

Phospho-NPM (Thr199) Antibody

✓ 100 µl
(10 Western mini-blot)

rev. 12/12/06

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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, IP, IHC, IC	H, M, R	38 kDa	Rabbit

Background: Nucleophosmin (NPM; also known as B23, numatrin or NO38) is an abundant phosphoprotein primarily found in nucleoli. It has been implicated in several distinct cellular functions, including assembly and transport of ribosomes, cytoplasmic/nuclear trafficking, regulation of DNA polymerase α activity, centrosome duplication and molecular chaperoning activities (1,2). The NPM gene is also known for its fusion with the anaplastic lymphoma kinase (ALK) receptor tyrosine kinase. The NPM portion contributes to transformation by providing a dimerization domain, which results in activation of the fused kinase (3,4).

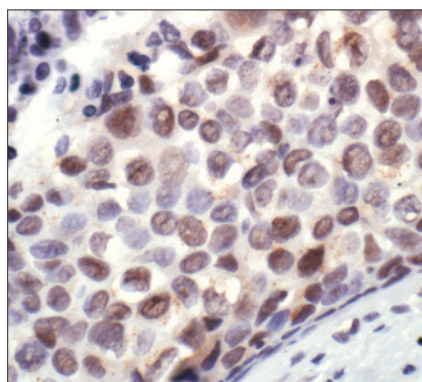
NPM associates with unduplicated centrosomes and is a direct substrate of Cdk2-cyclin E in centrosome duplication (4). Upon phosphorylation at Thr199 by Cdk2-cyclin E, NPM dissociates from centrosomes, and this dissociation is a prerequisite step for centrosome to initiate duplication (5).

Specificity/Sensitivity: Phospho-NPM (Thr199) Antibody detects endogenous levels of NPM only when phosphorylated at threonine 199.

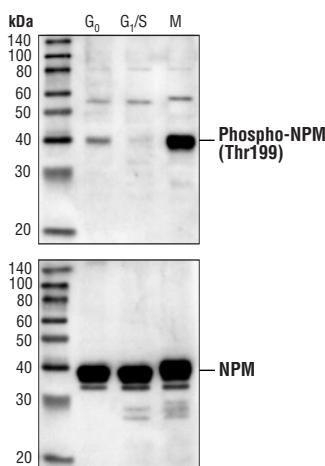
Source/Purification: Polyclonal antibodies are produced by immunizing rabbits with a synthetic phosphopeptide (KLH-coupled) corresponding to residues around Thr199 of human NPM. Antibodies are purified by protein A and peptide affinity chromatography.

Selected Application References:

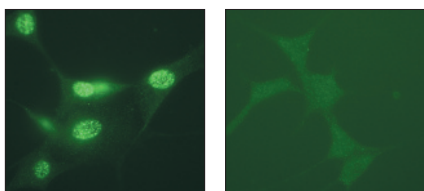
Saavedra, H.I. et al. (2003) Inactivation of E2F3 results in centrosome amplification. *Cancer Cell* 3, 333–346. Applications: W.



Immunohistochemical analysis of paraffin-embedded human breast carcinoma, showing nuclear localization, using Phospho-NPM (Thr199) Antibody.



Western blot analysis of extracts from HeLa cells synchronized at various stages of the cell cycle, using Phospho-NPM (Thr199) Antibody (upper) or NPM Antibody #3542 (lower).



Immunofluorescent analysis of NIH/3T3 cells, using Phospho-NPM (Thr199) Antibody (left) or the same antibody preincubated with phospho-NPM (Thr199) peptide (right).

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	1:100
Immunohistochemistry (Paraffin)	1:200
Immunocytochemistry (IF)	1:100

Companion Products:

NPM Antibody #3542

Phototope®-HRP Western Detection System:
Anti-rabbit IgG, HRP-linked Antibody #7074

Prestained Protein Marker, Broad Range (Premixed Format) #7720

Biotinylated Protein Ladder Detection Pack #7727

LumiGLO® Reagent and Peroxide #7003

Background References:

- (1) Okuda, M. et al. (2000) *Cell* 103, 127–140.
- (2) Takemura, M. et al. (1999) *J. Biochem. (Tokyo)* 125, 904–909.
- (3) Morris, S. W. et al. (1994) *Science* 263, 1281–1284.
- (4) Bischof, D. et al. (1997) *Mol. Cell. Biol.* 17, 2312–2325.
- (5) Tokuyama, Y. et al. (2001) *J. Biol. Chem.* 276, 21529–21537.

