# Rabbit (polyclonal) Anti-Inhibitor-2 [pT$^{72}$] Phosphospecific Antibody, Unconjugated

## PRODUCT ANALYSIS SHEET

| | |
|---|---|
| **Catalog Number:** | 44-1160G (10 mini-blot size) |
| **Lot Number:** | See product label |
| **Volume:** | 100 µL |
| **Form of Antibody:** | Rabbit polyclonal immunoglobulin in Dulbecco's phosphate buffered saline (without $Mg^{2+}$ and $Ca^{2+}$), pH 7.3 (+/- 0.1), 50% glycerol with 1.0 mg/mL BSA (IgG, protease free) as a carrier. |
| **Preservative:** | 0.05% sodium azide (Caution: sodium azide is a poisonous and hazardous substance. Handle with care and dispose of properly.) |
| **Purification:** | Purified from rabbit serum by sequential epitope-specific chromatography. The antibody has been negatively preadsorbed using a non-phosphopeptide corresponding to the site of phosphorylation to remove antibody that is reactive with non-phosphorylated Inhibitor-2. The final product is generated by affinity chromatography using an Inhibitor-2 -derived peptide that is phosphorylated at threonine 72. |
| **Immunogen:** | The antiserum was produced against a chemically synthesized phosphopeptide derived from the region of human Inhibitor-2 that contains threonine 72. This sequence is conserved in rabbit, dog, zebrafish, chimpanzee, cow, and chicken. |
| **Target Summary:** | Inhibitor-2 (I-2) exists as a heterodimer with protein phosphatase type-1 (PP1), termed MgATP-dependent phosphatase. I-2 binds and forms an inactive complex with PP1 in its unphosphorylated state. This complex is activated through a series of phosphorylations on serine and threonine residues in I-2 that increases phosphatase activity. Multiple kinases have been implicated in phosphorylation of I-2 at threonine 72, namely GSK-3, cdc2 and ERK1. Casein kinase II phosphorylates I-2 at serine residues, which in turn enhances threonine phosphorylation by GSK-3. Recent evidence has shown that phosphorylation at threonine 72 peaks during prophase of the cell cycle and is localized in the centrosomes. The I-2/PP1 complex also binds neurabin and the kinases Nek2, KPI-2, and Aurora-A. Regulation of I-2/PP1 has been shown to be important in cell cycle, gene expression, ion gating, and neuromodulation. |
| **Reactivity:** | Human or mouse (90% homologous). |
| **Applications:** | The antibody has been used in Western blotting and immunocytochemistry. |
| **Suggested Working Dilutions:** | For Western blotting applications, we recommend using the antibody at a 1:1000 starting dilution. For immunocytochemistry we recommend using the antibody at a 1:100 starting dilution. The optimal antibody concentration should be determined empirically for each specific application. |
| **Storage:** | Store at –20°C. We recommend a brief centrifugation before opening to settle vial contents. Then, apportion into working aliquots and store at –20°C. For shipment or short-term storage (up to one week), 2-8°C is sufficient. |
| **Expiration Date:** | Expires one year from date of receipt when stored as instructed. |
| **Positive Controls Used:** | HeLa or RAW 264.7 cells treated with nocodazole (200 ng/mL) for 16 hours. |

**This product is for research use only. Not for use in diagnostic procedures.**

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**Related Products:**

**Antibodies:**

CK2α [pT$^{344}$], Cat. # 44-1082G

CK2β [pS$^{209}$], Cat. # 44-1090G

GSK-3β [pS$^{9}$], Cat. # 44-600G

ERK1/2 [pTpY$^{185/187}$], Cat. # 44-680G

**References:**


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Leach, C., et al. (2003) Phosphorylation of phosphatase inhibitor-2 at centrosomes during mitosis. J. Biol. Chem. 278(28):26015-26020.

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Jaumot, M. and Hancock, J.F. (2001) Protein phosphatases 1 and 2A promote Raf-1 activation by regulating 14-3-3 interactions. Oncogene 20(30):3949-3958.

Wang, Q.M., et al. (1995) Phosphorylation and activation of the ATP-Mg-dependent protein phosphatase by the mitogen-activated protein kinase. J. Biol. Chem. 270(31):18352-18358.

