Extraction of Proteins from Formalin-Fixed, Paraffin-Embedded Tissue Using the Qproteome Extraction Technique and Preparation of Tryptic Peptides for Liquid Chromatography/ Mass Spectrometry Analysis

Thibault Geoui,[1] Henning Urlaub,[2] Uwe Plessmann,[2] and Peter Porschewski[1]

[1]QIAGEN GmbH, Hilden, Germany
[2]Max Planck Institute for Biophysical Chemistry, Göttingen, Germany

ABSTRACT

This unit provides a robust, reliable, and easy-to-use kit-based method for extraction of intact, non-degraded proteins from formalin-fixed, paraffin-embedded (FFPE) tissue, and their subsequent use for analysis by liquid chromatography/mass spectrometry (LC/MS). After deparaffinization, proteins are extracted from unstained sections of FFPE rat liver tissue. After a simple cleanup step using organic extraction, the sample is transferred into a buffer optimized for trypsin digestion of the extracted proteins. Subsequently, LC/MS is used to identify the proteins that gave rise to the tryptic peptides. Comparing formalin-fixed and frozen tissues, good correlation is observed in the mass spectrometric pattern attributable to the tryptic peptides and number of identified proteins. Since FFPE tissues are generally available in clinical practice, this method can be used to analyze biomarkers in different pathological situations (e.g., healthy vs. diseased). The method can also be used for protein extraction from fresh-frozen tissue. *Curr. Protoc. Mol. Biol.* 90:10.27.1-10.27.12. © 2010 by John Wiley & Sons, Inc.

Keywords: formalin-fixed tissue • mass spectrometry • proteomics • protein extraction

INTRODUCTION

Basic Protocol 1 describes the optimized extraction and cleanup of total protein from formalin-fixed and paraffin-embedded (FFPE) tissue. First, the tissue sections are deparaffinized and treated with lysis buffer at two temperatures to reverse formalin cross-linking and release the protein molecules. The solution is then centrifuged, and the supernatant is desalted to remove buffer components prior to proteolytic digestion.

Basic Protocol 2 describes the reconstitution of the protein sample for trypsin digestion and analysis by liquid chromatography/mass spectrometry (LC/MS). The separation and analysis of the peptides requires expertise in reversed-phase high-performance liquid chromatography (RP-HPLC) and mass spectrometry.

DEPARAFFINIZATION OF SECTIONS FROM AN FFPE TISSUE BLOCK AND EXTRACTION OF TOTAL PROTEIN

BASIC PROTOCOL 1

This protocol describes the preparation of deparaffinized tissue sections from an FFPE tissue block, the extraction of proteins from the sections, and the concentration and purification of the proteins from sample and kit compounds that can interfere with RP-HPLC and mass spectrometry. Standard formalin fixation and paraffin embedding (FFPE)

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procedures should be used for the preparation of FFPE tissue sections. To optimize protein recovery, it is important that the following criteria are met during fixation and embedding. (1) Tissue samples should be fixed in 4% to 10% formalin as quickly as possible after surgical removal. (2) The fixation time should be 14 to 24 hr, since longer fixation times will result in poor protein extraction efficiency. (3) Samples should be thoroughly dehydrated prior to paraffin embedding. Up to three sections, each with a thickness of up to 15 μm and an area of up to 100 mm^2, can be combined in one preparation. It is possible to use smaller sections ($\geq$25 mm^2) for one preparation. The yield of extracted protein may vary and depends on the amount and the nature of the starting material. If the amount of protein in the sample is not known, begin by using two sections per preparation (each 10 to 15 μm thick with an area of 100 mm^2) to determine protein content.

Because protein extraction efficiency is decreased when previously stained (e.g., Mayer's hematoxylin), slide-mounted sections are used (Becker et al., 2008), this method is not described in this unit (but see *UNIT 10.26*). This protocol can be used for extraction of proteins from fresh-frozen tissue, e.g., to make a direct comparison with FFPE tissue. For extraction of proteins from fresh-frozen tissue, skip steps 1-6 and start directly with step 7b.

Materials

FFPE tissue block: e.g., rat liver fixed for 24 hr in 10% neutral buffered formalin solution (PBS-buffered) prior to paraffin embedding
Fresh-frozen tissue (optional; stored at –80°C)
Qproteome FFPE Tissue 2D-Page Kit (#37633; QIAGEN) including:
  Extraction Buffer EXB
  FFPE solvents (including heptane, methanol, and chloroform)
  1.5-ml collection tubes with sealing clips
Protein quantification kit (e.g., Bio-Rad DC Protein Assay Kit 1 [#500-0001] for Lowry method *or* Pierce Micro BCA Protein Assay Kit [#23235])
Monoclonal antibody for protein assessment by immunoblotting (e.g., anti-α-tubulin, #MAB1501, Millipore, or anti-β-actin, #T5168, Sigma-Aldrich)
Acetone, HPLC grade

Microtome
100°C water bath or heating block
Thermomixer (e.g., Eppendorf)
0.5-ml microcentrifuge tubes (e.g., Eppendorf Safe-Lock tubes, colorless)

Additional reagents and equipment for 1-D SDS-PAGE (*UNIT 10.2A*) and immunoblotting (*UNIT 10.8*)

NOTE: When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheet (MSDS), available from the product supplier. Procedures using heptane, methanol, and chloroform should be performed in a well-ventilated fume hood. All organic solvents should be disposed of according to applicable environmental regulations.

