11) Mouse mAb #4051

Akt Control Cell Extracts #9273

IMPORTANT: 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.

Applications Key: **W**—Western **IP**—Immunoprecipitation **IHC**—Immunohistochemistry **IC**—Immunocytochemistry **IF**—Immunofluorescence **F**—Flow cytometry **E**—ELISA **D**—DELFIA®

Species Cross-Reactivity Key: **H**—human **M**—mouse **R**—rat **Hm**—hamster **Mk**—monkey **Mi**—mink **C**—chicken **X**—Xenopus **Z**—zebra fish **B**—bovine **All**—all species expected

Species enclosed in parentheses are predicted to react based on 100% sequence homology.

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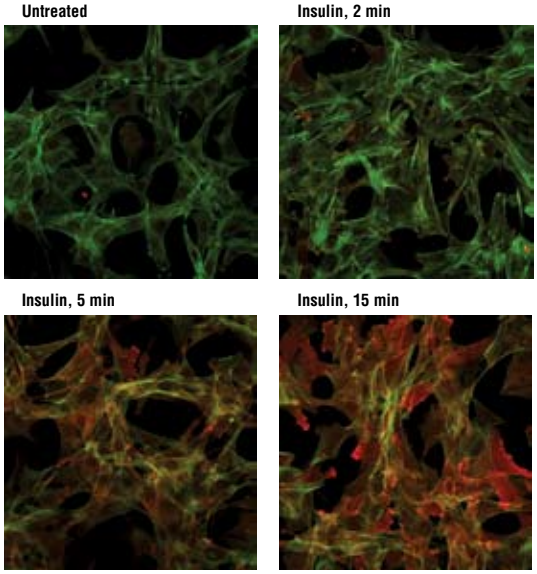
	Akt Antibody Comparision Table		Reactivity	WB	IP	Immunohistochemistry Paraffin	Immunohistochemistry Frozen	IF Cultured	Immunofluorescense In-cell Western
NEW	**#4685**	**Akt (pan) (11E7) Rabbit mAb**	H, M, R, Mk	+++	+++	+++	N/T	+++	+++	N/T
	#4051	Phospho-Akt (Ser473) (587F11) Mouse mAb	H, M, R, Hm	+++	+++	++	N/T	-	N/T	N/T
	#4058	Phospho-Akt (Ser473) (193H12) Rabbit mAb	H, M, R	+++	+++	-	N/T	+++	+++	+++
	#2336	Phospho-Akt (Ser473) (193H12) Rabbit mAb (Alexa Fluor® 488 Conjugate)	H, M, R	N/T	N/T	N/T	N/T	+++	N/T	N/T
	#2337	Phospho-Akt (Ser473) (193H12) Rabbit mAb (Alexa Fluor® 647 Conjugate)	H	N/T	N/T	N/T	N/T	+++	N/T	N/T
	#9271	Phospho-Akt (Ser473) Antibody	H, M, R, C, Hm	+++	++	-	N/T	+	++	+++
	#3787	Phospho-Akt (Ser473) (736E11) Rabbit mAb (IHC Specific)	H, M, (R predicted)	-	-	+++	+++	-	N/T	N/T
	#4056	Phospho-Akt (Thr308) (244F9) Rabbit mAb	H, M, R	+++	+++	-	N/T	-	N/T	+++
	#9266	Phospho-Akt (Thr308) (244F9H2) Rabbit mAb (IHC Specific)	H	N/T	N/T	+++	N/T	N/T	N/T	N/T
	#9275	Phospho-Akt (Thr308) Antibody	H, M, R, Hm, (B, C, X predicted)	+++	++	-	N/T	+++	N/T	N/T
	#2968	Phospho-Akt (Tyr326) Antibody	M, (H, R predicted)	+++	-	-	N/T	-	N/T	N/T
	#2966	Akt (5G3) Mouse mAb	H, M, R, Hm	-	+++	-	N/T	+++	+++	+++
	#9272	Akt Antibody	H, M, R, C, Dr, Hm	+++	+++	-	N/T	++	+++	+++
	#9279	Immobilized Akt (1G1) Mouse mAb	H, M, R, Hm	N/T	+++	N/T	N/T	N/T	N/T	N/T
	#2967	Akt1 (2H10) Mouse mAb	H, M, R	+++	+++	+++	N/T	+++	+++	+++
	#2962	Akt2 Antibody	H, M, R	+++	+++	-	N/T	-	N/T	N/T
	#2964	Akt2 (5B5) Rabbit mAb	H, M, R, Mk	+++	+++	-	N/T	-	N/T	N/T
	#4057	Akt2 (54G8) Rabbit mAb (IHC Specific)	H, (M, R predicted)	-	-	+++	N/T	-	N/T	N/T
	#4059	Akt3 Antibody	H M R	+++	+++	-	N/T	N/T	N/T	N/T

Table key: **+++** highly recommended / **++** recommended / **-** not recommended / **N/T** not tested



Confocal immunofluorescent images of C2C12 cells either serum starved or treated with insulin as indicated and labeled with Phospho-Akt (Ser473) (193H12) Rabbit mAb (red). Actin filaments have been labeled with fluorescein phalloidin.

