INSTRUCTIONS

ActivX® Serine Hydrolase Probes

88316 88317 88318

2347.0

Number	Description
88316	**ActivX Azido-FP Serine Hydrolase Probe**, 3.5µg Molecular Weight: 350.37
88317	**ActivX Desthiobiotin-FP Serine Hydrolase Probe**, 4.6µg Molecular Weight: 463.57
88318	**ActivX TAMRA-FP Serine Hydrolase Probe**, 6.8µg Molecular Weight: 679.76

Storage: Upon receipt store at -80°C. Product shipped with dry ice.

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Introduction

The Thermo Scientific ActivX FP Serine Hydrolase Probes enable selective labeling and enrichment of active serine hydrolases. Fluorophosphonate (FP) probes specifically label serines of enzymatically active serine hydrolases.[1-3] FP probes are also effective for screening small molecule inhibitors against enzymes derived from cell lysates, subcellular fractions, tissues and recombinant proteins. Depending on the active-site probe tag group and the enzyme class to be profiled, active site-labeled enzymes are detected and quantified by Western blot, fluorescent gel imaging or mass spectrometry. We offer three distinct probes for the following applications:

- Azido-FP probes are used in combination with phosphine- or alkyne-derivatized tags for detection or enrichment.
- Desthiobiotin-FP probes are used for enrichment and detection of active site-labeled proteins by Western blot or mass spectrometry.
- TAMRA-FP probes are used to label and detect serine hydrolase activity in samples using fluorescent gel imaging,[2] capillary electrophoresis or mass spectrometry.

The serine hydrolase superfamily is one of the largest, most diverse enzyme families in eukaryotic proteomes.[4] Serine hydrolases are generally grouped into two large 100+ member families: serine proteases (e.g., trypsin, elastase and thrombin) and metabolic serine hydrolases. Although many family members share a common catalytic active site, metabolic serine hydrolases are divided into multiple enzyme subclasses based on differences in structure, catalytic mechanism and substrate preference.

Assessment of active-site labeling can be accomplished by either Western blot or mass spectrometry (MS) (Figure 1). For the Western blot workflow, desthiobiotin-labeled proteins are enriched for SDS-PAGE analysis and subsequent detection with specific antibodies. For the MS workflow, desthiobiotin-labeled proteins are reduced, alkylated and enzymatically digested to peptides. Only the desthiobiotin-labeled, active-site peptides are enriched for analysis by LC-MS/MS. Both workflows are effective for determining inhibitor target binding, but only the MS workflow can identify global inhibitor targets and off-targets.[3,4]

Procedure Summary

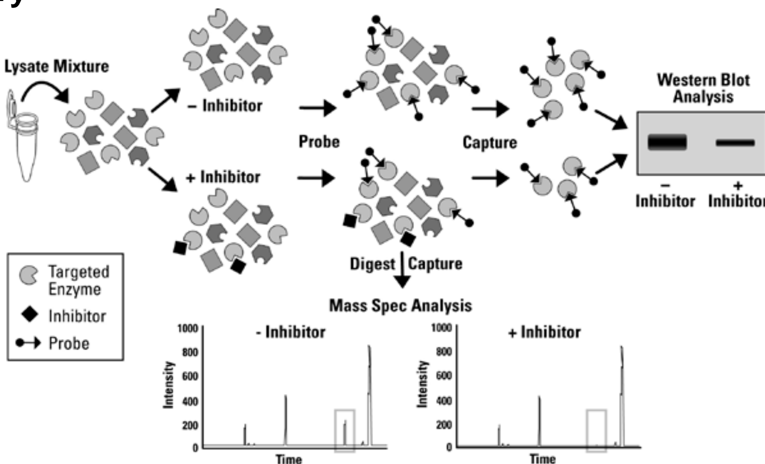

Figure 1. Western blot and mass spectrometry workflows enable targeted capture and analysis of enzymes using the active-site probes.

