Background
The ultimate goal of proteomics is
• to identify and quantify proteins that are relevant to a given biological state; and
• to unearth networks of interactions in an effort to understand that biological state at the molecular level.

A great deal of interest has been generated in the use of isotope coded affinity tags for the simultaneous quantification and identification of proteins in a complex sample. In this approach, protein samples from two states are labeled separately with heavy and light ICAT™ reagents (cysteine-specific reagents), combined, digested and then analyzed by mass spectrometry (MS). Specific isolation of ICAT reagent labeled peptides significantly reduces the complexity of peptide mixtures and increases the number of sequences that are identified in a single MS/MS experiment.

Cleavable ICAT Reagents
Cleavable ICAT reagents incorporate a number of key technological advances that significantly improve protein-expression analysis. Incorporation of an acid-cleavable linker into the ICAT molecule allows removal of the biotin affinity tag before MS and MS/MS analysis. This improves MS/MS performance and significantly increases the number of proteins identified and quantified with higher confidence scores in a single experiment. Incorporation of $^{13}$C rather than deuterium into the ICAT heavy reagent molecule promotes co-elution of the heavy and light isotopes in reversed phase chromatography, thereby increasing accuracy of quantification measurements by LC-ESI-MS, LC-MALDI-TOF-MS, and MALDI-TOF-MS. These significant improvements in the reagent design, along with the availability of ICAT software for data analysis, enable researchers to fully exploit the potential of this technology in protein-expression profiling studies.

Key Features/Benefits
Cleavable ICAT Reagents:
• Incorporates $^{13}$C rather than deuterium into the heavy reagent, which results in the co-elution of ICAT reagent labeled pairs from the reversed-phase column, which improves peptide quantification measurements.
• Incorporates an acid-cleavable site in the reagent, which lets you remove the biotin portion of the ICAT reagent tag prior to MS and MS/MS analysis.
• Reduces the overall size of the tag, enabling the analysis of larger peptides.
• Reduces tag fragmentation, which improves quality of MS/MS data and significantly increases the number of identified proteins, with high confidence per experiment.
• Improves reagent design, which reduces formation of ICAT/TCEP reaction products, byproducts of the alkylation reaction which improves overall MS data quality.
• Changes the mass difference between the heavy and light reagents from 8 to 9 Daltons, which avoids possible confusion between oxidized methionine and two cysteines labeled with ICAT reagents in LC-ESI-MS workflows.
• Lets you use Pro ICAT software for the QSTAR® System and GPS Explorer™ software for the 4700 Proteomics Analyzer which automates protein identification and quantitation.

Cleavable ICAT Reagent Structure
The ICAT cleavable reagent is composed of four main segments (Fig. 1):
1. A protein reactive group (Iodoacetamide): The reactive group covalently links the isotope-coded affinity tag to the protein by alkylation of free cysteines.
2. An affinity tag (biotin): This simplifies the analysis of the ICAT reagent-labeled peptides by selecting and concentrating the cysteine-containing peptides, thereby reducing the complexity of the peptide mixture.

3. An isotopically labeled linker (C\textsubscript{10}H\textsubscript{17}N\textsubscript{3}O\textsubscript{3}): The linker chain can substitute up to nine \textsuperscript{13}C atoms. Chemically the light and heavy molecules are identical—they merely differ in mass. The heavy reagent with nine \textsuperscript{13}C atoms is nine Daltons heavier than the light reagent. Mass spectrometric comparison of peptides labeled with heavy and light reagents provides a ratio of the concentration of the proteins in the original sample.

4. An acid cleavage site: Following avidin elution step, the ICAT-reagent-labeled peptides are removed from the affinity column. Cleave the biotin portion of the tag from the labeled peptides.

**ICAT Reagent Protocol**

The cleavable ICAT reagent protocol is detailed in Fig. 2. Proteins isolated from a control sample are treated with the light reagent, while proteins from the test sample are treated with the heavy reagent. The samples are mixed and the protein pool digested with trypsin. Following tryptic digestion of the pooled proteins, the peptides are separated from the byproducts of the labeling and digestion reactions on cation exchange chromatography. The ICAT-reagent-labeled peptides are then separated from the other peptides by avidin affinity chromatography.

