Rabbit (polyclonal)  
Anti-COT/Tpl2/MAP3K8 [pT\textsuperscript{290}]  
Phosphospecific Antibody, Unconjugated  

**PRODUCT ANALYSIS SHEET**

<table>
<thead>
<tr>
<th>Catalog Number:</th>
<th>441370G (10 mini-blot size)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lot Number:</td>
<td>See product label</td>
</tr>
<tr>
<td>Volume:</td>
<td>100 $\mu$L</td>
</tr>
<tr>
<td>Form of Antibody:</td>
<td>Rabbit polyclonal immunoglobulin in Dulbecco’s phosphate buffered saline (without Mg\textsuperscript{2+} and Ca\textsuperscript{2+}), pH 7.3 (+/- 0.1), 50% glycerol, with 1.0 mg/mL BSA (IgG, protease free) as a carrier.</td>
</tr>
<tr>
<td>Preservative:</td>
<td>0.05% sodium azide (Caution: sodium azide is a poisonous and hazardous substance. Handle with care and dispose of properly.)</td>
</tr>
<tr>
<td>Purification:</td>
<td>Purified from rabbit serum by sequential epitope-specific chromatography. The antibody has been negatively pre-adsorbed using a non-phosphopeptide corresponding to the site of phosphorylation to remove antibody that is reactive with non-phosphorylated COT. The final product is generated by affinity chromatography using a COT-derived peptide that is phosphorylated at threonine 290.</td>
</tr>
<tr>
<td>Immunogen:</td>
<td>The antiserum was produced against a chemically synthesized phosphopeptide derived from the region of human COT/Tpl2 that contains threonine 290.</td>
</tr>
<tr>
<td>Target Summary:</td>
<td>COT/Tpl2/MAP3K8 (also known as cancer Osaka thyroid oncogene) is a ~ 52 kDa MAPK kinase kinase that plays an important role in innate immunity and inflammation. COT regulates ERK1/2, JNK, p38 and NFkB signaling pathways in a cell- and stimulus–dependent manner, and is activated by several stimuli including IL-1, TNF-\alpha and liposaccharide. COT activation is initiated by its phosphorylation at threonine 290 in the activation loop which is required for its robust autophosphorylation. Phosphorylation of Tpl2 at threonine 290 regulates Tpl2 activation, degradation and its binding to NFkB1/p105.</td>
</tr>
<tr>
<td>Reactivity:</td>
<td>Human COT/Tpl2. Mouse (95% homologous) and rat (100% homologous) COT/Tpl2 are expected to react.</td>
</tr>
<tr>
<td>Applications:</td>
<td>The antibody has been used in Western blotting. Other applications may work but have not been tested.</td>
</tr>
<tr>
<td>Suggested Working Dilutions:</td>
<td>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.</td>
</tr>
<tr>
<td>Storage:</td>
<td>Store at $-20^\circ$C. We recommend a brief centrifugation before opening to settle vial contents. Then, apportion into working aliquots and store at $-20^\circ$C. For shipment or short-term storage (up to one week), 2 to 8°C is sufficient.</td>
</tr>
<tr>
<td>Expiration Date:</td>
<td>Expires one year from date of receipt when stored as instructed.</td>
</tr>
<tr>
<td>Positive Controls Used:</td>
<td>TNF-\alpha-treated Jurkat cells.</td>
</tr>
</tbody>
</table>

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Related Products:

- ERK1&2/MAPK [pTpY\(_{185/187}\)], Cat. # 44680G
- JNK1&2 [pTpY\(_{183/185}\)], Cat. # 44682G
- MAPK p38 [pTpY\(_{180/182}\)], Cat. # 44684G
- NF\(_{\kappa}B\) [pS\(_{529}\)], Cat. # 44711G
- NF\(_{\kappa}B\) [pS\(_{536}\)], Cat. # 44712G
- IKK\(_{\alpha}\) [pSpS\(_{176/180}\)], Cat. # 44714
- I\(_{\kappa}B\)\(_{\alpha}\) [pSpS\(_{32/36}\)], Cat. # 44726G
- CREB [pSpS\(_{129/133}\)], Cat. # 44297G
- MEK1 [pT\(_{292}\)], Cat. # 44458G
- MEK1 [pS\(_{298}\)], Cat. # 44460G
- Akt/PKB, Cat. # 44609G
- Akt/PKB [pT\(_{308}\)], Cat. # 44602G
- Akt/PKB [pS\(_{473}\)], Cat. # 44623G
- Syk [pY\(_{323}\)], Cat. # 44234G

References:


**Antibody-Peptide Competition and Phospho-regulation**

Extracts of Jurkat cells treated with TNF-\(\alpha\) (1-5) or purified active COT protein kinase (6) were resolved on a 10% Tris-glycine gel and transferred to PVDF. The membrane was either left untreated (1-4, 6) or treated with lambda phosphatase (5), blocked with a 5% BSA-TBST buffer for one hour at room temperature, and then incubated with the COT/Tpl2 [pT\(_{290}\)] antibody for two hours at room temperature in 3% BSA-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 a chemiluminescent method.

The data show that the COT/Tpl2 [pT\(_{290}\)] phosphosignal is blocked by the corresponding phosphopeptide and by lambda phosphatase treatment, indicating that the signal is phosphorylation site-specific.
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 ddH₂O 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. Incubate the blocked blot with primary antibody at a 1:1000 starting dilution in Tris buffered saline supplemented with 3% Ig-free BSA and 0.1% Tween 20 overnight at 4°C.

12. Following the transfer, rinse the membrane with Tris buffered saline supplemented with 3% Ig-free BSA and 0.1% Tween 20.

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 Na₄P₂O₇
- 2 mM Na₃VO₄
- 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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