1-D PAGE Cleavable ICAT® Reagent Applications Development Kit for Targeted Protein ID and Quantitation (Monoplex Version)

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

Protocol

1 Product Description

The 1-D PAGE Cleavable ICAT® Reagent Applications Development Kit for Protein Targeted Protein ID and Quantitation combines SDS PolyAcrylamide Gel Electrophoresis (PAGE), isotope-coded affinity tag chemistry, and cleavable-linker technology to facilitate the identification and quantification of differentially expressed proteins. 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.

The labeled proteins, products of the reactions generated by this kit, are separated by SDS-PAGE. The targeted bands are then excised, digested, and affinity isolated. The recovered labeled peptides are used for quantification by mass spectrometry.

This kit provides the following benefits:

- Designed for applications that require targeted protein identifications with quantitation for a specific molecular weight and/or known classes of proteins or complexes.
- Accommodates complex samples that require simplification using a protein workflow before enzymatic digestion.
- Allows convenient removal of ICAT reaction contaminants and low MW by-products from the labeling reactions.

When to Use

Use the 1-D PAGE Cleavable ICAT® Reagent Applications Development Kit when you are investigating 1) a protein or protein class with a known molecular weight range (for example, if you are investigating a protein class ~ 50 kDa, you can run the SDS-PAGE, then excise only the area surrounding 50 kDa region for ICAT labeling; or 2) if fractionation of a complex sample is required at the protein level prior to enzymatic digestion. For global protein expression analysis applications, the Cleavable ICAT Reagent Methods Development Kit is available (see Section 10, Ordering Information, for the part number).

1-D PAGE Cleavable ICAT® Reagent Kit Options

- **1-D PAGE Applications Development Kit** – Contains cleavable ICAT reagents, affinity buffers and cartridge, and cartridge/hardware accessories. Use this kit with your own 1-D SDS-PAGE system to get started with the 1-D PAGE Cleavable ICAT Reagent Kit application. This kit supports assay of up to 30 gel slices from a standard mini-gel format.
- **Bulk reagent kits** – Contain ICAT reagents only. After you set up your application using the applications development kit, order Cleavable ICAT Bulk Reagents for routine use. For information, see Section 10, Ordering 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.

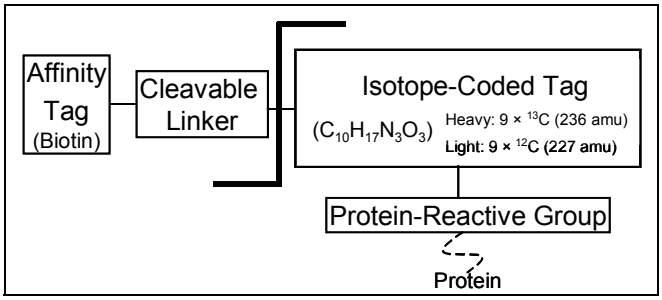


Figure 1 Cleavable ICAT Reagent Structure

Contents

- **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.
- **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 11, References.

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 1-D PAGE Cleavable ICAT Reagent Applications Development Kit

You can use the 1-D PAGE Cleavable ICAT Reagent Applications Development Kit to:

- Familiarize yourself with the ICAT protocol by labeling the 6-protein standard mixture provided in the kit.
- Ensure that expected ICAT reagent-labeled peptides are present in the 6-protein standard mixture provided in the kit.
- 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.

Figure 2 is an overview of the ICAT reagent protocol. Before running the protocol on real samples, test the protocol as described in Section 6.2, Testing the Protocol with the 6-Protein Standard Mix.

