Expression Pathology

Liquid Tissue® MS Protein Prep Kit

for the isolation of proteins and peptides from formalin fixed tissue in preparation for mass spectrometry analysis

Cat. No.	**10001-010**	**4 Preparations**
	10001-023	**10 Preparations**
	10001-053	**50 Preparations**
	10001-068	**Custom**

Store at -20°C

Instruction Manual

Version 1.46, August 2009

FOR RESEARCH USE ONLY - Not intended for animal or human diagnostic or therapeutic use. Refer to the following page for intended use.

Liquid Tissue® MS Protein Prep Kit is sold and distributed for evaluation and/or research purposes only and is not intended for re-sale, human or animal diagnostic or therapeutic use.

This product and its use is the subject of U.S. Patent No. 7,473,532 and patents pending and foreign counterparts owned by Expression Pathology Inc.

Purchaser Notification - Limited Use Label License

The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) to not transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. Expression Pathology will not assert a claim against the buyer of infringement of the above patents based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components were employed, provided that neither this product nor any of its components was used in the manufacture of such product. The buyer shall not determine the structure or otherwise reverse engineer this product. The use of this product by the buyer constitutes agreement with the terms of this limited use label license. If the purchaser is not willing to accept the limitations of this limited use statement, Expression Pathology is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Licensing Department at info@expressionpathology.com

Phone (301) 977-3654
Fax (301) 926-9283

Table of Contents:

Introduction

Expression Pathology has developed a unique methodology for the extraction and procurement of soluble and dilutable protein directly from formalin fixed paraffin embedded (FFPE) tissue samples, using *Liquid Tissue*® reagents and protocol. This patent pending technology forms the foundation for a collection of approaches for proteomic analysis of formalin fixed archival tissue, one of which is analysis of proteins by mass spectrometry.

Even though the proteomic complexity of a cell makes qualitative and quantitative analysis of a single protein difficult at best, observations carried out at the protein level are inherently attractive because of the cause and effect relationship of protein expression and biological function. Tissue based studies are also important for linking proteins directly to biological and disease processes because of informative pathologic context and relevance. Both fresh frozen tissue and formalin fixed archival tissue can be used for proteomic analysis. Fresh frozen tissue is difficult to obtain in large numbers, expensive to store, and difficult to process, whereas large collections of well defined formalin fixed archival tissue with clinical history, and often patient outcome, are readily available. However, effective and routine analysis of proteins in formalin fixed tissue has been limited to immunohistochemistry (IHC). This limitation is due to the chemical cross-linking properties of the common fixative reagent formalin that prevents standard protein preparation methodologies from extracting soluble protein from fixed tissue. Expression Pathology's *Liquid Tissue*® reagents provide for proteomic analysis of formalin fixed tissue not previously attainable.

Identification and quantitation of candidate biomarker proteins in large numbers of individual tissues is required to validate specific proteins, or panels of proteins, for clinical use as diagnostic, prognostic, toxicological, or therapeutic markers. Mass spectrometry provides exciting analytical methodology for this purpose. The *Liquid Tissue*® MS Protein Prep Kit allows the user to procure and prepare tryptic peptides from formalin fixed tissue for a multitude of mass spectrometry analyses across a variety of mass spectrometry platforms.

Tissue Processing

The process of cell selection and tissue dissection is important in obtaining high quality samples for analysis. Tissue sections between 5 and 10 microns thick, mounted on microscope slides, are the optimum for cell selection. Cells of interest can be removed from unwanted cells by macrodissection or microdissection depending on the technical capabilities and experience of the laboratory and the aim of the project. Macrodissection with a fine needle is used when the region of the tissue is large enough to be seen by the naked eye. Microdissection is more commonly used when the region of interest is too small for needle dissection and/or the cells of interest are interspersed with contaminating cells. A variety of tissue microdissection instruments are available for this procedure.

When making a *Liquid Tissue*® preparation, it is essential that any embedding compound (paraffin) within the tissue be completely removed prior to cell procurement. The standard protocol for deparaffinization of the tissue is included in the Procedures Section.

