INSTRUCTIONS

Pierce™ Kinase Enrichment Kits and ActivX™ Probes

2345.2

Number	Description
88310	**Pierce Kinase Enrichment Kit with ATP Probe**, contains sufficient reagents for 16 pull-downs **Kit Contents:** **ActivX Desthiobiotin-ATP Probe,** 16 × 12.6µg **Pierce IP Lysis Buffer**, 100mL **Reaction Buffer**, 125mL **Halt™ Protease/Phosphatase Inhibitor Cocktail (100X)**, 1mL **Zeba™ Spin Desalting Columns, 7K MWCO, 5mL**, 8 each **High Capacity Streptavidin Agarose Resin (50% slurry)**, 1mL **$MgCl_2$ (1M)**, 500µL **Urea**, 12g **Storage:** ActivX Desthiobiotin-ATP Probe is shipped separately with dry ice and stored at -80°C upon receipt. All other kit components are shipped at ambient temperature and stored at 4°C upon receipt.
88311	**ActivX Desthiobiotin-ATP Probe,** 16 × 12.6µg **Molecular Weight:** 1259.48 **Storage:** Upon receipt store at -80°C. Product is shipped with dry ice.
88312	**Pierce Kinase Enrichment Kit with ADP Probe**, contains sufficient reagents for 16 pull-downs **Kit Contents:** **ActivX Desthiobiotin-ADP Probe**, 16 × 9.9µg **Pierce IP Lysis Buffer**, 100mL **Reaction Buffer**, 125mL **Halt Protease/Phosphatase Inhibitor Cocktail (100X)**, 1mL **Zeba Spin Desalting Columns, 7K MWCO, 5mL**, 8 each **High Capacity Streptavidin Agarose (50% slurry)**, 1mL **$MgCl_2$ (1M)**, 500µL **Urea**, 12g **Storage:** ActivX Desthiobiotin-ADP Probe is shipped separately with dry ice and stored at -80°C upon receipt. All other kit components are shipped at ambient temperature and stored at 4°C upon receipt.
88313	**ActivX Desthiobiotin-ADP Probe**, 16 × 9.9µg **Molecular Weight:** 994.15 **Storage:** Upon receipt store at -80°C. Product is shipped with dry ice.

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Introduction

The Thermo Scientific™ Pierce™ Kinase Enrichment Kits with ActivX ATP and ADP Probes enable selective labeling and enrichment of ATPases including kinases, chaperones and metabolic enzymes. Thermo Scientific™ ActivX™ Desthiobiotin-ATP and -ADP Probes are nucleotide derivatives, which covalently modify the active site of enzymes with conserved lysine residues in the nucleotide-binding site.[1] The structure of desthiobiotin-ATP and -ADP consists of a modified biotin attached to the nucleotide by a labile acyl-phosphate bond (Figure 1). Depending on the position of the lysine within the enzyme active site, either desthiobiotin-ATP or -ADP may be better for labeling specific ATPases.

Both desthiobiotin-ATP and -ADP can selectively enrich, identify and profile target enzyme classes in samples or assess the specificity and affinity of enzyme inhibitors.[1,2] Many ATPases and other nucleotide-binding proteins bind nucleotides or inhibitors even when they are enzymatically inactive; these reagents bind both inactive and active enzymes in a complex sample. Preincubation of samples with small-molecule inhibitors that compete for active-site probes can be used to determine inhibitor binding affinity and target specificity.

Assessment of active-site labeling can be accomplished by either Western blot or mass spectrometry (MS) (Figure 2). 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 can be used for determining inhibitor target binding, but only the MS workflow can identify global inhibitor targets and off-targets.[1,3]

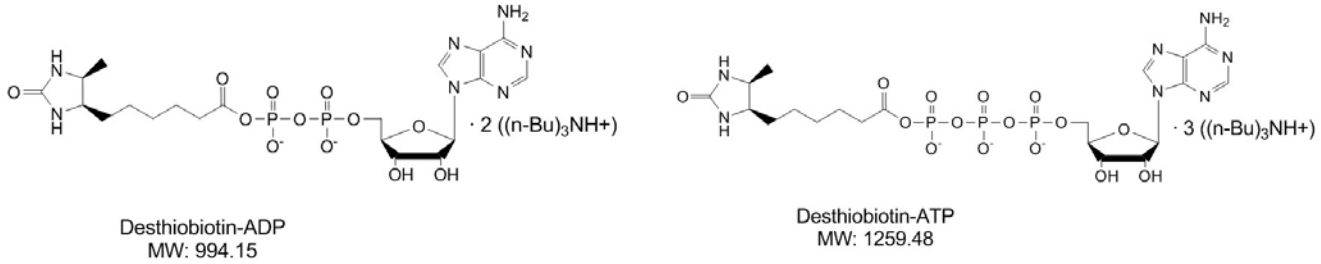

Figure 1. Chemical structures of the Thermo Scientific ActivX Desthiobiotin-ATP and -ADP Probes.

