iTRAQ® Reagents - 4plex Applications Kit - Protein

Amine-Modifying Labeling Reagents for Protein Sample Applications

Protocol

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Not for Use in Diagnostics

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Preface

This preface covers:

Safety

Safety Alert Words

Four safety alert words appear in our user documentation at points in the document where you need to be aware of relevant hazards. Each alert word—**IMPORTANT, CAUTION, WARNING, DANGER**—implies a particular level of observation or action, as defined below.

Definitions

IMPORTANT! – Indicates information that is necessary for proper instrument operation, accurate chemistry kit use, or safe use of a chemical.

CAUTION – Indicates a potentially hazardous situation that, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices.

WARNING – Indicates a potentially hazardous situation that, if not avoided, could result in death or serious injury.

DANGER – Indicates an imminently hazardous situation that, if not avoided, will result in death or serious injury. This signal word is to be limited to the most extreme situations.

Chemical Hazard Warning

WARNING **CHEMICAL HAZARD.** Some of the chemicals used with our instruments and protocols are potentially hazardous and can cause injury, illness, or death.

Chemical Safety Guidelines

To minimize the hazards of chemicals:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. (See "About SDSs" on page vi.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the SDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the SDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

About SDSs

Chemical manufacturers supply current Safety Data Sheets (SDSs) with shipments of hazardous chemicals to *new* customers. They also provide SDSs with the first shipment of a hazardous chemical to a customer after an SDS has been updated. SDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new SDS packaged with a hazardous chemical, be sure to replace the appropriate SDS in your files.

Obtaining SDSs

You can obtain the SDS for any chemical supplied with this kit at **sciex.com**.

Note: For the SDSs of chemicals not distributed with this kit, contact the chemical manufacturer.

Chemical Waste Hazards

CAUTION **HAZARDOUS WASTE.** Refer to Safety Data Sheets and local regulations for handling and disposal.

WARNING **CHEMICAL WASTE HAZARD.** Wastes produced by our instruments are potentially hazardous and can cause injury, illness, or death.

WARNING **CHEMICAL STORAGE HAZARD.** Never collect or store waste in a glass container because of the risk of breaking or shattering. Reagent and waste bottles can crack and leak. Each waste bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position. Wear appropriate eyewear, clothing, and gloves when handling reagent and waste bottles.

Chemical Waste Safety Guidelines

To minimize the hazards of chemical waste:

- Read and understand the Safety Data Sheets (SDSs) provided by the manufacturers of the chemicals in the waste container before you store, handle, or dispose of chemical waste.
- Provide primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the SDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood).For additional safety guidelines, consult the SDS.
- Handle chemical wastes in a fume hood.
- After emptying the waste container, seal it with the cap provided.
- Dispose of the contents of the waste tray and waste bottle in accordance with good laboratory practices and local, state/provincial, or national environmental and health regulations.

Waste Disposal

If potentially hazardous waste is generated when you operate the instrument, you must:

- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure the health and safety of all personnel in your laboratory.
- Ensure that the instrument waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.

IMPORTANT! Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological Hazard Safety

BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:

- U.S. Department of Health and Human Services guidelines published in *Biosafety in Microbiological and Biomedical Laboratories* (stock no. 017-040-00547-4)
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030).
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.

Additional information about biohazard guidelines is available at:

http://www.cdc.gov

How to Obtain More Information

Obtaining Information from the Software Help System

The Analyst® and ProteinPilot™ Software have Help systems that describe how to use each feature of the user interface. Access the Help system by doing one of the following:

- Click in the toolbar of the software window
- Select **Help**
- Press **F1**

How to Obtain Support

AB Sciex Pte. Ltd. is committed to meeting the needs of your research. Please go to **sciex.com** and go to the **Support** tab for local support information.

Contacting Technical Support in North America

To contact technical support:

- By telephone: Dial 1.877.740.2129
- By fax: Dial 1.650.627.2803

Introduction
iTRAQ® Reagents Chemistry

1

This chapter covers:

Overview

iTRAQ® Reagents are provided as a set of four isobaric (same mass) reagents:

- iTRAQ® Reagent - 4plex - 114 (for 25 mg protein)
- iTRAQ® Reagent - 4plex - 115 (for 25 mg protein)
- iTRAQ® Reagent - 4plex - 116 (for 25 mg protein)
- iTRAQ® Reagent - 4plex - 117 (for 25 mg protein)

The use of four reagents allows multiplexing of up to four different samples in a single SDS-PAGE experiment with subsequent LC/MS/MS analysis.