Mass Spectrometry and Proteomics

Liquid Tissue® protein preparations have been analyzed using multiple mass spectrometry platforms. Multidimensional liquid chromatography (LC) separation methods when combined with tandem mass spectrometry provide sufficient separation and resolution to resolve, detect, and quantitate individual and multiple proteins simultaneously while analyzing the entire proteome contained within a single *Liquid Tissue*® protein preparation. LC-ESI-MS/MS has been successfully utilized to positively identify hundreds of proteins simultaneously within a *Liquid Tissue*® preparation obtained from formalin fixed tissue. The list of identified proteins includes known cancer biomarkers as well as proteins that span the entire spectrum of molecular types and function including membrane receptors, cell signal pathway proteins, transcription factors, metabolic proteins, nuclear proteins, cytoplasmic proteins, and cytoskeletal proteins[3,4]. Quantitative comparison of isotopically labeled *Liquid Tissue*® protein preparations (O^{16} vs. O^{18}) from two different tissues by LC-ESI-MS/MS has positively identified dozens of differentially expressed proteins between the two tissues[3]. Surface Enhanced Laser Desorption/Ionization (SELDI) technology has also been successfully utilized to generate reproducible proteomic patterns from cells procured from formalin fixed tissue[2]. Results from mass spectrometry analysis of *Liquid Tissue*® protein preparations are of comparable quality to results obtained by mass spectrometry analysis of protein from fresh tissue, frozen tissue, cell lines, and serum.

Kit Information

Recommended Storage Conditions: Store at -20°C

Chemical Hazard — Handle with care, always wear gloves and follow standard safety precautions.

Kit Contents — The solutions in this kit should be clear. They should not be used when precipitates are present. The Reduction Reagent and Trypsin must be stored at -20°C when not in use and should be placed on ice during use. The *Liquid Tissue*® Buffer is stable at room temperature but can also be stored at -20°C.

Component	10001-010	10001-023	10001-053	10001-068
Liquid Tissue® Buffer	1 x 90 µl	1 x 200 µl	4 x 250 µl	6 x 250 µl
Reduction Reagent (100 mM DTT)	1 x 20 µl	1 x 40 µl	4 x 40 µl	6 x 40 µl
Trypsin, lyophilized (Proteomics Grade)	1 x 20 µg	1 x 20 µg	4 x 20 µg	6 x 20 µg
Trypsin Diluent	1 x 40 µl	1 x 40 µl	2 x 50 µl	2 x 50 µl
Reaction Tubes, (1.5 ml low protein binding)	4 tubes	10 tubes	50 tubes	0 Tubes
Product Manual	1 each	1 each	1 each	1 each

Additional Equipment and Materials Required

For Tissue Processing:

Oven, 58°C to 60°C
Microtome
Syringe
Xylene or Xylene Substitute
Dilute Alkaline Solution
Eosin
Dissecting Microscope or Magnifying Glass
Microscope Slides and Coverslips
Slide Jars, Plastic or Glass
30 Gauge Needle
100% Ethanol
Mayer's Hematoxylin Solution
Permanent Mounting Medium
DIRECTOR® Laser Microdissections Slides (Expression Pathology Inc.)
(For use with Leica LMD or P.A.L.M. Laser Microdissection Instruments)

For *Liquid Tissue*® Protocol:

Heating Block, 95°C
Waterbath, 37°C
Optional: Thermal Cycler with heated lid
Vortex Mixer
Microcentrifuge
PCR Tubes, 0.2 ml

General Lab Supplies and Equipment:

200 µl Pipettor
Disposable gloves
Timer
Water, double distilled or other high purity grade
10 µl Pipettor
Forceps

Procedures

Tissue Evaluation

1. Prior to the preparation of any *Liquid Tissue*® sample, it is important to evaluate tissue section microscopically to determine the appropriate cells to process. The histological detail of a tissue section can be seen by staining with hematoxylin and eosin (H&E).

    - Fresh solutions should be used each time tissue sections are processed. A maximum of five slides per 25 mls of solution can be processed before the solutions should be changed.

    - Slides should be transferred with forceps. Excess liquid can be removed by touching the slide to the side of the container and/or touching the end of the slide to a clean paper towel.

2. H&E Staining

    a. Cut a section of the formalin fixed paraffin embedded tissue on a microtome at 4 to 6 microns in thickness and place on standard glass microscope slide.

    b. Label 12 plastic slide jars as follows:

        Xylene #1
        Xylene #2
        100% Ethanol #1
        100% Ethanol #2
        85% Ethanol
        70% Ethanol
        Distilled Water
        Dilute Alkaline Solution
        95% Ethanol #1
        95% Ethanol #2
        95% Ethanol #3
        Eosin
        Xylene #3

    c. Fill the labeled plastic slide jars with the appropriate solution.

    d. Place the slide(s) to be processed in a 58° to 60°C oven for 30 to 60 min to melt the paraffin and allow contact of the tissue with the slide.

    e. Place the slide(s) in the plastic slide jar Xylene #1 for 5 min.

    f. Transfer the slide(s) to the plastic slide jar Xylene #2 for 5 min.

    g. Transfer the slide(s) to the plastic slide jar containing 100% Ethanol #1 for 5 min.