NOTE: Unless otherwise noted, use analytical-grade reagents for preparation of all buffers and solutions.

NOTE: For each pipetting step, use a fresh tip or a multi-step dispenser, depending on the number of samples.

Deparaffinize tissue

1. Using a microtome, cut up to four serial sections (10-15 μm thick and ~100 mm^2) from the same FFPE tissue block. For best results and to be sure that the tissue

includes the area of interest (e.g., cancerous versus cancer-free regions for comparison), use the first section of each paraffin block for hematoxylin/eosin staining and histological verification by an experienced pathologist. Leave subsequent sections (no. 2-4) from the same block unstained to use for protein extraction.

Optionally, an adjacent section (no. 5) can also be used for hematoxylin/eosin staining and histological verification to ensure that all areas of interest are included in the sample.

If the amount of protein in the sample is not known, begin with two sections per preparation, each 10 μm thick with an area of 100 mm^2.

2. Immediately place the unstained sections (nos. 2-4) in a 1.5-ml collection tube.

3. Pipet 0.5 ml heptane into the tube. Close tube tightly, vortex for 10 sec, and incubate for 1 hr at room temperature (15° to 25°C).

To avoid cross-contamination between samples, use a fresh pipet tip or multi-step dispenser for each pipetting step, depending on the number of samples.

The use of heptane instead of the standard solvent xylene for deparaffinization results in better protein extraction efficiency.

4. Add 25 μl methanol, close tube tightly, and vortex for 10 sec.

Addition of methanol improves the precipitation of the deparaffinized tissue during the centrifugation step.

5. Microcentrifuge at maximum speed for 2 min.

The tissue will form a pellet on the bottom of the tube.

6. Carefully remove the supernatant using a pipet. Discard the supernatant and air dry the pellet for 5 min.

Do not decant the supernatant and do not disturb the pellet. It is important to avoid over-drying the pellet, since dissolution becomes more difficult and thereby reduces the efficiency of extraction.

Extract protein

7a. *For FFPE tissue (from step 6):* Pipet 100 μl Extraction Buffer EXB into the tube containing the pellet and mix by vortexing. Carefully seal the collection tube with a sealing clip before proceeding.

7b. *For fresh-frozen tissue:* Use a scalpel to scrape a 3- to 5-mm-long splint of frozen tissue into a tube containing 100 μl Extraction Buffer EXB and mix by vortexing. Carefully seal the collection tube with a sealing clip before proceeding.

To identify areas of interest (e.g., cancerous versus cancer-free regions for comparison), it is recommended to prepare cryosections and have histological verification performed by an experienced pathologist.

8. Incubate on ice for 5 min. Mix by vortexing.

9. Incubate in a 100°C heating block for 20 min.

10. Using a Thermomixer, incubate the tube at 80°C for 2 hr with agitation at 750 rpm. Mix the tubes by inversion every 10 min.

Alternatively, a water bath can be used for incubation.

11. Cool the tube at 4°C for 1 min and remove the sealing clip.

It is important that the sealing clip be removed before proceeding to the next step.

12. Microcentrifuge the tube for 15 min at 14,000 × *g*, 4°C. Transfer the supernatant containing the extracted proteins to a new 1.5-ml collection tube.

13. Dilute an aliquot of extracted protein with an equal volume of distilled water and quantify the protein concentration using the Lowry (e.g., Bio-Rad DC Protein Assay Kit) or BCA method (e.g., Pierce Micro BCA Protein Assay Kit).

14. To assess protein extraction efficiency and possible degradation, add one-fifth volume of 5× SDS sample buffer to an aliquot of protein (15-20 µg) and analyze by 1-D SDS-PAGE (*UNIT 10.2A*) followed by immunoblotting (*UNIT 10.8*) using an antibody to a suitable marker protein, such as α-tubulin or β-actin.

    After quantification, extracted proteins can be stored for up to 1 week at 4°C. For longer storage, aliquot the extracted proteins and store up to 1 year at –20°C. Avoid repeated freeze-thaw cycles.

Purify protein sample

15. Dilute the protein sample with Extraction Buffer EXB to obtain a 0.5 µg/µl protein solution. Transfer 25 µg (i.e., 50 µl) to a 0.5-ml microcentrifuge tube.

16. Add 200 µl methanol, close the tube tightly, and vortex for 10 sec. Microcentrifuge at 9,000 × *g* for 10 sec.

17. Add 50 µl chloroform, close the tube tightly, and vortex for 10 sec. Microcentrifuge at 9,000 × *g* for 10 sec.

18. Add 150 µl water, close the tube tightly, and vortex for 10 sec (Fig. 10.27.1A). Microcentrifuge at 9000 × *g* for 1 min (Fig. 10.27.1B).

    After centrifugation, the sample separates into three layers: a lower, colorless, organic (chloroform) phase; a white interphase containing protein; and an upper, colorless, aqueous phase.

