Cleavable ICAT® Reagent Kit for Protein Labeling (Monoplex Version)

For Modifying Proteins with an Isotope-Labeled, Sulfhydryl-Modifying Biotinylation Reagent

Protocol

1 Product Description

The Cleavable ICAT® Reagent Kit for Protein Labeling (Monoplex Version) facilitates identification and quantification of differentially expressed proteins using isotope-coded affinity tag chemistry and cleavable-linker technology. The labeled peptides, products of the reactions generated by this kit, can be separated by capillary reversed-phase HPLC and analyzed by mass spectrometry.

By running a Control sample, (for example, a normal cell state) and a Test sample (for example, a diseased cell state), you obtain ratios of ICAT reagent-labeled peptides from which you can determine protein expression levels.

When to Use

Use the Cleavable ICAT® Reagent Methods Development Kit for global protein expression analysis, when you want to identify as many proteins as possible in a sample. For investigation of a protein or protein class with a known molecular weight range, or samples requiring protein pre-fractionation before analysis, the 1-D PAGE Cleavable ICAT Reagent Applications Development Kit for Targeted Protein ID and Quantitation is available (see Section 11, Ordering Information, for the part number).

Cleavable ICAT® Reagent Kit Options

- **Methods Development Kit** – Contains cleavable ICAT reagents, affinity and cation-exchange buffers and cartridges, and cartridge/hardware accessories. The Methods Development Kit contains extra vials of Light ICAT reagent for the development of a robust protocol with complex samples and to verify that your sample preparation protocol does not interfere with labeling and digestion. This kit supports methods development and two complete post-development assays.
- **10-Assay Kit** – Contains the same items as the Methods Development Kit, except for the cartridge/hardware accessories and the extra vials of Light ICAT reagent. This kit supports 10 complete assays.
- **Bulk reagent kits** – Contain ICAT reagents only. A variety of kits are available. Contact Applied Biosystems for information.

Enhancements to the Cleavable ICAT Reagents

- The Heavy ICAT reagent is now ^{13}C-based instead of deuterium based.
- Heavy- and Light-labeled peptides coelute, which allows quantification by mass spectrometry (MS).
- The mass difference between the Heavy and Light reagents is now 9 Da instead of 8 Da, eliminating potential confusion between oxidized methionine and doubly labeled peptides.
- The biotin portion of the ICAT reagent tag is cleaved with acid after the ICAT reagent-labeled peptides are eluted from the avidin cartridge. Biotin cleavage reduces the size of the reagent label on the peptide from 442 Da to 227 Da, which allows analysis of larger peptides.
- Tris(2-carboxyethyl)phosphine (TCEP)/ICAT reagent by-products of labeling are substantially reduced, which improves MS data quality.
- MS/MS sequence coverage is improved due to reduced fragmentation, which improves database searching and confidence in protein identifications.

Cleavable ICAT® Reagents

Heavy and Light cleavable ICAT reagents consist of four moieties as shown in Figure 1:

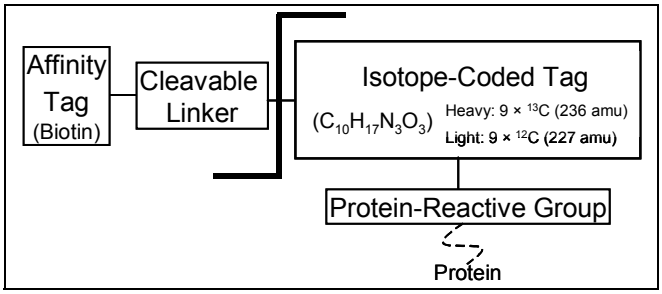

Figure 1 Cleavable ICAT Reagent Structure

- **Affinity tag (biotin)** – The affinity tag simplifies the analysis of the ICAT reagent-labeled peptides by enabling the selection and concentration of the cysteine-containing peptides, thereby reducing the complexity of the peptide mixture.
- **Cleavable linker** – After avidin purification of the ICAT reagent-labeled peptides, the biotin is removed by cleaving the linker with trifluoroacetic acid (TFA). Biotin removal reduces the overall mass of the tag on the peptides and improves the overall peptide fragmentation efficiency.

Contents

- **Isotope-coded tag** – The isotope-coded tag uniquely distinguishes labeled proteins (the Heavy reagent tag includes 9 heavy isotopes; the Light reagent tag contains no heavy isotopes). Chemically, the tags behave the same way, but differ in mass. This mass difference (9 Da) allows a mass spectrometric comparison of peptides labeled with Heavy and Light reagents and provides a ratio of the concentration of the proteins in the samples.
- **Protein reactive group (iodoacetamide)** – The reactive group covalently links the ICAT reagent to the protein by alkylation of free cysteines.

For additional information on protein expression profiling using ICAT reagents, refer to Section 12, References.

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Protocol Overview

Figure 2 is an overview of the ICAT reagent protocol. Before running the protocol on real samples, test the protocol as described in Section 6, Testing the Protocol.

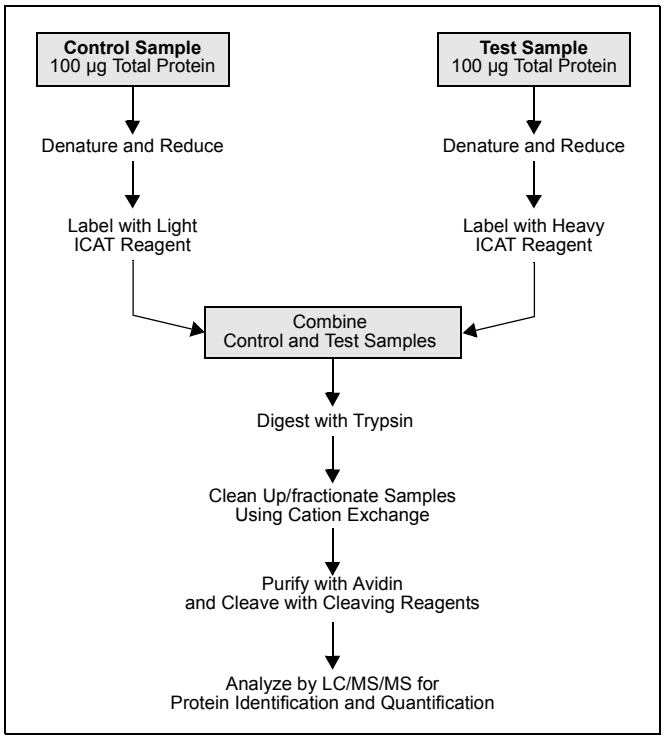


Figure 2 Overview of the Protocol

Laminin Peptide Standard Specifications

- Sequence: Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg
- Composition: $C_{40}H_{62}N_{12}O_{14}S$
- Average molecular weight: 967.1
- Monoisotopic MH^+: 967.43
- CAS: 110590-60-8
- Monoisotopic MH^+ after Light derivitization before cleaving: 1,874.91
- Monoisotopic MH^+ after Light derivitization after cleaving: 1,194.56

ICAT Reagent Specifications

Specification	Light ICAT Reagent	Heavy ICAT Reagent
Composition	$C_{43}H_{70}N_7O_{12}SI$	$^{13}C_9C_{34}H_{70}O_{12}SI$
Average molecular weight	1,036.1	1,045.1
Monoisotopic MH^+	1,036.39	1,045.42
Monoisotopic MH^+ added to peptide before cleaving	907.47	916.50
Composition of tag after cleaving	$C_{10}H_{17}N_3O_3$	$^{13}C_9CH_{17}N_3O_3$
Monoisotopic MH^+ added to peptide after cleaving	227.13	236.16
Composition of modified cysteine after cleaving	$C_{13}H_{22}N_4O_4S$	$^{13}C_9C_4H_{22}N_4O_4S$
Monoisotopic MH^+ of ICAT-reagent-labeled cysteine	330.1362	339.1664

2 Using the Methods Development Kit

You can use the Methods Development Kit to:

- Test the ICAT protocol by labeling the laminin peptide standard provided in the kit.
- Verify that expected ICAT reagent-labeled peptides are present in a single protein or a mix of up to 6 proteins (known protein).
- Verify that your sample preparation protocol for complex samples does not interfere with labeling and digestion (Control sample).
- Identify and quantify differentially expressed proteins in Control and Test (experimental) samples.

Table 1 summarizes the types of experiments suitable for the Methods Development kit.

Note: Extra vials of Light ICAT reagent are provided in the Methods Development Kit for the development of a robust protocol with complex samples.

