

ICAT labeling protocol

Note: Handle samples on ice unless otherwise indicated.

1. **Solubilize samples in fresh labeling buffer:**
 - 0.05% SDS
 - 10-200 mM Tris pH 8.3*
 - 5 mM EDTA,
 - 6 M Urea.

* The Tris pH 8.3 concentration is given as a range. Ultimately, you will want the sample solution pH to remain ~ 8.3 during the reduction and labeling steps. Your samples' history (esp. regarding sample solution ingredients) should dictate what buffer capacity you should ideally use.

If solubilizing from a pellet:

- a) Add labeling solution to pellet and agitate pellet into solution.
- b) If pellet does not fully solubilize upon agitation then water bath sonicate sample until pellet is in solution. Make sure to keep water bath cool while sonicating.

If sample is previously in solution:

- a) If possible, add labeling buffer components until sample solution = labeling buffer.
- b) If sample solution is incompatible with labeling buffer ingredients (e.g. sample solution contains a relatively high concentration of SDS and you do not want to dilute your sample to achieve 0.05% SDS) then precipitate your sample and resolubilize resulting pellet with labeling buffer.

Example method of precipitation:

- i) Add 6.5 volumes of -20°C acetone to one volume of sample solution.
- ii) Mix acetone and sample and let sit on ice for 5 min.
- iii) Spin precipitated sample down at 14,000 rpm for 10 min. at 4°C .

2. **Quantify proteins in sample solutions if you have not already done so.**

Recommended: Use Bio-Rad (Bradford) Assay with titrated Bovine IgG as standard curve.

Note: Complex mixtures of proteins have so far been successfully labeled at total protein concentrations as high as 4 mg/ml.

Note: You should ideally label samples-to-be-compared under identical conditions (i.e. similar protein conc., sample volume, etc.).

3. Reduce proteins.

Add Tributylphosphine (TBP; Aldrich cat. # 24,704-9) to a 5 mM final concentration* in your sample solutions.

* Ultimately you will want the TBP concentration to be greater than your estimated free sulfhydryl concentration (see step 4 for estimation) to ensure complete reduction of all disulfide bridges. And so you may need to use a concentration of TBP higher than 5 mM. However, the higher the concentration of TBP, the greater the likelihood of TBP cross-reacting with the ICAT reagents.

Note: Alternative reducing reagents are b) Tris(2-carboxyethyl) phosphine (TCEP) and b) dithiothreitol (DTT).

TCEP is water soluble and less toxic than TBP. It is therefore more user-friendly than TBP. However, it is acidic and so your sample solution will require careful buffering to retain an ideal pH of ~ 8.3. TCEP may also be more prone to react with the iodoacetamide group of ICAT reagents than TBP. To counter these two potential problems you should use TCEP at a relatively low concentration (1 – 5 mM recommended) and check the pH of your solution, adjusting to ~8.3 if necessary. (5mM recommended)

DTT is also water soluble and less toxic than TBP. It contains free sulfhydryl groups which will readily react with ICAT reagent. Despite this hindrance, it can be used as a reducing reagent in ICAT experiments following this procedure:

- a) Reduce with DTT at 5-100 mM for 10-30 min. at 37°C.
- b) Precipitate proteins via example method listed in step 1.
- c) Resolubilize pellets in labeling buffer + 5 mM TBP.
- d) Continue on to labeling step.

Reducing with DTT will enable less cross-reactions of TBP with the ICAT reagent although it will mean some sample loss due to the precipitation step.

4. Add ICAT reagent.

A) First estimate molar amount of cysteines in samples. To do this:

- a) Assume an average MW of proteins in your sample. Having some proteomic data upon the species you are working on may bolster this assumption.
- b) Use your protein assay and the average MW of proteins to determine the molar amount of proteins in your samples.

e.g. 0.5 mg protein from Bio-Rad assay.
 $0.5 \times 10^{-3} \text{ g} / 50,000 \text{ g/mol} = 10 \times 10^{-9} \text{ mol protein}$

- c) Assume an average # of cysteines per protein and calculate molar amount of cysteines.

e.g. 6 cysteines per protein
There are therefore $60 \times 10^{-9} \text{ mol cysteine}$

B) Next estimate how much ICAT reagent you will need to use to achieve a final concentration of **1.2 mM**. **This is the recommended minimal concentration at which you should label complex mixtures and be assured constitutive labeling.**

e.g. Per 100 μg tube of ICAT there are 175 nmol of ICAT reagent. And so to achieve a 1.2 mM ICAT reagent concentration in 500 μl you will need to use 600 nmol or 3.43 tubes of ICAT reagent (or, for simplicity's sake, use 4 tubes, which will give you a concentration of ICAT slightly higher than 1.2 mM).

