New workflows for protein expression analysis

Applied Biosystems

ICAT™ Reagent Technology

Breakthrough technology for protein expression analysis

In protein-expression analysis studies, ICAT™ reagent technology provides more complete protein ID and quantification data than is possible with 2-D gels. Now you can increase proteome coverage, identify and quantify the proteins that matter, and accelerate your discovery efforts.

The key to understanding proteins and their behavior in biological systems lies in dramatically improving the analysis of the proteome. Researchers need better ways to separate, quantify, and identify proteins in order to speed the development of therapeutics. That means breakthroughs in throughput, automation, and in-depth proteome coverage.

Applied Biosystems offers researchers just such a breakthrough: isotope-coded affinity tag technology. Developed by Dr. Ruedi Aebersold of the Institute for Systems Biology and Dr. Michael Gelb of the University of Washington, ICAT™ reagent technology is a powerful mass-spectrometry-based method for separating and analyzing complex samples to identify component proteins and determine relative expression levels. Complex protein samples from both normal and diseased sources can be labeled separately and then combined, purified, and analyzed by mass spectrometry. This technology helps researchers compare proteins expressed under different conditions, measure changes at the individual protein level, and identify the proteins present. Such information can help discover targets for therapeutic intervention or markers for diagnostic or toxicity studies.

Improved Quantitation of a Wider Range of Proteins

Researchers typically use 2-D gels to separate proteins in a cell or tissue sample before analysis. Coomassie staining, silver staining, or fluorescent labeling enables visualization of the proteins and provides some quantitative information. Protein spots can be excised, digested with trypsin, and the resulting peptide fragments analyzed by mass spectrometry. Database searching typically identifies each protein. This 2-D gel method is labor intensive, time consuming, and expensive to automate. It has difficulty identifying certain classes of proteins including membrane, acidic, basic, and low-abundance proteins. In addition quantitative information from the 2-D gel technique is not always reliable. The ICAT reagent method was developed to address these limitations.

ICAT Reagent Technology Benefits

- *Identifies and quantifies important membrane and low-abundance proteins.*
- *Allows relative protein quantitation as well as identification using a mass spectrometer.*
- *Reduces sample complexity by selecting for cysteine-containing peptides, leading to a deeper analysis of a proteome.*
- *Provides automation of chromatography steps on Vision™ Workstation and of protein identification and quantitation using Pro ICAT software on API QSTAR™Pulsar Hybrid LC/MS/MS System.*

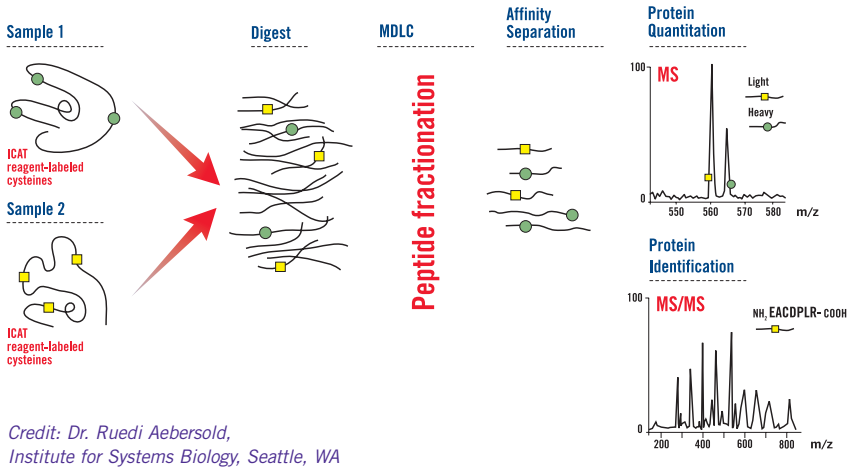
- ***Quantitative Protein Expression***
- ***Identification of Key Proteins***
- ***Automated Workflows***

How ICAT™ Reagents Work

The ICAT reagent, which labels protein samples, comprises three functional elements: 1) a protein reactive group that is specific for cysteine residues; 2) a linker that may be isotopically labeled; and 3) an affinity tag, biotin, that enables selective isolation of the ICAT reagent-labeled peptides.

