

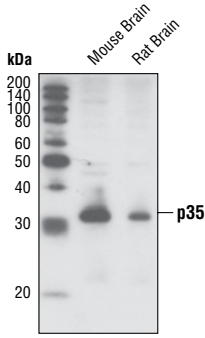
This product is for *in vitro* research use only and is not intended for use in humans or animals.

Applications	Species Cross-Reactivity	Molecular Wt.	Source
W, IP, IF-F	M, R, (H)	25-35 kDa	Rabbit

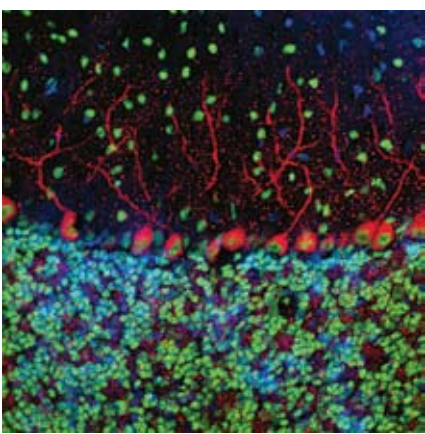
**Background:** Cyclin-dependent kinases (CDKs) are serine/threonine kinases that are activated by cyclins and govern eukaryotic cell cycle progression. While CDK5 shares high sequence homology with its family members, it is thought mainly to function in postmitotic neurons, regulating the cytoarchitecture of these cells. Analogous to cyclins, p35 and p39 associate with and activate CDK5 despite the lack of sequence homology. CDK5 is ubiquitously expressed, but high levels of kinase activity are detected primarily in the nervous system due to the narrow expression pattern of p35 and p39 in post-mitotic neurons. A large number of CDK5 substrates have been identified although no discrete substrates have been attributed as a function of p35 vs. p39. Amongst many, substrates of CDK5 include p35 and p39. p35 is rapidly degraded (T1/2 <20 min) by the ubiquitin-proteasome pathway (1). However, p35 stability increases as CDK5 kinase activity decreases, and this is likely a result of decreased phosphorylation of p35 at Thr138 by CDK5 (2). NGF activates Erk and EGR1, and induces p35 expression in PC12 cells (3). Proteolytic cleavage of p35 by calpain produces p25 upon neurotoxic insult, resulting in prolonged activation of CDK5 by p25. Accumulation of p25 is found in neurodegenerative diseases such as Alzheimer+s disease and Amyotrophic Lateral Sclerosis (ALS) (4–5).

**Specificity/Sensitivity:** p35 Antibody detects endogenous levels of total p35 protein. Calpain proteolytically cleaves p35 to p25 upon neurotoxic insult and p35 Antibody can detect p25.

**Source/Purification:** Polyclonal antibodies are produced by immunizing rabbits with a synthetic peptide (KLH-coupled) corresponding to the sequence of human p35/p25. Antibodies are purified by protein A and peptide affinity chromatography.



Western blot analysis of extracts from mouse and rat brain, using p35 Antibody.



Confocal immunofluorescent image of mouse cerebellum labeled with p35 Antibody (red) and CREB (86B10) #9104 (green). Blue pseudocolor = DRAQ5™ (fluorescent DNA dye).

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

**Recommended Antibody Dilutions:**

Western blotting	1:1000
Immunoprecipitation	1:50
Immunofluorescence (IF-F)	1:50

- Companion Products:**
- Calpain 1 Large Subunit (Mu-type) Antibody #2556
  - Calpain 2 Large Subunit (M-type) Antibody #2539
  - Phospho-APP (Thr668) Antibody #2451
  - APP Antibody #2452
  - beta-Amyloid Antibody #2454
  - Tau (Tau46) Mouse mAb #4019
  - EGR1 Antibody #4152
  - Phospho-Erk1/2 Pathway Sampler Kit #9911
  - Phototope®-HRP Western Blot Detection System, Anti-rabbit IgG, HRP-linked Antibody #7071
  - 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

- Background References:**
- (1) Dhavan, R. and Tsai, L.H. (2001) *Nat. Rev. Mol. Cell Biol.* 2, 749–759.
  - (2) Patrick, G.N. et al. (1998) *J. Biol. Chem.* 273, 24057–24064.
  - (3) Harada, T. et al. (2001) *Nat. Cell Biol.* 3, 453–459.
  - (4) Lee, M.S. et al. (2000) *Nature* 405, 360–364.
  - (5) Kusakawa, G. et al. (2000) *J. Biol. Chem.* 275, 17166–17172.

**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.**

## 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<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.

## 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  $\beta$ -glycerophosphate, 1 mM  $\text{Na}_3\text{VO}_4$ , 1  $\mu\text{g/ml}$  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-cleaning step to reduce non-specific binding to the Protein A/G agarose beads (See section below).

1. Take 200  $\mu\text{l}$  cell lysate and add primary antibody. Incubate with gentle rocking overnight at 4°C.
2. Add either protein A or G agarose beads (20  $\mu\text{l}$  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  $\mu\text{l}$  of 1X cell lysis buffer. Keep on ice during washes.
4. Resuspend the pellet with 20  $\mu\text{l}$  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  $\mu\text{l}$ ) on SDS-PAGE gel (12–15%).
7. Analyze sample by Western blotting (see Western Immunoblotting Protocol).

### Cell Lysate Pre-Cleaning (Optional)

1. Take 200  $\mu\text{l}$  cell lysate and add to either Protein A or G agarose beads (20  $\mu\text{l}$  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.

# 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® 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® 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® 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.