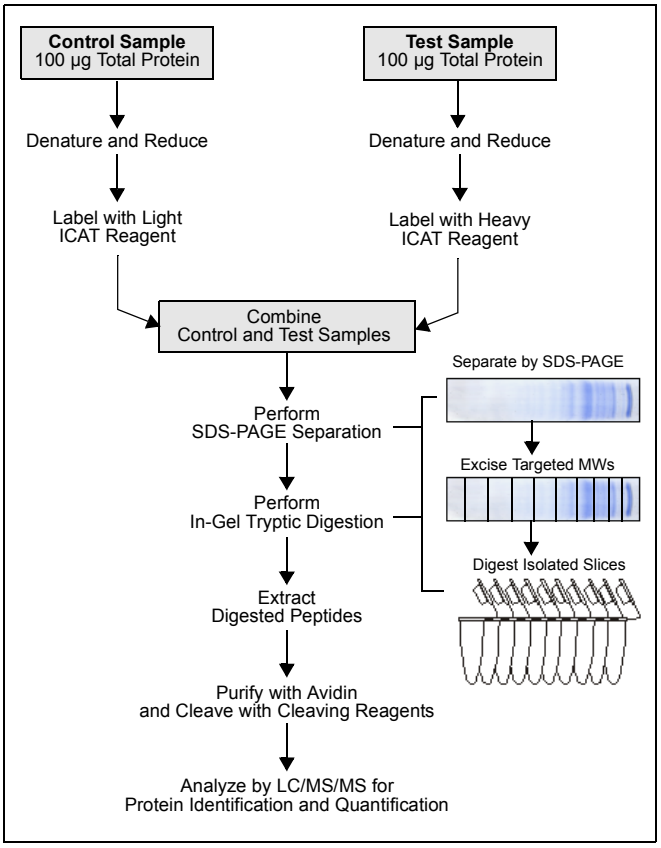


Figure 2 Overview of the 1-D PAGE Protocol

3 Materials

This section describes:

- User-supplied materials
- Kit materials

3.1 User-Supplied Materials

Item	Volume or Quantity per Assay
1-D SDS-PAGE gel system Note: This protocol is written for a 1-mm mini-gel system.	1
1-mm tris-glycine mini-gels	As needed
Gel running buffer	As needed
2× SDS-PAGE sample buffer (for example, Novex Tricine SDS Sample Buffer from Invitrogen)	As needed
Coomassie blue aqueous gel staining solution (for example, SimplyBlue SafeStain from Invitrogen)	As needed
Gel dehydration solution: 100% acetonitrile (ACN)	50 mL
Gel washing buffer: 50% ACN in 100 mM ammonium bicarbonate (NH_4HCO_3), pH 8.0	50 mL
Extraction solvent: 50% ACN containing 0.1% TFA	50 mL
100 mM ammonium bicarbonate (NH_4HCO_3) to reconstitute trypsin	1 mL per vial of trypsin
Clean, single-edged razor blade or scalpel for excising gel bands	1
Sonic water bath for use when extracting peptides from gel	1
Platform rocker for destaining gel	1
Clean container for destaining gel	1
Eppendorf tubes, 1.5-mL	As needed
Disposable gloves	As needed
Pipettors and tips suitable for: • 1 µL to 1 mL • Gel loading	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
Test sample (for example, a diseased cell state)	100 µg
Control sample (for example, a normal cell state)	100 µg
pH paper (pH 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 1-D PAGE Cleavable ICAT® Reagent Applications Development Kit (reagents, buffers, cartridges, and hardware).

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. Affinity buffer packs with cartridges and cartridge hardware

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 1 on page 4.

Table 1 on page 4 lists the kit materials and their recommended storage conditions.

Table 1 Kit Materials and Storage Conditions

Item	1-D PAGE Kit Volume or Qty.	Description	Shipping Box/ Storage Conditions
Cleavable ICAT Reagent Heavy	3 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	3 vials, 1 unit/vial[a]	Sulfhydryl-modifying biotinylation Light reagent, used to label the Control sample.	
6-protein standard mix	2 vials	Standard peptide to test the kit.	
Trypsin with $CaCl_2$	3 vials	Cleaves peptide bonds on the carboxyl side of lysine and arginine residues.	
Denaturing Buffer (pH 8.5)	1 vial, 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	Reduces the disulfide bonds of the proteins. Contains 50 mM TCEP.	
Cleaving Reagent B	1 vial, 500 µL/vial	Contains a scavenger that reduces side reactions during the cleaving reaction.	
1-D PAGE Cleavable ICAT Reagent Applications Development Kit for Protein Labeling Protocol	1	This document.	
1-D PAGE Cleavable ICAT Reagent Applications Development Kit for Protein Labeling Quick Reference	1	Laminated card that provides a quick reference to the steps in this protocol.	
Cleaving Reagent A	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–Avidin	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 gel slices.	Box 3: Avidin Cartridge and Affinity Buffers. Store at 2 to 8 °C
Affinity Buffer–Elute (30% acetonitrile + 0.4% TFA)	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	Phosphate buffer that adjusts the pH to approximately 7.2.	
Affinity Buffer–Wash 1 (1× PBS, pH 7.2)	100 mL	Phosphate buffer that decreases the salt concentration.	
Affinity Buffer–Wash 2 (50 mM v ammonium bicarbonate [NH_4HCO_3]/ 20% methanol, pH 8.3)	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 + 0.02% sodium azide [NaN_3])	100 mL	Phosphate buffer with sodium azide that maintains the proper pH and prevents growth of microorganisms.	
Cartridge holder	1 (for 200-µL cartridges)	Reusable bayonet-style holder for 200-µL cation-exchange and avidin cartridges.	Box 3: Cartridge/hardware accessories (Applications Development Kit only). Store at room temperature.
Needle-port adapter	1	Provides a secure connection for the HPLC syringe needle (while injecting onto the cartridge).	
Outlet connector	1	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 compare before- and after-labeling samples.
After adding ICAT reagents	
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 Running the Protocol

