

PhosphoPlus[®] cdc2 (Tyr15) Antibody Kit

✓ (10 Western mini-blot)



Cell Signaling
TECHNOLOGY[®]

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This product is for *in vitro* research use only and is not intended for use in humans or animals.

Kit Components:

■ Phospho-cdc2 (Tyr15) Antibody

rabbit polyclonal IgG, affinity purified
#9111 100 µl

■ Cdc2 Antibody

rabbit polyclonal IgG, affinity purified
#9112 100 µl

■ Cdc2 (Tyr15) Control Proteins

Cdc2 fusion protein (negative control) and extract from hydroxyurea-treated SK-N-MC cells (positive control)
#9113 4 Western mini blots

■ Phototope[®]-HRP Western Detection System

Anti-rabbit IgG, HRP-linked Antibody
#7074 50 µl

Anti-biotin, HRP-linked Antibody
#7075 100 µl

20X Lumiglo[®] Reagent and Peroxide
#7003 5 ml each

Biotinylated Protein Ladder Detection Pack
#7727 100 µl

Antibody Buffer: Antibodies are supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100 µg/ml BSA and 50% glycerol.

Antibody Storage: Store at -20°C. Do not aliquot the antibodies.

Control Proteins Buffer: Cdc2 (Tyr15) Control Proteins are supplied in 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.

Control Proteins Storage: Store at -20°C.

Protein Ladder Buffer: Supplied in 65 mM Tris-HCl (pH 7.0 at 25°C), 35 mM NaCl, 1 mM Na₂EDTA, 2% SDS (w/v), 1 mM Na₂S₂O₃, 40 mM dithiothreitol (DTT), 0.01% (w/v) phenol red and 10% glycerol.

Protein Ladder Storage: Store at -20°C.

Description: The PhosphoPlus[®] cdc2 (Tyr15) Antibody Kit provides reagents and protocols for the rapid analysis of the phosphorylation status of cdc2 (Tyr15).

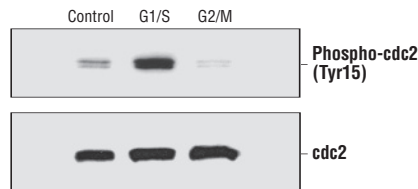
Cdc2 (Tyr15) Control Proteins: A cdc2 fusion protein serves as a negative control. Extract prepared from SK-N-MC cells 20 hours following hydroxyurea treatment serves as a positive control.

PhosphoPlus[®] Biotinylated Protein Ladder: Includes a mixture of purified proteins covalently coupled to biotin that resolve to 10 bands that have a range of 10-200 kDa.

Source/Purification: Polyclonal antibodies are produced by immunizing rabbits with a synthetic phosphopeptide (KLH-coupled) corresponding to residues surrounding Tyr15 of human cdc2 (Phospho-cdc2 (Tyr15) Antibody), or with a synthetic peptide derived from residues surrounding Tyr15 of human cdc2 (Cdc2 Antibody). Antibodies are purified by protein A and peptide affinity chromatography.

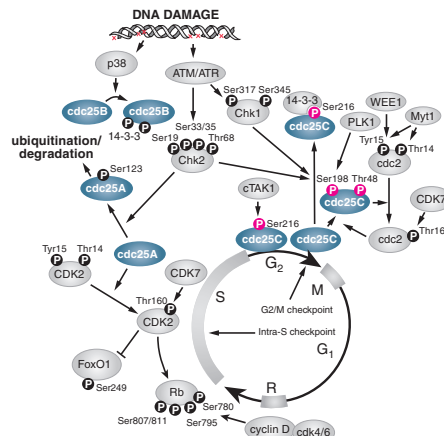
Specificity/Sensitivity: Phospho-cdc2 (Tyr15) Antibody detects endogenous levels of cdc2, cdk2 and cdk5 only when phosphorylated at Tyr15. It also detects the yeast ortholog, cdc28, when phosphorylated at Tyr19. It does not cross-react with cdk4, cdk6 or cdk7. Cdc2 Antibody detects endogenous levels of total cdc2 protein.