Table 1 Recommended Experiments Using the Methods Development Kit

Purpose	Sample	Cleavable ICAT Reagent	Described In
Check labeling and cation and avidin cartridges.	Laminin (provided in kit)	Label with Light reagent only	Section 6, Testing the Protocol
Check labeling, digestion, chromatography, and quantitative analysis.	Known protein (for example, 25 µg BSA or 100 µg bovine lactalbumin) or simple protein mix of up to 6 proteins	Label with Light and Heavy reagents	
Check sample preparation. If this step does not yield acceptable results, clean up sample or modify sample-prep protocol.	Control sample (for example, a normal cell state) **IMPORTANT!** This is an optional step that you can perform if you have an extra quantity of Control sample or known protein in sample buffer.	Label with Light reagent only	
Identify and quantify differentially expressed proteins	Control and Test samples (for example, a diseased cell state and a normal cell state)	Label with Light and Heavy reagents	Section 7, Running the Protocol, through Section 9, Evaluating Results

3 Materials

This section describes:

- User-supplied materials
- Kit Materials

3.1 User-Supplied Materials

Item	Volume or Quantity per Assay
Disposable gloves	As needed
Pipettors and tips suitable for 1 µL to 1 mL	As needed
Syringe (2-inch blunt needle, 22-gauge, 2.5-mL)	1
Fraction-collection tubes and rack	As needed
Screw-cap tubes, 2-mL	2 per assay
1.5-mL and >4-mL tubes, for cation-exchange step	As needed
Test sample (for example, a diseased cell state)	100 µg
Control sample (for example, a normal cell state)	100 µg
Known protein for testing protocol, for example: • Bovine serum albumin (BSA) • Bovine lactalbumin	 25 µg 100 µg
High-resolution cation-exchange column, if analyzing complex samples and you determine that fractionation is required (for example, PolySulfoethyl A Column, 5 micron 300 Å bead, from PolyLC, Inc., 2.1 × 200 mm, PN 202SE0503. Select a column size with the appropriate binding capacity for your sample size.)	1
pH paper: • pH range 2.5 to 4.5 – To check pH of sample before loading on the cation-exchange cartridge. • pH range 6 to 8 – To check pH of sample before loading on the avidin cartridge.	As needed
Milli-Q® water or equivalent (minimum 18.2 MOhms water, conductivity maximum 0.05 µS/0.05 µMho)	50 mL
Heating block, 37 °C	1
Heating block or water bath, 100 °C	1
Bench-top centrifuge	1
Vortexer	1
Centrifugal vacuum concentrator	1
Mass spectrometer with ICAT analysis software (for example, Applied Biosystems Pro ICAT Software and GPS Explorer™ Software)	1
Capillary reversed-phase HPLC system	1
If you analyze using Nanospray™ ESI mass spectrometry, either of the following tips: • New Objective, Inc. coated fused-silica PicoTips™ (coating applied to tip end; Cat. #FS360-20-10-CE-20). Also requires tubing fitting from LC Packings (Cat. #TF-250/350). • New Objective, Inc. distal coated fused-silica PicoTips™ (Cat. #FS360-20-10-D-20).	1

3.2 Kit Materials

This section describes the materials provided in and the storage conditions for the:

- Methods Development Kit (reagents, buffers, cartridges, and hardware)
- 10-Assay Cleavable ICAT Reagent Kit (reagents, buffers, and cartridges only)

The Cleavable ICAT Reagent Kits are shipped to you in three boxes containing:

1. Reagents (except Cleaving Reagent A) and instructions
2. Cleaving Reagent A
3. Cation-exchange and affinity buffer packs with cartridges and cartridge hardware (hardware included in Methods Development Kit only)

IMPORTANT! When you receive shipping container #1 of 3, immediately remove the Reagent Box 1 from the container and store it at –15 to –25 °C. Store items in the remaining two shipping containers as specified in Table 2 on page 4.

Table 2 on page 4 lists the kit materials and their recommended storage conditions.

Table 2 Kit Materials and Storage Conditions

Item	Methods Development Kit Volume/Qty.	10-Assay Kit Volume/Qty.	Description	Shipping Box/ Storage Conditions
Cleavable ICAT Reagent Heavy	3 vials, 1 unit/vial[a]	10 vials, 1 unit/vial[a]	Sulfhydryl-modifying biotinylation Heavy reagent, used to label the Test sample.	Box 1: Reagents. Store at –15 to –25 °C
Cleavable ICAT Reagent Light	6 vials, 1 unit/vial[a]	11 vials, 1 unit/vial[a]	Sulfhydryl-modifying biotinylation Light reagent, used to label the Control sample.	
Laminin Peptide Standard	1 vial	1 vial	Standard peptide to test the kit.	
Trypsin with $CaCl_2$	5 vials	10 vials	Cleaves peptide bonds on the carboxyl side of lysine and arginine residues.	
Denaturing Buffer (pH 8.5)	1 vial, 1.5 mL/vial	2 vials, 1.5 mL/vial	Disrupts the hydrogen, hydrophobic, and electrostatic bonds of the proteins. Contains 50 mM Tris and 0.1% SDS.	
Reducing Reagent	1 vial, 100 µL/vial	1 vial, 100 µL/vial	Reduces the disulfide bonds of the proteins. Contains 50 mM TCEP.	
Cleaving Reagent B	1 vial, 500 µL/vial	1 vial, 500 µL/vial	Contains a scavenger that reduces side reactions during the cleaving reaction.	
Cleavable ICAT Reagent Kit for Protein Labeling Protocol	1	1	This document.	
Cleavable ICAT Reagent Kit for Protein Labeling Quick Reference	1	1	Laminated card that provides a quick reference to the steps in this protocol.	
Cleaving Reagent A	10 mL	10 mL	Cleaves the biotin from the labeled peptide. Contains concentrated TFA.	Box 2: Cleaving Reagent A. Store at room temperature in a fume hood or vented cabinet.
ICAT Cartridge–Cation Exchange	one 200-µL cartridge	one 200-µL cartridge	Contains POROS® 50 HS, 50-µm particle size. (4.0 mm × 15 mm; Identified by a white band). Can be cleaned and reused up to 50 times.	Box 3: Avidin Cartridge and Affinity Buffers, Cation Exchange Cartridge and Buffers. Store at 2 to 8 °C
Cation Exchange Buffer–Load (10 mM potassium phosphate [KH_2PO_4]/25% acetonitrile, pH 3.0)	100 mL	100 mL	Phosphate buffer with acetonitrile that adjusts the pH and lowers the salt concentration.	
Cation Exchange Buffer–Elute (10 mM potassium phosphate [KH_2PO_4]/25% acetonitrile/ 350 mM KCl, pH 3.0)	100 mL	100 mL	Phosphate buffer with acetonitrile and salt that raises the salt concentration to elute the peptides.	
Cation Exchange Buffer–Clean (10 mM potassium phosphate [KH_2PO_4]/25% acetonitrile/1 M KCl, pH 3.0)	100 mL	100 mL	Phosphate buffer with acetonitrile and high salt concentration that cleans the cation-exchange cartridge after peptide elution.	
Cation Exchange Buffer–Storage (10 mM potassium phosphate [KH_2PO_4]/25% acetonitrile, pH 3.0, + 0.1% NaN_3)	100 mL	100 mL	Phosphate buffer with acetonitrile and sodium azide that maintains the proper pH and prevents growth of microorganisms.	
ICAT Cartridge–Avidin	one 200-µL cartridge	one 200-µL cartridge	Purifies biotinylated molecules. (4.0 mm × 15 mm; identified by a black band). Can be cleaned, activated, and reused to process up to 50 cation-exchange fractions.	
Affinity Buffer–Elute (30% Acetonitrile + 0.4% TFA)	100 mL	100 mL	Conditions the affinity cartridge and elutes ICAT reagent-labeled peptides. Contains 0.4% trifluoroacetic acid and 30% acetonitrile.	
Affinity Buffer–Load (2× PBS, pH 7.2) Note: The formulation for 1× PBS is 10 mM sodium phosphate (NaH_2PO_4), 150 mM NaCl.	100 mL	100 mL	Phosphate buffer that adjusts the pH to approximately 7.2.	
Affinity Buffer–Wash 1 (1× PBS, pH 7.2)	100 mL	100 mL	Phosphate buffer that decreases the salt concentration.	
Affinity Buffer–Wash 2 (50 mM ammonium bicarbonate [NH_4HCO_3]/ 20% methanol, pH 8.3)	100 mL	100 mL	Bicarbonate solution with methanol that decreases the salt concentration and reduces nonspecifically bound peptides.	
Affinity Buffer–Storage (pH 7.2) (2× PBS, pH 7.2 + 0.1% NaN_3)	100 mL	100 mL	Phosphate buffer with sodium azide that maintains the proper pH and prevents growth of microorganisms.	

Table 2 Kit Materials and Storage Conditions (Continued)

Item	Methods Development Kit Volume/Qty.	10-Assay Kit Volume/Qty.	Description	Shipping Box/ Storage Conditions
Cartridge holder	1 (for 200-µL cartridges)	Not included	Reusable bayonet-style holder for 200-µL cation-exchange and avidin cartridges.	Box 3: Cartridge/hardware accessories (Methods Development Kit only). Store at room temperature.
Needle-port adapter	1	Not included	Provides a secure connection for the HPLC syringe needle (while injecting onto the cartridge).	
Outlet connector	1	Not included	1/16-inch O.D. PEEK™ tubing and 10-32 compression screw for connecting to the outlet side of the cartridge holder.	

a. One unit of reagent labels 100 µg of protein.

4 Safety

Safety Alert Words

Four safety alert words appear in Applied Biosystems user documentation at points in the document where you need to be aware of relevant hazards. Each alert word–IMPORTANT, CAUTION, WARNING, and DANGER–implies a particular level of observation or action, as defined below:

IMPORTANT! – Indicates information that is necessary for proper instrument operation, accurate chemistry kit use, or safe use of a chemical.

CAUTION – Indicates a potentially hazardous situation that, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices.

WARNING – Indicates a potentially hazardous situation that, if not avoided, could result in death or serious injury.

DANGER – Indicates an imminently hazardous situation that, if not avoided, will result in death or serious injury. This signal word is to be limited to the most extreme situations.

Chemical Hazard Warning

WARNING **CHEMICAL HAZARD**. Some of the chemicals used with Applied Biosystems instruments and protocols are potentially hazardous and can cause injury, illness, or death.

