

SignalStain® Phospho-Stat IHC Sampler Kit

✓ 1 Kit
(3 X 40 µl)

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This product is intended for research purposes only. This product is not intended to be used for therapeutic or diagnostic purposes in humans or animals.

Products Included	Product #	Quantity	Antigen Retrieval/Diluent	Isotype
Phospho-Stat1 (Tyr701) (58D6) Rabbit mAb	9167	40 µl	EDTA/SignalStain® Antibody Diluent #8112	Rabbit IgG
Phospho-Stat3 (Tyr705) (D3A7) XP™ Rabbit mAb	9145	40 µl	EDTA/SignalStain® Antibody Diluent #8112	Rabbit IgG
Phospho-Stat5 (Tyr694) (C11C5) Rabbit mAb	9359	40 µl	EDTA/SignalStain® Antibody Diluent #8112	Rabbit IgG
*SignalStain® Antibody Diluent	8112	25 ml		
*SignalSlide® Phospho-Stat1/3/5 IHC Controls	8105	1 Pack		

See www.cellsignal.com for individual component applications, species cross-reactivity, and additional application protocols.

Description: The SignalStain® Phospho-Stat IHC Sampler Kit from Cell Signaling Technology allows the researcher to examine paraffin-embedded tissues or cells with antibodies that will detect phosphorylated Stat proteins. Each antibody is validated for use in immunohistochemical assays using multiple approaches. Also included in the kit are control slides that can be used to verify the performance of each antibody and a primary antibody diluent. Please see table above for the recommended antibody diluent for each antibody provided in the kit.

Background: Stat proteins serve as transcription factors in growth and survival pathways stimulated by growth factor and cytokine activation of receptor proteins. Receptor activation promotes tyrosine phosphorylation of Stat proteins, resulting in Stat dimerization and translocation to the nucleus where they regulate expression of numerous proteins that control cell growth, survival, differentiation and pathogen resistance (1). Stat1 is essential in IFN-α and IFN-γ stimulated pathways and is abnormally activated in many tumors (2,3). Both Stat1α (91 kDa) and Stat1β (84 kDa) isoforms are activated by IFN-α but only Stat1α responds to IFN-γ. Phosphorylation of Stat1 at Tyr701 induces Stat1 dimerization, nuclear translocation and DNA binding (4). Transcription factor Stat3 possesses oncogenic potential and anti-apoptotic activities; a number of human tumors display constitutively activated Stat3 (5,6). Activation of Stat3 follows phosphorylation at Tyr705, resulting in dimerization, nuclear translocation and DNA binding (7). Expression of Stat3α (86 kDa) and Stat3β (79 kDa) isoforms correlates with cell type, ligand and cell maturation stage (8). Phosphorylation of Stat5a at Tyr694 is essential for transcription factor activation (9). Stat5 is activated by interleukins and other cytokines (i.e. CSF1); presence of phosphorylated Stat5 is in some IL-3-treated cells suggests a role in angiogenesis and cell motility (10). Altered Stat5 expression and activity is associated with abnormal cell proliferation and apoptosis in some forms of leukemia (11).

Specificity/Sensitivity: Each antibody in the SignalStain® Phospho-Stat IHC Sampler Kit detects endogenous levels of its target protein. Phospho-Stat1 (Tyr701) (58D6) Rabbit mAb detects endogenous levels of Stat1 only when phosphorylated at Tyr701; p91 Stat1 phosphorylated at Tyr701 and the p84 splice variant will also be detected by this antibody. It does not cross-react with the corresponding phospho-tyrosines of other Stat proteins. Phospho-Stat3 (Tyr705) (D3A7) XP™ Rabbit mAb detects endogenous levels of Stat3 only when phosphorylated at Tyr705. This antibody does not cross-react with phospho-EGFR or the corresponding phospho-tyrosines of other Stat proteins. Phospho-Stat5 (Tyr694) (C11C5) Rabbit mAb detects endogenous Stat5a only when phosphorylated at Tyr694 and Stat5b when phosphorylated at Tyr699.

Source/Purification: Monoclonal antibody is produced by immunizing animals with a synthetic phosphopeptide corresponding to residues surrounding Tyr701 of human Stat1, Tyr705 of mouse Stat3, or Tyr694 of Stat5a.

Storage: Antibodies are supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100 µg/ml BSA, 50% glycerol and less than 0.02% sodium azide. Store at -20°C.

*SignalStain® Antibody diluent is supplied as a working solution and should be stored at 4°C (packaged separately).