    h. Transfer the slide(s) to the plastic slide jar containing 100% Ethanol #2 for 5 min.

    i. Transfer the slide(s) to the plastic slide jar containing 85% Ethanol for 1 min.

    j. Transfer the slide(s) to the plastic slide jar 70% Ethanol for 1 min.

    k. Transfer the slide(s) to the plastic slide jar containing Distilled Water for 1 min.

    l. Apply Mayer's Hematoxylin Solution to the tissue section so that it covers the entire section. Stain with Mayer's Hematoxylin for 5 to 15 min. Staining time may be varied for individual color preference.

    m. Rinse the slide in Distilled Water for 30 sec.

    n. Dip slide in Dilute Alkaline Solution (50 ml distilled water, 0.5 ml of 0.5 M NaOH) until the hematoxylin turns a bright blue (3 to 5 times).

    o. Rinse the slide in fresh Distilled Water for 30 sec.

    p. Rinse the slide in 95% Ethanol #1 for 30 sec.

    q. Eosin Counterstain

        (1) Dip slide in Working Eosin Solution 3 to 5 times.

        (2) Dip slide in 95% Ethanol #2 to remove excess stain.

        (3) Dip slide 5 times in 95% Ethanol #3.

        (4) Rinse in Xylene #3 for 30 sec.

        (5) Apply a coverslip to the slide with permanent mounting medium.

    r. Evaluate the tissue section with a microscope to locate the cells to be collected for processing. The cells can be identified for reference by indicating the area on the slide with a marker (on the coverslip of the slide).

Cell Procurement

a. A standard *Liquid Tissue*® preparation has been optimized for approximately 30,000 cells (maximum 45,000 cells) in a 20 µl reaction volume. The proportion of cells to reaction volume must be kept constant. For larger amounts of tissue the *Liquid Tissue*® Buffer, Trypsin, and Reduction Reagent volumes must be scaled proportionally.

b. As a rule of thumb, there are approximately 382,000 cells per cubic millimeter of tissue and approximately 30,000 cells are contained in a volume of 0.079 cubic millimeters.

c. Determine the number of cells that will be collected based on the reference area(s) identified on the H&E slide and the thickness of sections cut for procuring the cells. Use the Chart of Areas (on the following page) to determine the approximate number of cells being collected.

Chart of Areas

Section Thickness	Area Containing Approximately 30,000 cells	Perimeter Containing Approximately 30,000 cells	Circle Diameter Containing Approximately 30,000 cells
5 µm	16 mm²	4.0 x 4.0 mm	4.5 mm
7 µm	12 mm²	4.0 x 3.0 mm	3.9 mm
10 µm	8 mm²	4.0 x 2.0 mm	3.2 mm
15 µm	5.3 mm²	3.5 x 1.5 mm	2.6 mm

- To avoid introducing contamination, work in a clean area and wear clean gloves throughout the dissection protocol. Avoid touching the clean gloves to surfaces that may introduce foreign particles. Reduce the exposure of samples, reagents, equipment and supplies to contamination sources, such as skin, hair, clothing, dust and particulates.
- Section(s) of the formalin fixed, paraffin embedded tissue are cut on a microtome at 5 µm to 15 µm in thickness and placed on an appropriate slide (refer to the protocols below for further information).
- The appropriate subset of cells can be isolated by needle dissection, laser microdisscton or laser capture microdissection (LCM).
- There may be some samples where the tissue is not dissected but the entire section is taken for processing if all of the cells are appropriate for collection.
- IMPORTANT: It is strongly recommended that tissue sections be placed on a slide for dissection. If sections are cut and placed directly into a reaction tube, it is difficult to calculate the amount (i.e. area) of tissue being processed, and the preparation will almost always require a larger reaction volume. It is also more difficult to remove the paraffin and properly prepare the tissue sections for processing.

1. Tissue Preparation for Needle Dissection and Laser Microdissection

a. Label 7 plastic slide jars as follows:

Xylene #1
Xylene #2
100% Ethanol #1
100% Ethanol #2
85% Ethanol
70% Ethanol
Water, double distilled or other high purity grade

b. Fill the appropriately labeled plastic slide jars with freshly prepared reagents. Prepare 85% and 70% Ethanol with double distilled or other high purity water.

c. Place the slides to be processed in a 58° to 60°C oven for 30 to 60 min to melt the paraffin.

d. Deparaffinization and Rehydration

- Fresh solutions should be used each time tissue sections are processed. A maximum of five slides per 25 mls of solution can be processed before the solutions should be changed.
- Slides should be transferred with forceps. Excess liquid can be removed by touching the slide to the side of the container and/or touching the end of the slide to a clean paper towel.