Important Product Information

- Azido-FP and desthiobiotin-FP probes might not be visible in the vial. Store the TAMRA-FP probe and stock solutions protected from light.
- Dissolve the serine hydrolase FP probes in dry water-miscible organic solvent, such as DMSO, before diluting to < 10% solvent in final aqueous reaction buffer. Stock solutions in DMSO can be stored at -20°C for up to 6 months and can tolerate multiple freeze-thaw cycles.
- The serine hydrolase FP probes only label active serine hydrolases and are membrane permeable, allowing for labeling within intact cells. Do not use protease inhibitors containing PMSF or AEBSF in cell lysate preparations. Avoid denaturing detergents, such as SDS, in cell lysis buffers.
- For best results, perform labeling reactions using 1-5µM of probe for protein staining or enrichment. At 2µM probe final concentration, each product can label 100 reactions of 50µL for staining or 10 reactions of 500µL for enrichment.
- The Azido-FP Serine Hydrolase Probe reacts with phosphine reagents (Product No. 88900, 88901, 88906, 88907, 88908, 88909) in Staudinger ligations or with alkynes in Huisgen cycloaddition (i.e., Click) reactions.
- For the TAMRA-FP Serine Hydrolase Probe, use fluorescent imagers with Cy®3 filters. Green (532) laser Ex/Em: 552/575nm. The molar extinction coefficient is > 80,000$M^{-1}cm^{-1}$.
- Preclearing lysates with streptavidin agarose to remove endogenous biotinylated proteins may be required before labeling and detection using the Desthiobiotin-FP Serine Hydrolase Probe.
- Adding urea to samples after labeling is required to denature proteins before capture using streptavidin agarose. Prepare urea buffers the same day as the experiment.
- Desalting labeling reactions using the Thermo Scientific Zeba Spin Desalting Columns or equivalent is required to remove excess non-reacted FP probe before tryptic digestion and mass spectrometry analysis.
- Desthiobiotin-FP modification of serine-containing active-site peptides results in a monoisotopic mass increase of 443.2913 Da. TAMRA-FP modification of serine-containing active-site peptides results in a monoisotopic mass increase of 659.7514 Da.

Procedure for Protein Labeling and Detection

Note: This protocol is for labeling 100µg of sample (50µL at 2mg/mL). Scale the procedure accordingly for other amounts.

A. Additional Materials Required

- Dry dimethylsulfoxide (DMSO) (Product No. 20688)
- Ice-cold phosphate-buffered saline (PBS): 0.1M sodium phosphate, 0.15M sodium chloride; pH 7.2 (Product No. 28372)
- Protein Assay: Thermo Scientific Pierce BCA Protein Assay Reagent Kit (Product No. 23224) or Pierce 660nm Protein Assay Reagent (Product No. 22660)
- Lysis Buffer: Thermo Scientific Pierce IP Lysis Buffer (Product No. 87787), T-PER Tissue Protein Extraction Reagent (Product No. 78510), M-PER Mammalian Protein Extraction Reagent (Product No. 78501) or equivalent
- Microcentrifuge
- 1.7mL microcentrifuge tubes or equivalent
- 2X Laemmli reducing sample buffer (Product No. 84788 or equivalent)
- Optional: Thermo Scientific Halt Phosphatase Inhibitor Cocktail (Product No. 78420)

B. Cell Lysis

1. For adherent cells, harvest with trypsin-EDTA and then centrifuge at 500 × *g* for 5 minutes. For suspension cells, harvest by centrifuging at 500 × *g* for 5 minutes. For tissues, cut 5-20mg of tissue into small pieces.
2. Wash cells by suspending the cell pellet with ice-cold PBS.
3. Transfer 2-4 × 10^6 cells to a 1.5mL microcentrifuge tube. Pellet cells by centrifugation at 500 × *g* for 2-3 minutes and remove the PBS.
4. Add 100µL of Lysis Buffer containing phosphatase inhibitors (1:100) and incubate on ice for 10 minutes with periodic mixing. Homogenize tissue using a Dounce homogenizer or tissue grinder.