19. Carefully remove and discard the upper aqueous phase. Do not disturb the interphase or lower phase (Fig. 10.27.1C).

20. Add 150 µl methanol, close the tube tightly, and vortex vigorously for 10 sec (Fig. 10.27.1D). Microcentrifuge at 9,000 × *g* for 2 min.

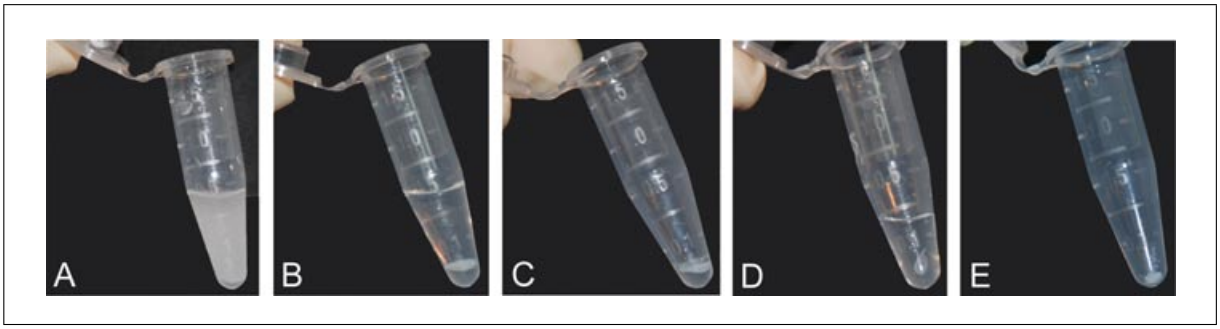

Figure 10.27.1 Steps for removing contaminants from an FFPE-extracted protein mixture. **(A)** After sequential addition of methanol, chloroform, and water to the protein sample in Extraction Buffer EXB followed by vortexing, the solution becomes turbid, which results from the mixing of organic solvents with the aqueous extraction buffer. **(B)** Centrifugation separates the sample into three phases: a lower, colorless, organic (chloroform) phase; a white interphase containing extracted proteins; and an upper, colorless, aqueous/methanol phase. **(C)** After removal of the upper aqueous phase, a negligible amount of the upper phase remains above the white protein-containing interphase. It is important to leave a small amount of upper phase rather than lose extracted protein. **(D)** After the addition of methanol, proteins appear as a floating precipitate, which disappears after vortexing. The chloroform/methanol mixture is miscible as a single phase that may be easily removed. **(E)** After removing the chloroform/methanol, the protein pellet forms a transparent or white gel-like pellet at the bottom of the tube. After washing the pellet with acetone and centrifugation, it has the same appearance. For the color version of this figure, go to *http://www.currentprotocol.com/protocol/mb1027*.

21. Carefully remove and discard the supernatant (Fig. 10.27.1E).

    The protein pellet is visible as a transparent or white gel-like pellet at the bottom of the tube.

22. Wash the pellet by adding 1 ml acetone and microcentrifuge at 9,000× *g* for 2 min.

23. Carefully remove the supernatant using a pipet. Discard the supernatant and air dry the pellet for 5 min.

    The samples can be stored for up to 1 week at 4°C. Longer storage is not recommended.

TRYPTIC DIGESTION OF EXTRACTED PROTEINS FOR MASS SPECTROMETRY

BASIC PROTOCOL 2

This method describes an efficient enzymatic (i.e., trypsin) digestion of the protein sample purified in Basic Protocol 1. The proteins are reconstituted in an appropriate buffer for efficient trypsin digestion (i.e., pH ∼8). After digestion, the resulting tryptic peptides are separated by reversed-phase liquid chromatography coupled to an electrospray ionization (ESI) mass spectrometer that fragments the individually separated peptides. The fragment spectra of the peptides, together with their intact mass ('precursor mass'), are subjected to database search using appropriate software (e.g., Mascot, *http://www.matrixscience.com*). The corresponding proteins are identified in the database search by comparing the experimentally determined peptide sequence (from the fragment spectra) and the precursor mass with all *in silico*−generated peptide sequences and masses derived from the proteins in a particular database.

Materials

Extracted protein sample (25 µg protein pellet; see Basic Protocol 1)
25 mM NH_4HCO_3, analytical grade
0.1% (w/v) RapiGest SF (Waters) in 25 mM NH_4HCO_3
50 mM dithiothreitol (DTT) in 25 mM NH_4HCO_3 (Calbiochem), analytical grade
100 mM iodoacetamide in 25 mM NH_4HCO_3 (Sigma-Aldrich), analytical grade
0.25 µg/µl trypsin in 25 mM NH_4HCO_3 (sequencing-grade trypsin, Promega)
5% (v/v) trifluoroacetic acid (TFA; Sigma-Aldrich), analytical grade
HPLC-grade acetonitrile (ACN, Acetonitrile LiChrosolv, gradient grade #1.00030.2500, Merck KGgA)
HPLC-grade formic acid (FA, for LC/MS, Sigma-Aldrich)
HPLC-grade H_2O (Water LiChrosolv #1.15333.2500, Merck KGgA)
Chromatography solvent A: 0.1 % (v/v) FA in H_2O
Chromatography solvent B: 95% (v/v) ACN/0.1% (v/v) FA