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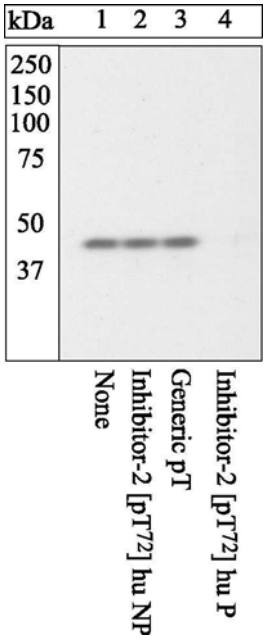


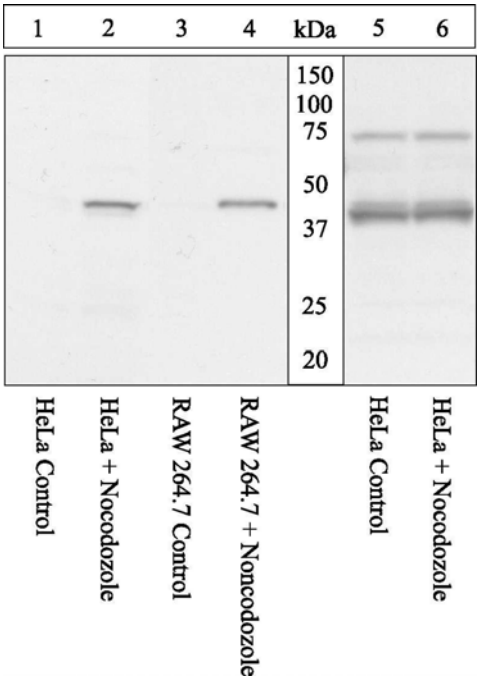


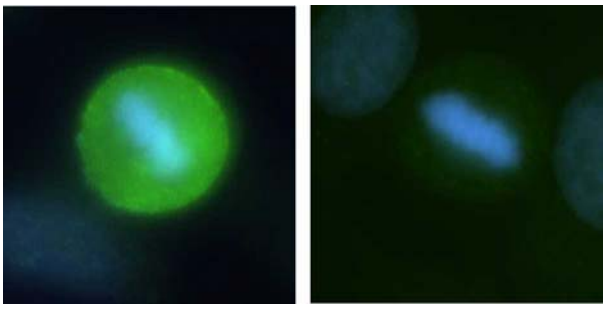

**Western Blotting**

**Left:** Extracts of RAW 264.7 cells treated (1-4) with 200 ng/mL nocodazole for 16 hours were resolved by SDS-PAGE on a 10% polyacrylamide gel and transferred to PVDF. The membrane was blocked with a 3% milk-TBST buffer for one hour at room temperature, then incubated with the Inhibitor-2 [pT$^{72}$] antibody applied at 4°C in a 3% milk-TBST buffer, following prior incubation with: no peptide (1), the non-phosphopeptide corresponding to the phosphopeptide immunogen (2), a generic phosphothreonine-containing peptide (3), or the phosphopeptide immunogen (4). After washing, the membrane was incubated with goat F(ab')$_2$ anti-rabbit IgG HRP conjugate (Cat. # ALI4404) and signals were detected using the Pierce SuperSignal™ method.

**Right:** Western blot using Inhibitor-2 [pT$^{72}$] antibody on extracts of HeLa or RAW 264.7 cells either untreated (1,3) or treated (2, 4) with 200 ng/mL nocodazole for 16 hours. The blot was reprobed (5, 6) with an antibody recognizing total human I-2 protein.

The data show that only the phosphopeptide corresponding to I-2 [pT$^{72}$] blocks the antibody signal, demonstrating the specificity of the antibody. The data also show the induction of Inhibitor-2 [pT$^{72}$] phosphorylation by the addition of nocodazole in both human and mouse cell systems.

**Immunocytochemistry**

HeLa cells grown on a chamber slide were fixed in cold 10% trichloroacetic acid for 10 minutes, rinsed in TBS and permeabilized with 0.1% Triton-X-100 for 15 minutes. Slides were blocked in 3% BSA-TBS buffer for 30 minutes, then incubated with the I-2 [pT$^{72}$] antibody applied at 4°C in a 1% BSA-TBST buffer, following prior incubation with: no peptide (left) or the phosphopeptide immunogen (right). After washing, the slides were incubated with anti-rabbit IgG FITC conjugate and counterstained with DAPI.



