# Rabbit (polyclonal) Anti-c-Met [pYpYpY$^{1230/1234/1235}$] Phosphospecific Antibody, Unconjugated

## PRODUCT ANALYSIS SHEET

**Catalog Number:** 44888G (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 c-Met protein. The final product is generated by affinity chromatography using a c-Met-derived peptide that is phosphorylated at tyrosines 1230, 1234 and 1235.

**Immunogen:** The antiserum was produced against a chemically synthesized phosphopeptide derived from the region of human c-Met that contains tyrosines 1230, 1234 and 1235. The sequence is conserved in mouse and rat.

**Target Summary:** Binding of scatter factor (SF)/hepatocyte growth factor (HGF) to the c-Met receptor tyrosine kinase (RTK) triggers receptor dimerization and phosphorylation on multiple residues within the juxtamembrane, catalytic core and cytoplasmic tail domains, thereby regulating receptor internalization, catalytic activity and multisubstrate docking. c-Met contains three tyrosines (Tyr-x-x-x-Tyr-Tyr motif) within the activation loop of the catalytic domain. This is also seen with the insulin receptor, insulin-like growth factor (IGF1) receptor and nerve growth factor (NGF) receptors/Trks, for which phosphorylation of all three tyrosines is required for full activation. With c-Met (and the related family member, RON) phosphorylation of tyrosines 1234 and 1235 has been shown to be important in receptor activation. Activation of the c-Met receptor results in binding and/or phosphorylation of many intracellular signaling proteins including multiple adaptor proteins (e.g., Grb2, Shc, Cbl, Crk, cortactin, paxillin, and GAB1), and a variety of other signal transducers (e.g., PI 3-kinase, FAK, Src, Erk1&2, JNK, PLC-γ, and STAT3).

**Reactivity:** Human and mouse c-Met. Rat (100% homlogous) c-Met has not been tested. The phosphospecific antibody that has been generated does not distinguish between the dually (pYpY$^{1234/1235}$) and triply (pYpYpY$^{1230/1234/1235}$) phosphorylated forms of c-Met, both of which are likely to represent activated forms of this receptor.

**Applications:** The antibody has been used in Western blotting. Previous lots of this antibody have been used in immunostaining. Other applications may work but have not been tested at Invitrogen.

**Suggested Working Dilutions:** For Western blotting applications, we recommend using the antibody at a 1:1000 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.

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

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**Expiration Date:** Expires one year from date of receipt when stored as instructed.

**Positive Controls Used:** Human epidermoid carcinoma A431 cells + EGF; 293T + HGF

**Related Products:**

**Antibodies:**

c-Met [pY$^{1003}$], Cat. # 44882G
c-Met [pY$^{1349}$], Cat. # 44892G
c-Met [pY$^{1365}$], Cat. # 44896
Cortactin [pY$^{421}$] mouse, Cat. # 44854G
STAT3 [pY$^{705}$], Cat. # 44380G
JNK1&2 [pTpY$^{183/185}$], Cat. # 44682G
Pro-Growth Sampler Pack, Cat. # 44587G

**ELISAs:** c-Met [pYpYpY$^{1230/1234/1235}$], Cat. # KHO0281

**References:**


Ma, P.C., et al. (2005) Functional expression and mutations of c-Met and its therapeutic inhibition with SU11274 and small interfering RNA in non-small cell lung cancer. Cancer Res. 65(4):1479-1488 (cites the use of this antibody).

Carbone, M., et al. (2003) Different susceptibility of human mesothelial cells to polyoma virus infection and malignant transformation. Cancer Res. 63(19):6125-6129 (cites the use of this antibody).

Christensen, J.G., et al. (2003) A selective small molecule inhibitor of c-Met kinase inhibits c-Met-dependent phenotypes *in vitro* and exhibits cytoreductive antitumor activity *in vivo*. Cancer Res. 63(21): 7345-7355 (cites the use of this antibody).

Palka, H.L., et al. (2003) Hepatocyte growth factor receptor tyrosine kinase met is a substrate of the receptor protein-tyrosine phosphatase DEP-1. J. Biol. Chem. 278(8):5728-5735 (cites the use of cat. # 44888G and 44896).

Vadnais, J., et al. (2002) Autocrine activation of the hepatocyte growth factor receptor/Met tyrosine kinase induces tumor cell motility by regulating pseudopodial protrusion. J. Biol. Chem. 277(50):48342-48350 (cites the use of cat. # 44888G, 44892G and 44896).

Fan, S., et al. (2001) The multisubstrate adapter Gab1 regulates hepatocyte growth factor (scatter factor)-c-Met signaling for cell survival and DNA repair. Mol. Cell. Biol. 21(15):4968-4984.

Crostella, L., et al. (2001) Hepatocyte Growth Factor/scatter factor-induces phosphorylation of cortactin in A431 cells in a Src kinase-independent manner. Oncogene 20(28):3735-3745.

Parr, C., et al. (2001) The HGF/SF-induced phosphorylation of paxillin, matrix adhesion, and invasion of prostate cancer cells were suppressed by NK4, an HGF/SF variant. Biochem. Biophys. Res. Commun. 285(5):1330-1337.

Guiton, M., et al. (1994) Identification of in vivo brain-derived neurotrophic factor-stimulated autophosphorylation sites on the TrkB receptor tyrosine kinase by site-directed mutagenesis. J. Biol. Chem. 269(48):30370-30377.





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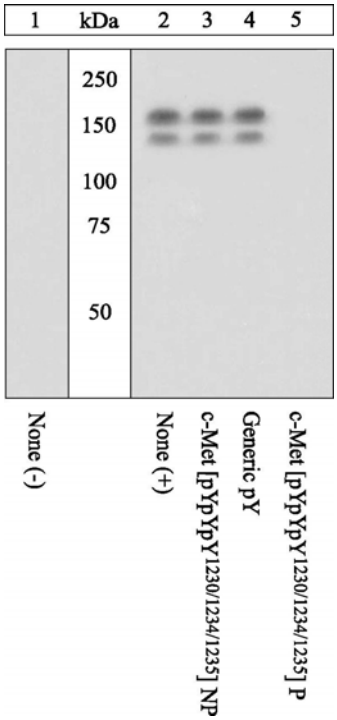


**Upregulation and Antibody-Peptide Competition**

Extracts of 293T cells transiently transfected with human Met cDNA unstimulated (1) or stimulated with 100 ng/mL HGF for 15 minutes (2-5) were resolved by SDS-PAGE on a 10% Tris-glycine gel and transferred to PVDF. The membrane was blocked with a 5% BSA-TBST buffer overnight at 4°C, then incubated with the c-Met [pYpYpY$^{1230/1234/1235}$] antibody in a 1% BSA-TBST buffer for two hours at room temperature, following prior incubation with: no peptide (1, 2), the non-phosphopeptide corresponding to the phosphopeptide immunogen (3), a generic phosphotyrosine-containing peptide (4), or the phosphopeptide immunogen (5). 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.

The data show that only the phosphopeptide corresponding to c-Met [pYpYpY$^{1230/1234/1235}$] blocks the antibody signal, demonstrating the specificity of the antibody. The data also show upregulation of phosphorylation at these sites upon HGF treatment to this cell system.

*Note*: There are three isoforms of c-Met, two of which are recognized by this antibody in this cell system.



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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 membrane 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) overnight at 4°C or 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 1% Ig-free BSA and 0.1% Tween 20 overnight at 4°C or for two hours at room temperature.
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
5 gm Ig-free BSA
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 at 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 signal 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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