C) Next make sure that you have a molar excess of ICAT reagent over free sulfhydryls at a 1.2 mM concentration of ICAT reagent.

e.g. For 0.5 mg in 500 μl of labeling solution you will have $60 \times 10^{-9} \text{ mol cysteine}$ and use $600 \times 10^{-9} \text{ mol ICAT reagent}$ (see above examples). This constitutes a 10-fold molar excess of reagent over free sulfhydryls.

D) If a 1.2 mM concentration of ICAT reagent in your sample volume will not give you a molar excess of reagent over free sulfhydryls you should add sufficient ICAT to achieve at least a 2-fold molar excess of reagent over free sulfhydryls in your sample volume.

E) Add your reagent to your samples, and agitate your sample in the dark, at 37C temperature, for 2 hours.

5. **Quench the reaction with a 10-fold molar excess of DTT over reagent.**
Agitate sample and let sample sit at room temperature for 5 minutes after adding DTT.

e.g. add DTT to 12 mM final concentration if your ICAT reagent concentration = 1.2 mM.

6. **Combine L and H labeled samples and dilute combined sample down with water to a final Urea concentration of 1 M.**
7. **Add trypsin 1:100 to 1:50 w/w (trypsin/sample) and let sample digest overnight at 37°C.**

Recommended: Promega sequencing grade trypsin (catalogue # V511A).

8. **Check that digestion is complete** (can check via gel electrophoresis and silver staining and/or MS).
9. **Proceed to cation exchange separation and/or cleanup, followed by avidin purification.**
10. Dry fractions down in speedvac
11. Add 95uL cleaving reagent A and 5uL cleaving reagent B to each avidin purified fraction, vortex, spin down and incubate at 37C for 2 hours.

Note: Cleaving reagent A is 95%TFA, use in hood with glass syringe/vials

12. After 2 hour incubation, dry samples down again in speedvac
13. Add 8ul of 0.2-0.4% acetic acid to each fraction, vortex, and spin down at 14,000rpm for 2 minutes. Remove and transfer supernatant to an autosampler vial for each fraction for MS analysis.

Note: Applied Biosystems (AB) offers all reagents, accessories, and relevant protocols necessary to perform an ICAT experiment. The protocol written above is slightly different from that recommended by AB. As yet we have insufficient data to concur whether our in-house protocol is more or less successful than that offered by AB. Information on AB's ICAT reagents etc. can be found at:
<http://www.appliedbiosystems.com/products/productdetail.cfm?ID=153>

For cation exchange clean up and separation we recommend using Polysulfoethyl Aspartamide columns (PolysulfoethylA™) offered by PolyLC, Inc (PolyLC@aol.com)

and developing column with a salt (KCl) gradient as recommended by PolyLC, while collecting fractions at a suitable interval (1 min. recommended).

For avidin purification we recommend using the AB avidin purification kit and accompanying protocol.

For MS analysis we separate our avidin-purified samples by a reverse-phase system in front of and in line with MS/MS (using LCQ-Deca machine). Resulting MS/MS data is then searched against appropriate database(s) using Sequest™.

Reverse phase components:

Stationary phase: Magic C18, MichromBioresources, Auburn, CA

Column: 100 um I.D. x 10 cm long

Running buffer (A): 0.005% Heptafluorobutyric acid, 0.4% Acetic acid, in water.

Eluting buffer (B): 0.4% Acetic acid in Acetonitrile.

Gradient:

- 0-2 min: 2% B (constant)
- 2-10 min: 2%-10% B (gradient)
- 10-65 min: 10%-40% B (gradient)
- 65-66 min: 40%-80% B (gradient)
- 66-76 min: 80% B (constant)
- 76-77 min: 80%-2% (re-equilibration)
- 77-98 min: 2% B (re-equilibration)