Following the avidin elution step, the ICAT-reagent-labeled peptides are evaporated to dryness and reconstituted in concentrated trifluoroacetic acid (TFA) to cleave the biotin portion of the tag from the labeled peptides. The reaction mix is kept at 37°C for two hours and is followed by a second evaporation step to remove the acid. The peptides are then placed in an autosampler for reversed-phase capillary LC/MS/MS analysis.

### Protocol Validation

#### Efficiency of Labeling and Reduction in Adduct Formation

To determine efficiency of labeling, bovine serum albumin (BSA) and a six-protein mix were labeled with the cleavable ICAT reagent-light. The alkylation reaction was allowed to proceed for two hours at 37°C before an aliquot of the heavy reagent was added. No peptides with the heavy label were detected; therefore the labeling with the light reagent was complete even on peptides with multiple cysteines (Fig. 3).

### ICAT/TCEP Reaction Product

Besides its reaction with cysteine-containing peptides, cleavable ICAT reagent also couples to residual TCEP reducing agent. This side reaction product and excess ICAT reagent are removed from peptide mixtures by cation exchange chromatography.

HPLC analysis of a laminin/ICAT reagent reaction mixture and other complex samples demonstrated the efficient removal of these byproducts on the cation exchange cartridge. (Fig. 4).

### Efficiency of the Acid Cleavage Process

The incorporation of an acid cleavage step in the protocol reduces the overall mass of the tag on the labeled peptides, and improves sequence information content of MS-MS spectra. A six-protein mix was used to evaluate the completeness of the acid cleavage step. The avidin-eluted...
peptides from the six-protein mix were subjected to the cleavage conditions—2 hrs at 37°C with concentrated TFA—which removed the biotin portion of the tag from the labeled peptides. MS analysis showed that after acid cleavage, all of the peptides decreased in molecular weight by the mass of the leaving group and the number of modified residues. The signals at the original masses before cleavage were undetectable.

Elution of Cleaved Portion of Tag on cLC Reversed-Phase Chromatography

Because the acid cleavage reaction is performed in-solution, it is important to know where the biotin portion of the tag (cleavage product) elutes on a reversed-phase chromatography column. Ideally, the cleaved portion of the tag should elute early or late in the reversed phase LC gradient. We labeled a sample of BSA using the cleavable reagents and protocol. Following cleavage, we analyzed the digest by LC/MS/MS. The cleaved portion of the ICAT reagent eluted late in the reversed-phase gradient and did not complicate the elution profile of the labeled peptides (Fig. 5).

With more complex hydrophobic samples, peptides may co-elute with the biotin portion of the molecule. Since the latter is singly charged, this does not interfere with automatic collection of peptide MS/MS spectra by LC-ESI-MS/MS, since peptides eluting late in the LC gradient tend to be larger and thereby multiply charged. Additionally, singly charged precursor ions need not be selected in an information-dependent acquisition (IDA) experiment. Likewise, in the LC-MALDI-TOF-MS workflow, the mass of the biotin molecule is below the mass ranges that are typically used for peptide mapping and sequencing studies.

Co-elution of Isotopes

One of the main limitations of the original ICAT reagents was the partial separation of the heavy- and light-tagged peptides on reversed-phase chromatography. This separation effect compromised the quantification of ICAT-reagent-labeled pairs. The cleavable ICAT reagents address this limitation by incorporating $^{13}$C instead of deuterium in the heavy reagent, thus eliminating isotope separation that occurs in reversed-phase chromatography.

We demonstrated co-elution of the isotopes with the cleavable reagents by labeling BSA with both the original and new cleavable ICAT reagents. An elution profile using the original ICAT reagents

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**Figure 4. Removal of ICAT/TCEP reaction products on cation exchange chromatography. Byproducts are in the CEX flow through fraction.**

**Figure 5. Elution profile of biotin fragment on reversed phase LC.**
reagents (Fig. 6a) clearly shows the separation of light and heavy isotopes. The same peptide labeled with cleavable ICAT reagents demonstrates the co-elution of the isotopes (Fig. 6b). We observed this throughout the LCMS run for every peptide we studied (data not shown).

Co-elution of the isotopes is critical in LC-MS MALDI analysis, because separation of the resolved pairs across different wells of a sample plate can lead to quantification errors. An elution profile of an ICAT reagent pair labeled with the cleavable reagents and analyzed via LC-MS MALDI is shown in Fig. 7. You can see that the heavy/light ratios remain constant across all wells.