(1) Place the slides in the plastic slide jar containing Xylene #1 for 5 min.

(2) Transfer the slides to the plastic slide jar containing Xylene #2 for 5 min.

(3) Transfer the slides to the plastic slide jar containing 100% Ethanol #1 for 5 min.

(4) Transfer the slides to the plastic slide jar containing 100% Ethanol #2 for 5 min.

(5) Transfer the slides to the plastic slide jar containing 85% Ethanol for 1 min.

(6) Transfer the slides to the plastic slide jar containing 70% Ethanol for 1 min.

(7) Transfer the slides to the plastic slide jar containing high-purity water for 1 min.

(8) Optional Step: Hematoxylin Staining

(a) Apply Mayer's Hematoxylin Solution to the tissue section so that it covers the entire section.

(b) Stain for 1 min to 5 min at room temperature. Staining time may vary depending on tissue type.

(c) Tap off excess stain before proceeding with the following steps.

(d) Rinse slide in double distilled or other high purity grade water for 30 sec.

(e) Rinse in fresh double distilled or other high purity grade water for 30 sec.

(9) Optional Step: Dehydration for Laser Microdissection Protocol (follows)

(a) Transfer the slides to the plastic slide jar containing 95% Ethanol for 30 sec.

(b) Transfer the slides to the plastic slide jar containing 100% Ethanol for 30 sec. and air dry.

2. Needle Dissection Protocol

- This procedure should be practiced to become familiar with the technique before it is attempted with precious samples.
- Recommended Slide: untreated, standard glass microscope slide.
- A tissue section from the same tissue block, previously stained with H&E, can be aligned with the unstained section and used as a guide for selecting which cells to collect.

a. Sample Collection

(1) Thaw the *Liquid Tissue*® Buffer and mix briefly to ensure the solution is uniform.

(2) For each 30,000 cells collected, 20 µl of *Liquid Tissue*® Buffer is required. Pipet the required amount into a 1.5 ml low protein binding Reaction Tube and close the tube.

(3) Place a new 30 gauge needle on a syringe.

(4) Remove the slide from the distilled water container and use a low-lint wipe to remove the water from the back of the slide and around the tissue section. A minimal amount of water should remain on the tissue. Do not let the tissue completely dry out.

(5) Use the areas on the H&E stained slide to direct the location of the cells for needle dissection. If necessary for better visualization, place the slide on a dissecting microscope or visualize under a magnifying glass.

(6) Gently scrape the areas of the tissue to be removed with the needle.

(7) The dissected cells will become detached from the slide and form small clumps of tissue that can be collected on the needle by electrostatic attraction. Collection of an initial tissue fragment on the tip of the needle will assist in collecting more tissue.

(8) The tip of the needle with the clumped tissue fragments should be carefully placed into the appropriately labeled Reaction Tube containing *Liquid Tissue*® Buffer.

(9) Gently shaking the needle will ensure the tissue detaches from the tip of the needle into the buffer.

(10) DO NOT aspirate the sample into the needle to avoid losing a portion of it.

(11) Microcentrifuge the tube(s) containing the cells in *Liquid Tissue*® Buffer at 10,000 rcf for 1 min.

3. Laser Microdissection Protocol

- This protocol requires the use of either a Leica LMD or P.A.L.M Microlaser instrument.
- Recommended slide: Expression Pathology Inc. DIRECTOR® Laser Microdissection Slide (contact us for further details). Additionally, both systems can be used with several types of plastic membrane slides, refer to manufacturers' information for specific details.
- It is recommended that 10 µm thick tissue sections be cut from formalin fixed paraffin embedded tissue blocks and placed on the appropriate microdissection slides (DIRECTOR® , PEN, PET or POL microdissection slides).
- Prepare the tissue sections according to the procedure above (Page 9 - Tissue Preparation for Needle and Laser Microdissection). NOTE: The plastic membrane slides are sensitive to Xylenes - refer to manufacturers' instructions for specifc details regarding the removal of paraffin from the sections.

a. Laser Microdissection Sample Collection

(1) Samples are collected into the cap of a low protein binding 0.2 ml PCR tube

(2) Thaw the *Liquid Tissue*® Buffer and mix briefly to ensure the solution is uniform.

(3) Pipet 20 µl of the *Liquid Tissue*® Buffer into the cap. Use the pipet tip to evenly distribute the buffer in the cap.

(4) Perform the laser microdissection to collect approximately 30,000 cells. Consult the instrument's User Guide for detailed instructions.