Chemical Safety Guidelines

To minimize the hazards of chemicals:

- Read and understand the Material Safety Data Sheets (MSDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. (See “About MSDSs” below).
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the MSDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer’s cleanup procedures as recommended on the MSDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

Chemical Waste Hazard

WARNING **CHEMICAL WASTE HAZ**ARD. Some wastes produced by the operation of the instrument or system or protocol are potentially hazardous and can cause injury, illness, or death.

Chemical Waste Safety Guidelines

To minimize the hazards of chemical waste:

- Read and understand the Material Safety Data Sheets (MSDSs) provided by the manufacturers of the chemicals in the waste container before you store, handle, or dispose of chemical waste.
- Provide primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the MSDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).For additional safety guidelines, consult the MSDS.
- Handle chemical wastes in a fume hood.
- After emptying the waste container, seal it with the cap provided.
- Dispose of the contents of the waste tray and waste bottle in accordance with good laboratory practices and local, state/provincial, or national environmental and health regulations.

About MSDSs

Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to *new* customers. They also provide MSDSs with the first shipment of a hazardous chemical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new MSDS packaged with a hazardous chemical, be sure to replace the appropriate MSDS in your files.

Obtaining MSDSs

You can obtain from Applied Biosystems the MSDS for any chemical supplied by Applied Biosystems. This service is free and available 24 hours a day.

To obtain MSDSs:

1. Go to **https://docs.appliedbiosystems.com/msdssearch.html**
2. In the Search field, type in the chemical name, part number, or other information that appears in the MSDS of interest. Select the language of your choice, then click **Search**.
3. Find the document of interest, right-click the document title, then select any of the following:
   - **Open** – To view the document
   - **Print Target** – To print the document
   - **Save Target As** – To download a PDF version of the document to a destination that you choose
4. To have a copy of a document sent by fax or e-mail, select **Fax** or **Email** to the left of the document title in the Search Results page, then click **RETRIEVE DOCUMENTS** at the end of the document list.
5. After you enter the required information, click **View/Deliver Selected Documents Now**.

5 Monitoring the Process

IMPORTANT! It is good practice to remove "process-monitoring aliquots" at several points during an assay. Then, if you have problems with the final analysis of your sample, you can analyze the process-monitoring aliquots to troubleshoot the protocol and determine where in the protocol the expected reactions failed to occur.

This protocol instructs you to remove process-monitoring aliquots at the points indicated below:

When to remove a process-monitoring aliquot	What to do with the process-monitoring aliquot
Before adding ICAT reagents	Run a gel to confirm labeling and digestion. **IMPORTANT!** For the laminin peptide standard test, analyze by MS instead of gel to confirm labeling.
After adding ICAT reagents	
After trypsin digestion (does not apply to the laminin standard test)	
Before loading on the avidin cartridge	Analyze by MS to confirm the presence of ICAT reagent-labeled peptides.
After eluting from the avidin cartridge	

6 Testing the Protocol

Before running the protocol on real samples, test the protocol as described in the following sections:

- Testing with the laminin peptide standard
- Testing with a known protein
- Testing the Control Sample with Light ICAT Reagent (if you have sufficient sample)

6.1 Testing with the Laminin Peptide Standard

Before using the kit for the first time, run the protocol with the laminin peptide standard supplied in this kit.

6.1.1 Denaturing and Reducing the Laminin Peptide Standard

WARNING **CHEMICAL HAZARD. Reducing Reagent** causes eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

1. Add 80 µL of the Denaturing Buffer to the laminin peptide standard vial.
2. Add 2 µL of the Reducing Reagent to the vial.
3. Vortex to mix, then centrifuge for a few seconds to bring all solution to the bottom of the tube.

   Note: In this and all subsequent procedures, when instructed to centrifuge, centrifuge at no more than 14,000 x g.
4. Place the vial in a boiling water bath for 10 minutes.
5. Vortex to mix, then centrifuge for 1 to 2 minutes to cool the vial.
6. Remove a 1-µL process-monitoring aliquot from the vial, and label as "unlabeled". For more information, see Section 5, Monitoring the Process.

6.1.2 Labeling the Laminin Peptide Standard with Cleavable ICAT Reagents

WARNING **CHEMICAL HAZARD**. Read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
Cleavable ICAT® Reagent Heavy and Cleavable ICAT® Reagent Light cause eye, skin, and respiratory tract irritation. Exposure may cause an allergic reaction.
Acetonitrile (ACN) is a flammable liquid and vapor. Exposure may cause eye and respiratory tract irritation and blood system damage.

1. Bring to room temperature a vial of Cleavable ICAT Reagent Light.
2. Centrifuge the reagent to bring all powder to the bottom of the vial.
3. Add 20 µL of acetonitrile to the ICAT Reagent Light vial.
4. Vortex to mix, then centrifuge for a few seconds to bring all solution to the bottom of the tube. All of the ICAT reagent may not dissolve.
5. Transfer the solution from the laminin vial to the ICAT Reagent Light vial containing the acetonitrile.
6. Vortex to mix, then centrifuge for a few seconds to bring all solution to the bottom of the tube.
7. Incubate for 2 hours at 37 °C.
8. Vortex to mix, then centrifuge for a few seconds to bring all solution to the bottom of the tube. All of the ICAT reagent should dissolve.
9. Remove a 1-µL process-monitoring aliquot, and label as "labeled".

6.1.3 Purifying and Cleaving the Laminin Peptide Standard

Purify the remaining reaction mixture by performing the procedures in:

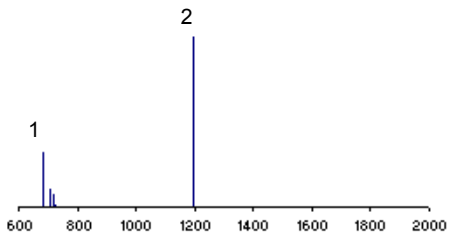
- Section 7.1.5, Cleaning Up the Peptides Using Cation Exchange, on page 11
- Section 7.2, Purifying the Biotinylated Peptides and Cleaving Biotin, on page 13

6.1.4 Analyzing the Laminin Peptide Standard

1. Dissolve the cleaved, dried samples in 500 µL of the solvent appropriate for analyzing the labeled peptides based on your analysis method (for example, HPLC loading buffer).
2. For MALDI analysis, mix a 1-µL aliquot of the cleaved labeled laminin sample with an appropriate matrix (for example, alpha-cyano-4-hydroxycinnamic acid) in a 1:1 ratio (v/v), then analyze by MS. For more information, see Section 8.5, Analyzing by MALDI.

   For electrospray analysis, see Section 8.3, Analyzing by Electrospray.

Figure 3 shows the expected peaks and masses for the laminin standard after cleaving. In MALDI spectra, an intense m/z 515.3 fragment peak (not shown in Figure 3) is also present in the spectrum. The m/z 515.3 peak, which corresponds to a laser-induced fragment of the affinity tag/linker, may interfere with observation of the peaks shown in Figure 3. To observe the peaks shown in Figure 3, eliminate the masses below m/z 600.



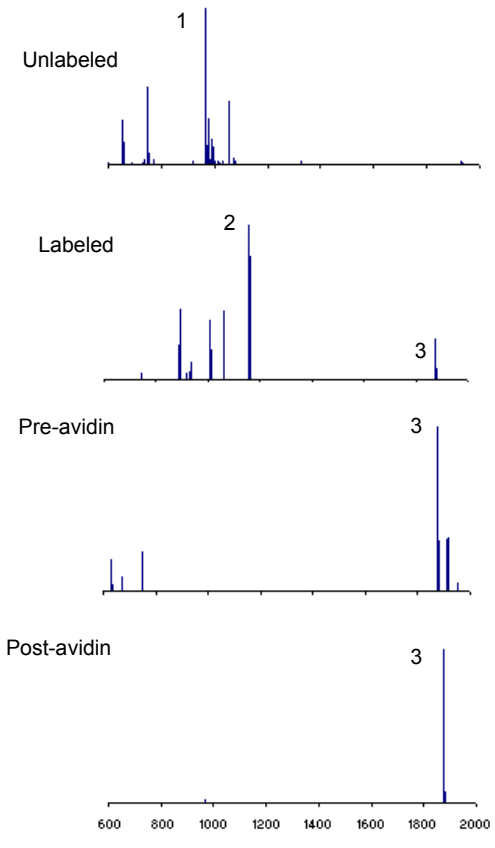
1. Cleaved biotin fragment (m/z 683.4)
2. Cleaved labeled laminin (m/z 1,194.6)

Figure 3 Laminin Standard After Cleaving

Troubleshooting

If you do not observe the expected peaks and masses, analyze the process-monitoring aliquots to determine at which point in the protocol the problem occurred. For information, see Section 5, Monitoring the Process.

Figure 4 illustrates spectra for each process-monitoring aliquot. In MALDI spectra, an intense m/z 515.3 fragment peak (not shown in Figure 4) is also present in all process-monitoring spectra. This m/z 515.3 peak, which corresponds to a laser-induced fragment of the affinity tag/linker, may interfere with observation of the peaks shown in Figure 4. To observe the peaks shown in Figure 4, eliminate the masses below m/z 600.



1. Laminin (m/z 967.4)
2. Reducing Reagent/ICAT (m/z 1,158.5)
3. Uncleaved labeled laminin (m/z 1,874.9)

Figure 4 Laminin Peptide Standard Process-Monitoring Spectra

6.2 Testing with a Known Protein

Before you run complex samples for the first time, test the protocol with a well-characterized known protein or with a mixture of up to 6 proteins that contain multiple cysteines (for example, 25 µg of bovine serum albumin or 100 µg of bovine lactalbumin). Use a pure sample that does not contain reducing reagents, denaturants, detergents, or high salt.

