Cleavable ICAT® Reagent Kit for Protein Labeling

(Monoplex Version)

Applied Biosystems

Quick Reference

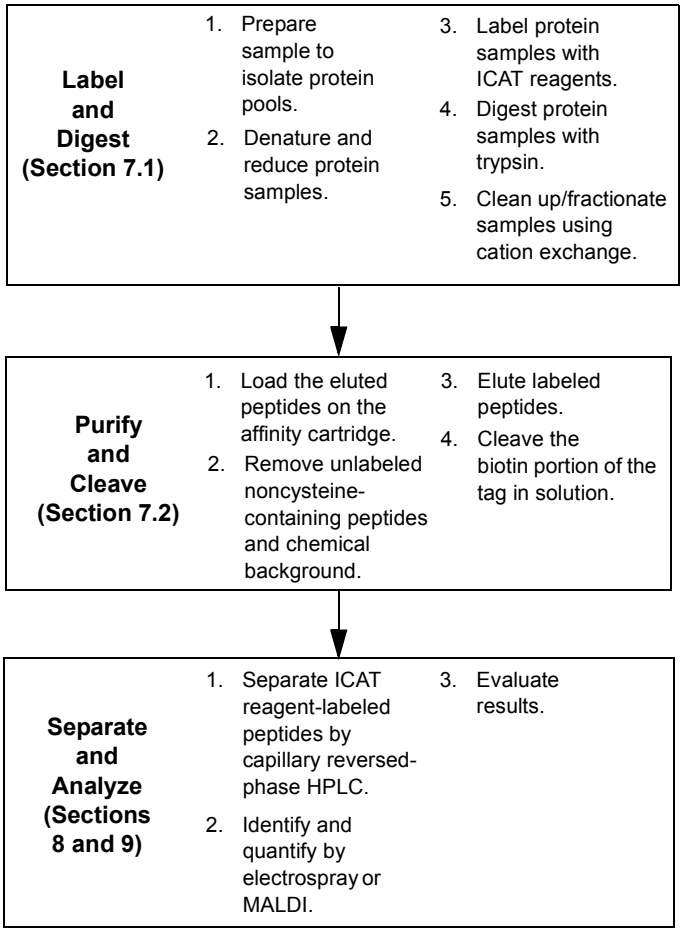
This Quick Reference provides abbreviated procedures you can refer to when you use the Cleavable ICAT® Reagent Kit for Protein Labeling (Monoplex Version). For general chemical safety information, background information, and more detailed procedures, refer to the protocol provided with the kit.

Note: Use this quick reference only after you perform the experiment at least one time using the complete protocol.

Chemical Safety

WARNING **CHEMICAL HAZARD.** Before handling any chemicals, refer to the Material Safety Data Sheet (MSDS) provided by the manufacturer, and observe all relevant precautions.

Protocol Overview



1 Testing the Protocol

Applied Biosystems strongly recommends that, before running samples for the first time, you test the protocol with the following:

- Laminin Peptide Standard supplied in this kit.
- Known protein that contains multiple cysteines (for example, 25 to 50 µg of bovine serum albumin or 100 µg of bovine lactalbumin).
- Control sample, if you have sufficient sample, to verify that your sample preparation protocol does not interfere with labeling and digestion.

Refer to the *Cleavable ICAT Kit for Protein Labeling Protocol,* Section 6, Testing the Protocol.

2 Labeling with Cleavable ICAT Reagents and Digesting with Trypsin

This section describes:

- Denaturing and reducing the proteins
- Labeling with the Cleavable ICAT Reagents
- Digesting with trypsin
- Preparing the cation-exchange cartridge
- Loading sample on the cation-exchange cartridge
- Cleaning and storing the cation-exchange cartridge

2.1 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. Prepare sample as described in *Cleavable ICAT Kit for Protein Labeling Protocol,* Section 7.1.1, Preparing Sample.
2. 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.
3. 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.
4. Add 2 µL of the Reducing Reagent to both the Control and Test sample tubes.
5. 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.
6. Place the Control and Test samples in a boiling water bath for 10 minutes.

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

8. Remove an optional 1-µL process-monitoring aliquot from each vial, and label as “unlabeled”. For more information, see the *Cleavable ICAT Kit for Protein Labeling Protocol,* Section 5, Monitoring the Process.

2.2 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 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 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 an optional 1-µL process-monitoring aliquot from each vial, and label as “labeled”. For more information, see the *Cleavable ICAT Kit for Protein Labeling Protocol,* Section 5, Monitoring the Process.

2.3 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.

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 the *Cleavable ICAT Kit for Protein Labeling Protocol,* Section 5, Monitoring the Process.

2.4 Preparing the Cation-Exchange Cartridge

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.
3. Connect the needle-port adapter to the inlet end of the cartridge holder.
4. Mark the inlet and outlet ends of the cartridge (or mark with a directional arrow) for future use. Use the same flow direction 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.

2.5 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 (containing 200 µg total protein) a tube with a capacity greater than 4 mL.

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 original sample tube.

   (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.

2.6 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 2.5 for each sample.
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.

3 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.

3.1 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.

3.2 Loading Sample on the Avidin Cartridge

1. Neutralize each cation-exchange fraction (from step 8 in Section 2.5, Loading Sample on the Cation-Exchange Cartridge) 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 the *Cleavable ICAT Kit for Protein Labeling Protocol,* 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/second) the neutralized fraction onto the avidin cartridge and collect the flow-through into tube #1 (Flow-Through).

3.3 Removing Non-Labeled Material

WARNING **CHEMICAL HAZARD. Affinity Buffer–Wash 2** contains **methanol**, a flammable liquid and vapor. Exposure may cause eye, skin, and respiratory tract irritation, central nervous system depression, and blindness. 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.

   (Keep tube #1 until you confirm that loading on the avidin cartridge is successful. If loading fails, you can repeat loading using tube #1 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. Divert the remaining 500 µL to waste.
4. Inject 1 mL of Milli-Q® water or equivalent. Divert to waste.

3.4 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/second) 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 after eluting from the avidin cartridge, and label as “post-avidin”. For more information, see the *Cleavable ICAT Kit for Protein Labeling Protocol,* Section 5, Monitoring the Process.

6. If you have additional cation-exchange fractions, repeat the steps in Section 3.1, Activating the Avidin Cartridge, through Section 3.4, Eluting ICAT Reagent-Labeled Peptides, for each fraction. (Start with step 3 in Section 3.1.)

3.5 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 3.4, 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.

3.6 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. Work in a fume hood.

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).

4 Separating and Analyzing

For information on MALDI and electrospray analysis, refer to the *Cleavable ICAT Kit for Protein Labeling Protocol,* Section 8, Separating and Analyzing the Fractions and Peptides, and Section 9, Evaluating Results.

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