   Note: For maximal labeling of serine hydrolases, do not add protease inhibitors that contain PMSF or AEBSF.
5. Centrifuge tube at 16,000 × *g* at 4°C for 5 minutes.
6. Transfer the supernatant (total lysate) to a new tube.

C. Sample Labeling

1. Perform a protein assay to measure the lysate's protein concentration.
2. Dilute lysate with Lysis Buffer to 2mg/mL and transfer 50µL (100µg) to a microcentrifuge tube.
3. Equilibrate serine hydrolase FP probe to room temperature in pouch with desiccant.
4. Dissolve serine hydrolase FP probe in 100µL of DMSO to make a 0.1mM stock solution.

   Note: Store unused stock solution at -20°C for up to 6 months.
5. Add 1µL of serine hydrolase FP probe stock to each sample for a final concentration 2µM probe and mix.

   Note: To determine serine hydrolase FP probe specificity, denature samples by boiling in sample buffer for 5 minutes before adding the FP probe.
6. Incubate sample for 15-30 minutes at room temperature. Longer incubation times might be necessary to label some serine hydrolases.
7. Stop reactions by adding 2X Laemmli reducing sample buffer and boiling for 5 minutes.
8. Analyze labeled proteins by SDS-PAGE followed by fluorescent gel scanning (TAMRA-FP probe) or streptavidin-HRP Western blotting (Desthiobiotin-FP probe).

Procedure for Protein Labeling and Enrichment

Note: This protocol is for labeling 1mg of sample at 2mg/mL. Scale the procedure accordingly for other amounts. Perform Sections A-B from Procedure for Protein Labeling and Detection at 10X scale and then proceed with the following protocol.

A. Additional Materials Required

- Urea, Sequanal grade (Product No. 29700)
- Pierce® IP Lysis Buffer (Product No. 87787) or equivalent lysis buffer
- Thermo Scientific High Capacity Streptavidin Agarose (Product No. 20357)

B. Material Preparation

10M Urea/ Lysis Buffer	Dissolve 0.9g of urea with 1.5mL of Lysis Buffer for each labeling reaction. Buffer may require heating to 37°C to fully dissolve urea.
5M Urea/ Lysis Buffer	Dilute 1mL of 10M Urea/IP Lysis Buffer with 1mL of Lysis Buffer for each labeling reaction.

C. Sample Labeling

1. Dilute lysate with Lysis Buffer to 2mg/mL and transfer 500µL (1mg) to a microcentrifuge tube.
2. Equilibrate serine hydrolase FP probe to room temperature in pouch with desiccant.
3. Dissolve serine hydrolase FP probe in 100µL DMSO to make a 0.1mM stock solution.

   Note: Store unused probe at -20°C for up to 6 months.
4. Add 10µL of serine hydrolase FP probe stock to each sample for a final mix concentration of 2µM.

   Note: To determine serine hydrolase FP probe specificity, denature samples by boiling for 5 minutes or adding 500µL of 10M Urea/Lysis Buffer before adding FP probe.
5. Incubate sample for 15-30 minutes at room temperature. Longer incubation times might be necessary to label some serine hydrolases.

D. Labeled Protein Capture and Elution

1. Add 500µL of 10M Urea/Lysis Buffer to each reaction for a total volume of 1mL.
2. Add 50µL of 50% High Capacity Streptavidin Agarose resin slurry to each sample and incubate for 1 hour at room temperature with constant mixing on a rotator.

   Note: Removal of the agarose storage buffer is not necessary. Mix agarose thoroughly and use a wide-bore pipette tip to transfer equal amounts of resin to each sample.
3. Centrifuge samples at 1000 × *g* for 1 minute to pellet resin. Remove supernatant.
4. Add 500µL of 5M Urea/Lysis Buffer and vortex briefly to mix. Centrifuge samples at 1000 × *g* for 1 minute to pellet resin. Repeat this step two additional times, discarding buffer after each wash.
5. Elute bound proteins by adding 2X Laemmli reducing sample buffer and boiling for 5 minutes.
6. Analyze eluted proteins by SDS-PAGE and Western blot.