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**Western Blotting Procedure**

1. Lyse approximately $10^7$ cells in 0.5 mL of ice cold Cell Lysis Buffer (formulation provided below). This buffer, a modified RIPA buffer, is suitable for recovery of most proteins, including membrane receptors, cytoskeletal-associated proteins, and soluble proteins. This cell lysis buffer formulation is available as a separate product which requires supplementation with protease inhibitors immediately prior to use (Cat. # FNN0011). Other cell lysis buffer formulations, such as Laemmli sample buffer and Triton-X 100 buffer, are also compatible with this procedure. Additional optimization of the cell stimulation protocol and cell lysis procedure may be required for each specific application.
2. Remove the cellular debris by centrifuging the lysates at 14,000 x g for 10 minutes. Alternatively, lysates may be ultracentrifuged at 100,000 x g for 30 minutes for greater clarification.
3. Carefully decant the clarified cell lysates into clean tubes and determine the protein concentration using a suitable method, such as the Bradford assay. Polypropylene tubes are recommended for storing cell lysates.
4. React an aliquot of the lysate with an equal volume of 2x Laemmli Sample Buffer (125 mM Tris, pH 6.8, 10% glycerol, 10% SDS, 0.006% bromophenol blue, and 130 mM dithiothreitol [DTT]) and boil the mixture for 90 seconds at 100°C.
5. Load 10-30 µg of the cell lysate into the wells of an appropriate single percentage or gradient minigel and resolve the proteins by SDS-PAGE.
6. In preparation for the Western transfer, cut a piece of PVDF slightly larger than the gel. Soak the membrane in methanol for 1 minute, then rinse with $ddH_2O$ for 5 minutes. Alternatively, nitrocellulose may be used.
7. Soak the membrane, 2 pieces of Whatman paper, and Western apparatus sponges in transfer buffer (formulation provided below) for 2 minutes.
8. Assemble the gel and membrane into the sandwich apparatus.
9. Transfer the proteins at 140 mA for 60-90 minutes at room temperature.
10. Following the transfer, rinse the membrane with Tris buffered saline for 2 minutes.
11. Block the membrane with blocking buffer (formulation provided below) for one hour at room temperature.
12. Incubate the blocked blot with primary antibody at a 1:1000 starting dilution in Tris buffered saline supplemented with 3% non-fat milk and 0.1% Tween-20 overnight at 4°C.
13. Wash the blot with several changes of Tris buffered saline supplemented with 0.1% Tween 20.
14. Detect the antibody band using an appropriate secondary antibody, such as goat $F(ab')_2$ anti-rabbit IgG alkaline phosphatase conjugate (Cat. # ALI4405) or goat $F(ab')_2$ anti-rabbit IgG horseradish peroxidase conjugate (Cat. # ALI4404) in conjunction with your chemiluminescence reagents and instrumentation.

**Cell Lysis Buffer Formulation:**
10 mM Tris, pH 7.4
100 mM NaCl
1 mM EDTA
1 mM EGTA
1 mM NaF
20 mM $Na_4P_2O_7$
2 mM $Na_3VO_4$
0.1% SDS
0.5% sodium deoxycholate
1% Triton-X 100
10% glycerol
1 mM PMSF (made from a 0.3 M stock in DMSO) or 1 mM AEBSF (water soluble version of PMSF)
60 µg/mL aprotinin
10 µg/mL leupeptin
1 µg/mL pepstatin
(alternatively, protease inhibitor cocktail such as Sigma Cat. # P2714 may be used)

**Transfer Buffer Formulation:**
2.4 gm Tris base
14.2 gm glycine
200 mL methanol
Q.S. to 1 liter, then add
1 mL 10% SDS.
Cool to 4°C prior to use.

**Tris Buffered Saline Formulation:**
20 mM Tris-HCl, pH 7.4
0.9% NaCl

**Blocking Buffer Formulation:**
100 mL Tris buffered saline
3 gm non-fat milk
0.1 mL Tween 20



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**Peptide Competition Experiment**

Invitrogen's Phosphorylation Site Specific Antibodies (PSSAs) have been developed to enable the specific and sensitive detection of phosphorylation of particular amino acid residues in target proteins, while circumventing the need for protein purification, phosphopeptide mapping or handling radioactivity. The specificity of a PSSA in each experimental system can be confirmed through peptide competition. In this technique, aliquots of antibody are pre-incubated with peptide containing the sequence of the phosphopeptide immunogen used to raise the PSSA and the corresponding non-phosphopeptide. Following preincubation with the peptide, each antibody preparation is then used as a probe in antibody-based detection methods, such as Western blotting, immunocytochemistry, flow cytometry, or ELISA. With a PSSA specific for the phosphorylated target protein, pre-incubation with an excess of peptide containing the sequence of the phosphopeptide immunogen will block all antigen binding sites, while pre-incubation with the corresponding non-phosphopeptide will not affect the antibody.