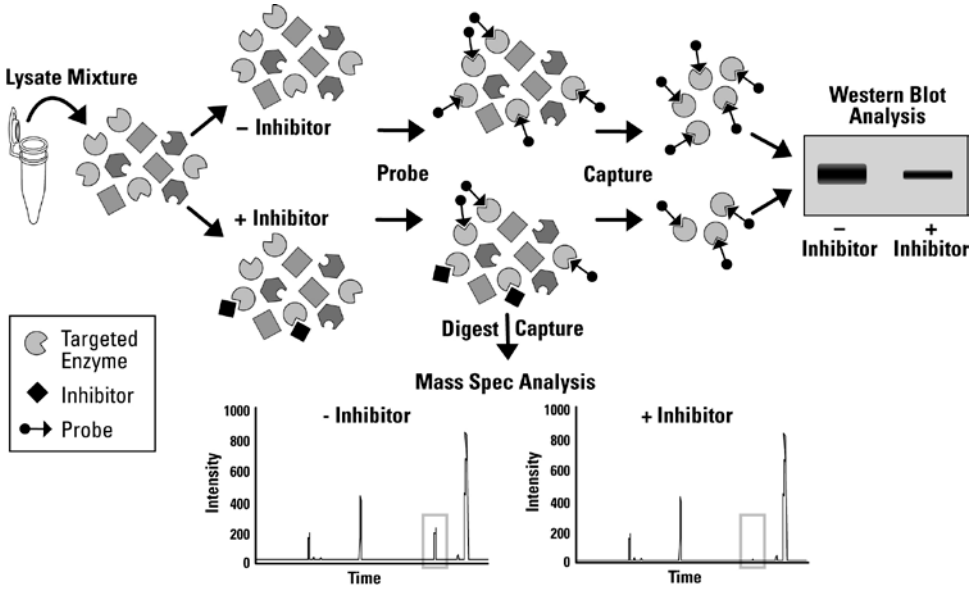

Figure 2. Western blot and mass spectrometry workflows enable targeted capture and analysis of enzymes using the active-site probes.

Important Product Information

- Desthiobiotin-ATP and -ADP are supplied in the Thermo Scientific™ No-Weigh™ Format, which enables single-use preparations. Note that the product is typically not visible in the vial.
- Desthiobiotin-ATP and -ADP are moisture-sensitive. To avoid moisture condensation onto the product, equilibrate vial to room temperature before opening. Prepare the labeling reagents immediately before use. The acyl-phosphate linkage readily hydrolyzes and becomes non-reactive; therefore, do not prepare stock solutions for storage. Discard any unused reconstituted compound.
- Desthiobiotin-ATP and -ADP are temperature-sensitive. Minimize exposure of product to ambient temperatures by returning unused product to -80°C between uses.
- Desthiobiotin-ATP and -ADP typically label accessible enzyme active sites, regardless of activity; however, some active enzymes might be preferentially labeled.
- The Pierce IP Lysis Buffer is effective for lysing cultured mammalian cells from both plated cells and cells pelleted from suspension cultures. Sonication is not required but might be necessary to fully lyse some cell types. For tissues, perform mechanical homogenization.
- Desalting of lysates using the Thermo Scientific™ Zeba™ Spin Desalting Columns or equivalent is required to remove endogenous ATP for optimal labeling.
- Adding $MgCl_2$ is required for labeling. $MnCl_2$ may be substituted for $MgCl_2$ in labeling reactions for active-site peptide labeling; however, proteins might precipitate.
- Adding urea to samples after labeling is required to denature proteins before capture using streptavidin agarose. Make urea buffers the same day as the experiment.
- For best results, perform labeling reactions using 5µM of desthiobiotin-ATP or -ADP when assessing inhibitor binding. Add up to 20µM for maximal protein enrichment. Labeling reactions using concentrations > 20µM require additional desalting to remove non-reacted probe before streptavidin capture.
- Desthiobiotin modification of lysine-containing active-site peptides results in a monoisotopic mass increase of 196.1212 Da.

Procedure for Protein Labeling and Enrichment

Note: This protocol is for labeling 2 × 1mg samples with 5-20µM of probe. Scale the procedure accordingly for other amounts.

A. Additional Materials Required

- 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)
- Scissors
- Variable-speed centrifuge
- 15mL conical collection tubes or equivalent
- Rotary mixer
- Microcentrifuge
- 1.7mL microcentrifuge tubes or equivalent
- 2X Laemmli reducing sample buffer (Product No. 84788 or equivalent)
- Optional: 1M $MnCl_2$

B. Material Preparation

8M Urea/IP Lysis Buffer	Dissolve 0.75g of urea with 1.5mL of IP Lysis Buffer for each labeling reaction.
4M Urea/IP Lysis Buffer	Dilute 1mL of 8M Urea/IP Lysis Buffer with 1mL of Pierce IP Lysis Buffer for each labeling reaction.