Thermomixer (e.g., Eppendorf)
0.5-ml microcentrifuge tubes (e.g., Eppendorf Safe-Lock tubes, colorless)
Speedvac evaporator (with rotor/adapters for 0.5-ml tubes)
Agilent 1100 chromatography system (Agilent Technologies) or equivalent, with:
- In-house-packed pre-columns (10–20 × 0.15 mm, C_{18}, 5 µm, 120 Å) *or* purchased pre-columns (e.g., #r15aq.v000.3T, 10 × 0.3 mm; Dr Maisch HPLC GmbH, *http://www.dr-maisch.com*)
- In-house-packed analytical columns (100 × 0.075 mm, C_{18}, 5 µm, 120 Å) *or* purchased analytical columns (e.g., #r15aq.s.1500075, 150 × 0.075 mm; Dr Maisch HPLC GmbH)

Linear Trap Quadrupole (LTQ) Orbitrap XL mass spectrometer (Thermo Fisher Scientific)
Mascot Daemon version 2.2.2 (*http://www.matrixscience.com*)
Mascot version 2.2.06 (*http://www.matrixscience.com*)
NCBInr FASTA database (*http://blast.ncbi.nlm.nih.gov*)

NOTE: Unless otherwise noted, use HPLC-grade water (e.g., Water LiChrosolv) and analytical-grade reagents for preparation of all buffers and solutions.

Digest proteins

1. Dissolve protein pellet (25 μg) in 10 μl of 1% (w/v) RapiGest SF in 25 mM NH_4HCO_3.
2. Add 10 μl of 50 mM DTT in 25 mM NH_4HCO_3.

   Dithiothreitol is a reducing agent capable of converting the sulfhydryl bonds of cystine to the free sulfhydryl groups of cysteine.
3. Using a Thermomixer, incubate the tube at 37°C for 1 hr with agitation at 950 rpm.
4. Add 10 μl of 100 mM iodoacetamide in 25 mM NH_4HCO_3.

   Iodoacetamide selectively alkylates the free sulfhydryl groups of cysteine residues to form the carboxyamidomethyl cysteine that is incapable of being oxidized to cystine. The resulting reduced and alkylated protein chain, no longer cross-linked by disulfide bonds, is more accessible for enzymic digestion.
5. Using a Thermomixer, incubate the tube at 37°C for 1 hr with agitation at 950 rpm. Mix the tube every 10 min by vortexing briefly.

   A heating block may also be used for incubation.
6. Add 90 μl of 25 mM NH_4HCO_3.
7. Add 20 μl of 0.25 μg/μl trypsin in 25 mM NH_4HCO_3.

   The enzyme/substrate ratio is 1:5 (w/w).

   Promega trypsin is supplied lyophilized and must first be dissolved in the supplied reconstitution buffer according to the manufacturer's instructions to obtain a 1 mg/ml stock solution. This stock solution can be stored up to 6 months at –20°C. Prior to use, the stock solution is diluted to a concentration of 0.25 μg/μl using 25 mM NH_4HCO_3.
8. Incubate at 37°C overnight (i.e., minimum 16 hr).
9. Stop the digestion by adding 20 μl of 5% (v/v) TFA.

   The addition of TFA reduces the pH from ~8 (the optimal pH for trypsin digestion) to ~3 (a pH where trypsin is unable to cleave proteins).
10. Using a Thermomixer, incubate the tube at 37°C for 1 hr with agitation at 950 rpm.
11. Microcentrifuge at 13,000 rpm for 30 min at room temperature (15° to 25°C).
12. Transfer the supernatant containing the tryptic peptides to a new 0.5-ml microcentrifuge tube and dry under vacuum using a Speedvac evaporator with adaptors for 0.5-ml microcentrifuge tubes.

    Alternatively, use empty 1.5-ml reaction tubes without lids as adaptors.

    Dried peptides should be analyzed as soon as possible. Avoid long-term storage (4 weeks or longer).

Perform mass spectrometry

13. Dissolve the pelleted peptides in 25 μl of 5% (v/v) ACN/1% (v/v) FA.
14. Load 5 μl (1/10 volume, corresponding to 5 μg of tryptic peptides from the extracted proteins) on an Agilent 1100 chromatography system (or equivalent) coupled on-line to an LTQ Orbitrap XL mass spectrometer. Run the samples at a flow rate of 200 nl/min using the following gradient:

    0% (v/v) solvent B for 5 min
    7% to 40% (v/v) solvent B over 40, 140, or 220 min

40% to 90% (v/v) solvent B over 1 min
90% (v/v) solvent B for 7 min
90% to 0% (v/v) solvent B over 0 min.

15. Analyze the tryptic peptides by mass spectrometry using mass spectrometric software to attribute specific tissue proteins as the source of the analyzed tryptic peptides.

An example of an approach that may be used is described below. Other equipment and approaches may be used, depending on the use of different chemical or enzymic cleavage methods, the availability of a different mass spectrometer, and the choice of other analysis software. However, electrospray ionization tandem mass spectrometry (ESI-MS/MS) is generally the method of choice, regardless of the instrument manufacturer.

