

Phospho-Syk Antibody Sampler Kit

- ✓ 4 x 40 µl
(4 Western mini-blot
per primary antibody)

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

Products Included	Product #	Quantity	Applications	Species Cross-Reactivity	Mol. Wt.	Source
Phospho-Syk (Tyr323) Antibody	2715	40 µl	W, IP	H, M	72	Rabbit
Phospho-Syk (Tyr525/526) Antibody	2711	40 µl	W	H, M	72	Rabbit
Syk Antibody	2712	40 µl	W	H, M	72	Rabbit
Phospho-Zap-70 (Tyr319)/Syk (Tyr352) Antibody	2701	40 µl	W, IP, IF-IC, F	H, M	70 kDa (Zap-70), 72 kDa (Syk)	Rabbit
Anti-rabbit IgG, HRP-linked Antibody	7074	100 µl				Goat

Background: Syk, a protein tyrosine kinase, is widely expressed and plays an important role in intracellular signal transduction in hematopoietic cells (1-3). Syk interacts with immunoreceptor tyrosine-based activation motifs (ITAMs) located in the cytoplasmic domains of immune receptors (4). It couples the activated immunoreceptors to downstream signaling events that mediate diverse cellular responses, including proliferation, differentiation and phagocytosis (4). There is also evidence of a role for Syk in nonimmune cells, and Syk is a potential tumor suppressor in human breast carcinomas (5). Tyr323 is a negative regulatory phosphorylation site within the SH2-kinase linker region in Syk. Phosphorylation of Tyr323 provides a direct binding site to the TKB domain of Cbl (6,7). Tyrosine 352 of Syk is involved in the association of PLC-γ1 (8). Tyrosines 525 and 526 are located in the activation loop of the Syk kinase domain, and phosphorylation of Tyr525/526 of human Syk (equivalent to the Tyr519/520 of mouse Syk) is essential for Syk function (9).

Description: Phospho-Syk Sampler Kit provides an economical means to evaluate the activation status of Syk, including the phosphorylation of Tyr323, Tyr352 and Tyr525/526. The control Syk Antibody is also included. The kit contains enough primary and secondary antibodies for four mini-blot experiments.

Specificity/Sensitivity: Phospho-Syk (Ser323) and Phospho-Syk (Tyr525/526) Antibodies detect transfected, Tyr323 and Tyr 525/526 phosphorylated human Syk. Syk Antibody and Phospho-Zap-70 (Tyr319)/Syk (Tyr352) Antibodies detect endogenous levels of total human Syk and Tyr352 phosphorylated Syk, respectively. Each phospho-Syk antibody recognizes only the specific phosphorylated form of Syk. The control Syk Antibody recognizes both nonphosphorylated and phosphorylated forms of Syk. Phospho-Zap-70 (Tyr319)/Syk (Tyr352) Antibody cross-reacts with the phosphorylated form of Zap-70. All other antibodies in the kit do not cross-react with phosphorylated or nonphosphorylated forms of other related family members.

Source/Purification: Polyclonal antibodies are produced by immunizing rabbits with synthetic phospho-peptides (KLH-coupled) corresponding to residues surrounding Tyr323, Tyr352 or Tyr525/526 of human Syk. The control Syk Antibody is produced by immunizing rabbits with a synthetic nonphospho-peptide (KLH-coupled) corresponding to the carboxy terminal sequence of human Syk. Polyclonal antibodies are purified by protein A and peptide affinity chromatography.

Background References:

- (1) Cheng, A.M. and Chan, A.C. (1997) *Curr. Opin. Immunol.* 9, 528–533.
- (2) Kurosaki, T. et al. (1997) *Curr. Opin. Immunol.* 9, 309–318.
- (3) Chu, D.H. et al. (1998) *Immunol. Rev.* 165, 167–180.
- (4) Turner, M. et al. (2000) *Immunol. Today* 21, 148–154.
- (5) Coopman, P.J. et al. (2000) *Nature* 406, 742–747.
- (6) Decker, M. et al. (1998) *J. Biol. Chem.* 273, 8867–8874.
- (7) Rao, N. et al. (2001) *EMBO J.* 20, 7085–7095.
- (8) Law, C.L. et al. (1996) *Mol. Cell. Biol.* 16, 1305–1315.
- (9) Zhang, J. et al. (2000) *J. Biol. Chem.* 275, 35442–35447.

Storage: Supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100 µg/ml BSA and 50% glycerol. Store at -20°C. *Do not aliquot the antibody.*

Recommended Antibody Dilutions:

See table below.