C. 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 50-100mg of tissue into small pieces.
2. Wash cells by suspending the cell pellet with ice-cold PBS.
3. Transfer 2-4 × 10^7 cells to a 1.5mL microcentrifuge tube. Pellet cells by centrifugation at 500 × *g* for 2-3 minutes and remove the PBS.
4. Add 1mL of Pierce IP Lysis Buffer containing protease and phosphatase inhibitors (1:100) and incubate on ice for 10 minutes with periodic mixing. Homogenize tissue using a Dounce homogenizer or tissue grinder.
5. Centrifuge tube at 16,000 × *g* at 4°C for 5 minutes.
6. Transfer the supernatant (total lysate) to a new tube.

D. Lysate 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 Reaction Buffer to the column. Centrifuge at 1000 × *g* for 2 minute 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 1000 × *g* for an additional 2-3 minutes.
4. Place column in a new collection tube and slowly apply 1mL of lysate to the center of the compact resin bed.
5. Centrifuge at 1000 × *g* for 2 minutes to collect the sample. Discard column after use.
6. Add more protease/phosphatase inhibitor cocktail to sample (1:100) and place on ice until labeling (Section E).

   Note: The samples may be snap-frozen with liquid nitrogen and stored at -80°C.

E. Sample Labeling

1. Perform a protein assay to measure the lysate's protein concentration.
2. Dilute lysate with Reaction Buffer to 2mg/mL and transfer 500µL (1mg) to a microcentrifuge tube.
3. Add 10µL of 1M $MgCl_2$ to each sample, mix and incubate for 1 minute at room temperature.

   Note: For best results, use $MnCl_2$ for active-site peptide labeling using the MS workflow.
4. If profiling an ATPase active-site inhibitor, add inhibitor to sample, mix and incubate 10 minutes at room temperature.
5. Equilibrate desthiobiotin-ATP or -ADP reagent to room temperature in pouch with desiccant.
6. Use scissors to cut off the single-use tubes needed and immediately return unused tubes to -80°C.
7. For a 5µM reaction (see Important Product Information), reconstitute reagent by adding 40µL of ultrapure water to make a 0.25mM stock solution.

   Note: For 20µM reaction, reconstitute reagent by adding 10µL of ultrapure water to make a 1mM stock solution.
8. Add 10µL of desthiobiotin-ATP or -ADP stock to each sample and incubate for 10 minutes at room temperature.

F. Labeled Protein Capture and Elution

1. Add 500µL of 8M Urea/IP 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 4M Urea/IP 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.

Procedure for Active-Site Peptide Enrichment

Note: This protocol is a method to generate and enrich active site-labeled peptides for MS analysis. Perform Sections C-E 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/IP Lysis Buffer	Dissolve 0.9g of urea with 1.5mL of Pierce IP 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/IP 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 for 30 minutes protected from light.

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 minute 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 the 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. 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 kinase (ATPase) captured	Insufficient amount of probe was used	Increase probe concentration to 20µM
	Probe was degraded	Store probes at -80°C and minimize exposure to moisture and elevated temperatures
	The optimal probe was not used	Specific kinases (ATPases) may label better with the ATP or ADP probe
	Insufficient lysate was used	Increase protein amount > 2mg/mL in labeling reaction
	Lysis was incomplete	Sonicate lysate or add additional non-denaturing detergents
	Lysate was not desalted	Desalt lysate to remove endogenous ATP
	$MgCl_2$ or probe was not added	Add $MgCl_2$ or probe to labeling reactions
	Proteins were not fully denatured after labeling	Increase urea final concentration to 6M before streptavidin enrichment
No inhibition of kinase (ATPase) when inhibitor was used	Too much probe was used	Decrease probe concentration to 5µM
	Insufficient inhibitor was used	Increase inhibitor concentration
	Inhibitor was added after probe	Pretreat lysates with inhibitors before probe 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
	Peptides were lost during sample handling	Use low protein binding tubes for lyophilization

Related Thermo Scientific Products

20357 **High Capacity Streptavidin Agarose Resin,** 2mL

78440 **Halt Protease and Phosphatase Inhibitor Cocktail (100X),** 1mL

87787 **Pierce IP Lysis Buffer,** 100mL

89891 **Zeba Spin Desalting Columns, 7K MWCO, 5mL,** 5 ea

Cited References

1. Patricelli, M.P., *et al.* (2007). Functional interrogation of the kinome using nucleotide acyl phosphates. *Biochemistry* **46:**350-8.
2. Cravatt, B.F., *et al.* (2008). Activity-based protein profiling: From enzyme chemistry to proteomic chemistry. *Annu Rev Biochem* **77:**383-414.
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.

These products and product applications are covered by U.S. and foreign patents and patents pending including: US Patent 7,365,178 B2, Australian Patent: 2004227362(AU), Canadian Patent: 2,521,130, Japanese Patent: 2006-509592,Singapore Patent: 200506345-8,European Application: 04758736.5, andHong Kong Application: 06103807.1.

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