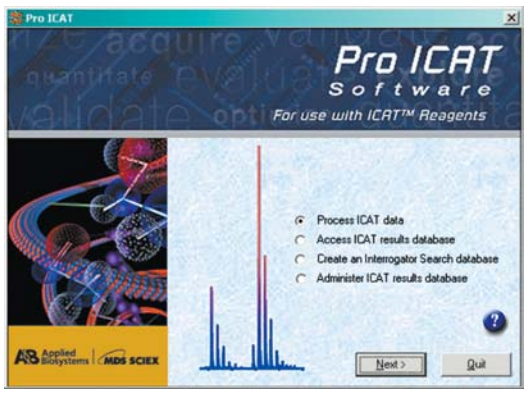
In this approach proteins are first covalently tagged with the ICAT™ reagents, followed by proteolysis of the combined-labeled protein samples. Depending upon sample complexity, peptides can be separated into 30 fractions on a high-resolution cation exchange column. The resulting fractions are then purified on an avidin affinity media to isolate the ICAT reagent-labeled peptides. In turn, each fraction is analyzed by capillary or nanoflow HPLC MS/MS and a new software program, Pro ICAT, is used to automatically quantify and identify the differentially expressed proteins.

Process Workflow for Expression Analysis Studies



Credit: Dr. Ruedi Aebersold, Institute for Systems Biology, Seattle, WA

New Pro ICAT Software

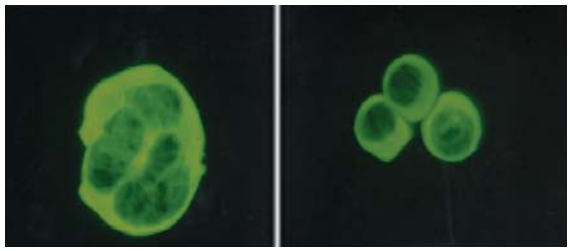


With the introduction of Pro ICAT software, Applied Biosystems now provides an automated implementation of the ICAT reagent-based proteomic workflow using the Vision™ WorkStation and API QSTAR™ Pulsar LC/MS/MS systems.

protein expression

The power of ICAT™ reagents is detailed here with a protein expression analysis workflow for the identification and quantification of proteins from Jurkat cells.

1 Sample Preparation, Labeling and Digestion



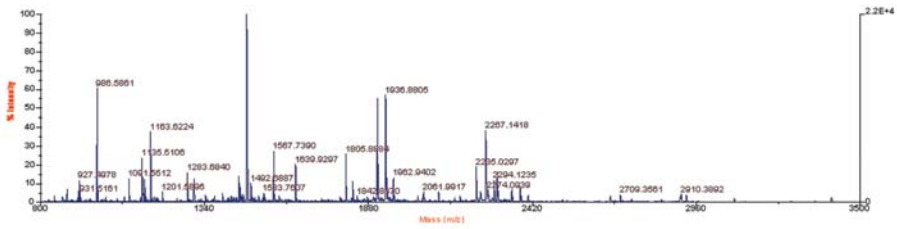
The Jurkat control and experimental cells were prepared as follows:

- Removed cytoskeletal and nuclei components by centrifugation at 4000g.
- Isolated microsomal fraction containing plasma membrane, golgi, endoplasmic reticulum, mitochondria, lysosomes, and other membrane bound vesicles by centrifugation at 100,000g.
- Dissolved microsomal pellets in labeling buffer (50mM Tris, 0.1% SDS)

