Store at 4C and -20C

#8111

SignalStain® Phospho-ErbB Family IHC Sampler Kit

☑ 1 Kit

Cell Signaling TECHNOLOGY®

Orders ■ 877-616-CELL (2355)
orders@cellsignal.com
Support ■ 877-678-TECH (8324)
info@cellsignal.com
Web ■ www.cellsignal.com

rev. 02/15/17

For Research Use Only. Not For Use In Diagnostic Procedures.

Products Included	Product #	Quantity	Antigen Retrieval/Diluent	Isotype
Phospho-EGF Receptor (Tyr1068) (D7A5) XP® Rabbit mAb	3777	40 µl	EDTA/SignalStain® Antibody Diluent #8112	Rabbit IgG
Phospho-HER2/ErbB2 (Tyr1221/1222) (6B12) Rabbit mAb	2243	40 µl	EDTA/SignalStain® Antibody Diluent #8112	Rabbit IgG
Phopho-HER3/ErbB3 (Tyr1289) (D1B5) Rabbit mAb	2842	40 µl	EDTA/SignalStain® Antibody Diluent #8112	Rabbit IgG
EGF Receptor (D38B1) Rabbit mAb	4267	40 µl	EDTA/ SignalStain® Antibody Diluent #8112	Rabbit IgG
*SignalStain® Antibody Diluent	8112	25 ml		
*SignalSlide® Phopsho-ErbB Family IHC Controls	8117	1 Pack		

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

Description: The SignalStain® Phospho-ErbB Family IHC Sampler Kit from Cell Signaling Technology allows the researcher to examine paraffin-embedded tissues or cells with antibodies that will detect active ErbB 1, 2 and 3 as well as total epidermal growth factor receptor (EGFR). 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. See the table above for the recommended antibody diluent for each antibody provided in the kit.

Background: The epidermal growth factor (EGF) receptor is a 170 kDa transmembrane tyrosine kinase that belongs to the HER/ErbB protein family. Ligand binding results in receptor dimerization, autophosphorylation, activation of downstream signaling and lysosomal degradation (1,2). EGFR is phosphorylated on multiple tyrosine residues, each of which leads to activation of a specific downstream pathway. Major residues involved in EGFR signaling include: Tyr845, Tyr992, Tyr1045, Tyr1068, Tyr1148 and Tyr1173 (2-9). Phosphorylation of EGFR at specific serine and threonine residues attenuates EGFR kinase activity. EGFR carboxy-terminal residues Ser1046 and Ser1047 are phosphorylated by CaM kinase II; a mutation to either of these serines results in upregulated EGFR tyrosine autophosphorylation (10).

The ErbB2 (HER2) proto-oncogene encodes a 185 kDa transmembrane, receptor-like glycoprotein with intrinsic tyrosine kinase activity (11). While ErbB2 lacks an identified ligand, ErbB2 kinase activity can be activated in the absence of a ligand when overexpressed and through heteromeric associations with other ErbB family members (12). Amplification of the ErbB2 gene and overexpression of its product are detected in almost 40% of human breast cancers, as such it is a key therapeutic target (13). ErbB2 has several key residues that are phosphorylated upon its activation including Tyr877, Tyr1221/1222 and Tyr1248 (11,14).

HER3/ErbB3 is a member of the ErbB receptor protein tyrosine kinase family, but lacks tyrosine kinase activity. Tyrosine phosphorylation of ErbB3 depends on its association with other ErbB tyrosine kinases. Ligand binding promotes formation of a heterodimer containing ErbB3 and another ErbB protein and subsequent tyrosine phosphorylation of ErbB3 by the activated ErbB kinase (15,16). At least nine putative carboxy-terminal tail tyrosine phosphorylation sites are found in ErbB3, including Tyr1222 and Tyr1289 (17). ErbB3 may function as an oncogenic unit together with other ErbB members in tumor development; ErbB2 requires ErbB3 to drive breast tumor cell proliferation (18). A novel anti-tumor strategy involves inhibiting the interaction between ErbB3 and ErbB tyrosine kinases.