Example: A tryptic peptide mixture was analyzed by ESI-MS/MS using an LTQ Orbitrap XL mass spectrometer with data-dependent acquisition (DDA). The MS scan (350-1,800 m/z) was performed in 500 msec using a Fourier transform (FT) analyzer at 30,000 full-width at half-maximum (FWHM) resolution. For MS/MS, the six most abundant precursors (i.e., peaks in the MS data with the largest area under the peak) per MS scan were selected and fragmented in the linear trap quadrupole (LTQ). MS/MS scan time was maximally 100 msec at a maximum fill rate of 10,000 ions. Data from two MS/MS scans were combined and averaged.

Raw data were processed using Mascot Daemon software that generated the corresponding peak list and submitted the peak list to a database search. The database search of the peak list derived from the product ion spectra was performed against the NCBInr database using Mascot as the search engine with the following search criteria: taxonomy mammalian, two missed cleavages allowed, MS precursor accuracy 5 ppm, MS/MS accuracy 0.5 Da, and carbamidomethylation of cysteines and oxidation of methionine as variable modifications.

Methionine residues are readily oxidized during sample preparation and/or upon ionization. Therefore, the possible oxidation of methionine (resulting in a mass shift of +15.999 Da) was taken into account in the database search.

The false-positive rate was determined with Mascot against a decoy database that was generated from the NCBInr FASTA database by the Mascot program. A decoy database contains randomly scrambled artificial protein FASTA sequences and thus serves as a control by identifying the percentage of false positive hits. In the database search, only proteins that showed one bold red peptide were considered. The use of red and bold typefaces is intended to highlight the most logical assignment of peptides to proteins. The first time a peptide match to a query appears in the report, it is shown in bold typeface. Whenever the top ranking peptide match appears, it is shown in red. This means that protein hits with peptide matches that are both bold and red are the most likely assignments to a protein (see http://www.matrixscience.com for details). For comparison of the results derived from FFPE and fresh liver sample analysis, the Scaffold program (http://www.proteomesoftware.com) was used for the generation of Venn diagrams (Fig. 10.27.3). This parallel extraction was done to demonstrate that there is no significant difference in the protein extraction efficiency between fresh-frozen and FFPE tissue. Previously, many researchers believed that protein cross-linking due to formalin fixation prevents protein profiling by immunoblotting or protein microarray analysis. However, both sample types can be used in the same workflow.

COMMENTARY

Background Information

Formalin fixation and paraffin embedding of patient biopsy tissue (FFPE tissue) has been the standard clinical procedure for preserving morphological details for histo- or immunohistopathological diagnosis for many decades. Typically, tissues from FFPE tissue blocks are used for immunohistochemistry or fluorescence *in situ* hybridization (FISH), although both methods are difficult to quantify. These procedures yield semi-quantitative information about protein expression or gene amplification of relevant diagnostic markers, which allows clinicians to make diagnostic and therapeutic decisions. Unfortunately, the extensive protein cross-linking that occurs

Table 10.27.1 Protein Yields from Different FFPE Tissues

Tissue	Total size (mm^2)	Number of sections	Protein yield (μg)
Heart (rat)[a]	~150	3	40
Liver (rat)[a]	~150	3	80
Colon (rat)[a]	~100-150	3	20
Brain (rat)[a]	~150	3	60
Breast cancer (human)[b]	~100	1	25-80

[a]Proteins were extracted from FFPE tissue sections (10 μm) that were directly cut from an FFPE tissue sample block using a microtome.

[b]A reference section of tissue routinely stained with hematoxylin/eosin was verified by a pathologist. The areas of interest were marked with a pen and used to locate the areas of interest in a subsequent unstained section. Proteins were extracted from two areas with different morphological structures (one containing healthy tissue without lesions and one containing cancerous cells) in the same unstained FFPE tissue section (10 μm) mounted on a microscope slide. The areas were scraped individually from the mounting using a needle. The samples were carefully transferred into separate 1.5-ml reaction tubes and processed according to Basic Protocol 1. The two areas were processed separately to determine the expression level of several markers in healthy as well as tumor areas.

during formalin fixation means that this tissue is unsuitable for proteomic studies (Ahram et al., 2003), so that additional processing, described in the protocols in this unit, is required. The identification of differentially expressed proteins specific to a given tumor could provide a means to classify tumor types or monitor cancer progression.

Recently, a method was developed to extract non-degraded, full-length proteins from FFPE tissue, which allows the quantification of well-characterized tumor markers by immunoblotting (*UNIT 10.8*) or reverse-phase protein microarrays. Reverse-phase protein microarrays (also called protein lysate microarrays) consist of lysates immobilized in a grid format onto nitrocellulose-coated slides. Several samples can be analyzed in parallel by using, for example, a target-specific antibody (Becker et al., 2007). Moreover, improvements of this method allow analysis of FFPE-extracted protein lysates by 2-D gel electrophoresis (*http://www1.qiagen.com/Products/QproteomeFFPETissue2D-PAGEKit.aspx*). After a simple cleanup step of the extracted tissue protein mixture, the protein lysate can be enzymatically digested, followed by separation and analysis of the resulting peptides using reversed-phase HPLC coupled to ESI-MS/MS.