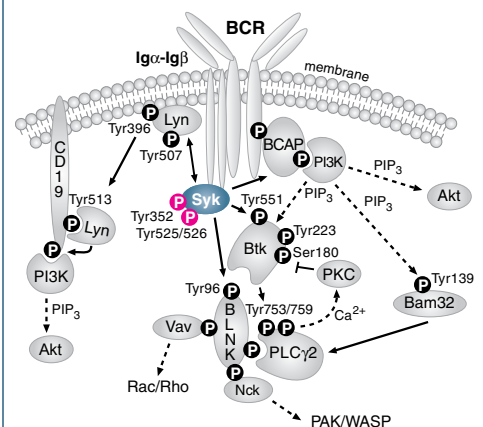
Companion Products:

Anti-rabbit IgG, HRP-linked Antibody #7074

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

Biotinylated Protein Ladder Detection Pack #7727

20X LumiGLO® Reagent and 20X Peroxide #7003



Phospho-Syk Signaling Pathway

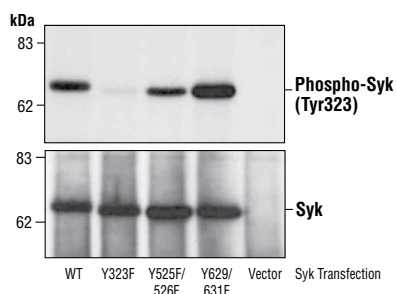
Applications Key: W—Western IP—Immunoprecipitation IHC—Immunohistochemistry IC—Immunocytochemistry IF—Immunofluorescence F—Flow cytometry E—ELISA D—DELFIATM

Species Cross-Reactivity Key: H—human M—mouse R—rat Hm—hamster Mk—monkey Mi—mink C—chicken X—Xenopus Z—zebra fish B—bovine All—all species expected
Species enclosed in parentheses are predicted to react based on 100% sequence homology.

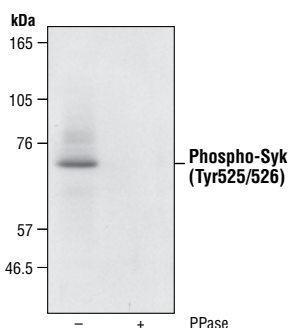
Recommended Antibody Dilutions

Product Name	Product #	Western blot	Immuno-precipitation	Immuno-histochemistry	Immuno-fluorescence	Immuno-cytochemistry	Flow Cytometry
Phospho-Syk (Tyr323) Antibody	2715	1:1000	1:50	NT	NT	NT	NT
Phospho-Syk (Tyr525/526) Antibody	2711	1:1000	Neg.	Neg.	NT	NT	NT
Syk Antibody	2712	1:1000	Neg.	NT	Neg.	NT	NT
Phospho-Zap-70 (Tyr319)/Syk (Tyr352) Antibody	2701	1:1000	1:100	NT	1:100 ^{IF-IC}	NT	1:25

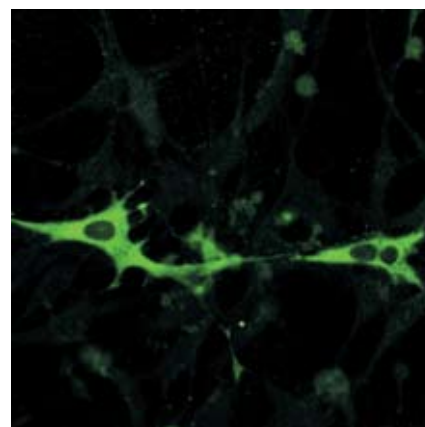
P = Paraffin IF-IC = Cultured Cell Lines ABC = ABC Detection N/A = Not Applicable NT = Not Tested Neg. = Negative
All antibodies were successfully tested for use in ELISA (dilution 1:1000)



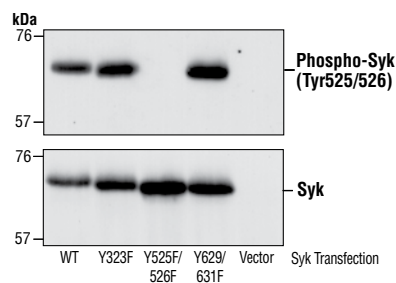
Phospho-Syk (Tyr323) Antibody: Western blot analysis of cell lysates from 293T cells expressing recombinant Wild-type and mutant Syk proteins (which were activated by cotransfected CD8), using Phospho-Syk (Tyr323) Antibody #2715 (upper) and Syk Antibody #2712 (lower). (Provided by Dr. Alagarsamy L. Reddi, laboratory of Dr. Hamid Band, Harvard University, Massachusetts.)