Specificity/Sensitivity: Each antibody in the SignalStain® Phospho-ErbB Family IHC Sampler Kit detects endogenous levels of its target protein. Phospho-EGF Receptor (Tyr1068) (D7A5) XP® Rabbit mAb detects endogenous levels of EGFR protein only when phosphorylated at Tyr1068. Phospho-HER2/ErbB2 (Tyr1221/1222) (6B12) Rabbit mAb detects endogenous levels of ErbB2 only when phosphorylated at Tyr1221/1222. The antibody does not cross react with other activated ErbB family members or other tyrosine-phosphorylated proteins. Phopho-HER3/ErbB3 (Tyr1289) (D1B5) Rabbit mAb detects endogenous levels of HER3/ErbB3 protein only when phosphorylated at Tyr1289. The antibody does not cross-react with other phosphorylated receptor tyrosine kinases. EGF Receptor (D38B1) Rabbit mAb detects endogenous levels of total EGF receptor protein.

Source/Purification: Monoclonal antibodies are produced by immunizing animals with synthetic phosphopeptides (KLH-coupled) corresponding to residues surrounding Tyr1068 of human EGFR, Tyr1221/1222 of human ErbB2, or to Tyr1289 of human HER3/ErbB3. Monoclonal antibody to EGFR is produced by immunizing animals with a synthetic GST-fusion protein corresponding to residues containing the cytoplasmic domain of human EGF receptor.

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

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

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

Recommended Antibody Dilutions:

Phospho-EGF Receptor (Tyr1068) (D7A5) XP® Rabbit mAb #3777
Immunohistochemistry (Paraffin) 1:400
IHC Protocol: Unmasking buffer/Antibody diluent
EDTA/SignalStain® Antibody Diluent #8112

Phospho-HER2/ErbB2 (Tyr1221/1222) (6B12) Rabbit mAb #2243
Immunohistochemistry (Paraffin) 1:320
IHC Protocol: Unmasking buffer/Antibody diluent
EDTA/SignalStain® Antibody Diluent #8112
Immunohistochemistry (Frozen) 1:320
Fixative: 3% Formaldehyde/MeOH

Phopho-HER3/ErbB3 (Tyr1289) (D1B5) Rabbit mAb #2842
Immunohistochemistry (Paraffin) 1:1600
IHC Protocol: Unmasking buffer/Antibody diluent
EDTA/SignalStain® Antibody Diluent #8112

EGF Receptor (D38B1) Rabbit mAb #4267
Immunohistochemistry (Paraffin) 1:50
IHC protocol: Unmasking buffer/Antibody diluent
EDTA/ SignalStain® Antibody Diluent #8112

Please visit www.cellsignal.com for a complete listing of recommended companion products.