This section describes:

- Overview of steps in the protocol
- Testing the protocol with the 6-protein standard mix
- Labeling with cleavable ICAT reagents and digesting with trypsin
- Purifying the biotinylated peptides and cleaving biotin

6.1 Overview of Steps in this Protocol

Figure 3 outlines the steps in this protocol. Before beginning, test the protocol as described in Section 6.2, Testing the Protocol with the 6-Protein Standard Mix.

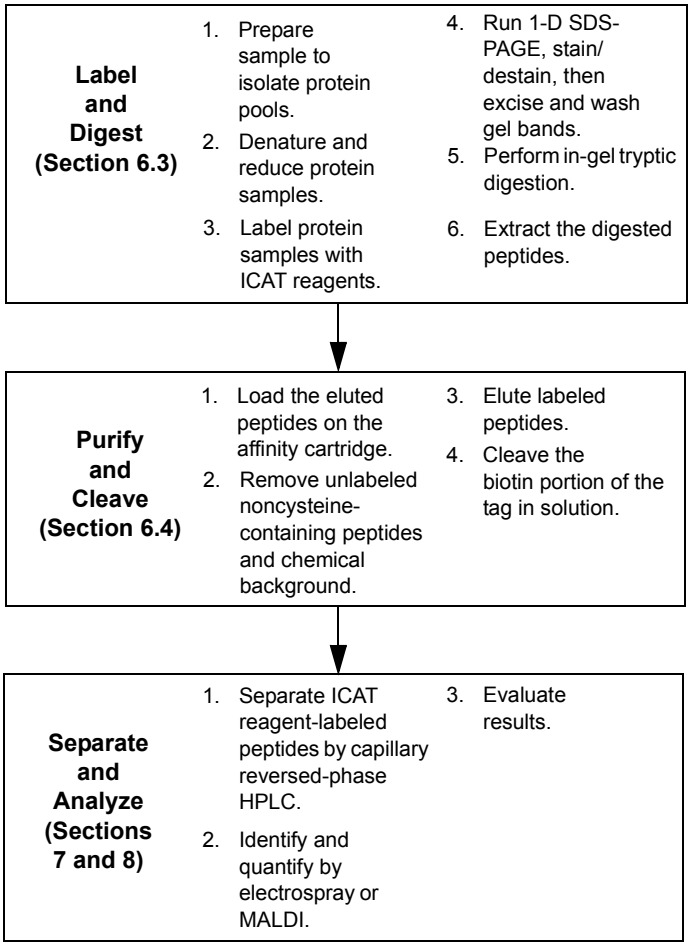


Figure 3 Steps in the Protocol

6.2 Testing the Protocol with the 6-Protein Standard Mix

Before you run your complex samples for the first time, test this protocol using the 6-protein standard mix provided in the kit.

You can use the protocol to quantitate a 1:1 (Light:Heavy) mix and a 1:2 (Light:Heavy) mix to within ±30% of expected values.

To test the protocol, perform the procedures in:

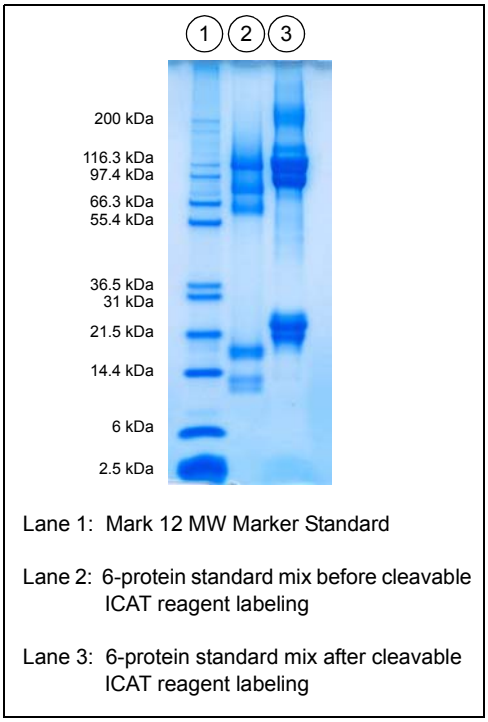
- Section 6.3, Labeling with Cleavable ICAT Reagents and Digesting with Trypsin
- Section 6.4, Purifying the Biotinylated Peptides and Cleaving Biotin
- Section 7, Separating and Analyzing the Fractions and Peptides

The 6-protein standard mix contains the following components:

Table 2 6-Protein Standard Mix Components

Component	Number of Cysteines	MW (kDa)	nmol/ Vial
β-Galactosidase	16	116.3	0.33
Apo- serotransferrin	38	75.2	0.33
Bovine Serum Albumin	35	66.4	0.33
β-Lactoglobulin	5	18.3	1.31
Lysozyme	8	14.3	0.70
α-Lactalbumin	8	14.1	0.70
Combined Total Protein	110	—	3.71

Figure 4 shows a 1-D gel separation of the 6-protein standard mix before and after ICAT reagent labeling.