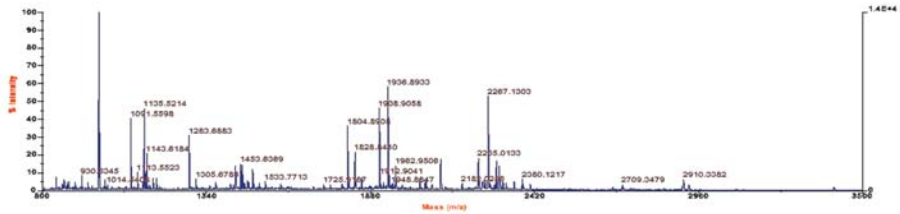
Sample Labeling

Equal amounts of control and experimental protein were reduced with 1mM TCEP for 10 minutes at 100˚C. The cysteine-containing proteins in the control and experimental samples were then labeled with the isotopically light and heavy versions of the ICAT reagent. The reduced and alkylated protein pools were combined and enzymatically cleaved with trypsin (overnight digestion) to generate peptide fragments.

Avidin Load: CEX Peptides



Avidin Elute: Eluted ICAT reagent-labeled peptides



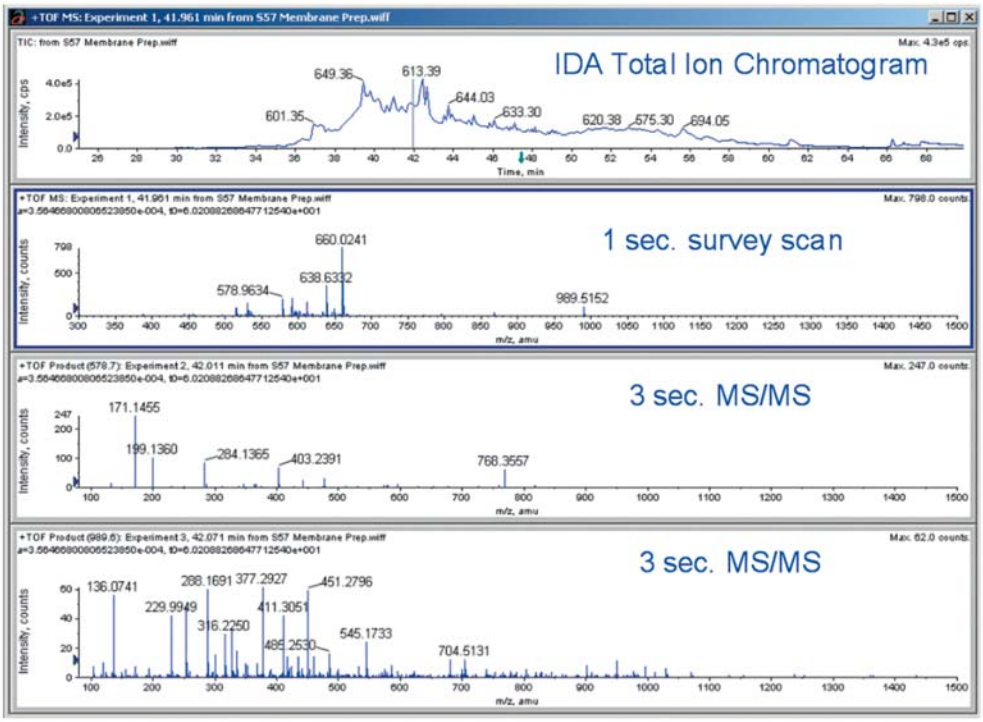
MALDI MS analysis of an ICAT reagent-labeled sample before and after avidin affinity chromatography. A reduction in overall sample complexity is achieved by isolating the ICAT reagent-labeled cysteine-containing peptides on the avidin affinity column. Non-cysteine containing peptides are in the flow through, as they are not labeled with the ICAT reagent.