U.S. Patent No. 5,675,063

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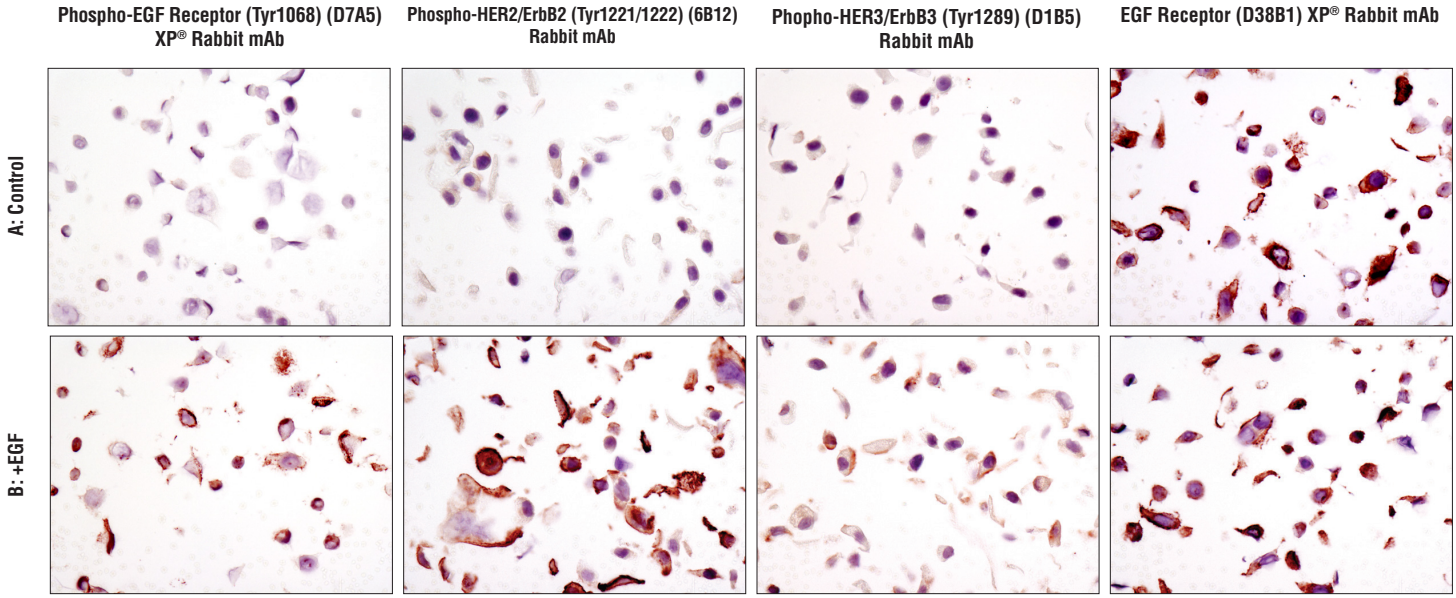
Applications Key: **W**—Western **IP**—Immunoprecipitation **IHC**—Immunohistochemistry **ChIP**—Chromatin Immunoprecipitation **IF**—Immunofluorescence **F**—Flow cytometry **E-P**—ELISA-Peptide

Species Cross-Reactivity Key: **H**—human **M**—mouse **R**—rat **Hm**—hamster **Mk**—monkey **Mi**—mink **C**—chicken **Dm**—D. melanogaster **X**—Xenopus **Z**—zebra fish **B**—bovine **Dg**—dog **Pg**—pig **Sc**—S. cerevisiae **All**—all species expected Species enclosed in parentheses are predicted to react based on 100% homology.



Immunohistochemical analysis of paraffin-embedded SK-BR-3 cell pellets, either untreated (upper) or EGF-treated (lower), using Phospho-EGF Receptor (Tyr1068) (D7A5) XP® Rabbit mAb #3777, Phospho-HER2/ErbB2 (Tyr1221/1222) (6B12) Rabbit mAb #2243, Phopho-HER3/ErbB3 (Tyr1289) (D1B5) Rabbit mAb #2842 or EGF Receptor (D38B1) XP® Rabbit mAb #4267. Cell pellets are provided in the SignalSlide® ErbB Family IHC Controls.

Background References:

(1) Hackel, P.O. et al. (1999) *Curr Opin Cell Biol* 11, 184–9.

(2) Zwick, E. et al. (1999) *Trends Pharmacol Sci* 20, 408–12.

(3) Cooper, J.A. and Howell, B. (1993) *Cell* 73, 1051–4.

(4) Hubbard, S.R. et al. *Nature* 372, 746–54.

(5) Biscardi, J.S. et al. (1999) *J Biol Chem* 274, 8335–43.

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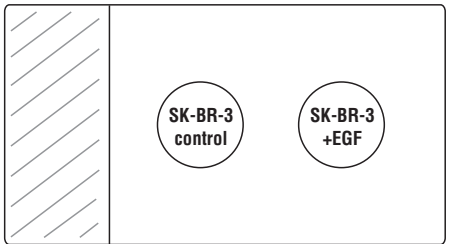

Schematic of placement of cell pellets on SignalSlide® Phospho-ErbB Family IHC Controls #8117.