Lane 1: Mark 12 MW Marker Standard

Lane 2: 6-protein standard mix before cleavable ICAT reagent labeling

Lane 3: 6-protein standard mix after cleavable ICAT reagent labeling

Figure 4 6-Protein Standard Mix Before and After ICAT Reagent Labeling

6.3 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
- Performing the SDS-PAGE separation
- Excising and washing the gel bands
- Performing in-gel trypsin digestion
- Extracting digested peptides

6.3.1 Preparing Sample

If you are running the 6-protein standard mix: Skip this section.

If you are running real sample: Before performing the ICAT Reagent experiment, ensure that:

- Your sample does not contain interfering contaminants. Table 3 lists potential interfering contaminants and potential adverse effects.
- You have at least 100 µg of protein in your sample as determined by performing a quantitative protein assay (for example, bicinchoninic acid [BCA] assay).

Table 3 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 acid, high salt, high detergent concentrations	Interfere with gel electrophoresis
High concentrations of charged species such as guanidine	

If necessary, clean up the sample 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.

6.3.2 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 you are running the 6-protein standard mix:*** Add 80 µL of the Denaturing Buffer to one vial of the 6-protein standard mix. Label this tube “Control”.

   If you are running real sample:

   - 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 you are running the 6-protein standard mix:*** Add 80 µL of the Denaturing Buffer to a second vial of the 6-protein standard mix. Label this tube “Test”.

   If you are running real sample:

   - 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 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.

6.3.3 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.

6.3.4 Performing the SDS-PAGE Separation

Note: This procedure is written for a 1-mm mini-gel system.

This section describes:

- Protein loading considerations
- Running the SDS-PAGE gel
- Staining and destaining the gel

Protein Loading Considerations

The maximum suggested protein load is 20 to 50 µg of combined heavy/light protein per gel lane, depending on the size of the wells.

The amount of the combined ICAT-reagent labeled samples is 200 µg. To accommodate the protein concentration in the labeled samples, you can do any of the following:

- Load multiple lanes with 20 to 50 µg of combined heavy/light protein, then combine the gel bands after SDS-PAGE.
- Use a formatted gel with fewer sample lanes to accommodate larger sample volumes and/or amounts.
- Consider a semi-preparative SDS-PAGE system.
- Concentrate sample to load more protein per lane. However, you may see:
  – Precipitation
  – Band spreading/gel swelling
  – Poor band separation

Running the SDS-PAGE Gel

1. ***If you are running the 6-protein standard mix:***

   a. 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.)

   b. 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.)

   If you are running real sample: Combine Control and Test samples into a single tube (tube now contains 200 µg total protein).

2. ***If you are running the 6-protein standard mix:*** Skip to step 3.

   If you are running real sample: Concentrate each Control/Test sample in a vacuum concentrator to an appropriate volume for sample introduction on to a SDS-PAGE system (for example, approximately 15 µL of combined sample for a typical 10-well mini-gel that accommodates a 30-µL total loading volume [sample and 2× SDS-PAGE sample loading buffer].)

   Note: When you concentrate the sample, excess ICAT reagent may precipitate as acetonitrile concentration is reduced. Remove precipitate by centrifuging the sample at minimum speed for 2 to 3 minutes, then pipetting the supernatant into a clean tube for use in step 3.

   Note: For complex sample preparations, you can perform a “scout run” on a mini-gel system to determine protein content and ability to separate targeted proteins. If you are analyzing larger protein loads to uncover low-level proteins that are not revealed with mini-gels, consider using a semi-prep gel system.

3. To the tubes containing the 6-protein standard mix or the combined Control/Test sample, add 2X SDS-PAGE sample buffer in a 1:1 ratio.

4. Place the tubes in a boiling water bath for 10 minutes.

5. Centrifuge for 30 seconds to cool the tubes.

6. Load an appropriate volume of supernatant onto the gel (for example, 30-µL total loading volume [sample and loading buffer] for a 10-well mini-gel).