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

Western Immunoblotting Protocol

For Western blots, incubate membrane with diluted anti-body in 5% BSA (for a polyclonal antibody) or 5% w/v nonfat dry milk (for a monoclonal antibody), 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.

- A1. ■ 1X Phosphate Buffered Saline (PBS)
 - A2. ■ 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
 - A3. ■ Transfer Buffer:
25 mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5)
 - A4. ■ 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).
 - A5. ■ Nonfat Dry Milk (weight to volume [w/v])
 - A6. ■ 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%).
 - A7. ■ Wash Buffer:
1X TBS, 0.1% Tween-20 (TBS/T)
 - A8. ■ Bovine Serum Albumin (BSA)
 - A9. ■ Primary Antibody Dilution Buffer:
1X TBS, 0.1% Tween-20 with 5% BSA (for a polyclonal antibody) or 5% nonfat dry milk (for a monoclonal); for 20 ml, add 2 ml 10X TBS to 18 ml water, mix. Add 1.0 g BSA (for a polyclonal antibody) or 1.0 g nonfat dry milk (for a monoclonal antibody) and mix well. While stirring, add 20 µl Tween-20 (100%).
- Note: See the specific product's data sheet for the preferred blocking agent. In general, BSA is recommended for polyclonal antibodies; nonfat dry milk is recommended for monoclonal antibodies.*
- A10. ■ Phototope®-HRP Western Blot Detection System #7074 or #7076: Includes biotinylated protein marker, secondary anti-rabbit (#7074) or secondary anti-mouse (#7076) antibody conjugated to horseradish peroxidase (HRP), anti-biotin antibody conjugated to HRP, LumiGLO® chemiluminescent reagent and peroxide.
 - A11. ■ Prestained Protein Marker, Broad Range (Premixed Format) #7720
 - A12. ■ Biotinylated Protein Marker Detection Pack #7726 or #7727
 - A13. ■ 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.

- B1. Treat cells by adding fresh media containing regulator for desired time.
- B2. Aspirate media from cultures; wash cells with 1X PBS; aspirate.
- B3. Lyse cells by adding 1X SDS sample buffer (100 µl per well of 6-well plate or 500 µl per plate of 10 cm plate). Immediately scrape the cells off the plate and transfer the extract to a microcentrifuge tube. Keep on ice.
- B4. Sonicate for 10–15 seconds to shear DNA and reduce sample viscosity.
- B5. Heat a 20 µl sample to 95–100°C for 5 minutes; cool on ice.
- B6. Microcentrifuge for 5 minutes.
- B7. 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 or #7727, 10 µl/lane) to determine molecular weights.

- B8. 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.

- C1. (Optional) After transfer, wash nitrocellulose membrane with 25 ml TBS for 5 minutes at room temperature.
- C2. Incubate membrane in 25 ml of blocking buffer for 1 hour at room temperature.
- C3. Wash three times for 5 minutes each with 15 ml of TBS/T.
- C4. Incubate membrane and primary antibody (at the appropriate dilution) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.
- C5. Wash three times for 5 minutes each with 15 ml of TBS/T.
- C6. 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.
- C7. Wash three times for 5 minutes each with 15 ml of TBS/T.

D. Detection of Proteins

- D1. 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.

- D2. 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.

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 $\mu\text{g}/\text{ml}$ 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 Agarose Beads:

(Can be stored for 2 weeks at 4°C.) Add 5 ml of 1X PBS to 1.5 g of protein A agarose beads. Shake 2 hours at 4°C; spin down. Wash pellet twice with PBS. Resuspend beads in 1 volume of PBS.