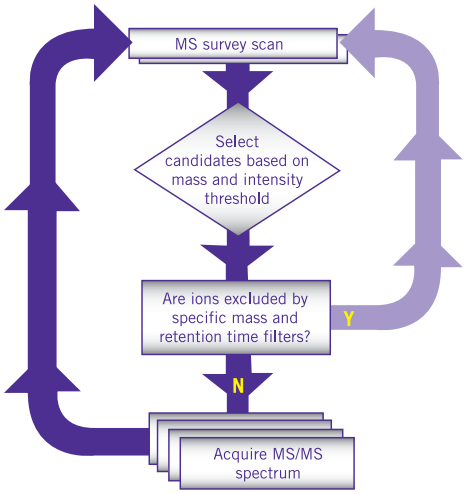
2 Peptide Separation and Purification

Following digestion, the resulting peptides were fractionated on a high-resolution cation exchange column into 28 fractions. This step also removes excess ICAT reagent, reducing reagent and trypsin from the sample. Using the robotic arm of the Vision™ Workstation, each fraction from the cation exchange column was automatically injected onto the avidin affinity column for purification of ICAT reagent-labeled peptides.

We wish to acknowledge Dr. David Han at the University of Connecticut for his work with Jurkat cells, as well as the work of Dr. Ruedi Aebersold and Dr. Tim Griffin at the Institute for Systems Biology.

3 Capillary LC/MS/MS Analysis of Avidin-Purified Fractions





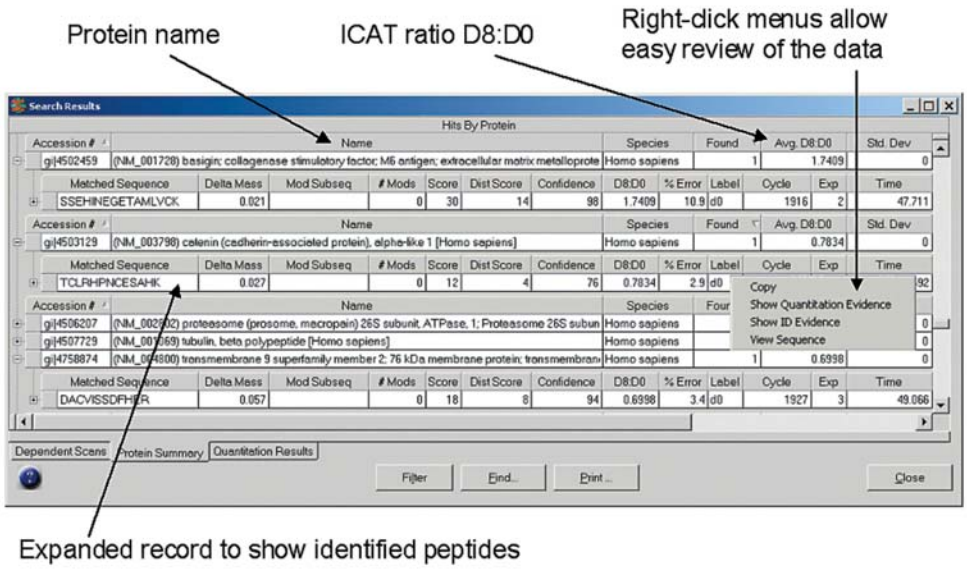
IDA enables intelligent, unattended data acquisition and processing based on user-selected criteria — rapidly acquiring and converting data to information.

Protein quantitation and identification studies with ICAT™ reagents require a mass spectrometer with MS and MS/MS capabilities along with Pro ICAT software for data analysis. Today, the most accurate, versatile MS/MS system is the API QSTAR™ Pulsar Hybrid LC/MS/MS System, the premier quadrupole time-of-flight system.

The peptides were further fractionated on a reversed phase column (75um ID x 7.5 cm) with a run time of 1 hour. The instrument automatically performed MS/MS on the most intense ions from the eluting peaks using Information Dependent Acquisition (IDA) maximizing both the MS and MS/MS information generated from a single run. In this example, the two most intense ions were subjected to MS/MS before the cycle was repeated.