The use of high-performance liquid chromatography (HPLC) or combinations of different types of HPLC (*UNIT 10.13*) sequentially coupled to mass spectrometry can increase the separation power and characterization of proteins extracted from FFPE tissue, and is an important development toward deeper and broader proteome coverage. One alternative for improved separation may be the use of multidimensional chromatography (MudPIT) with reversed-phase as the last dimension (Masuda et al., 2005), or other multidimensional methods (Guo et al., 2007). Furthermore, the continuous development of MS technologies provides rich opportunities to compare protein expression levels in normal and diseased tissues using protein expression profiling methods (Wright et al., 2005).

Critical Parameters and Troubleshooting

Deparaffinization of FFPE sections and protein extraction

Extraction efficiency relies heavily on the quality of the FFPE starting material and the nature of the sample, especially the protein content of the tissue used for the experiment (Table 10.27.1). Too little starting material can clearly result in low protein yield. In addition, yield depends on the amount and type(s) of tissue in the sections (e.g., the amount of normal versus diseased tissue, of fat, of epithelial versus mesenchymal tissue), and will vary among tumors, tumor locations, and tumor types. To make sure that the sections being analyzed contain the tissues of interest, use two consecutive sections (each 10 μm thick with an area of 100 mm^2), and use one for hematoxylin/eosin histological staining and verification by a pathologist. Subsequent unstained sections from the same FFPE sample are then used for protein extraction and determination of protein concentration (see Basic Protocol 1).

Tissue that is fixed for 14 to 24 hr immediately after surgical removal represents the optimal (or highest-quality) starting material. Tissue that has been fixed for longer than 24 hr is not poor quality per se, but will result in lower protein extraction efficiency. Conversely, too short a fixation time can lead to incomplete fixation of the proteins, resulting in protein degradation by autodigestion. It is also very important to fix the tissue sample as quickly as possible after surgical removal. In general, storing the starting material at room temperature or in a refrigerator prior to fixation will allow progressive protein degradation and reduce the amount of extractable protein.

Numerous protocols are available for fixation of tissues (Prophet, 1992; Hewitt et al., 2008; Carson and Hladik, 2009). For additional discussion of pre-analytical factors influencing tissue handling and processing (e.g., size and thickness of the tissue specimen, time from removal to fixation, duration of fixation, and volume of fixative), see Puchtler and Meloan (1985) and Werner et al. (2000).

Sample cleanup

The cleanup step using different organic solvents is suitable for removal of interfering buffer components and for concentrating (i.e., precipitating) the sample prior to dissolution in a buffer whose pH is optimized for maximally efficient trypsin digestion. The most common problem during organic solvent extraction is accidental removal of the protein-containing phase (see Fig. 10.27.1B). Take care when removing the upper aqueous phase containing the bulk of the contaminating buffer salts. If phase separation is insufficient, extend the centrifugation to 2 min at 9,000 $\times$ *g* and remove the upper phase. Separation is facilitated by addition of methanol, which precipitates the protein, causing the chloroform/methanol mixture to become a single phase that is easily removed without disrupting the protein pellet. After addition of methanol, mixing, and centrifugation, a transparent or white gel-like pellet should be visible at the bottom of the tube. A 25-μg protein pellet is usually visible to the naked eye. If the pellet accidentally detaches from the bottom during removal of the supernatant, repeat the centrifugation step and carefully remove the remaining supernatant. After the acetone wash step, do not over-dry the pellet. The pellet should be moist, as over-drying will lower the resolubilization efficiency in the digestion buffer. If required, sonicate the sample for 5 min at room temperature (15° to 25°C).

Protein digestion

The amount, pH, and composition of the digestion buffer described in the protocol are sufficient for solubilization of the cleaned-up protein pellet. Using too much protein (>25 μg) can lead to incomplete solubilization of the sample. This is correctable by moderately increasing the volume with additional digestion buffer.

LC/MS analysis

The number of identifiable peptides/proteins can be improved by changing several parameters. (1) The amount of enzyme (trypsin) can be increased (e.g., by changing the enzyme-to-substrate ratio). (2) The MS scans can be performed with mass segments representing *m/z* 350-500, 450-650, 600-900, 850-1250, and 1200-1500 in order to reduce the complexity of detectable precursors that elute into the MS. In this manner, more precursors are selected and sequenced (de Godoy et al., 2008). The raw data derived from the five MS scans are combined (to increase the signal-to-noise ratio) and submitted to the database search. (3) The HPLC gradient used for separation can be prolonged from 40 min to 140 min or even longer (up to 220 min; see Basic Protocol 2). (4) Longer analytical nano-columns (300 mm or more) can be used to increase the number of theoretical plates for separation and concomitantly increase the resolving power of the column. Note, however, that this requires column heating with an oven or a jacket to decrease back pressure. (5) A second separation step can be introduced to reduce the complexity of the sample (e.g., separation of peptides by strong cation-exchange chromatography [SCX] or by isoelectric focusing [IEF] of peptides derived after enzymatic digestion of FFPE-extracted proteins).