Product Capabilities

Using iTRAQ Reagents to label proteins allows you to:

- Analyze normal, diseased, and drug-treated states in the same experiment or time-course study
- Run duplicate or triplicate analyses of the same sample in one experiment
- Label multiple peptides per protein, including those from proteins with post-translational modifications, in 1 to 2 hours at room temperature.
- Label multiple peptides per protein, increasing confidence in identification and quantitation

Select AB SCIEX mass spectrometry systems provide software features designed for easy analysis of iTRAQ Reagent applications data for relative and absolute quantitation.

Kit Capacity

iTRAQ® Reagents - 4plex Applications Kit - Protein – Contains sufficient iTRAQ Reagents 114, 115, 116, and 117 (for 25 mg protein) to label 10 duplex, 6 three-plex, or 5 four-plex experiments.

Workflow

In the iTRAQ Reagent - 4plex (for 25 mg protein) protocol for protein applications, you reduce, cysteine block, and label each sample in a single tube. The single-tube process eliminates potential sample loss in individual samples that may cause inaccuracies in quantitation.

Then you combine all iTRAQ Reagent-labeled samples into one sample mixture for the SDS-PAGE separation, in-gel protein digestion, and LC/MS/MS analysis. If losses occur during analysis, each sample experiences the same loss and the peptide ratios are preserved.

Figure 1-1 summarizes the iTRAQ Reagents workflow. Up to four samples can be prepared and analyzed in a single experiment.