This test qualifies the protocol with a sample more complex than the laminin peptide, and verifies that you can use the protocol to quantitate a 1:1 (Light:Heavy) mix and a 2:1 (Light:Heavy) mix to within ±30% of expected values.

6.2.1 Denaturing and Reducing the Known Protein

WARNING **CHEMICAL HAZARD. Reducing Reagent** causes eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

1. Add 80 µL of the Denaturing Buffer to each of two tubes containing 100 µg of the known protein. (If working with a concentrated sample solution, add Denaturing Buffer to bring the volume up to 80 µL.)
2. Add 2 µL of the Reducing Reagent to each tube.
3. Vortex to mix, then centrifuge for a few seconds to bring all solution to the bottom of the tube.

   Note: In this and all subsequent procedures, when instructed to centrifuge, centrifuge at no more than 14,000 x g.
4. Place the tubes in a boiling water bath for 10 minutes.
5. Vortex to mix, then centrifuge for 1 to 2 minutes to cool the tubes.
6. Remove a 1-µL process-monitoring aliquot from each vial, and label as “unlabeled”. For more information, see Section 5, Monitoring the Process.

6.2.2 Labeling the Known Protein with Cleavable ICAT Reagents

WARNING **CHEMICAL HAZARD.** Read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
Cleavable ICAT® Reagent Heavy and Cleavable ICAT® Reagent Light cause eye, skin, and respiratory tract irritation. Exposure may cause an allergic reaction.
Acetonitrile (ACN) is a flammable liquid and vapor. Exposure may cause eye and respiratory tract irritation and blood system damage.

1. Bring to room temperature a vial of Cleavable ICAT Reagent Light and a vial of Cleavable ICAT Reagent Heavy.
2. Centrifuge the reagents to bring all powder to the bottom of each vial.
3. Add 20 µL of acetonitrile to each reagent vial.
4. Vortex to mix, then centrifuge for a few seconds to bring all solution to the bottom of the tube. All of the ICAT reagent may not dissolve.
5. Transfer the contents of one of the known protein vials to a vial of the Light reagent.
6. Transfer the contents of the other known protein vial to a vial of the Heavy reagent.
7. Vortex to mix, then centrifuge for a few seconds to bring all solution to the bottom of the tube. All of the ICAT reagent should dissolve.

8. Incubate for 2 hours at 37 °C.
9. Vortex to mix, then centrifuge for a few seconds to bring all solution to the bottom of the tube.
10. Remove a 1-µL process-monitoring aliquot from each vial, and label as “labeled”. For more information, see Section 5, Monitoring the Process.
11. In a fresh tube, mix a 39-µL aliquot of the Light-labeled sample with a 39-µL aliquot of the Heavy-labeled sample. (This is your 1:1 sample.)
12. In a second fresh tube, mix a 26-µL aliquot of the Light-labeled sample with a 52-µL aliquot of the Heavy-labeled sample. (This is your 1:2 sample.)

6.2.3 Digesting the Known Protein with Trypsin

WARNING **CHEMICAL HAZARD. Trypsin** causes eye, skin, and respiratory tract irritation. Exposure may cause an allergic reaction. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

1. Reconstitute a vial of trypsin with 200 µL of Milli-Q® water or equivalent.
2. Vortex to mix, then centrifuge for a few seconds to bring all solution to the bottom of the tube.
3. Add 80 µL of the trypsin solution to each of the 1:1 and 1:2 samples.
4. Vortex to mix, then centrifuge for a few seconds to bring all solution to the bottom of the tube.
5. Incubate 12 to 16 hours at 37 °C.
6. Vortex to mix, then centrifuge for a few seconds to bring all solution to the bottom of the tube.
7. Remove a 1-µL process-monitoring aliquot from each vial, and label as “post-trypsin”. For more information, see Section 5, Monitoring the Process.

6.2.4 Purifying and Cleaving the Known Protein

Purify the two samples by performing the procedures in:

- Section 7.1.5, Cleaning Up the Peptides Using Cation Exchange, on page 11
- Section 7.2, Purifying the Biotinylated Peptides and Cleaving Biotin, on page 13

6.2.5 Analyzing the Known Protein

1. Dissolve the cleaved, dried samples in 100 µL of the solvent appropriate for analyzing the labeled peptides based on your analysis method (for example, HPLC loading buffer).
2. For MALDI analysis, mix a 1-µL aliquot of the cleaved labeled known protein sample with an appropriate matrix (for example, alpha-cyano-4-hydroxycinnamic acid) in a 1:1 ratio (v/v), then analyze by MS. For more information, see Section 8, Separating and Analyzing the Fractions and Peptides.

   For electrospray analysis, see Section 8, Separating and Analyzing the Fractions and Peptides.

Table 3, “Theoretical ICAT Reagent-Labeled Cysteine Peptides in a BSA Trypsin Digest,” lists all possible peptides in a BSA trypsin digest. All theoretical peptides are listed for reference purposes only. You will not see all peptides in a single run.

Ensure that you see peaks with ratios of 1:1 (Light:Heavy) and 1:2 (Light:Heavy) ±30% in the appropriate samples.

If you do not see the expected peptides for BSA or for the known protein you used for testing, analyze the post-trypsin process-monitoring aliquot to determine if the peptides are present.

Table 3 Theoretical ICAT Reagent-Labeled Cysteine Peptides in a BSA Trypsin Digest

Sequence	No. of Cys	MH+ (m/z)	
		Light	Heavy
DVCK	1	691.3443	700.3744
CASIQK	1	876.4608	885.4909
GACLLPK	1	928.5284	937.5585
LCVLHEK	1	1,068.5870	1,077.6171
CCAADDK	2	1,179.5133	1,197.5735
NECFLSHK	1	1,204.5779	1,213.6080
QNCDQFEK	1	1,238.5470	1,247.5771
SHCIAEVEK	1	1,242.6147	1,251.6448
EACFAVEGPK	1	1,277.6194	1,286.6495
CCTESLVNR	2	1,478.7090	1,496.7692
CCTKPESER	2	1,506.7039	1,524.7641
SLHTLFGDELCK	1	1,589.7992	1,598.8293
YICDNQDTISSK	1	1,613.7476	1,622.7777
ECCDKPLLEK	2	1,631.8131	1,649.8733
DDPHACYSTVFDK	1	1,724.7584	1,733.7885
LKPDPNTLCDEFK	1	1,746.8731	1,755.9032
TCVADESHAGCEK	2	1,803.8000	1,821.8602
ETYGDMADCCEK	2	1,818.7343	1,836.7945
EYEATLEECCAK	2	1,842.8248	1,860.8850
MPCTEDYLSLILNR	1	1,894.9401	1,903.9702
RPCFSALTPDETYVPK	1	2,051.0266	2,060.0567
LFTFHADICTLPDTEK	1	2,078.0263	2,087.0564
YNGVFQECCQAEDK	2	2,087.9161	2,105.9763
ECCHGDLLECADDR	3	2,259.9791	2,287.0694
GLVLIAFSQYLQQCPFDEHVK	1	2,662.3697	2,671.3998

6.3 Testing the Control Sample with Light ICAT Reagent

A Control sample is an aliquot of your sample (for example, a normal cell state) that you run with your Test sample (for example, a diseased cell state) to yield ratios of ICAT reagent-labeled peptides from which you can determine protein expression levels.

If you have sufficient Control sample, Applied Biosystems recommends that you run your Control sample through the entire protocol with the Light reagent before you run Control and Test samples. A successful analysis of the Control sample with Light reagent verifies that your sample preparation protocol does not interfere with labeling and digestion.

IMPORTANT! Extra vials of Light ICAT reagent are provided in the Methods Development Kit to allow you to verify sample preparation using your Control sample.

Follow the steps in Section 7.1.1, Preparing Sample, through Section 7.1.4, Digesting with Trypsin, except:

- Use only the Control sample and label it with the Light reagent.
- In place of step 1 through step 4 in Section 7.1.4, reconstitute a vial of trypsin with 200 µL of Milli-Q® water or equivalent, then add 100 µL of the trypsin solution to the sample tube.

Perform the rest of the protocol (from step 5 in Section 7.1.4, Digesting with Trypsin, through Section 8, Separating and Analyzing the Fractions and Peptides).

7 Running the Protocol

Protocol Overview

Figure 2 outlines the steps in this protocol. Before beginning, test the protocol as described in Section 6, Testing the Protocol.

