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

Quick Reference

This Quick Reference provides abbreviated procedures you can refer to when you use the 1-D PAGE Cleavable ICAT® Reagent Applications Development Kit for Target Protein ID and Quantitation. 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.

1 Testing the Protocol

It is strongly recommended that, before running samples for the first time, you test the protocol with the 6-protein standard mix provided in the kit.

Refer to the *1-D PAGE Cleavable ICAT Reagent Applications Development Kit 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
- Performing the SDS-PAGE separation
- Excising and washing the gel bands
- Performing in-gel trypsin digestion
- Extracting digested peptides

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 the *1-D PAGE Cleavable ICAT Reagent Applications Development Kit Protocol,* Section 6.3.1, 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 a concentrated in Denaturing Buffer – Add Denaturing Buffer to bring the volume up to 80 µL.
3. If your Test sample is a precipitated pellet containing 100 µg of the Test sample – Add 80 µL of the Denaturing Buffer.

   If your sample is a 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 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 Control and Test tubes in a boiling water bath for 10 minutes.
7. Vortex to mix, then centrifuge the Control and Test tubes 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 *1-D PAGE Cleavable ICAT Reagent Applications Development Kit 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 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 the *1-D PAGE Cleavable ICAT Reagent Applications Development Kit Protocol,* Section 5, Monitoring the Process.

2.2.1 Performing the SDS-PAGE Separation

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

This section describes:

- Running the SDS-PAGE gel
- Staining and destaining the gel

For protein loading considerations, see the *1-D PAGE Cleavable ICAT Reagent Applications Development Kit Protocol.*

Running the SDS-PAGE Gel

1. Combine Control and Test samples into a single tube (tube now contains 200 µg total protein).
2. Concentrate each Control/Test sample in a vacuum concentrator to an appropriate volume for sample introduction onto an 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 for 2 to 3 minutes, then pipetting the supernatant into a clean tube for use in step 3.
3. To the tube containing the combined Control/Test sample, add 2× 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).

2.2.2 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 a vacuum concentrator for 10 minutes.

2.2.3 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), continue to 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 the 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.

2.2.4 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 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.

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

For information on making injections and assembling the cartridge, see the *1-D PAGE Cleavable ICAT Reagent Applications Development Kit Protocol*.

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.

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. To each sample (from step 8 in Section 2.2.4, Extracting Digested Peptides), 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 before loading on the avidin cartridge and label as “pre-avidin”. For more information, see the *1-D PAGE Cleavable ICAT Reagent Applications Development Kit Protocol,* 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).

3.3 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.

   (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/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 after eluting from the avidin cartridge, and label as “post-avidin”. For more information, see the *1-D PAGE Cleavable ICAT Reagent Applications Development Kit Protocol,* Section 5, Monitoring the Process.
6. If you have additional gel samples, 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.

When you finish eluting peptides from all gel samples as described in Section 3.4, 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.

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.

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 tubes 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 *1-D PAGE Cleavable ICAT Reagent Applications Development Kit Protocol,* Section 7, Separating and Analyzing the Fractions and Peptides, and Section 8, Evaluating Results.

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