Applications	Reactivity	Sensitivity	MW (kDa)	Source
W IHC-P	H M R (C)	Endogenous	60	Rabbit

Applications Key: W=Western Blotting IHC-P=Immunohistochemistry (Paraffin)

Reactivity Key: H=Human M=Mouse R=Rat C=Chicken

Species cross-reactivity is determined by western blot. Species enclosed in parentheses are predicted to react based on 100% sequence homology.

Protocols

Immunohistochemistry Protocol – Paraffin for SignalStain® Boost Detection Reagent

***IMPORTANT:** See product datasheet for the appropriate antibody diluent, dilution, and antigen unmasking procedure.

A. Solutions and Reagents

NOTE: Prepare solutions with purified water.

1. **Xylene**
2. **Ethanol**, anhydrous denatured, histological grade (100% and 95%).
3. **Deionized water** (dH_2O).
4. **Hematoxylin** (optional).
5. **Wash Buffer:**

- **1X TBS/0.1% Tween-20 (1X TBST):** To prepare 1 L, add 100 ml 10X TBS to 900 ml dH_2O. Add 1 ml Tween-20 and mix.
- **10X Tris Buffered Saline (TBS):** To prepare 1 L, add 24.2 g Trizma® base ($C_4H_{11}NO_3$) and 80 g sodium chloride (NaCl) to 1 L dH_2O. Adjust pH to 7.6 with concentrated HCl.

6. ***Antibody Diluent:**

a. **SignalStain® Antibody Diluent #8112**

b. **TBST/5% normal goat serum:** To 5 ml 1X TBST, add 250 µl normal goat serum (#5425).

c. **PBST/5% normal goat serum:** To 5 ml 1X PBST, add 250 µl normal goat serum (#5425).

1X PBS/0.1% Tween-20 (1X PBST): To prepare 1 L, add 100 ml 10X PBS to 900 ml dH_20. Add 1 ml Tween-20 and mix.

10X Phosphate Buffered Saline (PBS): To prepare 1 L, add 80 g sodium chloride (NaCl), 2 g potassium chloride (KCl), 14.4 g sodium phosphate, dibasic (Na_2HPO_4) and 2.4 g potassium phosphate, monobasic (KH_2PO_4) to 1 L dH_2O. Adjust pH to 7.4.

7. ***Antigen Unmasking:**

a. **Citrate:** 10 mM Sodium Citrate Buffer: To prepare 1 L, add 2.94 g sodium citrate trisodium salt dihydrate ($C_6H_5Na_3O_7•2H_2O$) to 1 L dH_2O. Adjust pH to 6.0.

b. **EDTA:** 1 mM EDTA: To prepare 1 L add 0.372 g EDTA ($C_{10}H_{14}N_2O_8Na_2•2H_2O$) to 1 L dH_2O. Adjust pH to 8.0.

c. **TE:** 10 mM Tris/1 mM EDTA, pH 9.0: To prepare 1 L, add 1.21 g Trizma®base ($C_4H_{11}NO_3$) and 0.372 g EDTA ($C_{10}H_{14}N_20_8Na_2$) to 950 ml dH_2O. Adjust pH to 9.0, then adjust final volume to 1 L with dH_2O.

d. **Pepsin:** 1 mg/ml in Tris-HCl, pH 2.0.

8. **3% Hydrogen Peroxide:** To prepare, add 10 ml 30% H_2O_2 to 90 ml dH_2O.

9. **Blocking Solution:** TBST/5% normal goat serum: to 5 ml 1X TBST, add 250 µl normal goat serum (#5425).

10. **Detection System:** SignalStain® Boost IHC Detection Reagents (mouse #8125, rabbit #8114).

11. **Substrate:** SignalStain® DAB Substrate Kit (#8059).

B. Deparaffinization/Rehydration

NOTE: Do not allow slides to dry at any time during this procedure.

1. **Deparaffinize/hydrate sections:**

a. Incubate sections in three washes of xylene for 5 min each.

b. Incubate sections in two washes of 100% ethanol for 10 min each.

c. Incubate sections in two washes of 95% ethanol for 10 min each.