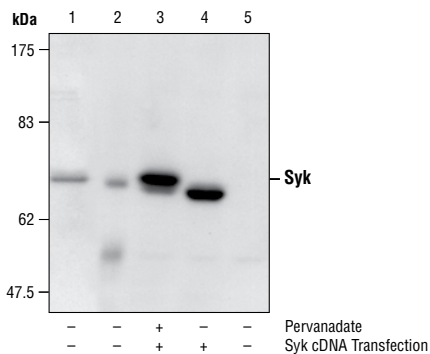
Phospho-Syk (Tyr525/526) Antibody: Dephosphorylation of phosphorylated Wild-type Syk abolished Phospho-Syk (Tyr525/526) Antibody's recognition of this protein.



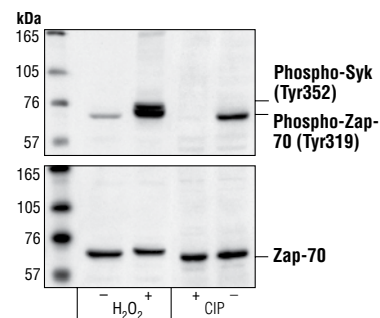
Phospho-Zap-70 (Tyr319)/Syk (Tyr352) Antibody: Immunocytochemical staining of NIH/3T3 cells transiently transfected with DNA construct expressing Syk, using Phospho-Zap-70 (Tyr319)/Syk (Tyr352) Antibody #2701.



Phospho-Syk (Tyr525/526) Antibody: Western blot analysis of cell lysates from 293T cells expressing recombinant Wild-type and mutant Syk proteins (which were activated by cotransfected CD8), using Phospho-Syk (Tyr525/526) Antibody #2711 (upper) and Syk Antibody #2712 (lower). (Provided by Dr. Hamid Band, Harvard University, Massachusetts.)



Syk Antibody: Western blot analysis of cell lysates from Jurkat cells (lane 1), THP1 cells (lane 2) and DT40 cells transfected with Syk cDNA expression construct (lanes 3-5), using Syk Antibody #2712.



Phospho-Zap-70 (Tyr319)/Syk (Tyr352) Antibody: Western blot analysis of extracts from Jurkat cells, starved for 16 hours, and treated with H₂O₂ (2 mM) or with calf intestinal alkaline phosphatase (CIP), using Phospho-Zap-70 (Tyr319)/Syk (Tyr352) Antibody #2701 (upper) or control Zap-70 Antibody #2702 (lower).

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°C with gentle shaking, overnight.

A Solutions and Reagents

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

- 1X Phosphate Buffered Saline (PBS)
- 1X 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
- Transfer Buffer:** 25 mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5)
- 10X Tris Buffered Saline (TBS):** 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).
- Nonfat Dry Milk (weight to volume [w/v])
- 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%).
- Wash Buffer:** 1X TBS, 0.1% Tween-20 (TBS/T)
- Bovine Serum Albumin (BSA)
- 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 System #7071:** Includes biotinylated protein ladder, secondary anti-rabbit (#7074) antibody conjugated to horseradish peroxidase (HRP), anti-biotin antibody conjugated to HRP, LumiGLO[®] chemiluminescent reagent and peroxide.
- Prestained Protein Marker, Broad Range (Premixed Format) #7720
- Biotinylated Protein Ladder Detection Pack #7727
- 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.

- Treat cells by adding fresh media containing regulator for desired time.
- Aspirate media from cultures; wash cells with 1X PBS; aspirate.
- 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.
- Sonicate for 10–15 seconds to shear DNA and reduce sample viscosity.
- Heat a 20 µl sample to 95–100°C for 5 minutes; cool on ice.
- Microcentrifuge for 5 minutes.
- 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.

- Electrotransfer to nitrocellulose or PVDF membrane.

C 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.

- (Optional) After transfer, wash nitrocellulose membrane with 25 ml TBS for 5 minutes at room temperature.
- Incubate membrane in 25 ml of blocking buffer for 1 hour at room temperature.
- Wash three times for 5 minutes each with 15 ml of TBS/T.
- Incubate membrane and primary antibody (at the appropriate dilution) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.
- Wash three times for 5 minutes each with 15 ml of TBS/T.
- 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.
- Wash three times for 5 minutes each with 15 ml of TBS/T.

D Detection of Proteins

- 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.

- 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.

Immunoprecipitation Protocol / (For Analysis By Western Immunoblotting)

A 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 -glycerophosphate, 1 mM Na_3VO_4 , 1 $\mu\text{g}/\text{ml}$ 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 or G Agarose Beads:** (Can be stored for 2 weeks at 4°C.) Please prepare according to manufacturer's instructions. Use Protein A for rabbit IgG pull down and Protein G for mouse IgG pull down.
- 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

B Preparing Cell Lysates

- Aspirate media. Treat cells by adding fresh media containing regulator for desired time.
- To harvest cells under nondenaturing conditions, remove media and rinse cells once with ice-cold PBS.