Background: Entry of all eukaryotic cells into M-phase of the cell cycle is regulated by activation of cdc2 kinase. Activation of cdc2 is controlled at several steps including cyclin binding and phosphorylation of Thr161 (1-4). However, the critical regulatory step in activating cdc2 during progression into mitosis appears to be dephosphorylation of Tyr15 and Thr14 (3,5). Phosphorylation at Tyr15 and inhibition of cdc2 is carried out by Wee1 and Myt1 protein kinases, while Tyr15 dephosphorylation and activation of cdc2 is carried out by the cdc25 phosphatase (3,4,6).



Western blot analysis of extracts from Saos cells, untreated, hydroxyurea-treated (G1/S) or nocodazole-treated (G2/M), using Phospho-cdc2 (Tyr15) Antibody #9111 (upper) or cdc2 Antibody #9112 (lower).

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Recommended Dilutions for Phospho-cdc2 (Tyr15) Antibody:

| | |
|--------------------------|--------|
| Western blotting | 1:1000 |
| Immunoprecipitation | 1:100 |
| Immunocytochemistry (IF) | 1:100 |

Recommended Dilutions for Cdc2 Antibody:

| | |
|------------------|--------|
| Western blotting | 1:1000 |
|------------------|--------|

Recommended Dilution for Detection of PhosphoPlus[®] Biotinylated Protein Ladder:

| | |
|----------------------------------|--------|
| Anti-biotin, HRP-linked Antibody | 1:1000 |
|----------------------------------|--------|

Companion Products:

Phospho-Chk1 (Ser345) Antibody #2341

Phospho-cdc2 (Thr161) Antibody #9114

cdc2 (POH1) mAb #9116

Anti-biotin, HRP-linked Antibody #7075

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

Selected Application References:

Dai, Y. et al. (2001) Pharmacological inhibitors of the mitogen-activated protein kinase (MAPK) kinase/MAPK cascade interact synergistically with UCN-01 to induce mitochondrial dysfunction and apoptosis in human leukemia cells. *Cancer Res.* 61, 5106–5115. Applications: W.

Misaki, K. et al. (2001) PKN delays mitotic timing by inhibition of Cdc25c: Possible involvement of PKN in the regulation of cell division. *Proc. Natl. Acad. Sci. USA* 98, 125–129. Applications: W

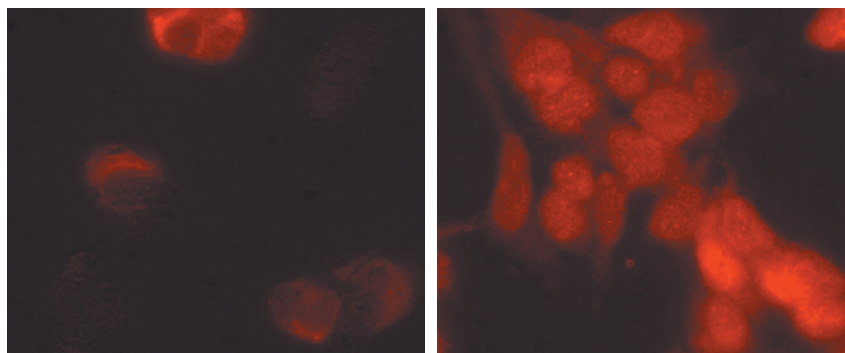
Yu, C. et al. (2002) Pharmacologic mitogen-activated protein/extracellular signal-regulated kinase kinase/ mitogen-activated protein kinase inhibitors interact synergistically with ST1571 to induce apoptosis in Bcr/Abl-expressing human leukemia cells. *Cancer Res.* 62, 188–199. Applications: W.