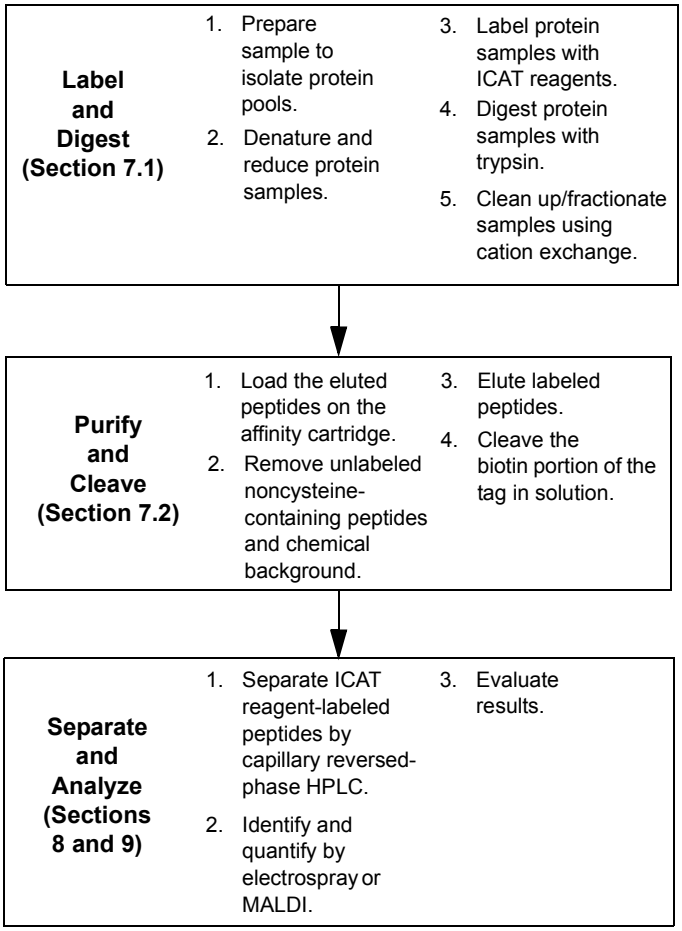


Figure 5 Steps in the Protocol

7.1 Labeling with Cleavable ICAT Reagents and Digesting with Trypsin

This section describes:

- Preparing sample
- Denaturing and reducing the proteins
- Labeling with the Cleavable ICAT Reagents
- Digesting with trypsin
- Cleaning up the peptides using cation exchange
- Fractionating complex samples with a high-resolution cation-exchange column

7.1.1 Preparing Sample

Before performing the ICAT Reagent experiment, ensure that there are no interfering sample contaminants and there is a sufficient quantity of sample.

Table 4 Potential Interfering Sample Contaminants

Potential Contaminant	Potential Adverse Effect
Reducing reagents (for example, mercaptoethanol and dithiothreitol)	React with the ICAT reagents, competing with protein derivitization
High amounts of detergents and denaturants (for example, SDS, urea, and guanidine)	Inactivate trypsin
High acid, salt, or detergent concentrations	Prevent peptides and proteins from binding to the cation-exchange cartridge

If necessary, clean up the samples by acetone precipitation (redissolve the precipitated pellet in the Denaturing Buffer provided in the kit). You can also use other techniques such as gel filtration chromatography, dialysis, or ultracentrifugation.

Make sure you have at least 100 µg of protein in your sample by performing a quantitative protein assay (for example, bicinchoninic acid [BCA] assay).

7.1.2 Denaturing and Reducing the Proteins

WARNING **CHEMICAL HAZARD. Reducing Reagent** causes eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

1. If your sample is a precipitated pellet containing 100 µg of the Control sample – Add 80 µL of the Denaturing Buffer.

   If your Control sample is concentrated in Denaturing Buffer – Add Denaturing Buffer to bring the volume up to 80 µL.

2. If your sample is a precipitated pellet containing 100 µg of the Test sample – Add 80 µL of the Denaturing Buffer.

   If your Test sample is concentrated in Denaturing Buffer – Add Denaturing Buffer to bring the volume up to 80 µL.

3. Add 2 µL of the Reducing Reagent to both the Control and Test sample tubes.

4. Vortex to mix, then centrifuge for a few seconds to bring all solution to the bottom of the tube.

   Note: In this and all subsequent procedures, when instructed to centrifuge, centrifuge at no more than 14,000 x g.

5. Place Control and Test tubes in a boiling water bath for 10 minutes.

6. Vortex to mix, then centrifuge the Control and Test tubes for 1 to 2 minutes to cool.

7. Remove an optional 1-µL process-monitoring aliquot from each tube, and label as “unlabeled”. For more information, see Section 5, Monitoring the Process.

7.1.3 Labeling with the Cleavable ICAT Reagents

WARNING **CHEMICAL HAZARD.** Read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
Cleavable ICAT® Reagent Heavy and Cleavable ICAT® Reagent Light cause eye, skin, and respiratory tract irritation. Exposure may cause an allergic reaction.
Acetonitrile (ACN) is a flammable liquid and vapor. Exposure may cause eye and respiratory tract irritation and blood system damage.

1. Bring to room temperature a vial of Cleavable ICAT Reagent Light and a vial of Cleavable ICAT Reagent Heavy.
2. Centrifuge the reagents to bring all powder to the bottom of each vial.
3. Add 20 µL of acetonitrile to each reagent vial.
4. Vortex each vial to mix, then centrifuge for a few seconds to bring all solution to the bottom of the tube. All of the ICAT reagent may not dissolve.
5. Transfer the entire contents of the Control sample to the vial of the Light reagent.
6. Transfer the entire contents of the Test sample to the vial of the Heavy reagent.
7. Vortex each vial to mix, then centrifuge for a few seconds to bring all solution to the bottom of the tube. All of the ICAT reagent should dissolve.
8. Incubate each vial for 2 hours at 37 °C.
9. Vortex each vial to mix, then centrifuge for a few seconds to bring all solution to the bottom of the tube.
10. Remove an optional 1-µL process-monitoring aliquot from each vial, and label as “labeled”. For more information, see Section 5, Monitoring the Process.

7.1.4 Digesting with Trypsin

WARNING **CHEMICAL HAZARD. Trypsin** causes eye, skin, and respiratory tract irritation. Exposure may cause an allergic reaction. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

IMPORTANT! If you are performing the procedure in Section 6.3, Testing the Control Sample with Light ICAT Reagent, skip step 1 through step 3 below. Instead, reconstitute a vial of trypsin with 200 µL of Milli-Q® water or equivalent, then add 100 µL of the trypsin solution to the Control sample tube.

1. Transfer the entire contents of the Control sample/Light Reagent to the vial containing Test sample/Heavy Reagent. Keep the empty Control sample/Light Reagent vial (you need it in step 3).
2. Dissolve a vial of trypsin in 200 µL of Milli-Q® water or equivalent.
3. Add the entire volume of the trypsin solution to the empty Control sample/Light Reagent vial, vortex to mix, then centrifuge for a few seconds to bring all solution to the bottom of the tube.
4. Transfer the trypsin solution from the Control sample/Light Reagent vial to the combined Control/Test mixture.
5. Vortex to mix, then centrifuge for a few seconds to bring all solution to the bottom of the tube.
6. Incubate 12 to 16 hours at 37 °C.
7. Vortex to mix, then centrifuge for a few seconds to bring all solution to the bottom of the tube.
8. Remove an optional 1-µL process-monitoring aliquot from the vial, and label as “post-trypsin”. For more information, see Section 5, Monitoring the Process.

7.1.5 Cleaning Up the Peptides Using Cation Exchange

Complex Sample Analysis

The first time you analyze a complex sample, use 100 µg of your sample to:

1. Perform all the procedures in this section (Section 7.1.5) and collect one cation-exchange fraction.
2. Run the single fraction through the remaining steps of the protocol (Section 7.2), then analyze and evaluate results (Section 8).

If you need a more detailed analysis of your sample (proteome) than the results on the single fraction provide, take another 100 µg of your sample and:

1. Instead of performing the procedure in this section, fractionate your complex sample as described in Section 7.1.6, Fractionating Complex Samples with a High-Resolution Cation-Exchange Column.
2. Run each fraction through the remaining steps of the protocol, then analyze and evaluate results.

Making an Injection

When instructed to inject a solution in the procedures in this section:

1. Fill a clean 2.5-mL syringe with the indicated solution.
2. Remove air bubbles.
3. Insert the syringe needle into the needle-port adapter, then securely tighten the adapter (no liquid should leak from the adapter).
4. Press the syringe plunger to inject.

General Injection Guidelines

- After each injection, wash the needle and syringe several times with Milli-Q® water or equivalent and once with the next solution before refilling the syringe for the next injection.
- For washing and conditioning steps, inject solution so that 2 to 3 drops/second flow from the outlet connector in the cartridge holder.
- For eluting and loading steps, inject solution so that approximately 1 drop/second flows from the outlet connector.

Assembling the Cation-Exchange Cartridge

Note: The cation-exchange cartridge can be used up to 50 times.

1. Assemble the cartridge holder provided in the Methods Development Kit.
2. Assemble the outlet connector: slide the PEEK tubing provided in the Methods Development Kit into a 10-32 compression screw, then finger-tighten the compression screw into the outlet end of the cartridge holder (Figure 6).
3. Connect the needle-port adapter to the inlet end of the cartridge holder (Figure 6).

4. Mark the inlet and outlet ends of the cartridge (or mark with a directional arrow) for future use. Use the same flow direction in all runs to prevent particles that may accumulate at the cartridge inlet from clogging the outlet tubing.
5. Unscrew the bayonet mount to open the cartridge holder, insert the cation-exchange cartridge, then close the holder.

