

**#3641** Store at -20°C

# Phospho- $\delta$ -Opioid Receptor (Ser363) Antibody

100  $\mu$ l  
 (10 Western mini-blot)



**Orders** ■ 877-616-CELL (2355)  
 orders@cellsignaling.com  
**Support** ■ 877-678-TECH (8324)  
 info@cellsignaling.com  
**Web** ■ www.cellsignaling.com

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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
W, IF-F, IF-IC Transfected	H, M	50 to 65 kDa	Rabbit

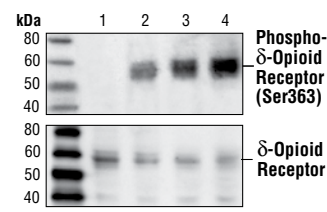
**Background:** Phosphorylation is an essential signal involved in desensitization and internalization of G-protein-coupled receptors (GPCRs) (1). The opioid receptors, like other GPCRs, are typically phosphorylated at multiple sites upon agonist binding (2,3). However, little is known about the specific role of each phosphorylation site in GPCR desensitization and internalization or about how the kinetics of phosphate addition affects these important processes. Phosphorylation of the  $\delta$ -opioid receptor (DOR) is hierarchical and involves at least two sites: threonine 358 and serine 363 (4). Serine 363 appears to be the primary phosphorylation site induced by the DOR-specific agonist DPDPE (4). Phosphorylation of DOR at serine 363 correlates with receptor internalization (4).

**Specificity/Sensitivity:** Phospho- $\delta$ -Opioid Receptor (Ser363) Antibody detects transfected  $\delta$ -opioid receptor only when phosphorylated at serine 363.

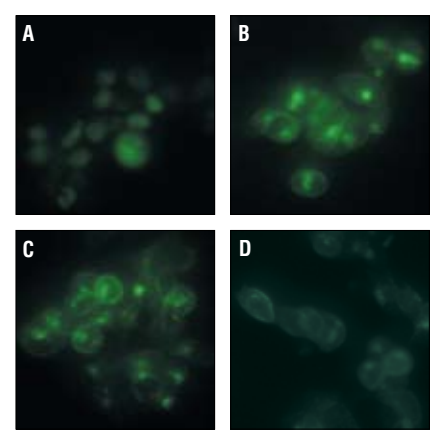
**Source/Purification:** Polyclonal antibodies are produced by immunizing rabbits with a synthetic phospho-peptide (KLH-coupled) corresponding to residues around serine 363 of human  $\delta$ -opioid receptor. Antibodies are purified by protein A and peptide affinity chromatography.

**Background References:**

- (1) Claing, A. et al. (2002) *Prog. Neurobiol.* 66, 61–79.
- (2) Arden, J.R. et al. (1995) *J. Neurochem.* 65, 1636–1645.
- (3) Pei, G. et al. (1995) *Mol. Pharmacol.* 48, 173–177.
- (4) Maestri-Ei Kouhen, O. et al. (2000) *J. Biol. Chem.* 275, 36659–36664.



Western blot analysis of extracts from flag-tagged  $\delta$ -opioid receptor stably transfected 293 cells untreated (lane 1), treated with 0.1  $\mu$ M DADLE (lane 2), 1  $\mu$ M MENK (lane 3) or 1  $\mu$ M DPDPE (lane 4) for 30 minutes each, using Phospho- $\delta$ -Opioid Receptor (Ser363) Antibody (upper) or flag-tagged antibody (lower).



Immunofluorescent staining of flag-tagged  $\delta$ -opioid receptor stably transfected 293 cells untreated (A) or treated with 0.1  $\mu$ M DADLE (B, C, D), using Phospho- $\delta$ -Opioid Receptor (Ser363) Antibody alone (A, B) or the same antibody preincubated with the corresponding nonphospho-antigenic peptide (C) or phospho-antigenic peptide (D).

**Entrez-Gene ID #** 4985  
**Swiss-Prot Acc. #** P41143

**Storage:** Supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100  $\mu$ g/ml BSA and 50% glycerol. Store at -20°C. Do not aliquot the antibody.

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

**Recommended Antibody Dilutions:**

Western blotting	1:1000
Immunofluorescence (IF-IC)	1:100
Immunofluorescence (IF-F)	1:100

**Companion Products:**

- Phospho- $\mu$ -Opioid Receptor (Ser375) Antibody #3451
- Anti-rabbit IgG, HRP-linked Antibody #7074
- Prestained Protein Marker, Broad Range (Premixed Format) #7720
- Biotinylated Protein Ladder Detection Pack #7727
- 20X LumiGLO® Reagent and 20X Peroxide #7003

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

## 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.

- 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<sup>®</sup>-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<sup>®</sup> 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 cm<sup>2</sup>) 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<sup>®</sup> (0.5 ml 20X LumiGLO<sup>®</sup>, 0.5 ml 20X Peroxide and 9.0 ml Milli-Q water) with gentle agitation for 1 minute at room temperature.

**NOTE:** LumiGLO<sup>®</sup> 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<sup>®</sup> incubation and declines over the following 2 hours.

# 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<sub>2</sub>HPO<sub>4</sub>) and 2.4 g potassium phosphate, monobasic (KH<sub>2</sub>PO<sub>4</sub>) to 1 L dH<sub>2</sub>O. 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<sub>2</sub>O)
- 1X PBS/0.3% Triton X-100 (PBS/Triton):** To prepare 1 L, add 100 ml 10X PBS to 900 ml dH<sub>2</sub>O. 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 (C<sub>6</sub>H<sub>5</sub>Na<sub>3</sub>O<sub>7</sub>•2H<sub>2</sub>O) to 1 L dH<sub>2</sub>O. 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<sub>2</sub>O. 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.

- Prolong<sup>®</sup> 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.

- 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.

**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.

- 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<sub>2</sub>O 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<sub>2</sub>O 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<sup>®</sup> 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 Prolong<sup>®</sup> Gold Antifade Reagent 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.