4 Experimental Results with Pro ICAT Software

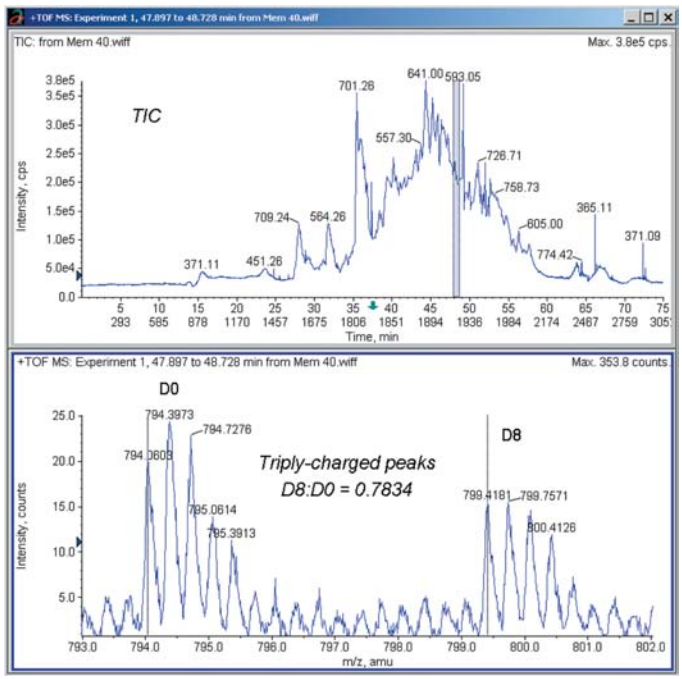
Pro ICAT software incorporates several proprietary algorithms that provide a new standard of speed and accuracy for protein identification and quantitation. The novel Interrogator™ database search engine provides high-throughput protein identification, while the LC/MS reconstruct quantitation algorithm determines ICAT™ reagent ratios using a sophisticated three-dimensional peak-finding technique. The results of every sample are stored in the software's results database for future retrieval and mining. In addition, a second expression-dependent MS/MS analysis method can also be created from the quantitation results. This can be used to identify only those proteins that are changing in an expression analysis experiment.



The protein summary (see above) automatically sorts the proteins by the number of times a peptide was identified for that protein, starting with the most frequently identified protein. The top level lists the protein accession number, the name, the species, the number of times found, and the weighted average D8:D0 ICAT reagent ratio calculated from all found peptides. Clicking the "+" sign expands the record to show the identified peptides, the Interrogator "Score" and "Confidence" statistics that were found, along with the D8:D0 ICAT reagent ratio calculated for each peptide.

The command "Show ID Evidence" opens the selected peptide sequence in a fragment pane viewer (data not shown) and automatically matches the theoretical fragments to the experimental data.

The command "Show Quantitation Evidence" from any peptide displays the averaged MS data from the most intense charge state identified for the D8:D0 ICAT reagent pair. However, all charge states are used to determine the ratio. Typical coefficients of variation (CV) are within the range 10% to 20%.



Results and Summary

From the 28 cation exchange fractions analyzed, a total of 841 proteins were quantified and identified using the Pro ICAT data analysis software. The data detailed in the table at right demonstrates that this approach generates a significant amount of unique protein information from fraction to fraction. In this example, only two proteins were common to fractions 24 and 25, as indicated by the red asterisks. This would not be the case if ICAT™ reagent tagging strategy had not been used. The process flow clearly shows how sample complexity can be reduced by selecting for cysteine-containing peptides, leading to a deeper analysis of a proteome.