Background References:

- (1) Norbury, C. and Nurse, P. (1992) *Annu. Rev. Biochem.* 61, 441–470.
- (2) Atherton-Fessler, S. et al. (1993) *Mol. Cell. Biol.* 13, 1675–1685.
- (3) Watanabe, N. et al. (1995) *EMBO J.* 14, 1878–1891.
- (4) Galaktionov, K. et al. (1995) *Genes Dev.* 9, 1046–1058.
- (5) Hunter, T. (1995) *Cell* 80, 225–236.
- (6) McGowan, C.H. and Russell, P. (1993) *EMBO J.* 12, 75–85.

| No. | Antibody | W | IP | IC | Species Cross-reactivity | Mol. Weight | Source |
|-------|-------------------------|--------|-------|-------|--------------------------|-------------|--------|
| #9111 | Phospho-cdc2 (Tyr15) Ab | 1:1000 | 1:100 | 1:100 | H, M, R, Mk, X | 34 | Rabbit |
| #9112 | Cdc2 Antibody | 1:1000 | - | - | H, M, R | 34 | Rabbit |

W=Western Blotting IP=Immunoprecipitation IC=Immunocytochemistry

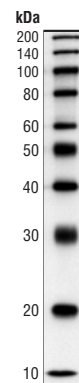


Immunofluorescent analysis of SK-N-MC cells, untreated (left) or hydroxyurea-treated (right), using Phospho-cdc2 (Tyr15) #9111 Antibody.

PhosphoPlus® Biotinylated Protein Ladder

Notes On Use:

- (a) Thaw protein ladder on ice. Mix well and aliquot the desired amount of the protein ladder (10 µl for mini-gels and 20 µl for full size gels) into a separate tube.
- (b) Heat the ladder to 95–100°C for 2 minutes.
- (c) Microcentrifuge briefly and, load directly onto gel. To ensure uniform mobility, load an equal volume of 1X Reducing SDS Loading Buffer into any unused wells.



11% SDS-PAGE

Western Immunoblotting Protocol

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

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% BSA; 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%).

Phototope[®]-HRP Western Blot Detection:

Biotinylated protein marker, secondary anti-rabbit antibody conjugated to horseradish peroxidase (HRP), anti-biotin antibody conjugated to HRP, LumiGLO[®] chemiluminescent reagent, peroxide

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).

Note: CST recommends loading prestained molecular weight markers (#7720, 10 µl/lane) to verify electrotransfer and biotinylated protein markers (#7726, 10 µl/lane) to determine molecular weights.

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 (1:2000) 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.
7. Wash 3 times for 5 minutes each with 15 ml of TBS/T.

Detection of Proteins

1. 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.

2. Drain membrane of excess developing solution, do not let dry, wrap in plastic wrap and expose to x-ray film. An initial ten-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.

Immunoprecipitation / Western Immunoblotting Protocol

Solutions and Reagents

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

■ 1X Phosphate Buffered Saline (PBS)

■ 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 $\mu\text{g/ml}$ Leupeptin

Note: CST recommends adding 1 mM PMSF before use.*

■ Transfer Buffer

- 25 mM Tris base, 0.2 mM glycine, 20% methanol (pH 8.5)

■ 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.

■ 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

Preparing Cell Lysates

1. Aspirate media. Treat cells by adding fresh media containing regulator for desired time.
2. To harvest cells under nondenaturing 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 four 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 five 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*).

Immunocytochemistry Protocol for Immunofluorescence

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.

Solutions and Reagents

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

- 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, use 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.
- 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.
- 100% Methanol
- Tris Buffered Saline (TBS):
50 mM Tris-HCl (pH 7.4), 150 mM NaCl
- 0.1% Sodium Borohydride:
Dissolve in PBS on day of use.
- 0.2% Triton X-100:
Prepare stock of 20% Triton in PBS; rotate tube overnight to dissolve. Dilute to 0.2%
- 1% Bovine Serum Albumin (BSA)
- Blocking Buffer:
10% horse or goat serum, 1% BSA, 0.02% NaN_3 , 1X PBS
- 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.

Fixation

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

Methanol (protein precipitation) Fixation

- a. Immerse coverslips in -20°C 100% methanol for 10 minutes.
- b. Rinse slips three times for 5 minutes each with room temperature TBS.
- c. Quench cells in fresh 0.1% sodium borohydride in TBS for 5 minutes.
- d. 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.

Paraformaldehyde (cross-linking) Fixation

- a. Immerse coverslips in 4% paraformaldehyde at room temperature for 10 minutes.
- b. 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.
- c. Wash slips three times for 5 minutes each with TBS at room temperature.
- d. 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.