7. Run the SDS-PAGE gel according to the manufacturer’s recommendations.

Staining and Destaining the Gel

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.

1. Rinse the gel with running Milli-Q® water or equivalent for 2 minutes.
2. Place the gel in a shallow container filled with clean Milli-Q water or equivalent, then soak for 20 minutes with gentle rocking.
3. Repeat step 2 two more times (for a total soaking time of 1 hour).
4. Place the gel in aqueous gel staining solution for about 5 minutes with gentle rocking. Stain for the shortest time that allows visualization of the protein bands.

   IMPORTANT! Do not overstain. Staining and destaining procedures may vary, depending on the type of staining solution you use.
5. As soon as the protein bands are visible, destain the gel:
   a. Rinse the gel with running Milli-Q water or equivalent for 2 minutes.
   b. Place the gel in a shallow container filled with clean Milli-Q water or equivalent, then soak for 20 minutes with gentle rocking.
   c. Repeat step 5b two more times (for a total soaking time of 1 hour).

6.3.5 Excising and Washing the Gel Bands

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

1. For each gel band that you excise, rinse a 1.5-mL Eppendorf tube 2 times with methanol, then 2 times with Milli-Q® water or equivalent.
2. Excise the bands or MW areas of interest from the gel.
3. Cut each excised gel band into small pieces (1 to 1.5 mm × 1 mm).
4. Transfer the gel pieces from each band into the rinsed 1.5-mL Eppendorf tubes.
5. Wash and further destain the gel pieces:
   a. To each tube, add 500 µL of gel washing buffer (50% ACN in 100 mM ammonium bicarbonate [NH_4HCO_3]).
   b. Vortex.
   c. Incubate at room temperature for 15 to 20 minutes.
   d. Pipette to remove, then discard the gel washing buffer.
6. Repeat step 5 one to two more times until the gel pieces are clear.
7. Dehydrate the gel pieces:
   a. Add 100 µL of gel dehydration solution (100% ACN) to each tube.
   b. Incubate at room temperature for 5 minutes or until the gel pieces turn white.
   c. Pipette to remove, then discard the gel dehydration solution.
8. Dry the gel pieces in a vacuum concentrator until completely dry (about 10 minutes).

6.3.6 Performing In-Gel Trypsin Digestion

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 1 mL of 100 mM ammonium bicarbonate [NH_4HCO_3].
2. Vortex to mix, then centrifuge for a few seconds to bring all solution to the bottom of the tube.
3. Add 50 µL of the trypsin solution to each tube containing dehydrated gel pieces.
4. Allow the gel pieces to rehydrate in the trypsin solution for 10 minutes.
5. Check the gel pieces. If any gel pieces are not uniformly clear (if they contain white areas), add 50 µL more of the trypsin solution to the tube until gel pieces are uniformly clear.

   Note: The volume of trypsin solution needed depends on the size and number of gel pieces in a tube.
6. If the gel pieces are not covered with liquid after adding 100 µL of trypsin solution, add a volume of 100 mM ammonium bicarbonate (NH_4HCO_3) to each tube to cover the gel pieces.

   IMPORTANT! Add just enough 100 mM ammonium bicarbonate (NH_4HCO_3) to cover the gel pieces.
7. Vortex gently to mix (avoid breaking the gel), then centrifuge for a few seconds to bring all solution to the bottom of the tube.
8. Incubate 12 to 16 hours at 37 °C.
9. Vortex to mix, then centrifuge for a few seconds to bring all solution to the bottom of the tube.

6.3.7 Extracting Digested Peptides

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.

1. Place the tubes containing the digested gel pieces (tube #1) in a sonic water bath for 20 minutes.
2. Transfer the supernatant from each tube #1 into a clean Eppendorf tube and retain (tube #2).
3. To the original tubes containing the digested gel pieces (tube #1), add 100 µL of the extraction solvent (50% ACN, 0.1% TFA).
4. Vortex to mix.
5. Place the tubes in a sonic water bath for 20 minutes.
6. Again transfer the supernatant from tube #1 to tube #2.
7. Repeat step 3 through step 6 two more times.
8. Place the tubes containing the combined extract for each sample (tube #2) in a vacuum concentrator and evaporate until dry.

6.4 Purifying the Biotinylated Peptides and Cleaving Biotin

This section describes:

- Before you begin
- 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

6.4.1 Before You Begin

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/5 second flows from the outlet connector.

Assembling the Avidin Cartridge System

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 isolates.

1. Assemble the cartridge holder provided in the Applications Development Kit.
2. Assemble the outlet connector: slide the PEEK tubing provided in the Applications Development Kit into a 10-32 compression screw, then finger-tighten the compression screw into the outlet end of the cartridge holder (Figure 5).
3. Connect the needle-port adapter to the inlet end of the cartridge holder (Figure 5).
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.

