Store at 4°C and -20°C

#8107

SignalStain® Akt Pathway IHC Sampler Kit

☑ 1 Kit

Cell Signaling TECHNOLOGY®

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rev. 02/21/17

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

Products Included	Product #	Quantity	Antigen Retrieval/Diluent	Isotype
Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb	4060	40 µl	Citrate/ SignalStain® Antibody Diluent #8112	Rabbit IgG
Phospho-S6 Ribosomal Protein (Ser235/236) (D57.2.2E) XP® Rabbit mAb	4858	40 µl	Citrate/ SignalStain® Antibody Diluent #8112	Rabbit IgG
Akt (pan) (C67E7) Rabbit mAb	4691	40 µl	Citrate/ SignalStain® Antibody Diluent #8112	Rabbit IgG
PTEN (D4.3) XP® Rabbit mAb	9188	40 µl	Citrate/ SignalStain® Antibody Diluent #8112	Rabbit IgG
*SignalStain® Antibody Diluent	8112	25 ml		
*SignalSlide® Akt Family IHC Controls	8115	1 Pack		

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

Description: SignalStain® Akt Pathway IHC Sampler Kit from Cell Signaling Technology allows the researcher to examine paraffin-embedded tissues or cells with antibodies directed against proteins involved in Akt signaling. Each antibody is validated for use in immunohistochemical assays using multiple approaches. The kit also contains control slides that can be used to verify the performance of each antibody, along with a primary antibody diluent. Please see table above for the recommended antibody diluent for each antibody provided in the kit.

Background: The protein kinase Akt (also called PKB or Rac) is activated by insulin and growth and survival factors to function in a wortmannin-sensitive pathway involving PI3 kinase (1,2). Akt is activated by phospholipid binding and activation loop phosphorylation at Thr308 by PDK1 (3) and by carboxy terminal phosphorylation at Ser473 by the mTORC2 complex composed of mammalian target of rapamycin (mTOR) in a complex with rictor and Sin1 (4,5). Akt signaling is negatively regulated via the PTEN phosphatase (6).

Akt's several functions include inhibition of apoptosis (7-9), regulation of glycogen synthesis via GSK-3 (10), and promotion of the cell cycle (11). Akt also regulates protein synthesis by phosphorylating mTOR in a rapamycin-sensitive complex containing raptor (mTORC1) (12). Akt also effects mTOR activity via phosphorylation and inhibition of PRAS40 (40 kDa, proline-rich protein), which binds to raptor in the mTORC1 complex and inhibits mTOR activity (13). Phosphorylation of PRAS40 by Akt at Thr246 relieves PRAS40 inhibition of mTORC1 (14), allowing protein synthesis to occur. Active of mTORC1 signals to p70 S6 kinase, which in turn phosphorylates S6 ribosomal protein. Phosphorylation of S6 ribosomal protein correlates with an increase in translation of a subset of mRNA transcripts that encode ribosomal proteins, translation elongation factors as well as regulators of cell cycle progression (15). Important S6 ribosomal protein phosphorylation sites include Ser235, Ser236, Ser240 and Ser244 within a small carboxy-terminal region (16).

Specificity/Sensitivity: Each antibody in the SignalStain® Akt Pathway IHC Sampler Kit detects endogenous levels of its target protein and does not cross-react with related proteins.

Source/Purification: Monoclonal antibodies are produced by immunizing animals with synthetic phosphopeptides (KLH-coupled) corresponding to residues surrounding the phosphorylation site(s) of interest, and synthetic peptides (KLH-coupled) derived from the carboxy-terminal sequence of mouse Akt or from the carboxy-terminal sequence of human PTEN.

Background References:

(1) Franke, T.F. et al. (1997) *Cell* 88, 435-7.

(2) Franke, T.F. et al. (1995) *Cell* 81, 727-36.

(3) Alessi, D.R. et al. (1996) *EMBO J* 15, 6541-51.

(4) Sarbassov, D.D. et al. (2005) *Science* 307, 1098-101.