2. Wash sections twice in dH_2O for 5 min each.

C. Antigen Unmasking*

NOTE: Consult product datasheet for specific recommendation for the unmasking solution.

1. **For Citrate:** Bring slides to a boil in 10 mM sodium citrate buffer, pH 6.0; maintain at a sub-boiling temperature for 10 min. Cool slides on bench top for 30 min.

2. **For EDTA:** Bring slides to a boil in 1 mM EDTA, pH 8.0: follow with 15 min at a sub-boiling temperature. No cooling is necessary.

3. **For TE:** Bring slides to a boil in 10 mM Tris/1 mM EDTA, pH 9.0: then maintain at a sub-boiling temperature for 18 min. Cool at room temperature for 30 min.

4. **For Pepsin:** Digest for 10 min at 37 ℃.

D. Staining

NOTE: Consult product datasheet for recommended antibody diluent.

1. Wash sections in dH_2O three times for 5 min each.

2. Incubate sections in 3% hydrogen peroxide for 10 min.

3. Wash sections in dH_2O twice for 5 min each.

4. Wash sections in wash buffer for 5 min.

5. Block each section with 100–400 µl blocking solution for 1 hr at room temperature.

6. Remove blocking solution and add 100–400 µl primary antibody diluted in recommended antibody diluent to each section*. Incubate overnight at 4 ℃.

7. Equilibrate SignalStain® Boost Detection Reagent to room temperature.

8. Remove antibody solution and wash sections in wash buffer three times for 5 min each.

9. Cover section with 1–3 drops SignalStain® Boost Detection Reagent as needed. Incubate in a humidified chamber for 30 min at room temperature.

10. Wash sections three times with wash buffer for 5 min each.

11. Add 1 drop (30 µl) SignalStain® DAB Chromogen Concentrate to 1 ml SignalStain®DAB Diluent and mix well before use.
12. Apply 100–400 µl SignalStain® DAB to each section and monitor closely. 1–10 minutes generally provides an acceptable staining intensity.
13. Immerse slides in dH_2O.
14. If desired, counterstain sections in hematoxylin per manufacturer's instructions.
15. Wash sections in dH_2O two times for 5 min each.
16. **Dehydrate sections:**

a. Incubate sections in 95% ethanol two times for 10 sec each.
b. Repeat in 100% ethanol, incubating sections two times for 10 sec each.
c. Repeat in xylene, incubating sections two times for 10 sec each.

17. Mount coverslips.

Western Immunoblotting Protocol (Primary Ab Incubation In BSA)

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

Products available from Cell Signaling Technology are linked by their respective catalog numbers.

A. Solutions and Reagents

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

1. **1X Phosphate Buffered Saline (PBS).**
2. **1X SDS Sample Buffer:** (#7722, #7723) 62.5 mM Tris-HCl (pH 6.8 at 25 ℃), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue or phenol red.
3. **Transfer Buffer:** 25 mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5).
4. **10X Tris Buffered Saline (TBS):** (#9997) 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).
5. **Nonfat Dry Milk:** (#9999) (weight to volume [w/v]).
6. **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%).
7. **Wash Buffer:** 1X TBS, 0.1% Tween-20 (TBS/T).
8. **Bovine Serum Albumin (BSA):** (#9998).
9. **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%).
10. **Phototope®-HRP Western Blot Detection System:** (#7071 anti-rabbit) or (#7072 anti-mouse) Includes biotinylated protein ladder, secondary (#7074 anti-rabbit) or (#7076 anti-mouse) antibody conjugated to horseradish peroxidase (HRP), anti-biotin antibody conjugated to HRP, LumiGLO® chemiluminescent reagent and peroxide.
11. **Prestained Protein Marker, Broad Range (Premixed Format):** (#7720).
12. **Biotinylated Protein Ladder Detection Pack:** (#7727).

13. **Blotting Membrane:** This protocol has been optimized for nitrocellulose membranes, which CST recommends. PVDF membranes may also be used.

B. 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 cm diameter 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 for complete cell lysis and to shear DNA (to reduce sample viscosity).
5. Heat a 20 µl sample to 95–100 ℃ 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 ladder (#7727, 10 µl/lane) to determine molecular weights.
8. Electrotransfer to nitrocellulose or PVDF membrane.