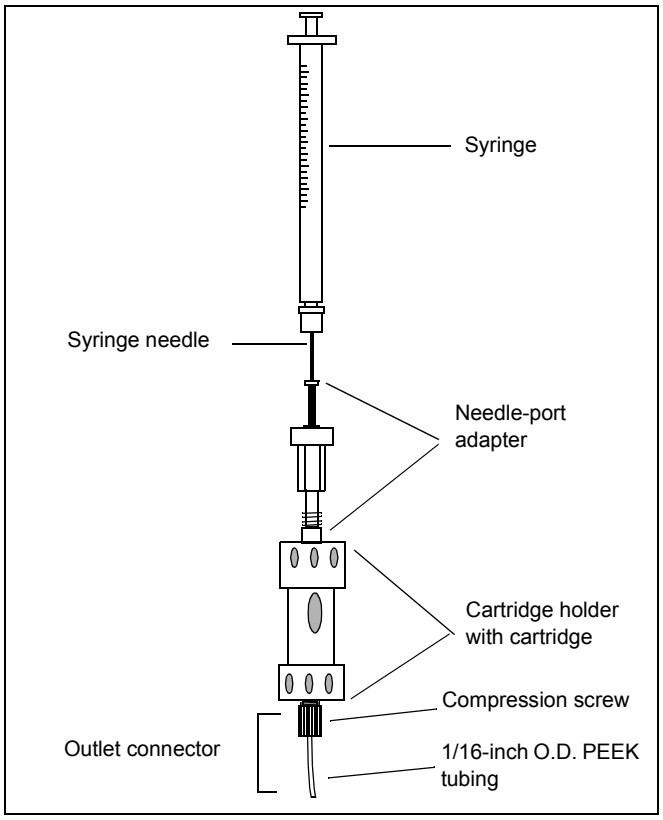


Figure 6 Cartridge Connection

Loading Sample on the Cation-Exchange Cartridge

WARNING **CHEMICAL HAZARD. Cation Exchange Buffer–Load and Cation Exchange Buffer–Elute contain acetonitrile**, a flammable liquid and vapor. Exposure causes eye, skin, and respiratory tract irritation and may cause blood damage. Keep away from heat, sparks, and flame. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

1. Transfer the entire contents of the Control/Test sample mixture to a tube with a capacity greater than 4 mL. The sample can be any of the following:
   - The laminin standard from Section 6.1.2, Labeling the Laminin Peptide Standard with Cleavable ICAT Reagents, step 8.
   - A known protein from Section 6.2.3, Digesting the Known Protein with Trypsin, step 6.
   - Control and Test samples from Section 7.1.4, Digesting with Trypsin, step 7.
2. Dilute the sample mixture by adding 4 mL of the Cation Exchange Buffer–Load.
3. Vortex to mix.
4. Check the pH using pH paper. If the pH is not between 2.5 and 3.3, adjust by adding more Cation Exchange Buffer–Load.
5. To condition the cartridge, inject 2 mL of the Cation Exchange Buffer–Load. Divert to waste.
6. Slowly inject (~1 drop/second) the diluted sample mixture onto the cation-exchange cartridge and collect the flow-through into a sample tube.
7. Inject 1 mL of the Cation Exchange Buffer–Load to wash the TCEP, SDS, and excess ICAT reagents from the cartridge. Collect the flow-through into the same sample tube used in step 6.

   (Keep the flow-through until you confirm that loading on the cation-exchange cartridge is successful. If loading fails, you can repeat loading using the flow-through after you troubleshoot the cause of the loading failure.)
8. To elute the peptides, slowly inject (~1 drop/second) 500 µL of the Cation Exchange Buffer–Elute. Capture the eluate in a fresh 1.5-mL tube. Collect the eluted peptides as a single fraction.
9. When you finish eluting all samples:
   - Clean and store the cartridge as described below.
   - Proceed to Section 7.2, Purifying the Biotinylated Peptides and Cleaving Biotin.

Cleaning and Storing the Cation-Exchange Cartridge

WARNING **CHEMICAL HAZARD. Cation Exchange Buffer–Clean, Cation Exchange Buffer–Load, and Cation Exchange Buffer–Storage contain acetonitrile**, a flammable liquid and vapor. Exposure causes eye, skin, and respiratory tract irritation and may cause blood damage. Keep away from heat, sparks, and flame. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

1. Wash the trypsin from the cation-exchange cartridge by injecting 1 mL of the Cation Exchange Buffer–Clean. Divert to waste.
2. If you have additional protein samples, repeat the steps in Section 7.1.5 for each sample. (Start with step 1 in "Loading Sample on the Cation-Exchange Cartridge" on page 12.)
3. If you do not have additional protein samples, inject 2 mL of the Cation Exchange Buffer–Storage.
4. Remove the cartridge, then seal the ends of the cartridge with the two end caps.
5. Record the number of times the cartridge has been used.
6. Store the cartridge at 2 to 8 °C.
7. Clean the needle-port adapter, outlet connector, and syringe with water.

7.1.6 Fractionating Complex Samples with a High-Resolution Cation-Exchange Column

If results for a highly complex sample are not satisfactory, fractionate to yield individual samples containing fewer peptides. Less complex samples are preferred in mass spec analysis for enhanced sensitivity and increased dynamic range.

The number of fractions you need depends primarily on the complexity of your investigation (for example, whether you are investigating the complete proteome of a cell or organism or investigating a known subset of proteins in a cell lysate or sample). Less complex samples such as immunoprecipitates require only a single fraction. Complex samples such as cell lysates may require more fractions.

High-Resolution Cation-Exchange Column

Use a PolySulfoethyl A Column, 5 micron 300 Å bead, from PolyLC, Inc., (for example, 2.1 × 200 mm, PN 202SE0503). Select a column size with the appropriate binding capacity for your sample size.

WARNING **CHEMICAL HAZARD. Acetonitrile (ACN)** is a flammable liquid and vapor. Exposure may cause eye and respiratory tract irritation and blood system damage. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

- **Buffer A** – 10 mM KH_2PO_4, 25% acetonitrile, pH 3 after the addition of acetonitrile
- **Buffer B** – 10 mM KH_2PO_4, 350 mM KCl, 25% acetonitrile, pH 3 after the addition of acetonitrile

Gradient Conditions

0 to 100% B in 1 hour, flow rate 0.2 mL/min (for a 2.1 × 200 mm column), room temperature

IMPORTANT! Optimize these suggested starting conditions for your sample. Do not exceed the column manufacturer's suggested pressure limit.

Collect the number of fractions and fraction size appropriate for the complexity of your sample, clean the column as described below, then proceed to Section 7.2, Purifying the Biotinylated Peptides and Cleaving Biotin.

Cleaning the Column

After collecting fractions, clean the column with Buffer A and 1 M KCl, pH 3 to remove trypsin. Reequilibrate the column with Buffer A.

7.2 Purifying the Biotinylated Peptides and Cleaving Biotin

This section describes:

- Activating the avidin cartridge
- Loading sample on the avidin cartridge
- Removing non-labeled material
- Eluting ICAT reagent-labeled peptides
- Cleaning and storing the avidin cartridge
- Cleaving the ICAT reagent-labeled peptides

IMPORTANT! The avidin cartridge has a maximum recommended load of 8 to 10 nmol for a nominal 1-kDa peptide. The avidin cartridge can be cleaned, activated, and reused for up to 50 cation-exchange fractions.

7.2.1 Activating the Avidin Cartridge

WARNING **CHEMICAL HAZARD. Affinity Buffer–Elute contains acetonitrile**, a flammable liquid and vapor. Exposure causes eye, skin, and respiratory tract irritation and may cause blood damage. Keep away from heat, sparks, and flame. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

1. Mark the inlet and outlet ends of the cartridge (or mark with a directional arrow) for future use. Use the same flow direction in all runs to prevent particles that may accumulate at the cartridge inlet from clogging the outlet tubing.
2. Insert the avidin cartridge into the cartridge holder.
3. Inject 2 mL of the Affinity Buffer–Elute. Divert to waste.

   Note: Injecting the Elute buffer before loading sample is required to free up low-affinity binding sites on the avidin cartridge.
4. Inject 2 mL of the Affinity Buffer–Load. Divert to waste.

7.2.2 Loading Sample on the Avidin Cartridge

1. Neutralize each cation-exchange fraction (from step 8 in Loading Sample on the Cation-Exchange Cartridgeon page 12) by adding 500 µL of the Affinity Buffer–Load.
2. Check the pH using pH paper. If the pH is not 7, adjust by adding more Affinity Buffer–Load.
3. Vortex to mix, then centrifuge for a few seconds to bring all solution to the bottom of the tube.
4. Remove an optional 1-µL process-monitoring aliquot and label as "pre-avidin". For more information, see Section 5, Monitoring the Process.
5. Label three fraction-collection tubes: **#1** (Flow-Through), **#2** (Wash), and **#3** (Elute), then place in a rack.
6. Slowly inject (~1 drop/5 seconds) of the neutralized fraction onto the avidin cartridge and collect the flow-through into tube #1 (Flow-Through).

   Note: Tube #1 (Flow-Through) contains unlabeled peptides. If needed, you can perform MS/MS analysis on this fraction for further protein coverage of sample. However, this analysis does not provide ICAT reagent-labeled peptide quantification information.

7.2.3 Removing Non-Labeled Material

WARNING **CHEMICAL HAZARD. Affinity Buffer–Wash 2 contains methanol**, a flammable liquid and vapor. Exposure causes eye, skin, and respiratory tract irritation, and may cause central nervous system depression and nerve damage. Keep away from heat, sparks, and flame. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

1. Inject 500 µL of Affinity Buffer–Load onto the cartridge and continue to collect in tube #1 (Flow-Through).

   (Keep tube #1 until you confirm that loading on the avidin cartridge is successful. If loading fails, you can repeat loading using tube #1 (Flow-Through) after you troubleshoot the cause of the loading failure.)
2. To reduce the salt concentration, inject 1 mL of Affinity Buffer–Wash 1. Divert the output to waste.
3. To remove nonspecifically bound peptides, inject 1 mL of Affinity Buffer–Wash 2. Collect the first 500 µL in tube #2 (Wash). Divert the remaining 500 µL to waste.
4. Inject 1 mL of Milli-Q® water or equivalent. Divert to waste.