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 plate on ice for 5 minutes.
- B4. Scrape cells off the plate and transfer to microcentrifuge tubes. Keep on ice.
- B5. Sonicate 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 protein A 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).

A. Solutions and Reagents

- A1. ■ 10X Phosphate Buffered Saline (PBS):
0.58 M sodium phosphate dibasic (Na_2HPO_4), 0.17 M sodium phosphate monobasic (NaH_2PO_4), 0.68 M NaCl. To prepare 1 liter of 10X PBS, combine 82.33 g Na_2HPO_4 , 23.45 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ and 40 g NaCl. Adjust pH to 7.4.
- A2. ■ 10 mM Sodium Citrate Buffer:
To prepare 1 liter, add 2.94 g sodium citrate to 1 liter distilled H_2O (dH_2O). Adjust pH to 6.0.
- A3. ■ EDTA (optional)*
- A4. ■ 1% Hydrogen Peroxide:
To prepare, add 10 ml 30% H_2O_2 to 290 ml dH_2O .
- A5. ■ Blocking Solution:
5% horse serum or goat serum in PBS
- A6. ■ ABC Reagent:
(Vectastain ABC Kit, Vector Laboratories, Inc., Burlingame, CA) Prepare according to manufacturer's instructions 30 minutes before use.
- A7. ■ DAB Reagent:
Add 6.7 μl of 30% hydrogen peroxide to 10 ml dH_2O ; add this mixture to 10 ml of 1 mg/ml DAB (diaminobenzidine tetrahydrochloride) in PBS, filter.

B. Protocol

- 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.
- B3. Wash sections in PBS for 5 minutes.
- B4. For antigen unmasking, heat sections in 10 mM sodium citrate buffer (pH 6.0) for 1 minute at full power followed by 9 minutes at medium power.* (Keep slides fully immersed in buffer and maintain temperature at or just below boiling.) Cool slides for 20 minutes after antigen unmasking.
- * Alternatively in Step B4, use 1 mM EDTA (pH 8.0), which gives superior results for EGF receptor and HER2/ErbB2 antibodies. See the Tyrosine Kinases/Docking Proteins section of the catalog for these products.
- B5. Wash sections in dH_2O three times for 5 minutes each.
- B6. Incubate sections in 1% Hydrogen Peroxide for 10 minutes.
- B7. Wash sections in dH_2O three times for 5 minutes each.
- B8. Wash section in PBS for 5 minutes.
- B9. Block each section with 100–400 μl blocking solution for 1 hour at room temperature.
- B10. Remove blocking solution and add 100–400 μl diluted primary antibody to each section. (Dilute antibody in blocking solution.) Incubate overnight at 4°C.
- B11. Remove antibody solution and wash sections in PBS three times for 5 minutes each.
- B12. Add 100–400 μl secondary antibody, diluted in blocking solution, to each section. Incubate 30 minutes at room temperature.
- B13. If using ABC avidin/biotin method, make ABC reagent according to the manufacturer's instructions and incubate solution for 30 minutes at room temperature.
- B14. Remove secondary antibody solution and wash sections three times with PBS for 5 minutes each.
- B15. Add 100–400 μl ABC reagent to each section and incubate for 30 minutes at room temperature.
- B16. Remove ABC reagent and wash sections three times in PBS for 5 minutes each.
- B17. Add 100–400 μl DAB reagent to each section and monitor staining closely.
- B18. As soon as the section turns brown, immerse slides in dH_2O .
- B19. If desired, counterstain sections in hematoxylin for 10 seconds.
- B20. Wash sections in dH_2O two times for 5 minutes each.
- B21. Dehydrate sections:
B21a. Incubate sections in 95% ethanol two times for 10 seconds each.
B21b. Repeat in 100% ethanol, incubating sections two times for 10 seconds each.
B21c. Repeat in xylene, incubating sections two times for 10 seconds each.
- B22. Mount coverslips.

This procedure works well when cells are grown in 6-well tissue culture plates containing sterile coverslips in the appropriate media and concentration of fetal bovine serum (FBS). If desired, provide extra coverslips for cell staining controls.