CEX Fraction 24	CEX Fraction 25
ribosomal protein S17	chaperonin containing t-complex polypeptide
proteasome (prosome, macropain) subunit, beta type	*heteroteneous ribonuclear particle protein U- human
Aspartyl-tRNA synthetase	(AJ224112) gamma-adaptin protein
HYPOTHETICAL PROTEIN KIAA0379	basigin; collagenase stimulatory factor; M6 antigen;
(AB063318) acute morphine dependence related protein	clathrin, heavy polypeptide-like 1 isoform a;
*heterogeneous nuclear ribonucleoprotein U,	riobosmal protein L3 [Homo sapiens]
*tubulin, beta polypeptide	(AF222689) protein arginine N-methyltransferase 1-variant 2
	guanine nucleotide binding protein (G protein)
	transmembrane 9 superfamily member 2;
	ubiquitin specific protease 14
	F-actin capping protein beta subunit
	TRIOSEPHOSPHATE ISOMERASE (TIM)
	(BC009408) CTP synthase
	(D80005) KIAA0183
	40S RIBOSOMAL PROTEIN S12
	(X61972) macropain subunit iota
	zinc finger protein 9
	*tubulin, beta, 5
	cofilin 1 (non-muscle)
	similar to ribosomal protein S3A

841 proteins identified from 28 fractions from the NCBI human-rodent database with confidence of 71 or higher. 572 of these were human.

Enabling New Workflows

An alternative to traditional separation approaches, ICAT™ reagent technology enables concurrent quantitation and identification of proteins in complex mixtures.

To demonstrate the power of ICAT™ reagents, we've detailed a protein expression analysis workflow for the identification and quantification of proteins from Jurkat cells using the ICAT™ reagents and other leading technologies from Applied Biosystems. This workflow integrates the ICAT™ reagents for sample labeling, the Vision™ WorkStation for automated fractionation and purification of ICAT reagent-labeled peptides and the API QSTAR™ Pulsar Hybrid LC/MS/MS System with Pro ICAT software for automated identification and quantification of labeled peptides.

Proteomic workflows can be further integrated through the Applied Biosystems Rapid Integration Solutions (RIS) program. Combining state-of-the-art software components and world-class professional services, we can deliver a complete, tailored informatics solution for integrating and automating a lab, helping researchers to track and manage all samples, robots, instruments, and data analysis. This powerful solution offers researchers a single integrated system that increases laboratory throughput, improves compliance, and elevates the quality of results.

Together, these technologies and workflows enable researchers to identify proteins more efficiently as targets for therapy or as disease markers.

Expression Dependent Identification

While the workflow described here for the Jurkat cells is an example of protein cataloging (identifying and quantifying as many proteins as possible), the goal in many protein expression analysis studies is to identify only those proteins that are changing. To demonstrate this point, we took a CEX fraction from a yeast workflow and detailed the number of proteins exhibiting expression differences. Of the 100 proteins identified from one CEX fraction, only 13 proteins showed expression level differences. Of these 13 proteins, only 5 proteins had ratios greater than 2:1 or less than 0.5:1.