Selected Application References:

Tu, Y. et al. (2007) Antiproliferative autoantigen CDA1 transcriptionally up-regulates p21(Waf1/Cip1) by activating p53 and MEK/ERK1/2 MAPK pathways. *J Biol Chem* 282, 11722–31. Application: W.

Radhakrishnan, S. et al. (2007) B7-DC/PD-L2 cross-linking induces NF-κB-dependent protection of dendritic cells from cell death. *J Immunol* 178, 1426–32. Applications: Flow Cytometry, W.

Simoncic, P.D. et al. (2006) T-cell protein tyrosine phosphatase (Tcptp) is a negative regulator of colony-stimulating factor 1 signaling and macrophage differentiation. *Mol Cell Biol* 26, 4149–60. Application: W.

Background References:

(1) Franke, T.F. et al. (1997) *Cell* 88, 435–7.

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(17) Navé, B.T. et al. (1999) *Biochem J* 344 Pt 2, 427–31.

(18) Inoki, K. et al. (2002) *Nat Cell Biol* 4, 648–57.

(19) Manning, B.D. et al. (2002) *Mol Cell* 10, 151–62.

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Western Immunoblotting Protocol (Primary Antibody 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.

1. 1X Phosphate Buffered Saline (PBS)
2. **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
3. **Transfer Buffer:** 25 mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5)
4. **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).
5. Nonfat Dry Milk (weight to volume [w/v])
6. **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%).
7. **Wash Buffer:** 1X TBS, 0.1% Tween-20 (TBS/T)
8. Bovine Serum Albumin (BSA)
9. **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%).
10. **Phototope®-HRP Western Blot Detection System** #7071**:** Includes biotinylated protein ladder, secondary anti-rabbit (#7074) antibody conjugated to horseradish peroxidase (HRP), anti-biotin antibody conjugated to HRP, LumiGLO® chemiluminescent reagent and peroxide.
11. Prestained Protein Marker, Broad Range (Premixed Format) #7720
12. Biotinylated Protein Ladder Detection Pack #7727
13. **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.

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 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 ladder (#7727, 10 µl/lane) to determine molecular weights.

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.

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.

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 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.

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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 µg/ml Leupeptin

NOTE: Add 1 mM PMSF immediately prior to use.

1. **Transfer Buffer:** 25 mM Tris base, 0.2 mM glycine, 20% methanol (pH 8.5)
2. **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.
3. **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 ice-cold 1X 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 three times for 5 seconds each.
6. Microcentrifuge for 10 minutes at 14,000 X g, 4°C, and transfer the supernatant to a new tube. If necessary, lysate can be stored at –80°C.

C Immunoprecipitation

Optional: It may be necessary to perform a lysate pre-clearing step to reduce non-specific binding to the Protein A/G agarose beads (See section below).

1. Take 200 µl cell lysate and add primary antibody. 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 and microcentrifuge for 1 minute at 14,000 X g.
6. Load the sample (15–30 µl) on SDS-PAGE gel (12–15%).
7. Analyze sample by Western blotting (see Western Immunoblotting Protocol).

Cell Lysate Pre-Clearing (Optional)

1. Take 200 µl cell lysate and add to either Protein A or G agarose beads (20 µl of 50% bead slurry).
2. Incubate at 4°C for 30 – 60 minutes.
3. Spin for 10 minutes at 4°C. Transfer the supernatant to a fresh tube.
4. Proceed to step 1 of Immunoprecipitation.

Flow Cytometry Protocol for Intracellular Staining Using Conjugated Secondary Antibodies

A Solutions and Reagents

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 distilled water (dH_2O). Adjust the pH to 7.4 with HCl and the volume to 1 liter. Store at room temperature.
2. Formaldehyde (methanol free)
3. **Incubation Buffer:** Dissolve 0.5 g bovine serum albumin (BSA) in 100mL 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 minutes at 37°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°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 0.5-1x10^6 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 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)

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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.

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, 16%, methanol free, Polysciences, Inc. (cat# 18814), use fresh, store opened vials at 4°C in dark, dilute in PBS for use.
3. Xylene
4. Ethanol, anhydrous denatured, histological grade, 100% and 95%
5. Distilled water (dH_2O)
6. **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.
7. **10 mM Sodium Citrate Buffer:** To prepare 1 L, add 2.94 g sodium citrate trisodium salt dihydrate ($C_6H_5Na_3O7 \bullet 2H_2O$) to 1 L dH_2O. Adjust pH to 6.0.
8. **1X PBS, high salt (0.4M) (high salt PBS):** To prepare 1L, add 100 ml 10X PBS to 900 ml dH20. Add 23.38 g NaCl and mix.
9. 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.

10. Prolong® Gold Antifade Reagent (Invitrogen, Eugene, OR, Cat# P36930)

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.

5.Methanol Permeabilization Step (if required, please refer to front page): 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.

6. 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.

1. 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.
2. 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).
3. 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.

4. Incubate overnight at 4°C.
5. 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.

6. 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.

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

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