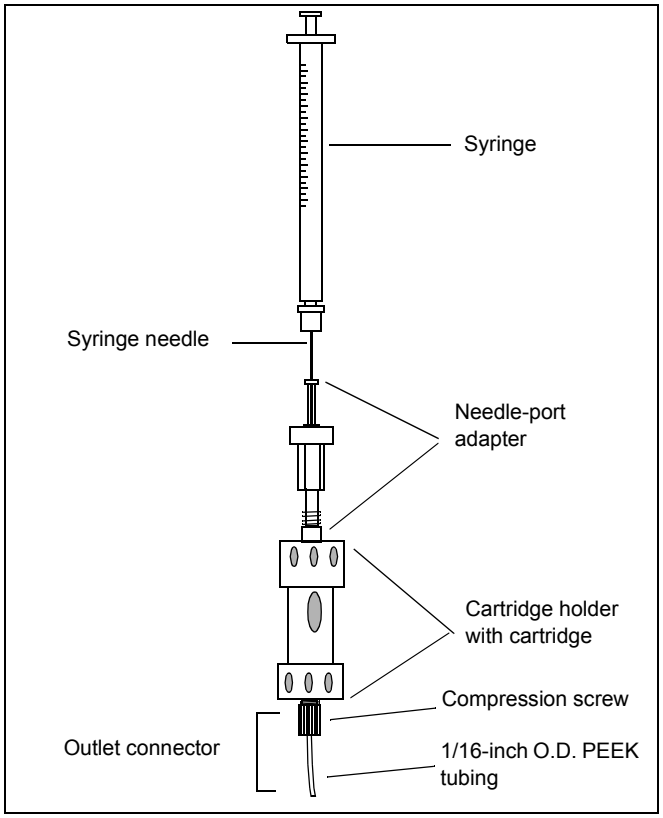


Figure 5 Cartridge Connection

6.4.2 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.

6.4.3 Loading Sample on the Avidin Cartridge

1. To each sample (from step 8 in Section 6.3.7, Extracting Digested Peptides on page 9), add 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. For each sample, label three Eppendorf tubes **#1** (Flow-Through), **#2** (Wash), and **#3** (Elute), then place in a rack.

6. Slowly inject (~1 drop/5 seconds) of the sample 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, analysis of the flow-through does not provide ICAT reagent-labeled peptide quantification information.

6.4.4 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.

6.4.5 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 gel samples, repeat the steps in Section 6.4.2, Activating the Avidin Cartridge, through Section 6.4.5, Eluting ICAT Reagent-Labeled Peptides, for each fraction. (Start with step 3 in Section 6.4.2.)

6.4.6 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.

When you finish eluting peptides from all gel samples as described in Section 6.4.5, Eluting ICAT Reagent-Labeled Peptides:

1. Reverse the direction of the avidin cartridge. Reversing direction before cleaning removes any gel from the inlet frit during cleaning.

2. Clean the cartridge by injecting 2 mL of the Affinity Buffer–Elute. Divert to waste.

3. Inject 2 mL of Affinity Buffer–Storage. Divert to waste.

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.

6.4.7 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. To each sample tube, add 90 µL of the freshly prepared cleaving reagent.

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 minutes).

9. Proceed to:

    - Section 7, Separating and Analyzing the Fractions and Peptides
    - Section 8, Evaluating Results

7 Separating and Analyzing the Fractions and Peptides

This section describes:

- Preparing sample or 6-protein standard mix
- Separating by capillary reversed-phase HPLC
- Analyzing by electrospray
- Analyzing by MALDI

7.1 Preparing Sample or 6-Protein Standard Mix

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).

7.2 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 7.3, Analyzing by Electrospray
- Section 7.5, Analyzing by MALDI

7.3 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 4 on page 13 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 4 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 5 provides suggested QSTAR System acquisition method settings for the acquisition of LC/MS/MS IDA (Information Dependent Acquisition) data.

IMPORTANT! The settings in Table 4 are for Analyst® QS Software with Service Pack 5.

Table 5 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

Table 5 QSTAR® System Acquisition Method Settings *(Continued)*

Parameter	Suggested Setting
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

7.4 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.

7.5 Analyzing by MALDI

This section provides guidelines and suggestions for MALDI analysis of the ICAT reagent-labeled peptides using an Applied Biosystems 4700 Proteomics Analyzer. 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 12.
- 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 6 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 6 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 Heavy-labeled to the Light-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.