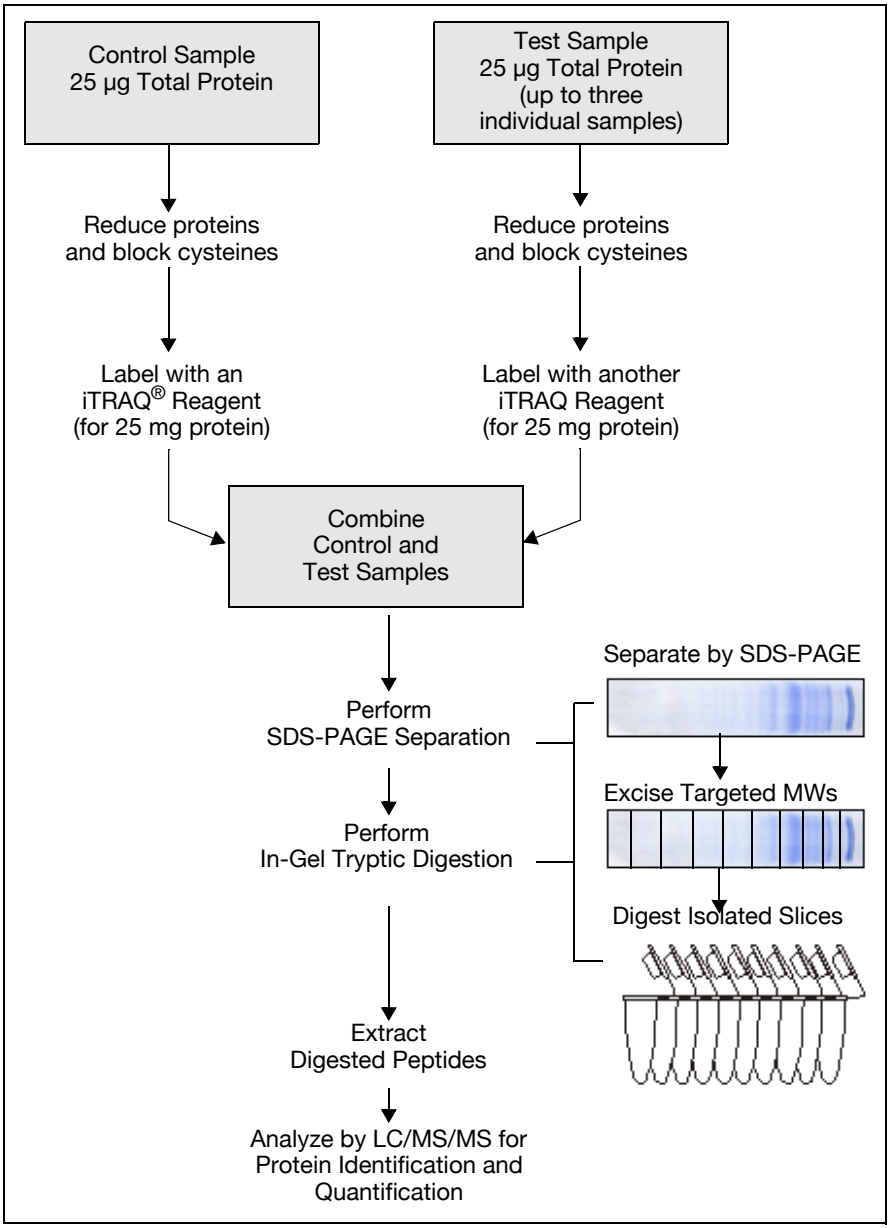

Figure 1-1 Overview of iTRAQ® Reagents - 4plex - protein method

Required Materials

User-Supplied Materials

WARNING CHEMICAL HAZARD. Some of the chemicals referred to in this protocol (such as those in Table 1-1) are not provided with your kit. When using chemicals not provided by or purchased from us. obtain the safety data sheets directly from their manufacturers. To receive additional copies of SDSs purchased from us at no extra cost, see "Obtaining SDSs" on page vi.

Table 1-1 User-supplied materials

Item	Quantity per Assay
Disposable gloves	As needed
Test sample	25 mg
Control sample	25 mg
Pipettors and tips suitable for: • 1 mL to 1 mL • Gel loading	As needed
Acetone, for acetone precipitation (optional)	As needed
pH paper with pH range 6.5 to 10 (to test the pH of the sample when labeling)	As needed
Milli-Q® water or equivalent (minimum 18.2 MOhms water, conductivity maximum 0.05 µS/0.05 µMho)	50 mL
Heating block, 60 °C	1
Incubator, 37 °C	1
Bench-top centrifuge	1
Vortexer	1
Centrifugal vacuum concentrator	1

Table 1-1 User-supplied materials

Item	Quantity per Assay
1-D SDS-PAGE 1-mm mini-gel system	As needed
Gel running buffer	As needed
SDS-PAGE sample buffer	As needed
Coomassie® blue aqueous gel staining solution	As needed
Gel dehydration solution: 100% acetonitrile (ACN)	50 mL
Gel washing buffer: 50% ACN in 100 mM ammonium bicarbonate (NH_4HCO_3), pH 8.0	50 mL
Extraction solvent: 50% ACN containing 0.1% TFA in Milli-Q® water or equivalent	50 mL
Ammonium bicarbonate (NH_4HCO_3), 100 mM, pH 8.5	As needed
Clean, single-edged razor blade or scalpel, for excising gel bands	1
Sonic water bath, for use when extracting peptides from gel	1
Platform rocker for destaining gel	1
Clean container for destaining gel	1
Eppendorf tubes, 1.5-mL	As needed
Nano reversed-phase HPLC system	1
If you analyze using Nanospray™ ESI mass spectrometry, either of the following tips: • New Objective, Inc. coated fused-silica PicoTips® (coating applied to tip end; Cat. #FS360-20-10-CE-20). Also requires tubing fitting from LC Packings (Cat. #TF-250/350). • New Objective, Inc. distal-coated fused silica PicoTips® (Cat. #FS360-20-10-D-20).	1
Aluminum foil (light protection during alkylation)	As needed
Iodoacetamide, for blocking reduced cysteines	As needed

Table 1-1 User-supplied materials

Item	Quantity per Assay
Trypsin or alternative enzyme [for example, our trypsin with $CaCl_2$, 10-pack (4352157) or our trypsin, TPCK, 8-pack (4370285)]	As needed

Kit Materials

WARNING CHEMICAL HAZARD. Some of the chemicals provided in your reagent kit may be hazardous. Before handling the reagents, read the safety data sheets (SDSs) that accompany your first shipment. Always follow the safety precautions (wearing appropriate protective eyewear, clothing, and gloves, etc.) presented in each SDS. To receive additional copies of SDSs at no extra cost, see For Research Use Only. Not for use in diagnostic procedures. on page vi.