- Remove PBS and add 0.5 ml 1X ice-cold cell lysis buffer plus 1 mM PMSF* to each plate (10 cm) and incubate the plates on ice for 5 minutes.
- Scrape cells off the plates and transfer to microcentrifuge tubes. Keep on ice.
- Sonicate samples on ice four times for 5 seconds each.
- Microcentrifuge for 10 minutes at 4°C, and transfer the supernatant to a new tube. If necessary, lysate can be stored at -80°C.

C Immunoprecipitation

- Take 200 μl cell lysate and add primary antibody. Incubate with gentle rocking overnight at 4°C.
- Add either protein A or G agarose beads (20 μl of 50% bead slurry). Incubate with gentle rocking for 1–3 hours at 4°C.
- Microcentrifuge for 30 seconds at 4°C. Wash pellet five times with 500 μl of 1X cell lysis buffer. Keep on ice during washes.
- Resuspend the pellet with 20 μl 3X SDS sample buffer. Vortex, then microcentrifuge for 30 seconds.
- Heat the sample to 95–100°C for 2–5 minutes.
- Load the sample (15–30 μl) on SDS-PAGE gel (12–15%).
- Analyze sample by Western blotting (see Western Immunoblotting Protocol).

ELISA Protocol

A Solutions and Reagents

- Carbonate Buffer:** 15 mM Na_2CO_3 , 35 mM NaHCO_3 , 0.2 g/L NaN_3 (pH 9.6). Use 1 μM synthetic peptide in carbonate buffer.
- 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.
- Wash Buffer:** 1X PBS containing 0.05% Tween-20 (PBST)
- Blocking Buffer:** 10 mg/ml bovine serum albumin (BSA) in PBST
- Antibody Dilution Buffer:** 1.0 mg/ml BSA in PBST
- Enzyme Substrate:** 1 mg/ml p-Nitrophenyl Phosphate (Sigma #N-9389) in 0.1 M diethanolamine buffer
- 0.1 M Diethanolamine Buffer:** 97 ml diethanolamine (Sigma #398179), 100 mg MgCl_2 and 0.2 g sodium azide; add 800 ml deionized water, adjust pH to 9.8 with 10 M HCl, then add deionized water to 1 liter.

B Coating Plates

- Coat the wells of a 96-well microtiter plate with 100 μl of 1 μM synthetic peptide in carbonate buffer by incubating overnight at 4°C or for 2 to 6 hours at 37°C. If the peptide does not bind or absorb, try other buffers in the pH 4–8 range.
- Discard the uncoated synthetic peptide; wash the coated wells at least three times with PBST.
- Block remaining active sites by incubating the plate with 200 $\mu\text{l}/\text{well}$ of blocking buffer for 1 hour at 37°C.
- Wash the wells at least three times with PBST.

C Protocol

- Prepare appropriate dilution of primary antibody with antibody dilution buffer. Add 100 μl to wells and incubate for 2 hours at 37°C or overnight at 4°C.
- Wash the wells at least three times with PBST.
- Dilute secondary antibody in PBST. Add 100 μl to wells and incubate for 1 hour at 37°C.
- Wash the wells at least three times with PBST.
- Add 100 μl enzyme substrate and incubate for 1–3 hours at 37°C.
- Read the absorbance at 405 nm with a microtiter plate reader.

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.

A Solutions and Reagents

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

1. Fetal Bovine Serum (FBS)
2. **10X Phosphate Buffered Saline (PBS):** To prepare 1L 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.
3. **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.
4. **Tris Buffered Saline (TBS):** 50 mM Tris-HCl (pH 7.4), 150 mM NaCl.
5. **0.1% Sodium Borohydride:** Dissolve in PBS on day of use.
6. **0.2% Triton X-100:** Prepare stock of 20% Triton in PBS; rotate tube overnight to dissolve. Dilute to 0.2%.
7. 1% Bovine Serum Albumin (BSA)
8. **Blocking Buffer:** 10% horse or goat serum, 1% BSA, 0.02% NaN₃, 1X PBS.
9. **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.

B 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.

C Methanol (protein precipitation) Fixation

1. Immerse coverslips in -20°C 100% methanol for 10 minutes.
2. Rinse slips three times for 5 minutes each with room temperature TBS.
3. Quench cells in fresh 0.1% sodium borohydride in TBS for 5 minutes.
4. 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 E1.

D Paraformaldehyde (cross-linking) Fixation

1. Immerse coverslips in 4% paraformaldehyde at room temperature for 10 minutes.
2. 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.
3. Wash slips three times for 5 minutes each with TBS at room temperature.
4. 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.

E Blocking

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

F 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 conditions.
5. Mount coverslips on slides using the ProLong™ Antifade Kit. Store slides at room temperature in the dark.