# Phospho-Cdc7 Thr376 Mouse Monoclonal Antibody (Clone TK-3H7)

**Cat# CY-M1021**

**100 µg (1 mg/ml x 100 µL)**

| Clone Name | Applications | Species Cross-reactivity | Molecular Wt. | Source Isotype |
|---|---|---|---|---|
| TK-3H7 | E | H | 67 kDa | Mouse IgG2b |

**Background:** Human Cdc7 encodes a catalytic subunit for Saccharomyces cerevisae Cdc7-related kinase complex of human. ASK, whose expression is cell cycle-regulated, binds and activates human Cdc7 kinase in a cell cycle-dependent manner. Human Cdc7 and ASK proteins can also be phosphorylated by Cdks in vitro. Among four possible Cdk phosphorylation sites of human Cdc7, replacement of Thr-376, corresponding to the activating threonine of Cdk, with alanine (T376A mutant) dramatically reduces kinase activity, indicative of kinase activation by phosphorylation of this residue. In vitro, Cdc2-Cyclin B, but not Cdk2-Cyclin E, Cdk2-Cyclin A, and Cdk4-Cyclin D1, phosphorylates the Thr-376 residue of human Cdc7, suggesting possible regulation of huCdc7 by Cdc2-Cyclin B at M phase.

**Specificity/Sensitivity:** Phospho-Cdc7 Thr376 Antibody detects phosphorylated recombinant Cdc7 by western blotting.

**Source/Purification:** Monoclonal antibody is produced by immunizing mice with a synthetic phosphopeptide corresponding to residues surrounding Thr376 of human Cdc7. IgG is purified by protein A-sepharose chromatography.

**Recommended Antibody Dilutions**: ELISA for detection of Cdc2-Cyclin B kinase activity: 1 ug/mL

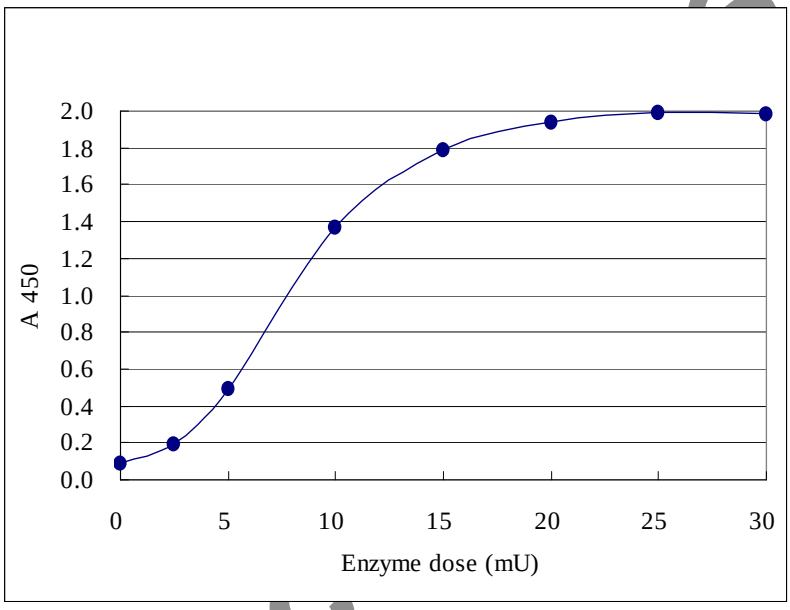
**Storage:** Supplied in 20 mM phosphate buffer (pH 7.5), 300 mM NaCl, 50 % glycerol. Store at –20°C.

**Applications Key: WB:**Western Blotting **IP:**Immunoprecipitation **IHC:**Immunohistochemistry **F:**Flow cytometry **E:**ELISA **FP:**Fluorescence Polarization assay
**Species Cross-Reactivity Key: H:**human **M:**mouse **R:**rat **Hm:**hamster **Mk:**monkey **Mi:**mink **C:**chicken **X:***Xenopus* **Z:**zebra fish **All:**all species expected Species enclosed in parentheses are predicted to react based on 100% sequence homology.

**Selected Application References:**

1. Masai H, Matsui E, You Z, Ishimi Y, Tamai K, Arai K. Human Cdc7-related kinase complex. In vitro phosphorylation of MCM by concerted actions of Cdks and Cdc7 and that of a criticial threonine residue of Cdc7 bY Cdks. *J Biol Chem*. **275**(37):29042-52, 2000
2. Masai H, Arai K. Regulation of DNA replication during the cell cycle: roles of Cdc7 kinase and coupling of replication, recombination, and repair in response to replication fork arrest. *IUBMB Life*. **49**(5):353-64, 2000
3. Masai H, Arai K. Cdc7 kinase complex: a key regulator in the initiation of DNA replication. *J Cell Physiol*. **190**(3):287-96, 2002

**Fig.1 ELISA for measurement of recombinant Cdc2-Cyclin B activity using Phospho-Cdc7 Thr376 Monoclonal antibody (TK-3H7)**




## Western Immunoblotting Protocol

**Solutions and Reagents**

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

**Transfer Buffer:** 25 mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5)
**SDS Sample Buffer (1X):** 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
**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%).
**10X TBS (Tris-buffered saline):** 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).
**Primary Antibody Dilution Buffer:** 1X TBS, 0.1% Tween-20 with 5% blocking agent; 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%).
**Chemiluminescent HRP Detection:** secondary anti-rabbit antibody conjugated to horseradish peroxidase (HRP), ECL™ chemiluminescent reagent (Amersham Pharmacia)

**Wash Buffer TBS/T:** 1X TBS, 0.1% Tween-20
**Blotting Membrane:** This protocol has been optimized for nitrocellulose membranes, which we recommend. PVDF membranes may also be used.