*Control slides should be stored at 4°C (packaged separately).

Recommended Antibody Dilutions:

Phospho-Stat3 (Tyr705) (D3A7) XP™ Rabbit mAb #9145

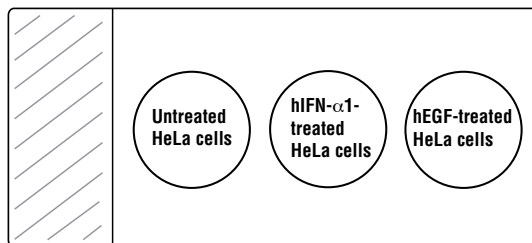
Immunohistochemistry (Paraffin) 1:50
Unmasking buffer: EDTA
Antibody diluent: SignalStain® Antibody Diluent #8112
Immunohistochemistry (Frozen) 1:100
Fixative: 3% Formaldehyde

Phospho-Stat1 (Tyr701) (58D6) Rabbit mAb #9167

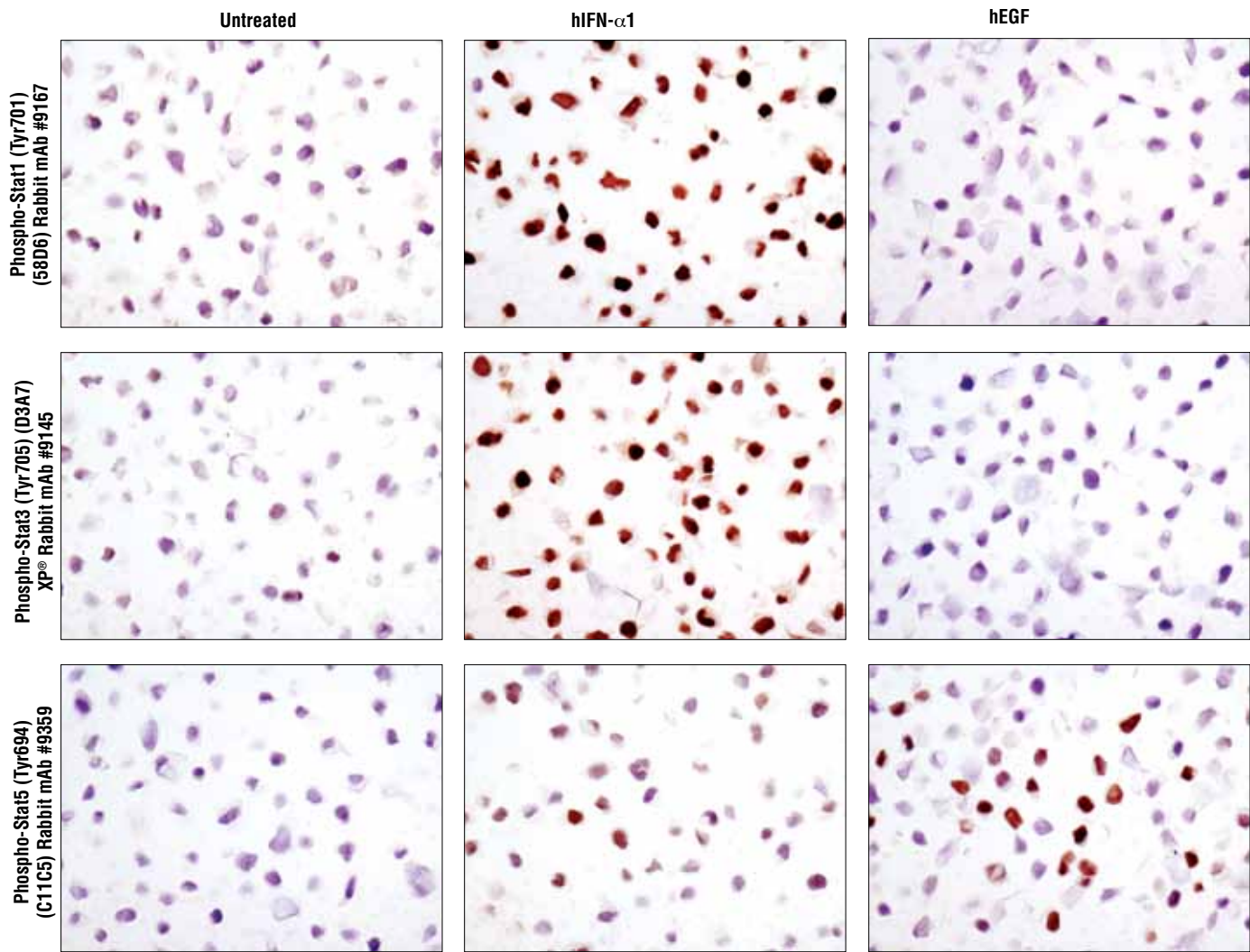
Immunohistochemistry (Paraffin) 1:100
Unmasking buffer: EDTA
Antibody diluent: SignalStain® Antibody Diluent #8112
Immunohistochemistry (Frozen) 1:200
Fixative: 10% Neutral buffered formalin