(5) Note: It is important to work reasonably quickly. Prolonged times for cell collection will result in the evaporation of the buffer due to the heat from the illumination light of the microscope. It is possible to lose 3 to 5 ul of the buffer as a result of evaporation over the period of time it takes to microdissect the tissue section.

(6) After collection of the cells, carefully place the tube over the cap and seal the cap to the tube. Microcentrifuge the tube(s) at 10,000 rcf for 1 min. The final volume of buffer should be approximately 15 to 20 ul (use another 0.2 ml PCR tube of the same type containing 20 µl of water for comparison to check for excessive evaporation). If the volume of buffer is significantly less, due to excessive evaporation, additional *Liquid Tissue*® Buffer may need to be added to the tube to bring the volume back to approximately 20 µl. DO NOT use any other reagent or water to adjust the volume.

(7) The 0.2 ml PCR tubes can be processed in a thermal cycler for the 95°C and 37°C incubation steps in the *Liquid Tissue*® Preparation Protocol. A heated lid should be used to minimize condensation under the cap during the incubations.be used to minimize condensation under the cap during the incubations.

4. Laser Capture Microdissection (LCM) Protocol

- This protocol is intended for use with an Arcturus or MMI Microdissection Instrument.
- Recommended slide: Refer to manufacturers' instructions
- Note: The preparation of the tissue sections for the LCM instrument requires a specific protocol which is outlined below.

a. Cut 7 µm thick sections from formalin fixed paraffin embedded tissue blocks and place on untreated microscope slides.

b. Label 10 plastic slide jars as follows:
   Xylene #1
   Xylene #2
   100% Ethanol #1
   85% Ethanol #1
   70% Ethanol #1
   Water, double distilled or other high purity grade
   70% Ethanol #2
   85% Ethanol #2
   100% Ethanol #2
   Xylene #3

c. Fill the appropriately labeled plastic slide jars with freshly prepared reagents. Prepare 85% and 70% Ethanol with double distilled or other high purity water.

d. Place the slides to be processed in a 58-60°C oven for 30 to 60 min to melt the p araffin.

e. Deparaffinization, Rehydration and Staining

- Fresh solutions should be used each time tissue sections are processed. A maximum of five slides per 25 ml of solution can be processed before the solutions should be changed.
- Slides should be transferred with forceps. Excess liquid can be removed by touching the slide to the side of the container and/or touching the end of the slide to a clean paper towel.

(1) Place the slides in the plastic slide jar containing Xylene #1 for 5 min.

(2) Transfer the slides to the plastic slide jar containing Xylene #2 for 5 min.

(3) Transfer the slides to the plastic slide jar containing 100% Ethanol #1 for 5 min.

(4) Transfer the slides to the plastic slide jar containing 85% Ethanol #1 for 1 min.

(5) Transfer the slides to the plastic slide jar containing 70% Ethanol #1 for 1 min.

(6) Transfer the slides to the plastic slide jar containing water for 1 min.

(7) Apply Mayer's Hematoxylin Solution to the tissue section so that it covers the entire section.

(8) Stain for 1 min to 5 min at room temperature. Staining time may vary depending on tissue type.

(9) Tap off excess stain before proceeding with the following steps.

(10) Rinse slide in double distilled or other high purity grade water for 30 sec.

(11) Place slide in fresh double distilled or other high purity grade water for 30 sec.

(12) Transfer the slides to plastic slide jar containing 70% Ethanol #2 for 1 min.

(13) Transfer the slides to plastic slide jar containing 85% Ethanol #2 for 1 min.

(14) Transfer the slides to plastic slide jar containing 100% Ethanol #2 for 1 min.

(15) Transfer the slides to plastic slide jar containing Xylene #3 for 1 min.

(16) Air dry.

f. Laser Capture Microdissection (LCM) Sample Collection

- NOTE: A maximum of 2 caps (i.e. the film from 2 caps) can be processed in a standard 20 µl reaction.

(1) Perform laser capture microdissection (LCM). Consult the User Guide for the instrument you will use for detailed instructions.

(2) For each 30,000 cells collected, 20 µl of *Liquid Tissue*® Buffer is required. Thaw the *Liquid Tissue*® Buffer and mix briefly to ensure the solution is uniform. Pipet the required amount into a 1.5 ml low protein binding Reaction Tube and close the tube.

(3) Transfer the LCM captured cells to a Reaction Tube as follows:

i. Wearing gloves and working in a clean area, place the LCM cap on a flat surface with the cells directed up.

ii. Using a new pipette tip, hold the cap with one hand and carefully push in from the edge of the cap toward the center with the pipette tip, working around the edge, to lift the film to which the cells are attached. The film should form a clump that can be transferred on the pipette tip into a Reaction Tube.

iii. Carefully transfer the film into the appropriately labeled Reaction Tube containing *Liquid Tissue*® Buffer using the pipette tip. Gently touch the film to the buffer to ensure it detaches from the pipette tip.

iv. Microcentrifuge the tube(s) containing the cells in *Liquid Tissue*® Buffer at 10,000 rcf for 1 min.