When you receive the shipping container, immediately remove the reagent box from the container and store it at –15 to –25 °C. See Table 1-2 for materials contained in the kit.

Table 1-2 iTRAQ® Reagents - 4plex Applications Kit - Protein (PN 4374321) kit materials

Item	Quantity	Description
Store at -15 to -25 °C		
iTRAQ® Reagent - 4plex - 114 (for 25 mg protein)	5 vials	Amine-modifying reagent, used for labeling. One vial of a reagent labels 25 mg of protein.
iTRAQ® Reagent - 4plex - 115 (for 25 mg protein)	5 vials	
iTRAQ® Reagent - 4plex - 116 (for 25 mg protein)	5 vials	
iTRAQ® Reagent - 4plex - 117 (for 25 mg protein)	5 vials	
6-Protein Mix	1 vial, 129 mg/vial	Used in testing the protocol. Contains: • Bovine serum albumin (22 mg) • a-lactalbumin (10 mg) • b-galactosidase (38 mg) • Lysozyme (10 mg) • Apotransferrin (25 mg) • a-lactoglobulin (24 mg)
Urea	5 vials, 100 mg/vial	Aids in solubilizing proteins.

Table 1-2 iTRAQ® Reagents - 4plex Applications Kit - Protein (PN 4374321) kit materials

Item	Quantity	Description
iTRAQ Dissolution Buffer	1 vial, 1.5 mL/vial	Dissolves the sample and buffers the reaction. Buffer pH is 8.5. Contains 0.5 M triethyammonium bicarbonate.
Denaturant (2% SDS)	1 vial, 50 µL/vial	Disrupts the hydrogen, hydrophobic, and electrostatic interactions of the proteins. Contains 2% SDS.
Reducing Reagent	1 vial, 100 mL/vial	Reduces the disulfide bonds of the proteins. Contains 50 mM tris-(2-carboxyethyl)phosphine (TCEP).
Ethanol	1 vial, 1.8 mL	Absolute, HPLC-grade or better. Used to dissolve the iTRAQ Reagents and optimize labeling.
Documents		
iTRAQ® Reagents - 4plex Applications Kit - Protein Protocol	1	Describes how to label protein samples with iTRAQ Reagents (for 25 mg protein) and to subsequently separate labeled proteins with SDS-PAGE.
Certificate of Analysis	1	Provides lot-purity information to enter in the ProteinPilot™ Software.

iTRAQ® Reagents - 4plex - Protein Protocol

2

This chapter covers:

Before You Begin

Required Materials

See "Required Materials" on page 1-5.

Removing Interfering Substances

Thiols and primary amines in your sample can interfere with the iTRAQ® Reagents - 4plex - Protein Protocol. If your sample contains any of the substances shown in Table 2-1, perform acetone precipitation (page 2-3) to clean up the sample.

Note: The process for labeling proteins as described in this protocol is less affected by interfering substances. In this protocol, the labeled proteins undergo SDS-PAGE separation, then tryptic digestion. Some of the substances that may interfere with tryptic digestion are removed during the SDS-PAGE separation.

Table 2-1 Substances that may interfere with the iTRAQ® Reagents reaction

Potential Interfering Substance	Potential Interference	When to Perform Acetone Precipitation
Thiols, for example, DTT and mercaptoethanol	Interfere with cysteine blocking.	Before beginning the protocol
Primary amines, for example, those in: • Ammonium acetate • Ammonium bicarbonate • Ammonium citrate • Ammonium tartrate • AMPD [2-amino-2-methyl-1,3-propanediol] • Aminoguanidine bicarbonate salt • AMP [2-amino-2-methyl-1-propanol] • Ethanolamine • Gly-gly • Tris buffers	React with iTRAQ® Reagents, interfering with labeling.	Before labeling with iTRAQ Reagents