Anticipated Results

In Basic Protocol 1, proteins are extracted from an FFPE tissue block using a simple and effective extraction methodology. Samples are processed for protein digestion by an efficient cleanup of the protein lysate, based on organic solvent extraction to remove interfering substances. In Basic Protocol 2, an acid-sensitive, detergent-containing buffer is used for optimal reconstitution and trypsin digestion. In Figure 10.27.2, samples derived from FFPE and freshly frozen tissue were analyzed by nano-RPLC separation coupled to nano-ESI-LTQ-MS/MS. A total of ~480 FFPE tissue

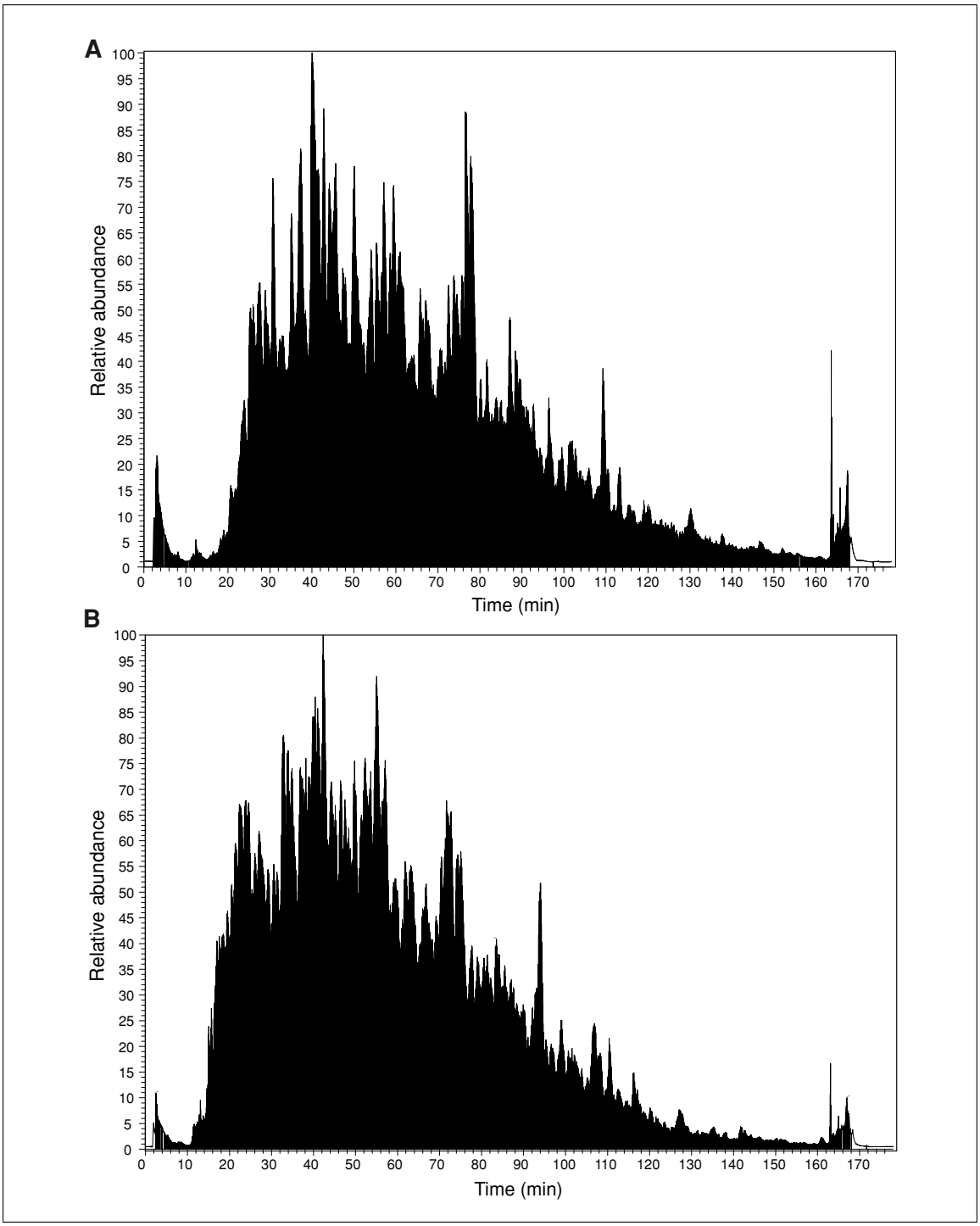

Figure 10.27.2 Total ion chromatogram of LC-coupled tandem MS analysis of hydrolyzed FFPE **(A)** and fresh **(B)** liver tissue. The LC gradient applied was 5% to 40% solvent B over 160 min (see Basic Protocol 2). The analysis was performed on an Orbitrap XL mass spectrometer (Thermo Fisher Scientific) by applying 5 μg of tryptic peptide mixture derived by enzymatic digestion of proteins extracted from FFPE (A) or fresh-frozen (B) tissue to an HPLC column coupled to the mass spectrometer.

