INSTRUCTIONS

# Pierce® GTPase Enrichment Kits and ActivX® Probes

88314 88315 2346.1

| Number | Description |
|---|---|
| **88314** | **Pierce GTPase Enrichment Kit with GTP Probe**, contains sufficient reagents for 16 pull-downs |
| | **Kit Contents:** |
| | **ActivX Desthiobiotin-GTP Probe,** 16 × 12.9µg |
| | **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 Resin (50% slurry)**, 1mL |
| | **$MgCl_2$ (1M)**, 500µL |
| | **EDTA (0.5M)**, 1mL |
| | **Urea**, 12g |
| | **Storage:** ActivX Desthiobiotin-GTP 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. |
| **88315** | **ActivX Desthiobiotin-GTP Probe,** 16 × 12.9µg |
| | **Molecular Weight:** 1275.48 |
| | **Storage:** Upon receipt store at -80°C. Product is shipped with dry ice. |

## Introduction

The Thermo Scientific Pierce GTPase Enrichment Kit with ActivX GTP Probe enables selective labeling and enrichment of small GTPases and large G-protein subunits. The Thermo Scientific ActivX Desthiobiotin-GTP Probe structure consists of a modified biotin attached to the nucleotide by a labile acyl-phosphate bond (Figure 1). After removal of GTP or GDP nucleotides from enzymes, the desthiobiotin-GTP probe can covalently modify conserved lysine residues in the GTPase nucleotide-binding site. Desthiobiotin-GTP can selectively enrich, identify and profile target enzyme classes in samples.[1,2] Preincubation of samples with small-molecule inhibitors that compete with 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]

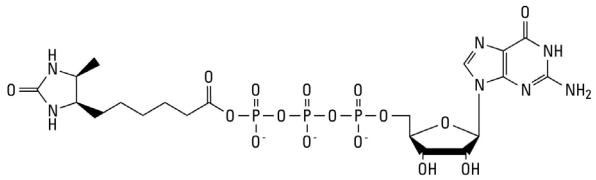

**Figure 1. Chemical structure of the Thermo Scientific ActivX Desthiobiotin-GTP Probe.**

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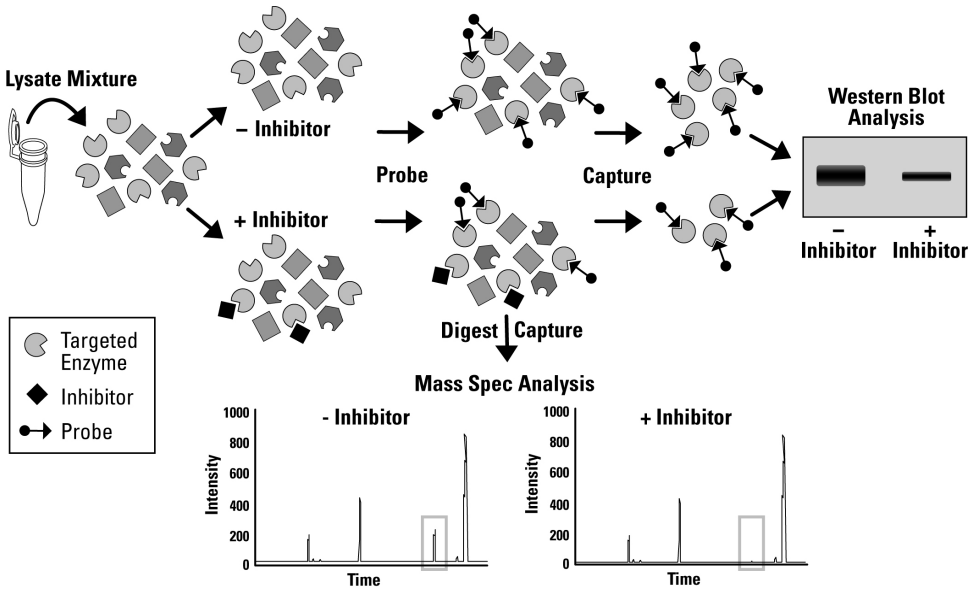


**Figure 2. Western blot and mass spectrometry workflows enable targeted capture and analysis of enzymes using an active-site probe.**

## Important Product Information

- Desthiobiotin-GTP is 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-GTP is moisture-sensitive. To avoid moisture condensation onto the product, equilibrate vial to room temperature before opening. Prepare the labeling reagent 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 reagent.
- Desthiobiotin-GTP is temperature-sensitive. Minimize exposure of product to ambient temperatures by returning unused product to -80°C between uses.
- Desthiobiotin-GTP typically labels 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 GTP for optimal labeling.
- Pretreatment of sample with EDTA is necessary to remove GTP/GDP from GTPase active sites before labeling. Adding $MgCl_2$ is required for probe labeling.
- 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 20µM probe for 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 at 2mg/mL. 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 660 nm Protein Assay Reagent (Product No. 22660)
- Scissors, variable-speed centrifuge, microcentrifuge and a rotary mixer
- 15mL conical collection tubes or equivalent
- 1.7mL microcentrifuge tubes or equivalent
- 2X Laemmli reducing sample buffer (Product No. 84788 or equivalent)
- Optional: 100mM GTPγS

### B. Material Preparation

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| 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 1 µL of 0.5M EDTA to each sample, mix and incubate for 5 minutes at room temperature.

   **Note:** If profiling a GTPase active-site inhibitor such as GTPγS, add inhibitor to sample, mix and incubate 10 minutes at room temperature.
4. Equilibrate desthiobiotin-GTP reagent to room temperature in pouch with desiccant.
5. Use scissors to cut off the single-use tubes needed and immediately return unused tubes to -80°C.
6. For 20µM reaction, reconstitute reagent by adding 10µL of ultrapure water to make a 1mM stock solution.
7. Add 10µL of desthiobiotin-GTP stock to each sample and mix.
8. Add 10µL of 1M $MgCl_2$ to each sample, mix and incubate for 10 minute at room temperature.

   **Note:** For negative control reactions, omit $MgCl_2$ from labeling reactions.

**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: Pierce Spin Columns (Product No. 69705)

## B. Material Preparation

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| 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. 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 compacted 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. Repeat this step two additional times.
6. Pool eluate fractions and freeze before lyophilizing. Lyophilize the samples in a vacuum concentrator. Store lyophilized samples at -20°C.
7. 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 GTPase captured | Insufficient amount of probe was used | Increase probe concentration |
| | Probe was degraded | Store probe at -80°C and minimize exposure to moisture and elevated temperatures |
| | 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 GTP/GDP |
| | EDTA, $MgCl_2$ or probe was not added or added in incorrect order | Add EDTA then probe then $MgCl_2$ to labeling reactions |
| | Proteins were not fully denatured after labeling | Increase urea final concentration to 6M before streptavidin enrichment |
| No inhibition of GTPase 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

**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

**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

### 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.





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