Phospho-Stat5 (Tyr694) (C11C5) Rabbit mAb #9359

Immunohistochemistry (Paraffin) 1:200
Unmasking buffer: EDTA
Antibody diluent: SignalStain® Antibody Diluent #8112



Selected rabbit monoclonal antibodies are produced under license (granting certain rights including those under U.S. Patent No. 5,675,063 and/or U.S.S.N. 11/476,277) from Epitomics, Inc. U.S.S.N. 11/476,277) from Epitomics, Inc.



Immunohistochemical analysis of paraffin-embedded HeLa cell pellets, untreated (left), Human Interferon- α 1 (hIFN- α 1)-treated #8927 (middle) or Human Epidermal Growth Factor (hEGF)-treated #8916 (right), using Phospho-Stat1 (Tyr701) (58D6) Rabbit mAb #9167 (top), Phospho-Stat3 (Tyr705) (D3A7) XP[®] Rabbit mAb #9145 (middle) or Phospho-Stat5 (Tyr694) (C11C5) Rabbit mAb #9359 (bottom). Immunohistochemical analysis of paraffin-embedded HeLa cell pellets, untreated (left), treated with Human Interferon- α 1 (hIFN- α 1) #8927 (middle), or treated with Human Epidermal Growth Factor (hEGF) #8916 (right), using Phospho-Stat1 (Tyr701) (58D6) Rabbit mAb #9167 (top), Phospho-Stat3 (Tyr705) (D3A7) XP[®] Rabbit mAb #9145 (middle), or Phospho-Stat5 (Tyr694) (C11C5) Rabbit mAb #9359 (bottom).

Background References:

- (1) Ihle, J.N. (2001) *Curr Opin Cell Biol* 13, 211–7.
- (2) Bromberg, J. (2002) *J Clin Invest* 109, 1139–42.
- (3) Frank, D.A. (1999) *Mol Med* 5, 432–56.
- (4) Ihle, J.N. et al. (1994) *Trends Biochem Sci* 19, 222–7.
- (5) Garcia, R. and Jove, R. (1998) *J Biomed Sci* 5, 79–85.
- (6) Cattlett-Falcone, R. et al. (1999) *Immunity* 10, 105–15.
- (7) Darnell, J.E. (1997) *Science* 277, 1630–5.
- (8) Biethahn, S. et al. (1999) *Exp Hematol* 27, 885–94.
- (9) Gouilleux, F. et al. (1994) *EMBO J* 13, 4361–9.
- (10) Buitenhuis, M. et al. (2004) *Int J Biochem Cell Biol* 36, 2120–4.
- (11) Bas'kiewicz-Masiuk, M. et al. (2003) *Cell Prolif* 36, 265–78.

Immunohistochemistry Protocol (Paraffin)

***IMPORTANT:** See product data sheet for the appropriate antibody diluent and antigen unmasking procedure. **IHC Protocol:** Unmasking buffer/antibody diluent.