8 Evaluating Results

8.1 Evaluating the 6-Protein Standard Mix

Theoretical peptides greater than 450 Da for the following proteins present in the 6-protein standard mix is listed in the corresponding table:

- β-Galactosidase – Table 7
- Apo-serotransferrin – Table 8
- Bovine serum albumin – Table 9
- β-Lactoglobulin – Table 10
- Lysozyme – Table 11
- α-Lactalbumin – Table 12

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 any of the expected peptides for the 6-protein standard mix, analyze the pre-avidin process-monitoring aliquot to determine if the peptides are present.

Table 7 Theoretical ICAT Reagent-Labeled Cysteine Peptides in 6-Protein Standard Mix Trypsin Digest – β-Galactosidase

Sequence	No. of Cys	MH^+	
		Light Reagent	Heavy Reagent
CGTR	1	663.3248	672.3550
LTAACFDR	1	1,123.5570	1,132.5872
YHYQLVWCQK	1	1,594.7840	1,603.8142
QFCMNGLVFADR	1	1,627.7725	1,636.8026
IGLNCQLAQVAER	1	1,641.8746	1,650.9048
AVLEAEVQMCGELR	1	1,774.8832	1,783.9133
IIFDGVNSAFHLWCNGR	1	2,176.0762	2,185.1064
CSHYPNHPLWYTLCDR	2	2,459.1389	2,477.1992
AVVELHTADGTLIEAEACDV GFR	1	2,643.3088	2,652.3390
SVDPSRPVQYEGGGADTT ATDIICPMYAR	1	3,297.5520	3,306.5822
WLSLPGETRPLILCEYAHA MGNSLGGFAK	1	3,358.7080	3,367.7382
LAENLSVTLPAASHAIPHLT TSEMDFCIELGNK	1	3,749.8883	3,758.9184
AAGHYQAEAALLQCTADTL ADAVLITTAHAWQHQGK	1	4,001.9932	4,011.0234
FAWFPAPEAVPESWLECD LPEADTVVVPSNWQMHGY DAPIYTNVTYPITVNPPFVP TENPTGCYSLTFNVDESWL QEGQTR	2	9,627.5234	9,645.5837

Table 8 Theoretical ICAT Reagent-Labeled Cysteine Peptides in 6-Protein Standard Mix Trypsin Digest – Apo-serotransferrin

Sequence	No. of Cys	MH^+	
		Light Reagent	Heavy Reagent
CLK	1	590.3336	599.3637
CLVEK	1	818.4446	827.4747
CQSFR	1	867.4147	876.4448
INHCR	1	869.4416	878.4717
DSSLCK	1	879.4246	888.4547
EACVHK	1	913.4566	922.4867
SCHTGLGR	1	1,057.5213	1,066.5514
SCHTAVGR	1	1,057.5213	1,066.5514
DDTVCLAK	1	1,091.5407	1,100.5708
ASYLDCIR	1	1,167.5832	1,176.6134
WCALSHHER	1	1,365.6486	1,374.6788

Table 8 Theoretical ICAT Reagent-Labeled Cysteine Peptides in 6-Protein Standard Mix Trypsin Digest – Apo-serotransferrin *(Continued)*

Sequence	No. of Cys	MH^+	
		Light Reagent	Heavy Reagent
CDEWSVNSVGK	1	1,450.6637	1,459.6938
WCAVSEHEATK	1	1,487.6953	1,496.7254
DYELLCLDGTR	1	1,524.7368	1,533.7670
SVIPSDGPSVACVK	1	1,585.8260	1,594.8561
CGLVPVLAENYNK	1	1,646.8576	1,655.8878
DQYELLCLDNTR	1	1,709.8169	1,718.8470
FDEFFSEGCAPGSK	1	1,747.7638	1,756.7939
KPVEEYANCHLAR	1	1,756.8805	1,765.9106
DCHLAQVPSHTVVAR	1	1,859.9550	1,868.9852
CSTSSLLEACTFR	2	1,871.8995	1,889.9598
LCMGSGLNLCEPNNK	2	2,046.9775	2,065.0378
IECVSAETTEDCIAK	2	2,065.9786	2,084.0389
EGTCPEAPTDECKPVK	2	2,158.0160	2,176.0763
SDNCEDTPEAGYFAVAVVK	1	2,242.0338	2,251.0639
SAGWNIPIGLLYCDLPEPR	1	2,341.2015	2,350.2316
QQQHLFGSNVTDCSGNFC LFR	2	2,855.3357	2,873.3960
AVANFFSGSCAPCADGTDF PQLCQLCPGCGCSTLNQY FGYSGAFK	6	6,008.7313	6,062.9122