**Sequence Coverage**

An important attribute of these cleavable reagents is overall improvement in sequence coverage. To demonstrate this, we labeled BSA with both the original and new cleavable ICAT reagents and analyzed it by MS/MS on an API QSTAR® Pulsar System. With the original ICAT reagents, we observed 46% coverage of “b” and “y” ions. With the cleavable reagents, the coverage increased to 83%. The improvement in sequence coverage was even more dramatic when we analyzed peptides with multiple cysteines or peptides with a cysteine in the middle of the sequence (Fig. 8). The “b” and “y” ion coverage increased from 13% to 69% with the cleavable reagents.

Overall improvements in sequence coverage can be directly attributed to a substantial reduction in tag fragmentation during MS-MS. Fragmentation resulting from the new ICAT tag is dramatically reduced to <5% of the ion current. However, this minimal fragmentation is enough to produce several diagnostic ions, which can be used to confirm an ICAT-reagent-labeled peptide.
Complex Sample Analysis

To demonstrate the improved performance of the cleavable ICAT reagents, we analyzed a much more challenging sample—the proteome of wild type and ΔUPF1 mutant yeast strains.

In yeast, the products of the UPF1 gene have been implicated in the nonsense-mediated mRNA decay (NMD) pathway. Mutations to these genes stabilize mRNAs containing early nonsense codons without affecting the decay rates of most other mRNAs. Nonsense-mediated mRNA decay (NMD) is a mechanism by which aberrant transcripts containing premature termination codons are recognized and efficiently degraded.

A comparison of five cation exchange fractions processed through the ICAT reagent workflow is shown in Table 1. It illustrates the improved protein identification with the cleavable ICAT reagents. Because the quality of the MS/MS data is much higher, approximately five times more proteins were identified over a 99% confidence threshold (as set by the Pro ICAT software) than with the original ICAT reagents (more proteins could be identified for the old reagent if the confidence threshold was lowered).

Software for Data Analysis

Software supporting the cleavable reagents is now available for both the Applied Biosystems/MDS SCIEX API QSTAR® Pulsar System (Pro ICAT Software) and Applied Biosystems 4700 Proteomics Analyzer (GPS Explorer™ Software).

Conclusions

The development of a next-generation ICAT reagent based on cleavable linker technology is a significant advance in expression-profiling studies. Incorporation of an acid-cleavable linker into the ICAT molecule lets you remove the biotin affinity tag before MS and MS/MS analysis. This results in improved sequence information in collected MS/MS spectra, increasing the number of proteins identified and quantified with higher confidence scores in a single experiment.

Incorporation of 13C rather than deuterium into the heavy ICAT reagent molecule enables co-elution of the heavy and light isotopes, providing increased accuracy of quantification measurements. Evaluation of these new reagents with a yeast model system generated a greater number of significant protein identifications with improved ICAT reagent ratio statistics compared to the original ICAT reagent.

These significant improvements in the quality of protein IDs and quantification, along with the complementary ICAT software for data analysis, lets you fully exploit the potential of this technology in your protein expression profiling studies.

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<th>Fraction Number</th>
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<td>12</td>
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<tr>
<td>2</td>
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<td>5</td>
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<tr>
<td>Total # of Unique Proteins</td>
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<td>85</td>
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Table 1. Comparison of cleavable ICAT and original ICAT reagents on a complex yeast sample.

Figure 8. Sequence coverage for BSA peptides using original ICAT (left panel) versus Cleavable ICAT (right panel) reagents.
Ordering Information
To place an order from the U.S. or Canada, dial 1.800.327.3002, then follow the voice instructions.

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<th>Description</th>
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<tr>
<td>Contains cleavable ICAT reagents, affinity and cation-exchange buffers and cartridges.</td>
<td></td>
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<tr>
<td>Cleavable ICAT Reagent 10-Assay Kit</td>
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<tr>
<td>Contains cleavable ICAT reagents and affinity and cation-exchange buffers and cartridges.</td>
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<tr>
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*Larger bulk quantities of ICAT Reagents Light and Heavy are available on request. Each unit of reagent labels 100 µg of protein.

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