A Solutions and Reagents

- Xylene
- Ethanol, anhydrous denatured, histological grade (100% and 95%)
- Deionized water (dH₂O)
- Hematoxylin (optional)
- Wash Buffer:**
1X TBS/0.1% Tween-20 (1X TBST): To prepare 1 L add 100 ml 10X TBS to 900 ml dH₂O. Add 1 ml Tween-20 and mix.
10X Tris Buffered Saline (TBS): To prepare 1 L add 24.2 g Trizma® base (C₄H₁₁NO₃) and 80 g sodium chloride (NaCl) to 1 L dH₂O. Adjust pH to 7.6 with concentrated HCl.
- *Antibody Diluent:**
 - SignalStain® Antibody Diluent #8112**
 - TBST/5% normal goat serum:** To 5 ml 1X TBST add 250 µl normal goat serum.
 - PBST/5% normal goat serum:** To 5 ml 1X PBST add 250 µl normal goat serum.
1X PBS/0.1% Tween-20 (1X PBST): To prepare 1L add 100 mL 10X PBS to 900 mL dH₂O. 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 (Na₂HPO₄) and 2.4 g potassium phosphate, monobasic (KH₂PO₄) to 1 L dH₂O. Adjust pH to 7.4.
- *Antigen Unmasking:**
 - Citrate:** 10 mM Sodium Citrate Buffer: To prepare 1 L add 2.94 g sodium citrate trisodium salt dihydrate (C₆H₅Na₃O₇•2H₂O) to 1 L dH₂O. Adjust pH to 6.0.
 - EDTA:** 1 mM EDTA: To prepare 1 L add 0.372 g EDTA (C₁₀H₁₄N₂O₈Na₂•2H₂O) to 1 L dH₂O. Adjust pH to 8.0.
 - TE:** 10 mM Tris/1 mM EDTA/0.05% Tween-20, pH 9.0: To prepare 1L add 1.21 g Trizma® base (C₄H₁₁NO₃) and 0.372 g EDTA (C₁₀H₁₄N₂O₈Na₂•2H₂O) to 950 ml dH₂O. Adjust pH to 9.0, add 0.5 ml Tween-20, then adjust final volume to 1000 ml with dH₂O.
 - Pepsin:** 1 mg/ml in Tris-HCl pH 2.0.
- 3% Hydrogen Peroxide:** To prepare, add 10 ml 30% H₂O₂ to 90 ml dH₂O.
- Blocking Solution:** TBST/5% normal goat serum: to 5ml 1X TBST add 250 µl normal goat serum.
- Biotinylated secondary antibody.
- ABC Reagent:** (Vectastain ABC Kit, Vector Laboratories, Inc., Burlingame, CA) Prepare according to manufacturer's instructions 30 minutes before use.
- DAB Reagent or suitable substrate:** Prepare according to manufacturer's recommendations.

B Deparaffinization/Rehydration

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

- Deparaffinize/hydrate sections:**
 - Incubate sections in three washes of xylene for 5 minutes each.
 - Incubate sections in two washes of 100% ethanol for 10 minutes each.
 - Incubate sections in two washes of 95% ethanol for 10 minutes each.
- Wash sections twice in dH₂O for 5 minutes each.

C *Antigen Unmasking

NOTE: Consult product data sheet for specific recommendation for the unmasking solution.

- For Citrate:** Bring slides to a boil in 10 mM sodium citrate buffer pH 6.0 then maintain at a sub-boiling temperature for 10 minutes. Cool slides on bench top for 30 minutes.
- For EDTA:** Bring slides to a boil in 1 mM EDTA pH 8.0 followed by 15 minutes at a sub-boiling temperature. No cooling is necessary.
- For TE:** Bring slides to a boil in 10 mM TE/1 mM EDTA/0.05% Tween-20, pH 9.0 then maintain at a sub-boiling temperature for 18 minutes. Cool on the bench for 30 minutes.
- For Pepsin:** Digest for 10 minutes at 37°C.

D Staining

- Wash sections in dH₂O three times for 5 minutes each.
- Incubate sections in 3% hydrogen peroxide for 10 minutes.
- Wash sections in dH₂O twice for 5 minutes each.

NOTE: Consult product data sheet for recommended antibody diluent.

- Wash section in wash buffer for 5 minutes.
- Block each section with 100-400 µl blocking solution for 1 hour at room temperature.
- Remove blocking solution and add 100-400 µl primary antibody diluted in recommended antibody diluent to each section. Incubate overnight at 4°C.
- Remove antibody solution and wash sections in wash buffer three times for 5 minutes each.
- Add 100-400 µl biotinylated secondary antibody, diluted in TBST per manufacturer's recommendation, to each section. Incubate 30 minutes at room temperature.
- If using ABC avidin/biotin method, prepare ABC reagent according to the manufacturer's instructions and incubate solution for 30 minutes at room temperature.
- Remove secondary antibody solution and wash sections three times with wash buffer for 5 minutes each.
- Add 100-400 µl ABC reagent to each section and incubate for 30 minutes at room temperature.
- Remove ABC reagent and wash sections three times in wash buffer for 5 minutes each.
- Add 100-400 µl DAB or suitable substrate to each section and monitor staining closely.
- As soon as the sections develop, immerse slides in dH₂O.
- If desired, counterstain sections in hematoxylin per manufacturer's instructions.
- Wash sections in dH₂O two times for 5 minutes each.
- Dehydrate sections:
 - Incubate sections in 95% ethanol two times for 10 seconds each.
 - Repeat in 100% ethanol, incubating sections two times for 10 seconds each.
 - Repeat in xylene, incubating sections two times for 10 seconds each.
- Mount coverslips.