7.2.4 Eluting ICAT Reagent-Labeled Peptides

WARNING **CHEMICAL HAZARD. Affinity Buffer–Elute contains acetonitrile**, a flammable liquid and vapor. Exposure causes eye, skin, and respiratory tract irritation and may cause blood damage. Keep away from heat, sparks, and flame. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

1. Fill a syringe with 800 µL of the Affinity Buffer–Elute.
2. To elute the labeled peptides, slowly inject (~1 drop/5 seconds) 50 µL of the Affinity Buffer–Elute and discard the eluate.
3. Inject the remaining 750 µL of Affinity Buffer–Elute and collect the eluate in tube #3 (Elute).
4. Vortex to mix, then centrifuge for a few seconds to bring all solution to the bottom of the tube.
5. Remove an optional 1-µL process-monitoring aliquot from the eluate in tube #3, and label as "post-avidin". For more information, see Section 5, Monitoring the Process.
6. If you have additional cation-exchange fractions, repeat the steps in Section 7.2.1, Activating the Avidin Cartridge, through Section 7.2.4, Eluting ICAT Reagent-Labeled Peptides, for each fraction. (Start with step 3 in Section 7.2.1.)

7.2.5 Cleaning and Storing the Avidin Cartridge

WARNING **CHEMICAL HAZARD. Affinity Buffer–Elute contains acetonitrile**, a flammable liquid and vapor. Exposure causes eye, skin, and respiratory tract irritation and may cause blood damage. Keep away from heat, sparks, and flame. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

1. When you finish eluting peptides from all cation-exchange fractions as described in Section 7.2.4, Eluting ICAT Reagent-Labeled Peptides, clean the cartridge by injecting 2 mL of the Affinity Buffer–Elute. Divert to waste.
2. Inject 2 mL of Affinity Buffer–Storage. Divert to waste.
3. Remove the cartridge, then seal the ends of the cartridge with the two end caps.
4. Record the number of times the cartridge has been used.
5. Store the cartridge at 2 to 8 °C.
6. Clean the needle-port adapter, outlet connector, and syringe with water.

7.2.6 Cleaving the ICAT Reagent-Labeled Peptides

DANGER **CHEMICAL HAZARD**. **Cleaving Reagent A contains trifluoroacetic acid**. Exposure causes eye, skin, and respiratory tract burns. It is harmful if inhaled. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

WARNING **CHEMICAL HAZARD**. **Cleaving Reagent B** is a flammable liquid and vapor. Exposure causes eye, skin, and respiratory tract irritation. Keep away from heat, sparks, and flame. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

1. Evaporate each affinity-eluted fraction to dryness in a centrifugal vacuum concentrator.
2. In a fresh tube, prepare the final cleaving reagent by combining Cleaving Reagent A and Cleaving Reagent B in a 95:5 ratio. You need ~ 90 µL of final cleaving reagent per sample.
3. Vortex to mix, then centrifuge for a few seconds to bring all solution to the bottom of the tube.
4. Transfer ~90 µL of freshly prepared cleaving reagent to each sample tube.
5. Vortex to mix, then centrifuge for a few seconds to bring all solution to the bottom of the tube.
6. Incubate for 2 hours at 37 °C.
7. Centrifuge the tube for a few seconds to bring all solution to the bottom of the tube.
8. Evaporate the sample to dryness in a centrifugal vacuum concentrator (~30 to 60 min).
9. Proceed to:
   - Section 8, Separating and Analyzing the Fractions and Peptides
   - Section 9, Evaluating Results

8 Separating and Analyzing the Fractions and Peptides

This section describes:

- Separating by capillary reversed-phase HPLC
- Analyzing by electrospray
- Analyzing by MALDI

8.1 Preparing Sample

Dissolve the cleaved, dried samples in 10 to 50 µL of the solvent appropriate for analyzing the labeled peptides based on your analysis method (for example, HPLC loading buffer).

8.2 Separating by Capillary Reversed-Phase HPLC

Connecting the HPLC System to the Mass Spectrometry System

- For electrospray analysis, connect to an Applied Biosystems/ MDS SCIEX QSTAR® Hybrid LC/MS/MS Quadrupole TOF System. For connection details, refer to the QSTAR System documentation on CD-ROM.
- For MALDI analysis, connect the HPLC to an automated MALDI plate spotter such as the PROBOT™ Micro Fraction Collector (available from LC Packings – a Dionex Company). Analyze the spotted plate using an Applied Biosystems (AB) 4700 Proteomics Analyzer.

Selecting the Capillary HPLC Column and Parameters

Based on the amount of peptide in the sample, use the following table to select the capillary HPLC column size, flow rate, and injection volume.

IMPORTANT! The suggested settings below are based on a capillary reversed-phase LC system using an LC Packings Ultimate™ Capillary/ Nano LC System with a Switchos™ Micro Column Switching Device.

Estimated Amount of Peptide Per Sample	Column Size (I.D.)	Column Name	Flow Rate	Injection Volume[a] (Pre-concen.)	Injection Volume[b] (*No* Pre-concen.)
0.2 to 5.0 pmol	300 µm	Capillary-300	4 µL/min	75 µL	1 to 10 µL
0.02 to 1.0 pmol	180 µm	Capillary-180	1 µL/min	50 µL	1 to 5 µL
2 to 500 fmol	75 µm	Nano-75	200 nL/min	50 µL	1 µL

a. Assumes that preconcentration is performed with the Switchos device immediately before injection onto the capillary LC column.

b. Assumes that *no* preconcentration is performed.

After connecting the HPLC system to the mass spectrometer system and selecting settings, proceed to the appropriate section:

- Section 8.3, Analyzing by Electrospray
- Section 8.5, Analyzing by MALDI

8.3 Analyzing by Electrospray

This section provides guidelines and suggestions for electrospray analysis of the ICAT reagent-labeled peptides. In this configuration, the capillary reversed-phase HPLC system is connected to a QSTAR system.

Selecting a Source for the QSTAR® System

Based on the capillary HPLC column size and its corresponding flow rate, use the following table to select the ESI source for the QSTAR System.

Column Size (I.D.)	Flow Rate	ESI Source for QSTAR System
300 µm	4 µL/min	IonSpray™ Source
180 µm	1 µL/min	MicroIonSpray™ Source
75 µm	200 nL/min	Protana NanoES (see below for more information)

With the Protana NanoES source, you can use two types of tips:

- Coated fused-silica PicoTips™ (coating applied to tip end) – New Objective, Inc. 360-µm O.D. coated fused-silica spray tip (Cat. #FS360-20-10-CE-20). Fit onto the normal tip holder of the source. To connect the 360-µm O.D. tip to the 280-µm O.D. HPLC tubing, use a Teflon® fitting from LC Packings (Cat. #TF-250/350).
- Distal-coated fused-silica PicoTips™ – New Objective, Inc. 20 µm pulled to 10 µm, (Cat. #FS360-20-10-D-20). Connect the fused silica outlet from the HPLC directly to the liquid junction (for example, a low-dead-volume Valco reducing union, Cat. #ZRU 1.5) using standard HPLC fittings.

HPLC Gradient Conditions for QSTAR System Analysis

Table 5 on page 15 provides suggested capillary HPLC gradient conditions for analyzing peptides using a QSTAR system. Gradient duration depends on the complexity of the sample.

CHEMICAL HAZARD. **Formic Acid** is a flammable liquid and vapor. Exposure causes eye, skin, and respiratory tract burns. It is harmful if inhaled, and may cause allergic reactions. Keep away from heat, sparks, and flame. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

CHEMICAL HAZARD. Acetonitrile (ACN) is a flammable liquid and vapor. Exposure may cause eye and respiratory tract irritation and blood system damage. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

IMPORTANT! For better sensitivity by ESI on the QSTAR System, use formic acid instead of TFA for capillary or nanoflow applications.

Table 5 Suggested HPLC Gradient Conditions for Electrospray Analysis

Parameter	Suggested Setting
Mobile phase A	0.1% formic acid in 5% acetonitrile, 95% Milli-Q® water or equivalent
Mobile phase B	0.1% formic acid in 95% acetonitrile, 5% Milli-Q® water or equivalent
Gradient	
• For complex samples (for example, unfractionated lysates)	2-hour (2 to 30% B)
• For crudely fractionated samples (for example, gel slice extractions or ion-exchange salt cuts)	1- to 2-hour (2 to 30% B)
• For highly fractionated samples (for example, gel bands or high-resolution ion-exchange fractionates)	1-hour (2 to 30% B)

QSTAR® System Acquisition Method Settings

Table 6 provides suggested QSTAR System acquisition method settings for the acquisition of LC/MS/MS IDA (Information Dependent Acquisition) data.

IMPORTANT! The settings in Table 6 are for Analyst® QS Software with Service Pack 5.