Table 9 Theoretical ICAT Reagent-Labeled Cysteine Peptides in 6-Protein Standard Mix Trypsin Digest – Bovine Serum Albumin

Sequence	No. of Cys	MH^+	
		Light Reagent	Heavy Reagent
DVCK	1	691.3449	700.3750
CASIQK	1	876.4613	885.4915
GACLLPK	1	928.5290	937.5591
LCVLHEK	1	1,068.5876	1,077.6177
CCAADDK	2	1,179.5138	1,197.5741
NECFLSHK	1	1,204.5785	1,213.6086
QNCDQFEK	1	1,238.5476	1,247.5777
SHCIAEVEK	1	1,242.6152	1,251.6454
EACFAVEGPK	1	1,277.6200	1,286.6501
CCTESLVNR	2	1,478.7096	1,496.7699
CCTKPESER	2	1,506.7045	1,524.7648
SLHTLFGDELCK	1	1,589.7998	1,598.8299

Table 9 Theoretical ICAT Reagent-Labeled Cysteine Peptides in 6-Protein Standard Mix Trypsin Digest – Bovine Serum Albumin *(Continued)*

Sequence	No. of Cys	MH^+	
		Light Reagent	Heavy Reagent
YICDNQDTISSK	1	1,613.7481	1,622.7783
ECCDKPLLEK	2	1,631.8137	1,649.8740
DDPHACYSTVFDK	1	1,724.7590	1,733.7892
LKPDPNTLCDEFK	1	1,746.8736	1,755.9038
TCVADESHAGCEK	2	1,803.8006	1,821.8609
ETYGDMADCCEK	2	1,818.7348	1,836.7951
EYEATLEECCAK	2	1,842.8254	1,860.8857
MPCTEDYLSLILNR	1	1,894.9407	1,903.9708
RPCFSALTPDETYVPK	1	2,051.0272	2,060.0573
LFTFHADICTLPDTEK	1	2,078.0269	2,087.0570
YNGVFQECCQAEDK	2	2,087.9167	2,105.9770
ECCHGDLLECADDR	3	2,259.9797	2,287.0701
GLVLIAFSQYLQQCPFDEHVK	1	2,662.3703	2,671.4005

Table 10 Theoretical ICAT Reagent-Labeled Cysteine Peptides in 6-Protein Standard Mix Trypsin Digest – β-Lactoglobulin

Sequence	No. of Cys	MH^+	
		Light Reagent	Heavy Reagent
WENGECAQK	1	1,291.5741	1,300.6043
LSFNPTQLEEQCHI	1	1,885.9118	1,894.9420
YLLFCMENSAEPEQSLACQCLVR	3	3,328.5838	3,355.6743

Table 11 Theoretical ICAT Reagent-Labeled Cysteine Peptides in 6-Protein Standard Mix Trypsin Digest – Lysozyme

Sequence	No. of Cys	MH^+	
		Light Reagent	Heavy Reagent
CK	1	477.2495	486.2797
GCR	1	562.2771	571.3073
CELAAAMK	1	1,063.5280	1,072.5582
WWCNDGR	1	1,163.5056	1,172.5358
GYSLGNWVCAAK	1	1,495.7368	1,504.7669
NLCNIPCSALLSSDITASVNCAK	3	3,018.5062	3,045.5967

Table 12 Theoretical ICAT Reagent-Labeled Cysteine Peptides in 6-Protein Standard Mix Trypsin Digest – α-Lactalbumin

Sequence	No. of Cys	MH^+	
		Light Reagent	Heavy Reagent
IWCK	1	776.4129	785.4431
ALCSEK	1	877.4453	886.4755
CEVFR	1	880.4351	889.4652
LDQWLCEK	1	1,261.6251	1,270.6552
FLDDDLTDDIMCVK	1	1,869.8614	1,878.8916
DDQNPHSSNICNISCDK	2	2,344.0298	2,362.0901
GYGGVSLPEWVCTTFHTSGYDTQAIVQNNDSTEYGLFQINNK	1	4,881.2743	4,890.3044

8.2 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.

8.3 ICAT Reagent Fragments Using MS/MS Analysis

MS/MS analysis of ICAT reagent-labeled peptides yields ICAT reagent-specific fragment ions. Table 13 lists the possible fragment masses of Light and Heavy ICAT reagents bound to a cysteine residue in a peptide.

Table 13 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

9 Technical Support

Applied Biosystems is committed to meeting the needs of your research through enabling technologies such as the 1-D PAGE Cleavable ICAT® Reagent Applications Development Kit . Our dedicated support staff is available to answer questions about using this product to its fullest potential.

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- By telephone: Dial **1**.**800**.**899**.**5858**
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10 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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