Invitrogen has developed a line of control peptides specifically for use in peptide competition experiments with our PSSAs. These peptides, available as separate Invitrogen catalog items, are provided in pairs which contain the sequences of the phosphopeptide immunogen and the corresponding non-phosphopeptide.

In performing the Peptide Competition Experiment, it is important to note that the optimal dilutions of both antibody and peptide should be determined empirically for each specific application. The optimal dilution of antibody in these procedures is below saturating, as determined by previous experiments in your system. If an optimal antibody dilution has not been determined in your system, please refer to the Suggested Working Dilution on the antibody Product Analysis Sheet for guidance on an appropriate starting dilution. The optimal dilution of peptide used in these procedures will depend on the overall affinity or avidity of the antibody, as well as the quantity of the target antigen. A 50-150 fold molar excess of peptide to antibody is found to be effective for most peptide competition experiments.

In the example presented below, the PSSA is used as a dilution of 1:1000 and the peptides are used at a concentration of 333 nM. The total volume of the phosphopeptide and non-phosphopeptide-pre-incubated antibody preparations is 2 mL, sufficient for probing Western blot strips, as well as for use in other antibody-based detection methods. Under these conditions, the molar excess of peptide to antibody is ≥50.

**Procedure:**

1. Prepare three *identical test samples*, such as identical PVDF or nitrocellulose strips to which the protein of interest has been transferred. The test samples should be blocked using a blocking buffer, such as Tris buffered saline supplemented with 0.1% Tween 20, and either 5% BSA or 5% non-fat dried milk.
2. Prepare 6.5 mL of *working antibody stock solution* (1:1000 in this example) by adding 6.5 µL of antibody stock solution to 6.5 mL of buffer containing blocking protein, such as TBS supplemented with 0.1% Tween 20, and either 3% BSA or 3% non-fat dried milk.
3. Apportion the unused PSSA into working aliquots and store at –20°C for future use (the stock PSSA contains 50% glycerol and will not freeze at this temperature).
4. Allow the *lyophilized control peptides* to reach room temperature, ideally under desiccation.
5. Reconstitute each of the control peptides (supplied at 0.1 mg/vial) to a concentration of 66.7 µM with nanopure water. For a peptide with a molecular mass of 1500 (stated on the peptide Product Analysis Sheet), reconstitution with 1 mL water yields a solution with a concentration of 66.7 µM.
6. Apportion the unused reconstituted peptide solutions into working aliquots and store at -20°C for future use.
7. Label 3 test tubes as follows:
   - tube 1: water only no peptide control
   - tube 2: phosphopeptide
   - tube 3: non-phosphopeptide
8. Into each tube, pipette the following components
   - tube 1: 2 mL diluted PSSA solution plus 10 µL nanopure water
   - tube 2: 2 mL diluted PSSA solution plus 10 µL phosphopeptide
   - tube 3: 2 mL diluted PSSA solution plus 10 µL non-phosphopeptide
9. Incubate the three tubes for 30 minutes at room temperature with gentle rocking. During this incubation, the peptides have the chance to bind to the combining site of the antibody.
10. At the end of the incubation step, transfer the contents of each of the three tubes to clean reaction vessels containing one of the three identical test samples.

    **For Western blotting strips:**
    - Incubate the strips with the pre-incubated antibody preparations for 1 hour at room temperature or overnight at 4°C.
    - Wash each strip four times, five minutes each, to remove unbound antibody.
    - Transfer each strip to a new solution containing a labeled secondary antibody [e.g., goat $F(ab')_2$ anti-rabbit IgG alkaline phosphatase conjugate (Cat. # ALI4405) or goat $F(ab')_2$ anti-rabbit IgG horseradish peroxidase conjugate (Cat. # ALI4404)].
    - Remove unbound secondary antibody by thorough washing, and develop the signal using your chemiluminescent reagents and instrumentation.

The signals obtained with antibody incubated with the "Water Only, No Peptide Control" (Tube 1), represents the maximum signal in the assay. This signal should be eliminated by pre-incubation with the "Phosphopeptide" (Tube 2), while pre-incubation with the "Non-Phosphopeptide" (Tube 3) should not impact the signal. If the "Phosphopeptide" only partially eliminates the signal, repeat the procedure using twice the volume of water or peptide solutions listed in Step 8. If partial competition is seen following pre-incubation with the "Non-Phosphopeptide", repeat the procedure using half the volumes of water or peptide solutions listed in Step 8.



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