Acetone Precipitation

WARNING CHEMICAL HAZARD. Acetone is a flammable liquid and vapor. Exposure may cause eye, skin, and upper respiratory tract irritation. Prolonged or repeated contact may dry the skin. Exposure may cause central nervous system depression. Keep away from heat, sparks, and flame. Read the SDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

To clean up a sample by acetone precipitation:

1. Chill acetone to –20 °C and the sample tube containing the sample to 4 °C.
2. Add six volumes of cold acetone to the cold sample tube.
3. Invert the tube three times.
4. Incubate the tube at –20 °C until a flocculent forms (30 minutes to 4 hours).
5. Spin at 6,000 ´ g for 10 minutes.
6. Decant the acetone. Do not dry.
7. Use the precipitated pellet as your sample in "Reducing the Proteins and Blocking Cysteine," step 1, page 2-5.

Testing the Protocol

To verify that your sample preparation protocol does not interfere with the iTRAQ Reagents labeling, it is strongly recommended that you run a control sample through the entire protocol before you run an actual experiment. If you lack enough control sample to test the protocol, prepare a sample using your sample conditions and the 6-Protein Mix provided in the kit. Label the 6-Protein Mix sample with one of the iTRAQ Reagents. One vial of 6-Protein Mix (containing 129 mg of protein) is enough for five iTRAQ Reagent (for 25 mg protein) labeling reactions.

Sample Solubility

If your sample is insoluble after adding Dissolution Buffer and Denaturant (steps 1 and 2 on page 2-5), choose an alternative detergent/denaturant or buffer from Table 2-2. These buffers are free of primary amines and can buffer at pH 8.0 to 8.5 when used at a concentration of at least 0.3 M. During the labeling reaction, the buffer concentration must be at a level of at least 0.06 M.

Table 2-2 Recommended alternative detergents/denaturants and buffers

Alternative Detergent/Denaturant	Alternative Buffer	
• SDS • OG (octyl B-D-glucopyranoside) • NP®-40 • Triton® X-100 • Tween® 20 • CHAPS • Urea (6 M) IMPORTANT! When using urea, always use a fresh solution. When reducing a sample containing urea, incubate the tubes at 37 °C for 1 hour (step 6 on page 2-5)	• BES • BICINE • Boric acid • CHES • DIPSO • EPPS	• HEPBS • HEPES • HEPPSO • MOBS • MOPS • Phosphate buffer • PIPES • POPSO

Modifications

If the protocol test fails, modify your sample preparation or the protocol.

Running the Labeling Protocol

Running the iTRAQ Reagents 4plex protocol for 25 mg protein applications involves:

- Denaturing the sample, reducing the sample, and blocking the cysteines
- Labeling the proteins with iTRAQ Reagents (for 25 mg protein)

Reducing the Proteins and Blocking Cysteine

WARNING CHEMICAL HAZARD. Read the SDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
Denaturant (2% SDS) causes eye and skin irritation.
Reducing Reagent causes eye, skin, and respiratory tract irritation.
Urea may cause eye, skin and respiratory tract irritation.

IMPORTANT! If your sample contains thiols, perform acetone precipitation (see "Acetone Precipitation" on page 2-3).

1. To each of up to four sample tubes containing 5 to 25 mg of sample, add 20 mL iTRAQ Dissolution Buffer.
2. Add 1 mL of Denaturant (2% SDS).
3. Vortex to mix.

   If the sample does not dissolve, see "Sample Solubility" on page 2-4.
4. To each sample tube, add 2 mL Reducing Reagent.
5. Vortex to mix, then spin.
6. Incubate the tubes at 60 °C for 1 hour.

   IMPORTANT! If your sample contains urea, incubate at 37 °C for 1 hour. The lower incubation temperature minimizes carbamylation.
7. Spin to bring the sample to the bottom of the tube.
8. Prepare a fresh solution of 200 mM iodoacetamide.

   For example, dissolve 37.0 mg of iodoacetamide in 1 mL Milli-Q water or equivalent. If you have the resources to accurately weigh smaller amounts, you can prepare a smaller volume.

   IMPORTANT! The 200 mM iodoacetamide solution must be freshly prepared to avoid the presence of the degradation by-product iodine that may cause your sample to oxidize.
9. To each tube, add 1 mL of 200 mM iodoacetamide.
10. Vortex to mix, then spin.
11. Incubate the tubes in the dark at room temperature for 30 min.