Liquid Tissue® Preparation

Note: The protocol that follows assumes the amount of *Liquid Tissue*® Buffer used during the cell procurement to be 20 µl, and that approximately 30,000 cells were collected. If the number of cells exceeds 45,000, the reagent volumes used must be scaled appropriately, i.e. if 60,000 cells were collected, the volume of Liquid Tissue buffer must be increased to 40 µl and the amounts of Trypsin and Reduction Reagent must also be doubled.

1. Heat the Reaction Tube(s) containing the procured cells in the *Liquid Tissue*® Buffer in a heating block at 95°C for 90 min. Make sure that the cap is on securely to help prevent evaporation.

2. Every 20 min, remove the tubes and shake down the buffer that has formed a condensation in the cap so that it covers the cells by flicking the tube in a downward motion with your wrist. Alternately, quickly microcentrifuge for 5 to 10 sec. at 10,000 rcf. Gently flick the tube to resuspend the cells in the buffer and immediately place the tube back into the heating block. DO NOT allow the tubes to cool down completely.

3. After 90 min at 95°C, microcentrifuge the tube(s) at 10,000 rcf for 1 min. (Note: If the sample was isolated using the LCM caps or plastic membrane slides, the film will have melted during the 95°C heating step and will pellet at the bottom of the tube.)

4. Cool the tube(s) on ice for 1 to 2 min.

5. Prepare the Trypsin Solution

   a. Reconstitute the lyophilized enzyme with 20 µl of Trypsin Diluent.

   b. Mix the vial briefly to ensure the Trypsin is dissolved. Place vial on ice.

   c. The reconstituted trypsin solution can be stored at -20°C for up to 4 weeks and is stable for at least 3 freeze thaw cycles.

6. Carefully open each Reaction Tube and add 1.0 µl of Trypsin Solution to each reaction tube (30,000 cells in 20 µl). Mix and briefly microcentrifuge to collect the solution at the bottom of the Reaction Tube.

7. Heat the Reaction Tube(s) in a waterbath at 37°C. For the first hour , every 20 min, remove the tubes, and vortex rigorously for 10 to 15 sec. Shake the buffer down to the bottom of the tube so that it covers the cells before placing the tube back into the waterbath.

8. Continue incubating at 37°C overnight (16 to 18 hours).

9. At the end of the 37°C incubation, microcentrifuge the tube(s) at 10,000 rcf for 1 min. Note: At this stage of the process, the sample should be completely solubilized.

10. Protein Determination: See Appendix A for information about determining the total protein concentration of the sample. An aliquot **must be removed prior to the addition of the Reduction Reagent** to avoid interference in the total protein determination assay.

11. Carefully open each tube and add 2 µl of Reduction Reagent (100 mM DTT) (30,000 cells in 20 µl). Mix and briefly microcentrifuge to collect the solution at the bottom of the Reaction Tube.

12. Heat the reaction tube(s) at 95°C for 5 min.

13. Microcentrifuge the tube(s) at 10,000 rcf for 1 min. Note the presence and size of any pellet.

14. Store the *Liquid Tissue*® preparation at -20°C until ready for analysis.

Appendix A

Micro BCA Protein Assay for *Liquid Tissue*® MS Preparations

1. Purpose:

Procedure for determination of Total Protein Concentration in *Liquid Tissue*® MS preparations, using the Pierce Micro BCA Protein Assay Reagent Kit. The kit is a detergent-compatible bicinchoninic acid formulation for the colorimetric detection and quantitation of total protein. The kit procedure has been modified for small sample volumes in a 96-well microplate format.

The *Liquid Tissue*® MS Protein Prep Kit Reduction Reagent, DTT, is not compatible with this protein determination assay. Sample preparations made with the *Liquid Tissue*® MS Protein Prep Kit can be tested in this assay using an aliquot taken prior to the addition of DTT to the preparation. Follow the instructions in the Methods Section below for collection of an appropriate *Liquid Tissue*® MS preparation aliquot for analysis.