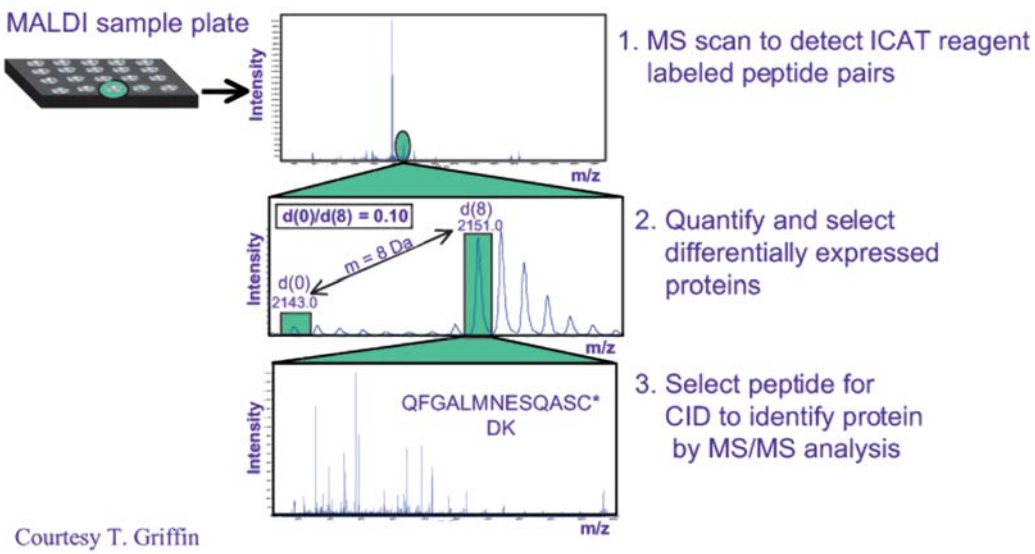
Yeast Table

CEX Fraction #	Proteins ID and Quantified	Expression Ratios 1:1	Expression Ratios >2 or <0.5
25	100	87	5

Workflows to perform expression-dependent protein identification can be accomplished by running two online capillary LC/MS runs in series. LC/MS is performed from a first injection of a sample, and Pro ICAT quantitates all D8:D0 pairs. From these results, a second LC/MS/MS IDA method is generated, containing an inclusion list for all of the ratios of interest. The intensity difference for a pair and a minimum quality score threshold (optional) are specified. Peaks that meet these criteria are then used for a second LC/MS/MS IDA analysis of the same sample and the parent proteins are identified.

Expression dependent identification can also be performed on the Applied Biosystems 4700 Proteomics Analyzer or when using the oMALDI™ source on the QSTAR™ Pulsar System. MALDI ionization has the advantage of being free of the online capillary HPLC time constraints inherent to ESI-LC/MS/MS. Instead, the capillary HPLC fractions are spotted onto a MALDI plate in one minute fractions with equal solution of matrix. MS analysis is performed on each spot on the plate, and D8:D0 ratios are calculated for all peptide pairs found. Based on user input for the D8:D0 ratio range of interest, the peptides from each spot are automatically fragmented and MS/MS spectra are obtained. The results of each MS/MS analysis are then automatically searched with the Mascot™ database search engine from Matrix Science to determine the ID of each quantitated component of interest.

Selective Identification of Differentially Expressed Proteins with the MALDI MS/MS



Courtesy T. Griffin

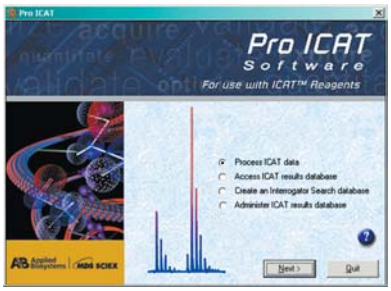
To help you fully understand the protein complement of the human genome, Applied Biosystems is committed to developing innovative, effective proteomics solutions. Further proof of our commitment is the Proteomics Research Center (PRC), a world-class, completely functional proteomics facility and network of global labs. In the PRC, we push all innovations to the limit, testing sensitivity, throughput, flexibility, accuracy. Our goal is to address entire workflows and identify and eliminate the gaps and bottlenecks, enabling researchers to do what they do best.

Applied Biosystems has a broad portfolio of technologies, products, and expertise to perform protein expression analysis and other proteomics experiments. Shown here are the key products used in the experiment detailed in this brochure.

ICAT™ Reagents
Find the proteins that matter.

Vision™ Workstation
For automation of the fractionation and purification of ICAT reagent-labeled peptides.

API QSTAR™ Pulsar Hybrid LC/MS/MS System
Highest level of flexibility for identifying and quantifying proteins.



Pro ICAT Software
A new standard of speed and accuracy for protein identification and quantification.

Rapid Integration Solutions
Tailored informatics solutions for integrating and automating your laboratory.

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Printed in the USA. Publication 114BR09-01, 2/2002, PJA