Blocking

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

Staining

1. 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.

2. Wash all slips three times for 5 minutes each with TBS.
3. 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.
4. Wash all slips three times for 5 minutes each with TBS in low lighting.
5. Mount coverslips on slides using the ProLong™ Antifade Kit. Store slides at room temperature in the dark.

Material Safety Data Sheet (MSDS) for LumiGLO® Reagent and Peroxide

I. Identification

Product name: 20X LumiGLO® Reagent and Peroxide

CAS number: N/A

Catalog number: 7003

II. Physical Data

Appearance: Clear to faint yellow colored solution.

Melting or Freezing point: <0°C/32°F (water)

Boiling Point: >100°C/212°F (water)

Solubility in water: Dilutable

III. Ingredients

20X LumiGLO®: Contains ≤20.0% weight % Dimethylsulfoxide (CAS number 67-68-5). This product is a mixture that may contain one or more hazardous chemicals. The hazardous ingredients listed above are only those as required by 29 CFR 1910.1200 g 2.C1.

Peroxide: Reagent is not considered to be a hazardous product. It contains less than 1.0% hazardous chemical and less than 0.1% carcinogenic chemical.

IV. Fire and Explosion Hazard Data

Extinguishing media: CO₂, dry chemical.

Special fire fighting procedures: If involved in fire, don NIOSH/MSHA-approved self-contained breathing apparatus, flame/chemical resistant.

Unusual fire and explosion hazards: May emit toxic fumes under fire conditions.

V. Health Hazard Data

Threshold Limit Value (TLV) and source: Data not available.

Acute effects of overexposure: To the best of our knowledge, the chemical, physical and toxicological properties have not been thoroughly investigated.

Swallowing: May be harmful if swallowed.

Skin absorption: May be harmful if absorbed through the skin.

Inhalation: May be harmful if inhaled. Material may be irritating to mucous membranes and upper respiratory tract.

Skin contact: May be harmful.

Eye contact: May cause eye irritation.

Chronic effects of overexposure: May be harmful.

Emergency and First Aid Procedures

Swallowing—Wash out mouth with water, provided person is conscious. Call a physician.

Skin—In case of contact, immediately wash skin with soap and copious amounts of water.

Inhalation—If inhaled, remove to fresh air. If breathing becomes difficult, call a physician.

Eyes—In case of contact with eyes, flush with copious amounts of water for at least 15 minutes. Assure adequate flushing by separating eyelids with fingers. Consult a physician.

VI. Reactivity Data

Stability/Conditions to avoid: Not reactive as far as is known.

Incompatibility/Materials to avoid: Strong oxidizing agents, acid chlorides, acid anhydrides.

Combustion/Decomposition products: Carbon monoxide, carbon dioxide, sulfur oxides.

Hazardous polymerization: Not susceptible to polymerization.

VII. Spill or Leak Procedures

Steps to be taken if material is spilled or released: Wear self-contained breathing apparatus, rubber boots and rubber gloves. Use Vermiculite or another suitable absorbent to clean up spill, place in a suitable closed container for disposal, then wash down spill site.

Waste disposal method: Dissolve the material in a combustible solvent and burn in an EPA-licensed chemical incinerator equipped with an after-burner and scrubber.

VIII. Special Protection Information

Respiratory protection: Avoid inhalation. Use NIOSH/MSHA-approved respirator.

Ventilation: Use mechanical exhaust.

Protective equipment: Wear suitable protective clothing, chemical resistant gloves and lab safety glasses.

IX. Special Precautions

Handling and storage: Store at 4°C.

Precautions to be taken in handling and storage: This compound is sold only for research use by personnel familiar with the toxicology of organic chemicals and who are well trained in good laboratory habits, such as avoiding spills, keeping hands clean at all times and not rubbing eyes with hands while working in the laboratory.

The above information is believed to be correct but does not purport to be all inclusive and shall be used only as a guide for experienced personnel. Cell Signaling Technology, Inc., shall not be held liable for any damage resulting from the handling of or from contact with the above product. The burden of safe use of this material rests entirely with the user.

Revised: April 2001

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