2. Equipment:

a. Pipet or Pipet device
b. Vortex mixer
c. 37°C incubator
d. Plate shaker
e. Microplate reader

3. Materials:

a. Micro BCA Protein Assay Reagent Kit
   Pierce Biotechnology, Inc. Catalog # 23235
b. BSA Standards, pre-diluted
   Concentrations: 2.0, 1.0, 0.8, 0.5, 0.3, 0.2, 0.1 µg/µl
c. Microplate, 96-well flat bottom UV transparent
   Corning Catalog #3635
d. High Purity Grade Water

4. Methods:

a. Collect *Liquid Tissue*® MS preparation aliquots for analysis:

(1) Immediately following the *Liquid Tissue*® Preparation protocol step of incubating at 37°C overnight with Trypsin in the *Liquid Tissue*® MS Protein Prep Kit manual:

(a) Microcentrifuge the tube(s) at 10,000 rcf for 1 min.

(b) Heat the reaction tube(s) at 95°C for 5 min.

(c) Microcentrifuge the tube(s) at 10,000 rcf for 1 min.

(2) Remove a 5 µl aliquot of the *Liquid Tissue*® MS preparation for analysis. Mark the aliquot with the preparation reference number and "No DTT". Store the *Liquid Tissue*® MS preparation remaining in the reaction tube at -20°C until the protein determination assay has been completed.

b. Prepare Micro BCA Working Reagent

(1) Determine the Total Volume required:

Total Volume =
(Number of Standards + Number of Samples) x (2 replicates) x (75 µl)

(2) Mix together Reagent A, Reagent B and Reagent C in the following proportions:

Reagent	Parts	Volume for 1 Well	Volume for 40 Wells
Reagent A	25	37.5 µl	1.50 ml (1500 µl)
Reagent B	24	36.0 µl	1.44 ml (1440 µl)
Reagent C	1	1.5 µl	0.06 ml (60 µl)
Final Volume		75.0 µl	3.00 ml (3000 µl)

c. Pipet 75 µl of High Purity Grade Water into the appropriate Blank wells in the microplate.

d. Pipet 73 µl of High Purity Grade Water into the appropriate number of BSA Standard wells in the microplate.

e. For samples, 1 or 2 µl can be run.

If using 1 µl of sample, pipet 74 µl of High Purity Grade Water into the appropriate number of Sample wells in the microplate.

If using 2 µl of sample, pipet 73 µl of High Purity Grade Water into the appropriate number of Sample wells in the microplate.

f. Pipet 2 µl of each BSA Standard replicate into the appropriate microplate wells containing High Purity Grade Water.

g. Pipet the appropriate volume of each Sample replicate (1 µl or 2 µl) into the appropriate microplate wells containing High Purity Grade Water.

h. Add 75 µl of the Micro BCA Working Reagent to each well and the mix the microplate thoroughly on a plate shaker for 30 sec.

i. Carefully put a plate cover on the microplate and incubate at 37°C for 2 hours.

Note: Limit incubation of the microplate to less than or equal to 37°C, otherwise high background and aberrant color development may result.

j. Cool the microplate to room temperature (approximately 10 min).

k. Immediately measure the absorbance at 562 nm on a microplate reader.

l. Calculations:

(1) Subtract the average 562 nm absorbance reading of the Blank replicates from the 562 nm reading of all other individual BSA Standard and Sample replicates.

(2) Prepare a standard curve by plotting the average Blank Corrected 562 nm reading for each BSA Standard vs. its concentration in µg/µl. Use the standard curve to determine the protein concentration of each unknown sample using the Blank Corrected 562 nm reading for the Sample.

Note: Results for 1 µl sample volumes need to be multiplied by 2 for the final concentration in µg/µl.

m. If no additional aliquots are required from the *Liquid Tissue*® MS preparation, add Reduction Reagent (100 mM DTT) to the remaining *Liquid Tissue*® MS preparation. Adjust the volume of 100 mM DTT to be added to the volume of *Liquid Tissue*® MS preparation remaining.

The final concentration of the DTT needs to be 10 mM, which is a 1:10 dilution of the stock reagent.

For example:
15 µl *Liquid Tissue*® MS preparation volume
1.5 µl Reduction Reagent (100 mM DTT) volume

(1). Add the appropriate volume of Reduction Reagent (100 mM DTT) to the *Liquid Tissue*® MS preparation remaining in the reaction tube. Mix and briefly microcentrifuge to collect the solution at the bottom of the Reaction Tube.

(2). Heat the reaction tube(s) at 95°C for 5 min.

(3). Microcentrifuge the tube(s) at 10,000 rcf for 1 min.

(4). Store the *Liquid Tissue*® MS preparation remaining in the Reaction Tube at -20°C until ready for mass spectrometry analysis.

Appendix B

Chromatography and Mass Spectrometery Parameters for Analysis of *Liquid Tissue*® MS Preparations

The following information provides guidelines for the chromatography and mass spectrometry parameters for a global protein analysis of *Liquid Tissue*® preparations on a Thermo Fisher Scientific LTQ mass spectrometry instrument.