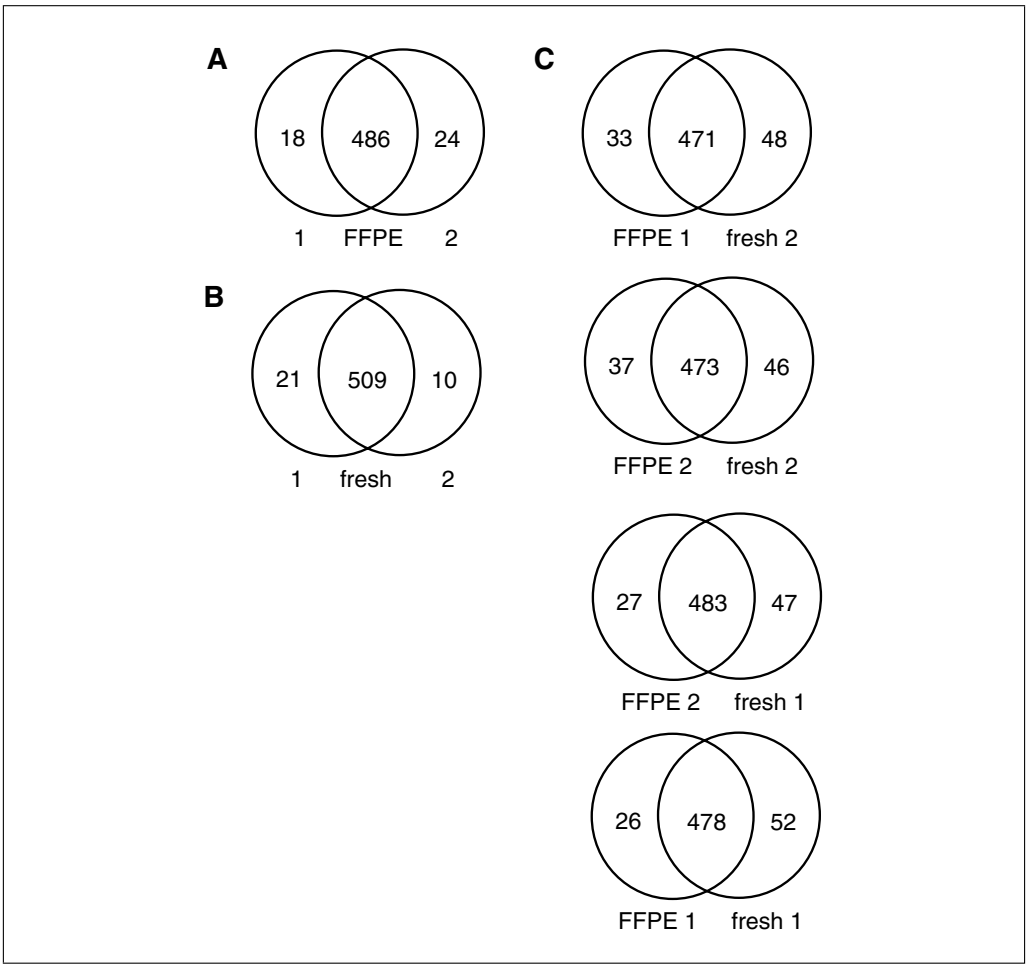

Figure 10.27.3 Venn diagrams of the LC/MS/MS analysis of two technical replicates of FFPE samples **(A)**, fresh samples **(B)**, and a comparison between FFPE and fresh samples **(C)**. The numbers indicate same and different proteins identified in each analysis.

proteins were identified from two replicates of the LC/MS/MS analysis (Fig. 10.27.3A). LC/MS/MS analysis of the same starting material (fresh sample) revealed >500 proteins in two replicates.

Approximately 86.7% of the total proteins were identified in both the freshly frozen and the FFPE tissue when the same amount of starting material was analyzed (Fig. 10.27.3C). In a comparison of MS results from freshly frozen and FFPE tissue samples, most proteins from the FFPE sample were also detected in the corresponding freshly frozen sample. Only ~31 and ~48 proteins, representing ~4.6% and ~8.7% of the total proteins identified, were unique to the FFPE or fresh sample, respectively.

Time Considerations

Deparaffinization of FFPE sections requires 15 min hands-on time and ~1.5 hr total time, including cutting of sections with a microtome. The protein extraction step requires 20 min hands-on time and ~2.5 hr total time. Protein quantification using a commercially available kit requires 10 min hands-on time and ~30 to 40 min total time, depending on the method used. Basic Protocol 1 can be completed within half a day, and the extracted protein mixture may be stored at –20°C until Basic Protocol 2 is carried out (within 2 to 3 days including LC/MS and depending on the complexity of the sample).

The time required for cleanup of the extracted proteins is 15 to 25 min, depending on the number of protein samples. Basic Protocol 1 describes the steps necessary to process one sample, but several samples can be processed in parallel, e.g., to compare samples from a healthy and diseased specimen, or from two different tissues. After completing the cleanup steps, the protein pellet may be stored at 4°C until the protein digestion (up to 1 week).

Protein digestion requires 15 min hands-on time, with 2.25 hr time overall for sample resolubilization/reconstitution and an overnight

incubation (~14 to 24 hr) for digestion. Reversed-phase HPLC separation and mass spectrometry takes ~2 hr per sample followed by MS analysis. The time needed for the HPLC gradient depends on the sample complexity. Because considerable technical expertise is required to use the coupled HPLC, mass spectrometer, and analysis software, these steps are best performed by an investigator who is experienced with these technologies.

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