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

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.
   c. **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 dH_2O. 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 phophate, 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 ($C_6H_5Na_3O_7 \bullet 2H_2O$) to 1 L dH_2O. Adjust pH to 6.0.
   b. **EDTA:** 1 mM EDTA: To prepare 1 L add 0.372 g EDTA ($C_{10}H_{14}N_2O_8Na_2 \bullet 2H_2O$) to 1 L dH_2O. Adjust pH to 8.0.
   c. **TE:** 10 mM Tris/1 mM EDTA/0.05% Tween–20, pH 9.0: To prepare 1L add 1.21 g Trizma® base ($C_4H_{11}NO_3$) and 0.372 g EDTA ($C_{10}H_{14}N_2O_8Na_2 \bullet 2H_2O$) to 950 ml dH_2O. Adjust pH to 9.0, add 0.5 ml Tween–20, then adjust final volume to 1000 ml 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 5ml 1X TBST add 250 µl normal goat serum.
10. Biotinylated secondary antibody.
11. **ABC Reagent:** (Vectastain ABC Kit, Vector Laboratories, Inc., Burlingame, CA) Prepare according to manufacturer's instructions 30 minutes before use.
12. **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.

1. **Deparaffinize/hydrate sections:**
   a. Incubate sections in three washes of xylene for 5 minutes each.
   b. Incubate sections in two washes of 100% ethanol for 10 minutes each.
   c. Incubate sections in two washes of 95% ethanol for 10 minutes each.
2. Wash sections twice in dH_2O for 5 minutes each.

C *Antigen Unmasking

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

1. **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.
2. **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.
3. **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.
4. **For Pepsin:** Digest for 10 minutes at 37°C.

D Staining

1. Wash sections in dH_2O three times for 5 minutes each.
2. Incubate sections in 3% hydrogen peroxide for 10 minutes.
3. Wash sections in dH_2O twice for 5 minutes each.

NOTE: Consult product data sheet for recommended antibody diluent.

4. Wash section in wash buffer for 5 minutes.
5. Block each section with 100-400 µl blocking solution for 1 hour 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°C.
7. Remove antibody solution and wash sections in wash buffer three times for 5 minutes each.
8. Add 100-400 µl biotinylated secondary antibody, diluted in TBST per manufacturer's recommendation, to each section. Incubate 30 minutes at room temperature.
9. If using ABC avidin/biotin method, prepare ABC reagent according to the manufacturer's instructions and incubate solution for 30 minutes at room temperature.
10. Remove secondary antibody solution and wash sections three times with wash buffer for 5 minutes each.
11. Add 100-400 µl ABC reagent to each section and incubate for 30 minutes at room temperature.
12. Remove ABC reagent and wash sections three times in wash buffer for 5 minutes each.
13. Add 100-400 µl DAB or suitable substrate to each section and monitor staining closely.
14. As soon as the sections develop, immerse slides in dH_2O.
15. If desired, counterstain sections in hematoxylin per manufacturer's instructions.
16. Wash sections in dH_2O two times for 5 minutes each.
17. Dehydrate sections:
    a. Incubate sections in 95% ethanol two times for 10 seconds each.
    b. Repeat in 100% ethanol, incubating sections two times for 10 seconds each.
    c. Repeat in xylene, incubating sections two times for 10 seconds each.
18. 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_20_2 to 90 ml methanol. Store at -20°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°C:** remove from freezer and equilibrate at -20°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°C. Air dry. Proceed with staining procedure immediately.
   - **1c. Methanol:** 10 minutes at –20°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, followed by 5 minutes in methanol at –20°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 in 3% H_20_2 diluted in methanol at room temperature.
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°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_20.
14. If desired, counterstain sections in Hematoxylin per manufacturer's instructions.
15. Wash sections in dH_20 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.