A. Solutions and Reagents

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

- A1. ■ Fetal Bovine Serum (FBS)
- A2. ■ 10X Phosphate Buffered Saline (PBS):
0.58 M sodium phosphate dibasic (Na_2HPO_4), 0.17 M sodium phosphate monobasic (NaH_2PO_4), 0.68 M NaCl. To prepare 1 liter of 10X PBS: 82.33 g Na_2HPO_4 , 23.45 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ and 40 g NaCl. Adjust pH to 7.4.
- A3. ■ 4% Paraformaldehyde:
Prepare day of use either from commercial 16% stock solution or by dissolving paraformaldehyde in 1X PBS by heating at 60°C and stirring until it dissolves. Cool before use.
- A4. ■ Tris Buffered Saline (TBS):
50 mM Tris-HCl (pH 7.4), 150 mM NaCl
- A5. ■ 0.1% Sodium Borohydride:
Dissolve in PBS on day of use.
- A6. ■ 0.2% Triton X-100:
Prepare stock of 20% Triton in PBS; rotate tube overnight to dissolve. Dilute to 0.2%
- A7. ■ 1% Bovine Serum Albumin (BSA)
- A8. ■ Blocking Buffer:
10% horse or goat serum, 1% BSA, 0.02% NaN_3 , 1X PBS
- A9. ■ ProLong™ Antifade Kit:
As directed by Molecular Probes (#P-7481), prepare just before use. Add approximately 1 ml of ProLong mounting medium to one vial of ProLong antifade reagent. Mix gently. Any unused mixture can be stored at -20°C (to slow reaction) for up to one month.

B. Fixation

- B1. Prepare 4% paraformaldehyde and/or cool 100% methanol to -20°C in a tightly sealed container.
- B2. Treat cells as desired.
- B3. Wash cells on coverslips once with cool or room temperature TBS.
- B4. Aspirate off TBS completely and immediately fix as appropriate for your samples.

C. Methanol (protein precipitation) Fixation

- C1. Immerse coverslips in -20°C 100% methanol for 10 minutes.
- C2. Rinse slips three times for 5 minutes each with room temperature TBS.

- C3. Quench cells in fresh 0.1% sodium borohydride in TBS for 5 minutes.
- C4. Wash slips three times for 5 minutes each with room-temperature TBS. Aspirate off completely and return slips to TBS only. Continue protocol at "Blocking" step E1.

D. Paraformaldehyde (cross-linking) Fixation

- D1. Immerse coverslips in 4% paraformaldehyde at room temperature for 10 minutes.
- D2. Wash coverslips once with TBS. Aspirate completely and then permeabilize cells on coverslips with 0.2% Triton X-100 for 5 minutes at room temperature or alternatively with -20°C methanol for 5 minutes.
- D3. Wash slips three times for 5 minutes each with TBS at room temperature.
- D4. Quench cells in fresh 0.1% sodium borohydride in TBS for 5 minutes. Aspirate off completely and return slips to TBS only. Continue protocol at blocking step.

E. Blocking

- E1. Block all slips with blocking buffer at room temperature for 45–60 minutes. Wash once for 5 minutes with TBS.

F. Staining

- F1. Dilute the primary antibody as appropriate in 1% BSA in TBS. Centrifuging the antibody for 20 minutes at 12,000 x g in a refrigerated microcentrifuge prior to use will remove any aggregated material, thereby reducing background. Apply the diluted antibody to the cells on coverslips and, most critically, incubate **overnight** at 4°C.

Note: When using any primary or fluorescence-labeled secondary antibody for the first time, titrate out the antibody to determine which dilution allows for the strongest specific signal with the least background for your sample.

Note: You may wish to leave one slip for a secondary antibody-only control.

- F2. Wash all slips three times for 5 minutes each with TBS.
- F3. Incubate all slips with a dilution of the fluorescence-labeled secondary antibody in 1% BSA in TBS for 30–45 minutes at room temperature in the dark.
- F4. Wash all slips three times for 5 minutes each with TBS in low lighting conditions.
- F5. Mount coverslips on slides using the ProLong™ Antifade Kit. Store slides at room temperature in the dark.