(5) Jacinto, E. et al. (2006) *Cell* 127, 125-37.

(6) Cantley, L.C. and Neel, B.G. (1999) *Proc Natl Acad Sci U S A* 96, 4240-5.

(7) Cardone, M.H. et al. (1998) *Science* 282, 1318-21.

(8) Brunet, A. et al. (1999) *Cell* 96, 857-68.

(9) Zimmermann, S. and Moelling, K. (1999) *Science* 286, 1741-4.

(10) Cross, D.A. et al. *Nature* 378, 785-9.

(11) Zhou, B.P. et al. (2001) *Nat Cell Biol* 3, 245-52.

(12) Navé, B.T. et al. (1999) *Biochem J* 344 Pt 2, 427-31.

(13) Vander Haar, E. et al. (2007) *Nat Cell Biol* 9, 316-23.

(14) Sancak, Y. et al. (2007) *Mol Cell* 25, 903-15.

(15) Peterson, R.T. and Schreiber, S.L. (1998) *Curr Biol* 8, R248-50.

(16) Ferrari, S. et al. (1991) *J Biol Chem* 266, 22770-5.

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.

*Control slides should be stored at 4°C.

Recommended Antibody Dilutions:

Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb #4060
Immunohistochemistry (Paraffin) 1:50
IHC protocol: Unmasking buffer/Antibody diluent Citrate/ SignalStain® Antibody Diluent #8112

Phospho-S6 Ribosomal Protein (Ser235/236) (D57.2.2E) XP® Rabbit mAb #4858
Immunohistochemistry (Paraffin) 1:50
IHC protocol: Unmasking buffer/Antibody diluent Citrate/ SignalStain® Antibody Diluent #8112
Immunohistochemistry (Frozen) 1:50
Fixative: 10% Neutral buffered formalin

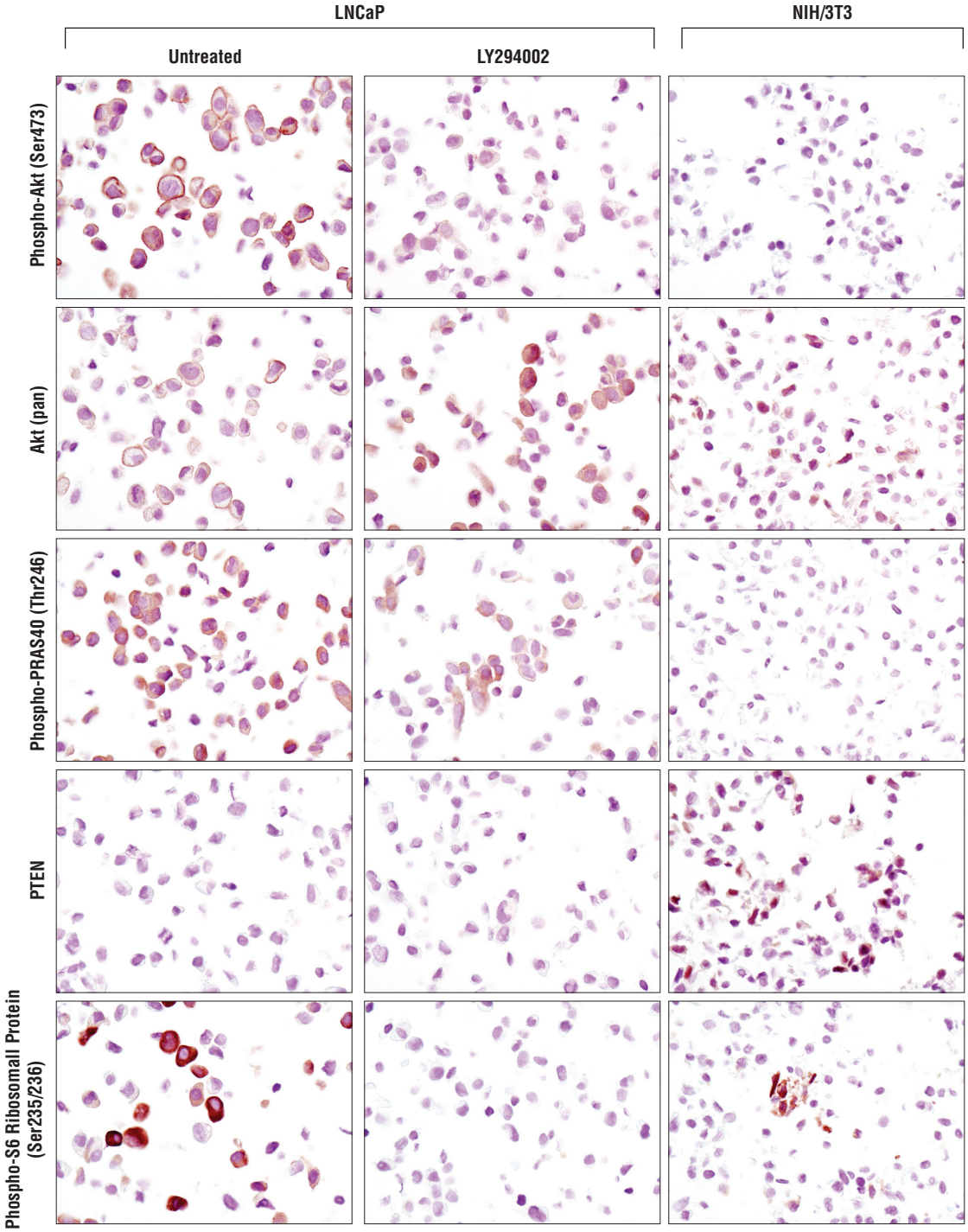
Phospho-PRAS40 (Thr246) (C77D7) Rabbit mAb #2997
Immunohistochemistry (Paraffin) 1:50
IHC protocol: Unmasking buffer/Antibody diluent Citrate/ SignalStain® Antibody Diluent #8112