1. Liquid Chromatograhy Parameters:

Column: 75 µm (ID) (360 µm OD) x 10 cm (length); Fused Silica
Polymicro Technologies, Phoenix, AZ

Chromatography Matrix: Reverse Phase, C-18 Silica, 3 µm; 300Å Pore Size
Source: Vydac, Hesperia, CA

Injection Amount: 1 µg total protein (in 0.1% Formic Acid)

Solvent A: 0.1% Formic Acid in Water (100%)

Solvent B: 0.1% Formic Acid in Acetonitrile (100%)

Chromatography:

Step	Type	% Solvent A/B @Start	% Solvent A/B @Finish	Time	Flow Rate
Loading	Static	98%/ 02%	98%/ 02%	30 min	500 nl/min
Elution 1	Linear	98%/ 02%	60%/ 40%	110 min	250 nl/min
	Linear	60%/ 40%	02%/ 98%	30 min	250 nl/min
Equilibration	Static	02%/ 98%	02%/ 98%	10 min	500 nl/min
	Linear	02%/ 98%	98%/ 02%	2 min	500 nl/min
	Static	98%/ 02%	98%/ 02%	15 min	500 nl/min

2. Thermo Fisher Scientific LTQ Mass Spectrometer Instrument Parameters:

Exclusion Duration	90 sec
Exclusion List	500
Scan Range	350 - 1800
Min Signal Req'd	100 - 500
Number of Scan Events	5 - 7 with no repeat
Zoom AGC Target	3,000
Full AGC Target	75,000
SIM AGC Target	30,000
MSn AGC Target	10,000
Zoom Micro Scans	3
Zoom Max Ion Time (ms)	500

Frequently Asked Questions

Question: **Is the protein purified from formalin fixed tissue with this kit functional?**
Answer: **No. The proteins have been digested with trypsin to render small predictable peptides intended only for mass spectrometry.**

Question: **Are the extracted proteins full length and can they be analyzed by Western blots?**
Answer: **No. The proteins have been trypsin digested to small peptides and do not reflect the actual size of the original intact proteins.**

Question: **If the protein preparation is used without performing the tryptic digestion are the proteins functional and can they be analyzed by Western blot?**
Answer: **No. Prior to tryptic digestion, the proteins are not functional nor can they be assayed using methods intended for full length intact proteins.**

Question: **What mass spectrometry instruments have been utilized for analysis of *Liquid Tissue*® preparations?**
Answer: **A range of instruments and methodologies have demonstrated utility for proteomic analysis of *Liquid Tissue*® preparations including LC-ESI-MS/MS, SELDI-TOF, MALDI-TOF, LC-MS/MS, and LC-FTICR-MS.**

Question: **Can the sample be directly analyzed without any further treatment?**
Answer: **Yes, the reagents and process for preparing *Liquid Tissue*® preparations with this kit have been optimized for mass spectrometry analysis. The preparations do not contain substances that would interfere with mass spectrometry analysis and therefore do not require any further treatment.**

Question: **How much of a *Liquid Tissue*® preparation should be run for mass spectrometry analysis?**
Answer: **Typically 1 µg of a *Liquid Tissue*® preparation is sufficient. Running larger amounts of sample can result in poor resolution and actually reduce the number of protein identifications.**

Question: **The cells to be processed were collected on LCM caps. How many caps (cells/film) can be processed in a reaction?**
Answer: **A maximum of two (2) caps (cells/film) can be processed in a standard 20 µl reaction. A 20 µl volume of reagent is not sufficient to cover the material from additional caps.**

References

1. Krizman, D.B. et al. Genetic Engineering News 25(12):30-31, 2005.
2. Prieto, D.A. et al. Biotechniques 38:S25-S28, 2005.
3. Hood, B.L. et al. Molecular and Cellular Proteomics 4(11):1741-1753, 2005.
4. Patel V. et al. Clinical Cancer Research 14: 1002-1014, 2008.

Patents

Liquid Tissue Technology is protected by U.S. Patent 7,473,532 and patents pending and foreign equivalents thereof.

DIRECTOR Technology is protected by U.S. Patent 7,294,367 and 7,381,440 and foreign equivalents thereof.

Trademarks

Liquid Tissue is a registered trademark of Expression Pathology Inc.
DIRECTOR is a registered trademark of Expression Pathology Inc.

Expression Pathology, Inc.
9620 Medical Center Drive
Rockville, MD 20850

Phone: 1.301.977.3654
e-mail: info@expressionpathology.com

http://www.expressionpathology.com