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Procedure for Active-site Peptide Enrichment

Note: This method generates and enriches active site-labeled peptides for MS analysis. Perform Sections A-C from Procedure for Protein Labeling and Enrichment and then proceed with the following protocol.

A. Additional Materials Required

- Zeba™ Spin Desalting Columns 7K MWCO, 5mL (Product No. 89891)
- 1M Tris•HCl, pH 8.0
- Phosphate-buffered saline (PBS): 0.1M sodium phosphate, 0.15M sodium chloride; pH 7.2 (Product No. 28372)
- Trypsin endoproteinase, modified, TPCK-treated, MS-Grade (Product No. 90055)
- DTT, No-Weigh™ Format (Product No. 20291)
- Iodoacetamide, Single-Use (Product No. 90034)
- LCMS-grade acetonitrile (ACN, Product No. 51101)
- LCMS-grade water (Product No. 51140)
- Trifluoroacetic acid (TFA, Product No. 28904)
- Optional: Thermo Scientific Pierce Spin Columns (Product No. 69705)

B. Material Preparation

10M Urea/Lysis Buffer	Dissolve 0.9g of urea with 1.5mL of Lysis Buffer for each labeling reaction.
Digestion Buffer (2M urea/20mM Tris, pH 8.0)	Dissolve 2.4g of urea with 0.4mL 1M Tris, pH 8.0 and 19.6mL of LCMS-grade water.
500mM DTT	Dissolve 7.7mg of DTT with 0.1mL water.
1M Iodoacetamide	Dissolve 18.4mg of iodoacetamide with 0.1mL water.
Elution Buffer (50% ACN, 0.1% TFA)	Dilute 10µL of TFA with 5mL of ACN and 5mL of LCMS-grade water.
0.1% TFA	Dilute 10µL of TFA with 10mL of LCMS-grade water.

C. Labeled Protein Reduction and Alkylation

1. Add 500µL of 10M Urea/ Lysis Buffer to each reaction for a total volume of 1mL.
2. Add 10µL of 500mM DTT to each sample and incubate at 65°C for 30 minutes.
3. Cool samples to room temperature, add 40µL of 1M iodoacetamide to each sample and incubate protected from light for 30 minutes.

D. Buffer Exchange

1. Twist off the Zeba Spin Desalting Column's bottom closure and loosen cap. Place column in a 15mL collection tube.
2. Centrifuge column at 1000 × *g* for 2 minutes at room temperature to remove storage solution.

   Note: Resin will appear compacted and dry after centrifugation.
3. Add 3mL of Digestion Buffer to the column. Centrifuge at 1000 × *g* for 2 minutes to remove buffer. Repeat this step two additional times, discarding buffer from the collection tube.

   Note: If buffer is not completely removed after final spin, centrifuge at 1000 × *g* for an additional 2-3 minutes.
4. Place column in a new collection tube and slowly apply 0.5mL of each reaction to the center of the compact resin bed.
5. Centrifuge at 1000 × *g* for 2 minutes to collect the sample. Discard column after use.

E. Labeled Protein Digestion

1. Transfer desalted proteins to a new microcentrifuge tube.
2. Reconstitute 20µg of MS-grade trypsin (1 vial) with 10µL of LCMS-grade water.
3. Add trypsin to sample and incubate at 37°C with shaking for 2 hours.

F. Labeled Peptide Capture and Elution

1. Add 50µL of 50% High Capacity Streptavidin Agarose resin slurry to each digested sample and incubate for 1 hour at room temperature with constant mixing on a rotator.