Labeling the Samples with iTRAQ® Reagents

WARNING CHEMICAL HAZARD. Read the SDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.
Ethanol is a flammable liquid and vapor. Exposure causes eye, skin, and respiratory tract irritation and may cause central nervous system depression and liver damage.
iTRAQ™ Reagents 114-117 cause eye and respiratory tract irritation. Exposure may cause blood damage.

1. Inspect sample tubes for precipitate.
   - If no precipitate is present, add 20 mL of Milli-Q water or equivalent.
   - If precipitate is present:

     a. Reconstitute 1 vial of urea in 250 mL of Milli-Q water or equivalent.

     b. Add 20 mL of the urea solution to the sample tube.
2. Vortex to mix, then spin. Ensure the sample is dissolved before completing step 7.

   IMPORTANT! The sample must be completely in dissolved before iTRAQ Reagent labeling.
3. Allow each required vial of iTRAQ Reagent - 4plex (for 25 mg protein) to reach room temperature.
4. Spin to bring the solution to the bottom of the tube.
5. Add 50 mL of ethanol to each vial of room-temperature iTRAQ Reagent - 4plex (for 25 mg protein).
6. Vortex each vial to mix, then spin.
7. Transfer the entire contents of an iTRAQ Reagent - 4plex (for 25 mg protein) to each sample tube.

   For example, for a duplex-type experiment, transfer the contents of the iTRAQ® Reagent - 4plex - 114 (for 25 mg protein) to sample 1 tube and transfer the contents of the iTRAQ® Reagent - 4plex - 117 (for 25 mg protein) to the sample 2 tube.
8. Vortex each tube to mix, then spin.
9. Incubate the tubes at room temperature for 2 hours.

Combining the iTRAQ® Reagents-Labeled Samples

WARNING CHEMICAL HAZARD.
iTRAQ™ Reagents 114-117 cause eye and respiratory tract irritation. Exposure may cause blood damage. Read the SDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

1. Spin each iTRAQ Reagent-labeled sample tube to bring the solution to the bottom of the tube.
2. Combine the contents of each iTRAQ Reagent-labeled sample tube into one tube.
3. Vortex to mix, then spin.
4. Dry the sample in a centrifugal vacuum concentrator.

SDS-PAGE Separation 3

This chapter covers:

This section describes:

- Separating the iTRAQ® Reagents-labeled samples using an SDS-PAGE 1-mm mini-gel system
- Performing in-gel tryptic digestion
- Extracting the protein digests and reconstituting the protein digests for LC/MS/MS analysis

Before You Begin

Required Materials

See “Required Materials” on page 1-5.

Using an Alternative Enzyme

The procedure for in-gel digestion in this protocol uses tryptic digestion, resulting in large molecular weight peptides and reduced sample complexity.

To generate a greater number of peptides and improve protein coverage, use an alternative digestion enzyme, such as chymotrypsin. See “Selecting an Alternative Enzyme” on page 3-5.

Performing SDS-PAGE Separation on a 1-mm Mini-gel System

Loading and Running the Gel

1. For loading sample on to the SDS-PAGE system, reconstitute the combined iTRAQ Reagent-labeled sample (from step 4 on page 2-7) in an appropriate volume of SDS-PAGE sample buffer.

   The maximum suggested protein load per gel lane is 50 to 100 µg of combined protein, depending on the size of the wells. The procedure in “Running the Labeling Protocol” on page 2-4 yields a sample with 20 to 100 µg of protein.

2. Load the SDS-PAGE gel with sample according to the manufacturer’s recommendations.

3. Run the gel according to the manufacturer’s recommendation.

Staining and Destaining the Gel

WARNING CHEMICAL HAZARD. Read the SDS 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.

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! Follow the manufacturer's instructions. Staining and destaining procedures may vary, depending on the type of staining solution you use. Do not overstain.
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).