**Protein Blotting**
A general protocol for sample preparation is described below.

1. Treat cells by adding fresh media containing regulator for desired time.
2. Aspirate media from cultures; wash cells with 1X PBS; aspirate.
3. Lyse cells by adding 1X SDS Sample Buffer (100 µl per well of 6-well plate or 500 µl per plate of 10 cm2 plate). Immediately scrape the cells off the plate and transfer the extract to a microcentrifuge tube. Keep on ice.
4. Sonicate for 10–15 seconds to shear DNA and reduce sample viscosity.
5. Heat a 20 µl sample to 95–100°C for 5 minutes; cool on ice.
6. Microcentrifuge for 5 minutes.
7. Load 20 µl onto SDS-PAGE gel (10 cm x 10 cm).
8. Electrotransfer to nitrocellulose membrane.

**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.*

1. (Optional) After transfer, wash nitrocellulose membrane with 25 ml TBS for 5 minutes at room temperature.
2. Incubate membrane in 25 ml of Blocking Buffer for 1 hour at room temperature.
3. Wash 3 times for 5 minutes each with 15 ml of TBS/T.
4. Incubate membrane and primary antibody (at the appropriate dilution) in 10 ml Primary Antibody Dilution Buffer with gentle agitation overnight at 4°C.
5. Wash 3 times for 5 minutes each with 15 ml of TBS/T.
6. Incubate membrane with HRP-conjugated secondary antibody (1:3000 in 10 ml of Blocking Buffer with gentle agitation for 1 hour at room temperature.
7. Wash 3 times for 5 minutes each with 15 ml of TBS/T.

**Detection of Proteins**
1. Incubate membrane with 4 ml ECL™ with gentle agitation for 1 minute at room temperature.
2. Drain membrane of excess developing solution, do not let dry, wrap in plastic wrap and expose to x-ray film. An initial ten seconds exposure should indicate the proper exposure time.

## Immunoprecipitation Followed by Western Immunoblotting Protocol

**Solutions and Reagents**
*Note: Prepare solutions with Milli-Q or equivalently purified water.*

**Cell Lysis Buffer (1X):** 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 Glycerolphosphate, 1 mM $Na_3VO_4$, 1 µg/ml Leupeptin
*Note: We recommend adding 1 mM PMSF before use.*
**Protein A Agarose Beads:** 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. (Can be stored for 2 weeks at 4°C)
**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,
**Transfer Buffer:**25 mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5)
**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%).
**10X TBS (Tris-buffered saline):** For 1 liter of 10X TBS, use 24.2 g Tris base and 80 g NaCl. Adjust pH to 7.6 with HCl (use at 1X).
**Primary Antibody Dilution Buffer:** 1X TBS, 0.05% Tween-20 with 5% nonfat dry milk. For 20 ml, add 2 ml 10X TBS to 18 ml water, mix. Add 1.0 g nonfat dry milk and mix well. While stirring, add 10 µl Tween-20 (100%).
**Wash Buffer TBS/T:** 1X TBS, 0.1% Tween-20
**Chemiluminescent HRP Detection:** secondary anti-rabbit antibody conjugated to horseradish peroxidase (HRP), ECL™ chemiluminescent reagent (Amersham Pharmacia)
**Wash Buffer TBS/T:** 1X TBS, 0.1% Tween-20
**Blotting Membrane:** This protocol has been optimized for nitrocellulose membranes, which we recommend. PVDF membranes may also be used.

**Preparing Cell Lysates**

1. Aspirate media. Treat cells by adding fresh media containing regulator for desired time.
2. To harvest cells under non-denaturing conditions, remove media and rinse cells once with ice-cold PBS.
3. Remove PBS and add 0.5 ml 1X ice-cold Cell Lysis Buffer plus 1 mM PMSF to each plate (10 cm2) and incubate the plate on ice for 5 minutes.
4. Scrape cells off the plate and transfer to microcentrifuge tubes. Keep on ice.
5. Sonicate 4 times for 5 seconds each on ice.
6. 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.

**Immunoprecipitation**

1. Take 200 µL cell lysate and add primary antibody; incubate with gentle rocking overnight at 4°C.
2. Add Protein A Agarose Beads (20 µL 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 2 times with 500 µL of 1X Cell Lysis Buffer. Keep on ice during washes.
4. Resuspend the pellet with 20 µL 3X SDS Sample Buffer. Vortex, then, microcentrifuge for 30 seconds.
5. Heat the sample to 95–100°C for 2–5 minutes.
6. Load the sample (15–30 µL) on SDS-PAGE gel (12–15%).
7. Analyze sample by Western blotting (see Western Immunoblotting Protocol).

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