Immunohistochemistry Frozen Section Protocol

A Solutions and Reagents

1. Xylene,
2. Ethanol (anhydrous denatured, histological grade 100% and 95%)
3. Hematoxylin (optional)
4. **Fixative: For optimal fixative, please refer to the product data sheet**
 - 4a. 10% neutral buffered formalin
 - 4b. Acetone
 - 4c. Methanol
 - 4d. 16% formaldehyde
 - 4d1. **3% formaldehyde:** To prepare, add 18.75 ml 16% formaldehyde to 81.25 ml 1X TBS.
5. **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. **Wash buffer:** 1X Tris Buffered Saline (TBS) To prepare 1 L add 100 ml 10X TBS to 900 ml dH_2O .
7. **Methanol/Peroxidase:** To prepare, add 10 mL 30% H_2O_2 to 90 ml methanol. Store at $-20^\circ C$.
8. **Blocking Solution:** 1X TBS/0.3% Triton-X 100/5% normal goat serum
To prepare: add 500 μl goat serum and 30 μl Triton-X 100 to 9.5 ml 1X TBS.
9. **Biotinylated Secondary Antibody.**
10. **ABC Reagent:** (Vectastain ABC Kit, Vector Laboratories, Inc., Burlingame, CA). Prepare according to manufacturer's instructions 30 minutes before use.
11. **DAB Reagent or suitable substrate:** Prepare according to manufacturer's recommendations.

B Sectioning

1. **For tissue stored at $-80^\circ C$:** remove from freezer and equilibrate at $-20^\circ C$ for approximately 15 minutes before attempting to section. This may prevent cracking of the block when sectioning.
2. Section tissue at a range of 6-8 μm and place on positively charged slides.
3. Allow sections to air dry on bench for a few minutes before fixing (this helps sections adhere to slides).

C Fixation

NOTE: Consult product data sheet to determine the optimal fixative.

1. After sections have dried on the slide, fix in optimal fixative as directed below.
 - 1a. **10% Neutral buffered formalin:** 10 minutes at room temperature. Proceed with staining procedure immediately.
 - 1b. **Cold acetone:** 10 minutes at $-20^\circ C$. Air dry. Proceed with staining procedure immediately.
 - 1c. **Methanol:** 10 minutes at $-20^\circ C$. Proceed with staining procedure immediately.
 - 1d. **3% Formaldehyde:** 15 minutes at room temperature. Proceed with staining procedure immediately.
 - 1e. **3% Formaldehyde/methanol:** 15 minutes at room temperature in 3% formaldehyde, followed by 5 minutes in methanol at $-20^\circ C$ (**do not rinse in between**). Proceed with staining procedure immediately.

D Staining

1. Wash sections in wash buffer twice for 5 minutes.
2. Incubate for 10 minutes at room temperature in 3% H_2O_2 diluted in methanol.
3. Wash sections in wash buffer twice for 5 minutes.
4. Block each section with blocking solution for one hour at room temperature.
5. Remove blocking solution and add 100-400 μl diluted primary antibody to each section. (Dilute antibody in blocking solution). Incubate overnight at $4^\circ C$.
**Refer to product datasheet to determine the recommended dilution.*
6. Remove antibody solution and wash sections three times with wash buffer for 5 minutes each.
7. Add 100-400 μl secondary antibody, diluted in blocking solution per manufacturer's recommendation, to each section. Incubate 30 minutes at room temperature.
8. If using ABC avidin/biotin method, make ABC reagent according to the manufacturer's instructions and incubate solution for 30 minutes at room temperature.
9. Remove secondary antibody solution and wash sections three times in wash buffer for 5 minutes each.
10. Add 100-400 μl ABC reagent to each section and incubate for 30 min. at room temperature.
11. Remove ABC reagent and wash sections three times in wash buffer for 5 minutes each.
12. Add 100-400 μl DAB or suitable substrate to each section and monitor staining closely.
13. As soon as the sections develop, 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 minutes each.
16. **Dehydrate sections:**
 - 16a. Incubate sections in 95% ethanol two times for 10 seconds each.
 - 16b. Repeat in 100% ethanol, incubating sections two times for 10 seconds each.
 - 16c. Repeat in xylene, incubating sections two times for 10 seconds each.
17. Mount coverslips.