Excising and Washing the Gel Bands

WARNING CHEMICAL HAZARD. Read the SDS 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. Prepare a digestion tube for each gel band that you excise.
   a. Rinse a 1.5-mL Eppendorf tube two times with methanol, then two times with Milli-Q water or equivalent.
   b. Label each tube as a digestion tube and indicate the gel band identification.
2. Excise the bands or molecular weight areas of interest from the gel.
3. Cut each excised gel band into 1 × 1.5 mm plugs.
4. Transfer the gel pieces from each band to the appropriate digestion tube.
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).
   b. Gently vortex each tube to mix, taking care to avoid breaking the gel.
   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 vacuum concentrator until completely dry (about 10 minutes).

Performing In-Gel Tryptic Digestion

Selecting an Alternative Enzyme

iTRAQ Reagents are amine reactive tags that label the N-terminal and lysine residues of proteins. The procedure for in-gel digestion presented here uses tryptic digestion. The labeled lysine residues are not recognized by trypsin, therefore cleavages occur at only arginine residues. For this reason, tryptic digestion of iTRAQ Reagent-labeled samples yields large molecular weight peptides and reduces sample complexity.

To generate a greater number of peptides and improve protein coverage, use an alternative digestion enzyme, such as chymotrypsin. When selecting an alternative digestion enzyme, consider the enzymes supported by the data analysis software. Enzymes supported by ProteinPilot™ Software include:

- Arg-C
- AspN
- Chymotrypsin
- Glu-C
- Lys-C
- Trypsin

Digesting the Labeled Proteins

WARNING CHEMICAL HAZARD. Trypsin causes eye, skin, and respiratory tract irritation. Exposure may cause an allergic reaction. Read the SDS, 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) pH 8.5.
2. Vortex to mix, then centrifuge for a few seconds to bring all solution to the bottom of the vial.
3. To each tube containing dehydrated gel pieces, add 50 µL of the trypsin solution.
4. Allow the gel pieces to rehydrate in the trypsin solution for 10 minutes.
5. Visually inspect the gel pieces.
   - If the gel pieces are uniformly clear, proceed to step 6.
   - If any gel pieces contain white areas, add an additional 50 µL of trypsin solution to the tube. Allow the gel pieces to rehydrate in the additional trypsin solution for 10 minutes.
   - If necessary, add only 100 mM ammonium bicarbonate to cover the gel pieces. Too much liquid may increase the number of autolytic peaks.
6. Gently vortex each tube to mix, taking care to avoid breaking the gel, then centrifuge for a few seconds to bring all solution to the bottom of the tube.
7. Incubate the tubes at 37 °C overnight (12 to 16 hours).

Extracting the Protein Digests

WARNING CHEMICAL HAZARD. Read the SDS 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.

1. Prepare a supernatant tube for each gel band.
   a. Rinse a 1.5-mL Eppendorf tube 2 times with methanol, then 2 times with Milli-Q water or equivalent.
   b. Label each tube as a supernatant tube and indicate the gel band identification (corresponding to the digestion tube).
2. Vortex each digestion tube (the tubes containing the digested gel pieces) to mix, then centrifuge for a few seconds to bring all solution to the bottom of the tube.
3. Place the digestion tubes in a sonic water bath for 20 minutes.
4. Transfer the supernatant from each digestion tube to the corresponding supernatant tube.
5. To the digested gel pieces remaining in each digestion tube, add 100 µL of the extraction solvent (50% ACN containing 0.1% TFA in Milli-Q® water or equivalent).
6. Gently vortex each tube to mix, taking care to avoid breaking the gel.
7. Place the tubes in a sonic water bath for 20 minutes.
8. Add the supernatant from each digestion tube to the corresponding supernatant tube.
9. Repeat steps 5 through 8 two more times.
10. Place the supernatant tubes (containing the combined extract for each sample) in a vacuum concentrator and evaporate until dry.

IMPORTANT! Unless you are immediately analyzing the samples, store the dried labeled samples at –15 to –25 °C.

LC/MS/MS Analysis

For protein identification and quantification using LC/MS/MS analysis, reconstitute each sample in 2% ACN containing 0.1% TFA in Milli-Q® water or equivalent.

AB Sciex Pte. Ltd.

500 Old Connecticut Path,
Framingham, MA 01701, USA
Phone: +1 877.740.2129
Fax: +1 650.627.2803

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