Table 6 QSTAR® System Acquisition Method Settings

Parameter	Suggested Setting
Acquisition Method Window (MS Tab)	
Experiment	1
Scan type	TOF MS
Accumulation time (for Experiment 1)	0.5 to 1.0 second
Experiment	2 and 3
Scan type (for Experiments 2 and 3)	Product Ion
Accumulation Time (for Experiments 2 and 3)	1 to 3 seconds
Enhance All	Enabled
Quad Resolution	Low
Collision Energy (CE) volts	0, which sets the method to use the IDA Collision Energy Parameters script. See the suggested settings below.
Switch Criteria Tab	
For ions greater than	400 amu
For ions smaller than	1200 amu
Charge state	2 to 4
Exceeds	10 counts
Use Advanced Settings	Enabled
Switch after	1 spectrum
Exclude former target ions for	60 seconds
Ignore peaks within	4 amu
Mass tolerance window	100 ppm
IDA Collision Energy Parameters (default values, may need to be optimized on your system by changing the Intercept values)	

Charge	Slope	Intercept
Unknown	0.0575	9
1	0.0575	9
2	0.0625	–3
3	0.0625	–5
4	0.0625	–6
5	0.0625	–6

Maximum Allowed CE: 80 V

8.4 Processing the Data Using Pro ICAT Software (API QSTAR® System Data Only)

After analysis by capillary or nanoflow HPLC MS/MS, you can use the Pro ICAT software to automatically quantify and identify the differentially expressed proteins from the LC/MS/MS IDA data. Pro ICAT software incorporates the LC/MS reconstruct quantitation algorithm to determine ICAT® reagent ratios using a three-dimensional peak-finding technique on the MS data. The Interrogator™ database search engine is then applied to the MS/MS data for protein identification, and the data is then correlated to the quantitation data. The results of every sample are stored in the Pro ICAT software Microsoft Access database for future retrieval and mining.

Expression-Dependent Protein Identification

After quantitation using the Pro ICAT software, you can run a script on the results in the Pro ICAT database to generate an inclusion list for the ratios of interest (to limit the inclusion list, you can specify the intensity difference for a pair and a minimum quality score threshold). You can then import the ratios into a new LC/MS/MS IDA QSTAR System method for analysis of the same sample. This approach can be used to identify only those proteins that are changing in an expression analysis experiment.

8.5 Analyzing by MALDI

This section provides guidelines and suggestions for MALDI analysis of the ICAT reagent-labeled peptides using an Applied Biosystems 4700 Proteomics Workstation. In this configuration, the capillary reversed-phase HPLC system is connected to a PROBOT™ Micro Fraction Collector (available from LC Packings – a Dionex Company). See PROBOT™ Micro Fraction Collector Guidelines below for more information.

PROBOT™ Micro Fraction Collector Guidelines

- Based on your capillary HPLC column size, set the PROBOT Micro Fraction Collector to collect 0.1- to 2.0-µL fractions. See “Selecting the Capillary HPLC Column and Parameters” on page 15.
- Optimize the HPLC gradient and the fraction collection according to the total elution time and total collection time for the peptides of interest. For example, if you collect 100 fractions at 1 sample per minute, select a gradient that elutes the peptides of interest within a 100-minute period.
- Refer to the installation chapter in the *PROBOT Sample Robot for HPLC and CZE Guide* for details on setting up and connecting the fraction collector.

HPLC Gradient Conditions for MALDI Analysis

Table 7 provides suggested capillary HPLC gradient conditions for analyzing peptides using a 4700 Proteomics Analyzer. Gradient duration depends on the complexity of the sample.

WARNING **CHEMICAL HAZARD. Acetonitrile (ACN)** is a flammable liquid and vapor. Exposure may cause eye and respiratory tract irritation and blood system damage. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

DANGER **CHEMICAL HAZARD. Trifluoroacetic acid (TFA)** causes eye, skin, and respiratory tract burns. It is harmful if inhaled. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Table 7 Suggested HPLC Gradient Conditions for MALDI Analysis

Parameter	Suggested Setting
Mobile phase A	0.1% trifluoroacetic acid in 5% acetonitrile, 95% Milli-Q® water or equivalent
Mobile phase B	0.1% trifluoroacetic acid in 95% acetonitrile, 5% Milli-Q® water or equivalent
Gradient	
• For complex samples (for example, unfractionated lysates)	2-hour (2 to 30% B)
• For crudely fractionated samples (for example, gel slice extractions or ion-exchange salt cuts)	1- to 2-hour (2 to 30% B)
• For highly fractionated samples (for example, gel bands or high-resolution ion-exchange fractionates)	1-hour (2 to 30% B)

AB 4700 Proteomics Analyzer Method Settings

To perform MS analyses on ICAT reagent-labeled samples, use standard reflector methods, but use a higher number of laser shots and search-pattern positions (for example, 50 shots/subspectra and 20 randomly selected search pattern positions). Settings such as these provide good reproducibility for measuring the relative abundances of peak pairs of ICAT reagent-labeled peptides.

To perform MS/MS analyses on ICAT reagent-labeled samples, use standard MS/MS acquisition methods. Optimize the timed ion selector (TIS) window to ensure that only one peak in a peak pair is selected for MS/MS (for example, use a window less than ±9 Da).

Set the acquisition method to eliminate the masses below m/z 600. In MALDI spectra, an intense m/z 515.3 fragment peak is present in spectra if you do not perform an LC/MS cleanup step after cleavage. This m/z 515.3 peak corresponds to a laser-induced fragment of the affinity tag/linker.

The AB 4700 Proteomics Analyzer first records the MS spectra for quantitation. Peak pairs representing the ICAT reagent-labeled peptides are recognized by their characteristic mass differences (multiples of 9.03 m/z, depending on the number of cysteine residues in the peptide). Relative expression of the corresponding protein is defined as the intensity ratio of the Light-labeled to the Heavy-labeled peptides (assuming you have labeled your Control sample with light reagent).

Precursor ions for MS/MS analysis (for protein identification) can be selected using:

- Interpretation methods in the AB 4700 Explorer™ Software
- Result-based analysis in the GPS Explorer™ Software version 2.0.

MS/MS analyses can be run in automated mode using either 4700 Explorer software or GPS Explorer Software version 2.0. The spot sets generated from ICAT reagent-labeled samples can be further processed using the GPS Explorer Software version 1.0 or 2.0. The GPS Explorer software allows you to identify proteins from MS/MS spectra of ICAT reagent-labeled peptides and generates quantitative information on the peak pairs.

9 Evaluating Results

9.1 Quantitation Notes

- You may see a small peak preceding the major peak that corresponds to the Heavy-labeled protein. ICAT Reagent Heavy is 90 to 95% isotopically pure (refer to the Certificate of Analysis for actual amounts).
- Quantitation for standard proteins or complex samples is typically within 30% of expected values.

9.2 ICAT Reagent Fragments Using MS/MS Analysis

MS/MS analysis of ICAT reagent-labeled peptides yields ICAT reagent-specific fragment ions. Table 8 lists the possible fragment masses of Light and Heavy ICAT reagents bound to a cysteine residue in a peptide.

Table 8 ICAT Reagent Fragment Masses

Electrospray		MALDI	
Light (m/z)	Heavy (m/z)	Light (m/z)	Heavy (m/z)
243.1	252.1	144.1	148.1
245.1	254.1	146.1	150.1
269.1	278.1	243.1	252.1
286.1	295.1	245.1	254.1
		269.1	278.1
		286.1	295.1

10 Technical Support

Applied Biosystems is committed to meeting the needs of your research through enabling technologies such as the Cleavable ICAT® Reagent Kit for Protein Labeling. Our dedicated support staff is available to answer questions about using this product to its fullest potential.

Contacting Technical Support in North America

To contact technical support:

- By telephone: Dial **1**.**800**.**899**.**5858**
- By fax: Dial **1**.**508**.**383**.**7855**

11 Ordering Information

To place an order from the U.S. or Canada, dial **1**.**800**.**327.3002**, then follow the voice instructions.

Description	Quantity	Part Number
1-D PAGE Cleavable ICAT® Reagent Applications Development Kit for Targeted Protein ID and Quantitation Contains cleavable ICAT reagents, Cleaving Reagents A and B, affinity buffers and cartridge, and cartridge/hardware accessories.	1 kit	4348367
Cleavable ICAT® Reagent Methods Development Kit for Protein Labeling Contains cleavable ICAT reagents, Cleaving Reagents A and B, affinity and cation-exchange buffers and cartridges, and cartridge/ hardware accessories. See page 4 for details.	1 kit	4339035
Cleavable ICAT® Reagent 10-Assay Kit Contains cleavable ICAT reagents, Cleaving Reagents A and B, and affinity and cation-exchange buffers and cartridges. See page 4 for details.	1 kit	4339036
Cleavable ICAT® Bulk Reagents (10 units) Contains Cleavable ICAT Reagents Light and Heavy, Cleaving Reagents A and B.	1 kit	4339038
Cleavable ICAT® Bulk Reagents (100 units) Contains Cleavable ICAT Reagents Light and Heavy, Cleaving Reagents A and B.	1 kit	4339039
Cleavable ICAT® Bulk Reagents (200 units) Contains Cleavable ICAT Reagents Light and Heavy, Cleaving Reagents A and B.	1 kit	4339040
ICAT Cartridge–Avidin	5 cartridges	4326694
ICAT Affinity Buffer Pack with Avidin Cartridge	1 pack	4326740
ICAT Cartridge Pack–Cation Exchange	5 cartridges	4326695
ICAT Cation Exchange Buffer Pack with Cation Exchange Cartridge	1 pack	4326747
Cleaving Reagent A (not needed if you order a kit)	1 vial	4338543
Cleaving Reagent B (not needed if you order a kit)	1 vial	4339052
Cartridge holder	1 holder	4326688
Needle-port adapter	1 adapter	4326689
Outlet tubing kit	1 kit	4326690

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