Akt (pan) (C67E7) Rabbit mAb #4691
Immunohistochemistry (Paraffin) 1:300
IHC protocol: Unmasking buffer/Antibody diluent Citrate/SignalStain® Antibody Diluent #8112

PTEN (D4.3) XP® Rabbit mAb #9188
Immunohistochemistry (Paraffin) 1:125
IHC protocol: Unmasking buffer/Antibody diluent Citrate/ SignalStain® Antibody Diluent #8112

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**—zebrafish **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 LNCaP cell pellets, either untreated (left) or LY294002-treated (middle) or NIH/3T3 cell pellets (right), using Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb, Phospho-S6 Ribosomal Protein (Ser235/236) (D57.2.2E) XP® Rabbit mAb, Phospho-PRAS40 (Thr246) (C77D7) Rabbit mAb, Akt (pan) (C67E7) Rabbit mAb and PTEN (D4.3) XP® Rabbit mAb. Cell pellets are provided in the SignalSlide® Akt Family IHC Controls.

Related Products:

Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb #4060

Phospho-S6 Ribosomal Protein (Ser235/236) (D57.2.2E) XP® Rabbit mAb #4858

Phospho-PRAS40 (Thr246) (C77D7) Rabbit mAb #2997

Akt (pan) (C67E7) Rabbit mAb #4691

PTEN (D4.3) XP® Rabbit mAb #9188

Normal Rabbit (DA1E) mAb IgG #3900

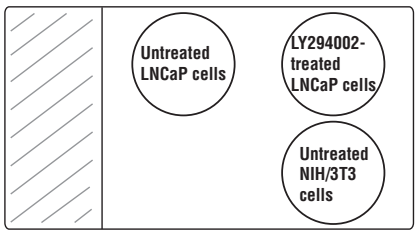
SignalStain® Antibody Diluent #8112

Phospho-Akt (Ser473) Blocking Peptide #1140

Phospho-S6 Ribosomal Protein (Ser235/236) Blocking Peptide #1220

Akt (pan) Blocking Peptide #1085

PTEN Blocking Peptide #1250



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_2O_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_2O_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_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.