   Note: For all subsequent steps, vortex briefly after adding buffer, centrifuge samples at 1000 × *g* for 1 minute to pellet resin and discard supernatant. Washing resin may be facilitated by transferring resin to an optional Pierce Spin Column.
2. Wash resin three times with 500µL of Pierce IP Lysis Buffer.
3. Wash resin four times with 500µL of PBS.
4. Wash resin four times with 500µL of LCMS-grade water.
5. Elute peptides by adding 75µL of Elution Buffer and incubating sample for 3 minutes. Transfer the eluate to a new microcentrifuge tube. Repeat this step two additional times.
6. Pool eluate fractions and freeze before lyophilizing.
7. Lyophilize the samples in a vacuum concentrator. Store lyophilized samples at -20°C.
8. Resuspend the samples in 25µL of 0.1% TFA and inject 1-5µL directly onto an LC-MS/MS system (e.g., Thermo Scientific LTQ or LTQ Orbitrap XL Mass Spectrometer) for analysis.

Troubleshooting

Problem	Possible Cause	Solution
No or low amount of serine hydrolase detected or captured	Insufficient amount of probe was used	Increase probe concentration
	Probe was degraded	Store probes at -80°C until reconstitution; store DMSO stocks at -20°C; minimize exposure to moisture and elevated temperatures
	Insufficient lysate was used	Increase protein to > 2mg/mL in labeling reaction
		Prefractionate lysates using Thermo Scientific Subcellular Protein Fractionation Kit (Product No. 78840)
	Lysis was incomplete	Sonicate lysate or add more non-denaturing detergents
	Serine hydrolase was inactive	Avoid serine protease inhibitors, denaturants and denaturing detergents in sample preparation
	Proteins were not fully denatured after labeling	Increase urea final concentration to 6M before streptavidin enrichment
	Endogenous biotinylated proteins were present	Pre-clear samples with streptavidin agarose before labeling
No inhibition of serine hydrolase when inhibitor was used	Too much probe was used	Decrease probe concentration to < 2µM
	Insufficient inhibitor was used	Increase inhibitor concentration and incubation
	Inhibitor was added after probe	Pretreat lysates with inhibitors before labeling
	Inhibitor does not bind active site	Use active-site inhibitors
No or low amount of active-site peptides captured	Protein digestion was incomplete	Increase trypsin amount and digestion incubation
		Use different MS-grade protease besides trypsin
	Peptides were lost during sample handling	Use low protein-binding tubes for lyophilization

Related Thermo Scientific Products

88310	**Pierce Kinase Enrichment Kit with ATP Probe**
88311	**ActivX Desthiobiotin-ATP Probe,** 16 × 12.6µg
88312	**Pierce Kinase Enrichment Kit with ADP Probe**
88313	**ActivX Desthiobiotin-ADP Probe,** 16 × 9.9µg
88314	**Pierce GTPase Enrichment Kit with GTP Probe**
88315	**ActivX Desthiobiotin-GTP Probe,** 16 × 12.9µg
20357	**High Capacity Streptavidin Agarose Resin,** 2mL
78420	**Halt Phosphatase Inhibitor Cocktail (100X),** 1mL
87787	**Pierce IP Lysis Buffer,** 100mL
89891	**Zeba Spin Desalting Columns, 7K MWCO, 5mL,** 5 ea
78840	**Subcellular Protein Fractionation Kit**

References

1. Liu,Y., *et al.* (1999). Activity-based protein profiling: The serine hydrolases. *Proc Natl Acad Sci USA* **96(26):**14694-9.
2. Patricelli, M.P., *et al.* (2001). Direct visualization of serine hydrolase activities in complex proteomes using fluorescent active site-directed probes. *Proteomic* **1:**1067-71.
3. Okerberg, E.S., *et al.* (2005). High-resolution functional proteomics by active-site peptide profiling. *Proc Natl Acad Sci USA* **102(14)**:4996-5001.
4. Simon, G.M. and Cravatt, B.F. (2010). Activity-based proteomics of enzyme superfamilies: Serine hydrolases as a case study. *JBC* **285(15):**11051-5.

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