C. Membrane Blocking and Antibody Incubations

NOTE: Volumes are for 10 cm x 10 cm (100 cm^2) 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 three 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 ℃.
5. Wash three times for 5 minutes each with 15 ml of TBS/T.

I. For Unconjugated Primary Antibodies

1. Incubate membrane with **appropriate** 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.
2. Wash three times for 5 minutes each with 15 ml of TBS/T.

II. For HRP Conjugated Primary Antibodies

Skip to Detection of Proteins (Step D).

III. For Biotinylated Primary Antibodies

1. Incubate membrane with HRP-Streptavidin (at the appropriate dilution) in milk for one hour with gentle agitation at room temperature.
2. Wash three times for 5 minutes each with 15 ml of TBS/T.

D. 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 10-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.

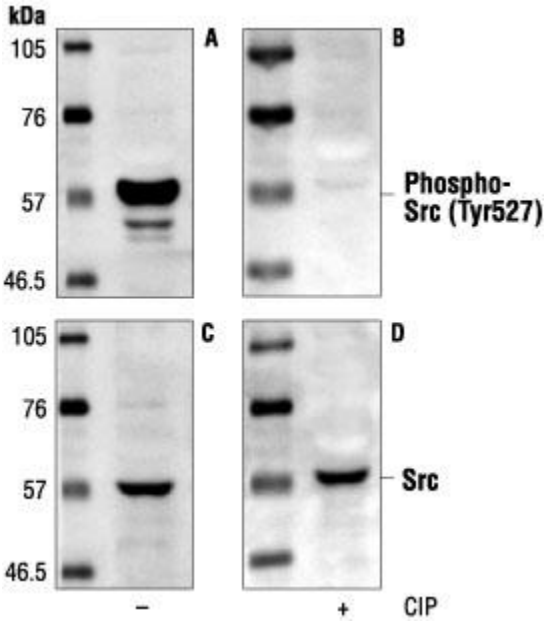
Specificity / Sensitivity

Phospho-Src (Tyr527) Antibody detects endogenous levels of Src only when phosphorylated at Tyr527. The antibody may cross-react with other Src family members such as Yes, Fyn, Fgr and Yrk when phosphorylated at the equivalent sites.

Source / Purification

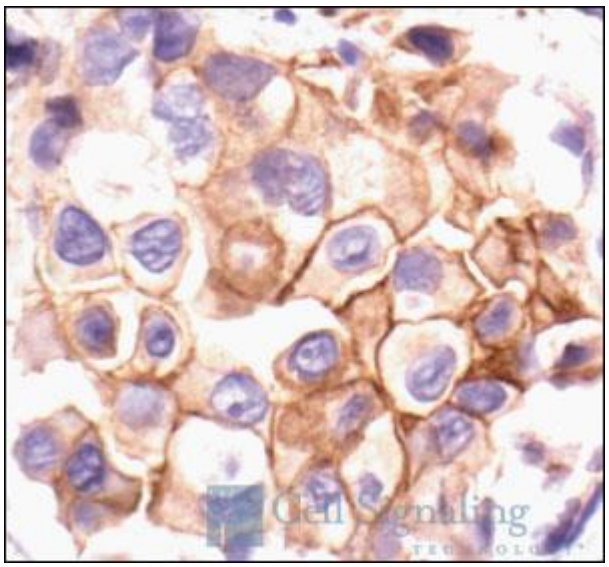
Polyclonal antibodies are produced by immunizing animals with a synthetic phosphopeptide corresponding to residues surrounding Tyr527 of human Src. Antibodies are purified by protein A and peptide affinity chromatography.

Western Blotting



Western blot analysis of extracts from NIH/3T3 cells, using Phospho-Src (Tyr527) Antibody (A,B) or v-Src antibody (C,D). The phospho-specificity of the antibody was confirmed by treating the membrane with calf intestinal alkaline phosphatase (CIP) (B,D) after Western transfer.

IHC-P (paraffin)



Immunohistochemical analysis of paraffin-embedded human breast carcinoma, showing membrane and cytoplasmic localization using Phospho-Src (Tyr527) Antibody.